ADVANCES IN
NEUROBLASTOMA
RESEARCH

ANR

COLOGNE 2014

ANR CONGRESS

13TH – 16TH MAY 2014

COLOGNE, GERMANY

INFORMATION BOOK

OVERVIEW. PROGRAM. ABSTRACTS.

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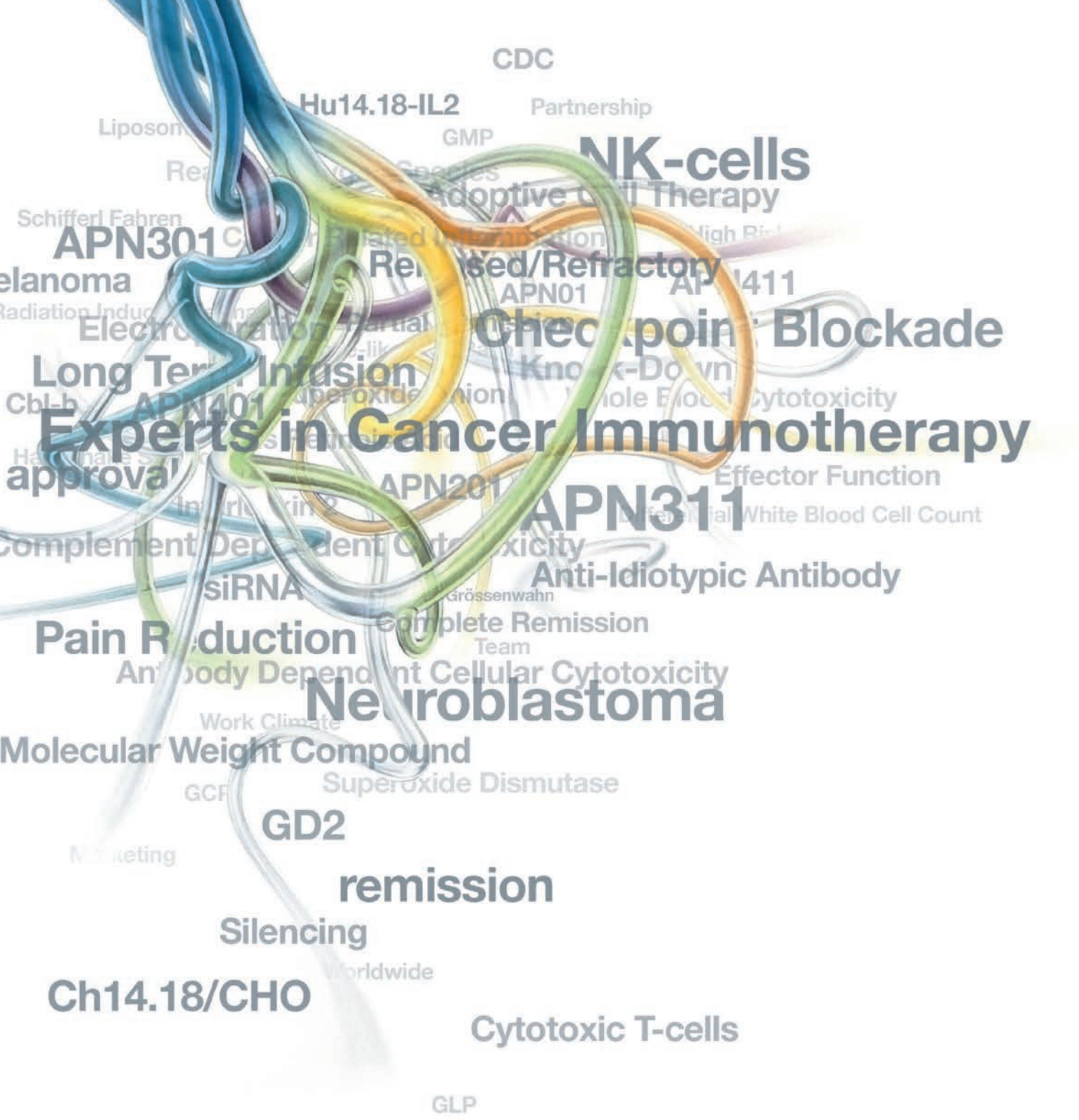
KÖLN



APEIRON BIOLOGICS

Experts in development of antibody-based immunotherapies for neuroblastoma

APEIRON Biologics AG
Campus-Vienna-Biocenter 5
1030 Vienna, Austria

www.apeiron-biologics.com

PROGRAM AT A GLANCE ANR 2014

13TH – 16TH MAY 2014

Monday, May 12th

- ANRA Steering Committee — Conference Room 2
- iINRGdb* — Conference Rooms 3/4/5
- VIP Reception at Köln Sky*

* Invitation only

Tuesday, May 13th

- Consortium of Neuroblastoma Foundations - Medical Advisory Board — Conference Rooms 3/4/5
- Break/Coffee
- Lunch
- Consortium of Neuroblastoma Foundations - Operational Meeting — Conference Rooms 3/4/5
- Consortium of Neuroblastoma Foundations - Reception — Conference Rooms 3/4/5
- Update Course — Europasaal
- Break/Coffee
- Update Course (cont.) — Europasaal
- Lunch
- Update Course (cont.) — Europasaal
- Break/Coffee
- Update Course (cont.) — Europasaal
- Workshop 1 Drug Discovery and Development for Neuroblastoma — Offenbachsaal
- Lunch
- Workshop 2 Stem Cell and Differentiation Workshop — Offenbachsaal
- Break/Coffee
- Workshop 3 Epigenetics in Neuroblastoma — Offenbachsaal
- INRG UHRG — Conference Room 1
- Poster Mounting
- Welcome Reception at the Historic Town Hall of Cologne

Wednesday, May 14th

- Welcome address — Congress Saal
- Keynote 1 Crest Development — Congress Saal
- Plenary 1 Basic Research I — Congress Saal
- Break/Coffee
- Plenary 2 Basic Research II Translational Research I — Congress Saal
- Lunch
- Parallel A1 Basic Research: Oncogenesis I — Offenbachsaal
- Parallel B1 Translational Research: Preclin. Exp. Therapies I — Europasaal
- Break/Coffee
- Parallel A2 Basic Research: Oncogenesis II — Offenbachsaal
- Parallel B2 Translational Research: Preclin. Exp. Therapie II — Europasaal
- Poster Viewing
- ANRA Advisory Board — Conference Rooms 3/4/5
- Philharmonic Concert at the Cologne Philarmonic Hall

Thursday, May 15th

- Keynote 2 Epigenetics in Neuroblastoma — Congress Saal
- Plenary 3 Translational Research II Clinical Research I — Congress Saal
- Break/Coffee
- Parallel A3 Basic Research: Oncogenesis III Biological Models — Offenbachsaal
- Parallel B3 Translational Research: Preclin. Exp. Therapie III — Europasaal
- Lunch
- Parallel A4 Basic Research: High Throughput Techniques — Offenbachsaal
- Parallel B4 Translational Research: Preclin. Exp. Therapie IV MRD — Europasaal
- Break/Coffee
- Parallel A5 Basic Research: Developmental Neuroblastoma Biology — Offenbachsaal
- Parallel B5 Clinical Research I — Europasaal
- Poster Viewing
- Gala Dinner at the Restaurant Tanzbrunnen & Rheinterrassen — until 0:30 am

Friday, May 16th

- Parallel A6 Basic Research, Translational Research, Clinical Research II — Europasaal
- Parallel B6 Clinical Research III — Offenbachsaal
- Break/Coffee
- Parallel A7 Translational Research: Molecular Markers — Europasaal
- Parallel B7 Clinical Research IV — Offenbachsaal
- Lunch
- Keynote 3 Cellular Immunology Preclin. Exp. Therapies I — Congress Saal
- Plenary 4 Clinical Research II — Congress Saal
- Lifetime Achievement Award — Congress Saal
- Farewell — Congress Saal
- Break/Coffee
- Poster Removal
- Parents' and Survivors' Meeting (in German) — Conference Rooms 3/4/5

Dear scientists, physicians, students, nurses, parents, patients, supporters, friends, dear all,

a warm WELCOME TO YOU
in Cologne and at the congress Advances in Neuroblastoma Research 2014.

We are so happy that you are here and that we can together continue the great tradition of conferences which started 1975 with less than 20 people in Philadelphia. In the past the meetings have been very influential as well for the practical medical care of the patients as for the biological concepts of the origin and the course of the disease.

The broad spectrum and the high quality of the submitted abstracts promise exciting presentations and discussions. More than 450 submissions were accepted by the reviewers for the scientific program. This year the main themes focus on the genomic and transcriptomic aberrations, the preclinically tested therapeutic agents and clinical trials.

An educational workshop on the pre-conference day May 13th, 2014 will summarize the current knowledge of the biology and the state of the art in the clinical. This is a perfect opportunity for an update, in particular for physicians to become more familiar with the experimental points of view and for experimental researchers to learn the most burning medical needs.

The three workshops will cover hot topics of current experimental cancer research from a broader perspective but with potential application to neuroblastoma. We are happy that top scientists agreed to discuss their cutting edge science with us.

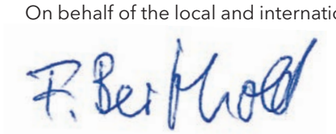
For the first time, a consortium of neuroblastoma research funding organisations from all-over the world meet and discuss the opportunities how to foster research most efficiently and to spread knowledge for serving the patient's needs throughout the various countries.

A parents' and survivor's meeting is also a new opening for German speaking families with neuroblastoma patients. Invited experts will report at their meeting about the relevant advances of medical sciences and listen to the experiences and needs of the families.

We are grateful to our sponsors and supporting organisations which demonstrated their devotion to our research activities and made the conference possible.

You can expect from this conference many new ideas, innovative tools for the care of your patients and general new insights in the cancer development. Enjoy the presentations, the discussions and the social program. I hope that you can find time to explore the flair and the beauty of Cologne, a modern city with more than 2000 years of history. It's not too fond to say that Cologne is one of the most vital and charming cities in Germany. The local people calls "Cologne is a feeling".

On behalf of the local and international organizing committee,



Prof. Dr. Frank Berthold
Director of Department of Pediatric Oncology and Hematology
University Hospital of Cologne

Welcome to ANR 2014

It is a great privilege, as President of Advances in Neuroblastoma Research Association, to welcome you to Advances in Neuroblastoma Research (ANR) 2014 in Cologne.

The aims of ANRA are to understand the pathogenesis of neuroblastoma and to improve the diagnosis and treatment of patients with neuroblastoma, through the advancement and integration of laboratory and clinical research. This results in the ultimate goal of cure for more children with neuroblastoma. ANR brings together people dedicated to the aims of ANRA from throughout the world and facilitates international interaction.

I want to congratulate Professor Frank Berthold and the Local Organising Committee for their enthusiasm, efficiency, hard work and vision in organising of ANR 2014.

I am very confident that ANR 2014 will be a landmark. A large number of proffered abstracts which highlight advances in the field will be presented at plenary, parallel or posters sessions.

We are very fortunate in having outstanding keynote lecturers and the organisers of the workshops have brought together investigators at the cutting edge of their field. On Tuesday there is an Educational Session which gives an overview of the current state of the art for neuroblastoma.

The Steering Committee and Regional Advisory Boards of ANRA have worked with the Local Organising Committee in suggesting the format of the meeting, topics and speakers.

A focus of ANR 2014 is to allow significant time for discussion of presentations and also in the Plenary Sessions we are introducing very brief overviews highlighting the relevance of the findings.

Another great innovation in ANR 2014 is the parallel meeting of the Consortium of Neuroblastoma Foundations on Tuesday 12th May. The Consortium has been recently formed and brings together, with one voice, the neuroblastoma foundations. We are privileged they are meeting at ANR and we are delighted that members will be at ANR 2014, interacting with other attendees.

As always the Steering Committee of ANRA is very open to suggestions so that ANRA can achieve its goal even more successfully.

In conclusion, I welcome you and look forward to a highly successful ANR 2014.
There will be outstanding presentations and interaction between researchers,
so we may be more effective curing more children with neuroblastoma.

Andy Pearson
Professor A D J Pearson
President, Advances in Neuroblastoma Research Association

Dear Participants of the ANR 2014 Congress in Cologne,

For many of us our childhood is a good time. Unfortunately, this is not true for all children. Above all not for those who are seriously ill - for example, if they have one or more neuroblastoma. This is one of the most common malignant tumours in babies and young children which still accounts for 11 per cent of cancer deaths in children. Thus, there is a great demand for further research into scientific findings as to how neuroblastoma originate, early diagnosis and possible treatment.

The international congress “Advances in Neuroblastoma Research” provides an important contribution to that. Medical specialists, scientists and students have the opportunity to “think outside the box” and discuss the latest developments in this field.

As the Lord Mayor of Cologne I am pleased that this congress is taking place here in the Rhine-side metropolis. As a traditional university city with a medical faculty Cologne provides an ideal venue for the event, especially since great importance is attached here to scientific research together with comprehensive health care.

I wish you both interesting and productive discussions and a successful congress. I also hope that besides the medical and scientific exchange of information and experience you will find sufficient time to enjoy the tourist attractions and cultural sights of Cologne.

Yours

Jürgen Roters
Lord Mayor of the City of Cologne

Dear ANR2014 participants,

The Medical Faculty of the University of Cologne is proud that the “Advances in Neuroblastoma Research Congress” is held this year in Cologne.

The members of the faculty wish you fruitful discussions that increase the understanding of the enigmatic characters of the various neuroblastoma types and that open new paths for the care of those children. The University of Cologne is a modern University amidst Europe. It was founded by the citizens of Cologne in 1388 and represents the fourth oldest University in Europe. Today the University of Cologne attracts and educates the highest number of students in Germany.

The Medical Faculty is particular devoted to progress in oncology. Pediatric oncology is one of the best examples how to proceed by national and international collaboration. Neuroblastoma represents the main research focus of the Department of Pediatric Oncology and Hematology. The highly visible research activities in this field are embedded in a vital academic scene provided by the Comprehensive Cancer Center (CIO) and the Center of Molecular Medicine (CMMC). Major challenges are ahead. The impressive increase of knowledge at the molecular level of the different neuroblastoma types could identify key players in small subsets only. The main paths for maturation from neuroblastoma to ganglioneuroma, for the initiation of regression and for the evolution of drug resistance are still hardly understood. It is also well appreciated how difficult it is to translate promising targeted therapies into clinical practice, in particular with a quite limited number of patients. The ANR congress is a perfect occasion to extent international collaboration.

I hope that in addition to the scientific use of the meeting you will enjoy the hospitality of your German friends and partners and that you can experience the beauty and vitality of the city and the country.

Prof. Dr. Dr. h.c. Thomas Krieg
Dean of the Medical Faculty

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The ANR2014 Congress would like to thank all of the generous sponsors and supporters who contributed to the realization of the congress and associated events.

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Een actie voor Villa Joep!
Het fonds tegen neuroblastoom kinderkanker

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Apeiron is a privately financed biotech company based in Vienna, Austria, developing immunologic therapies against cancer. Its portfolio consists of five clinical projects and some preclinical approaches.

Its lead project, APN311 (ch14.18/CHO), is a chimeric monoclonal antibody against the GD2 ganglioside abundandly expressed on neuroblastoma; GD2 also occurs on other tumors of neuroectodermal origin. Together with the internationally active SIOPEN study group, APN311 is clinically investigated in high-risk neuroblastoma, with more than 600 patients treated to date. A novel treatment modality has been elaborated with substantially improved tolerability and marked clinical activity in relapsed/refractory neuroblastoma patients. The project is close to submission for market authorization in the EU and US.
Apeiron's project APN301 is an anti-GD2 antibody-IL2 fusion protein (immunocytokine) which is currently being tested in a phase II trial in the US and Canada in neuroblastoma (together with COG) and in a separate trial in melanoma.

The recombinant human Angiotensin Converting Enzyme 2 (GSK2586881, APN01) was developed by Apeiron until end of Phase I, licensed to GlaxoSmithKline (GSK) in 2010 and is presently being investigated by GSK in an ongoing phase II study in Acute Lung Injury.
Furthermore, a broad program is pursued to develop therapies to selectively boost the immune system via checkpoint blockade to combat cancer: APN401 is a novel individualized adoptive cellular therapy based on the target cbl-b. Patient's PBMC are silenced ex-vivo for the cbl-b gene, thereby activating them, and then re-infused to the patient. A Phase I study in USA is presently being set up. The APN411 project aims for development of low molecular weight compounds to boost immune cells via novel checkpoint blockade mechanisms and is performed in collaboration with Evotec. APN201 (human Superoxide Dismutase) is pursued to cope with oxidative stress and associated inflammation with a focus on applications in oncology.

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APEIRON Biologics AG

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Agilent Technologies is a leading supplier of life science systems that enable scientists to study complex biological processes and disease mechanisms. Engineered for sensitivity and reproducibility, our Genomics product portfolio includes molecular biology reagents, PCR and qPCR instrumentation, a new scanner, microarrays and software for CGH+SNP, Gene expression, miRNA and methylation analysis, as well as the Bioanalyzer and TapeStation for sample Quality Control, SureSelect & Haloplex Target Enrichment Platforms for NGS and high-resolution SureFISH probes – Complete solutions, from sample preparation to data analysis, and automation, to optimize your workflow productivity.

United Therapeutics CORPORATION

United Therapeutics Europe Ltd.

Contact:

Unither House, Curfew Bell Road
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United Kingdom

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Gold Sponsor

United Therapeutics Corporation is a biotechnology company focused on the development and commercialization of unique products to address the unmet medical needs of patients with chronic and life-threatening conditions, including neuroblastoma.

United Therapeutics (UT) entered into a Cooperative Research and Development Agreement with the US National Cancer Institute (NCI) to collaborate on the late-stage development and regulatory agency submissions of ch14.18 for children with high-risk neuroblastoma. Results from NCI's pivotal phase III study were published in September 2010. This study was conducted by the Children's Oncology Group, a national consortium of researchers supported by the NCI. The NCI has also completed a second phase III trial in 105 children to define more clearly the safety and toxicity profile of ch14.18 immunotherapy and UT are developing the commercial production capability for the antibody.

These studies are the basis for a marketing authorization application UT filed with the European Medicines Agency in 2013 seeking approval of ch14.18 immunotherapy for the treatment of neuroblastoma. UT also expects to file a biologics license application with the FDA during 2014. UT has received orphan drug designation for ch14.18 from the FDA and the EMA.

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Alex's Lemonade Stand Foundation

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Gold Sponsor

Alex's Lemonade Stand Foundation (ALSF) shares the vision of our founder and creator, Alexandra "Alex" Scott - to find better treatments and cures for all childhood cancers. We recognize the importance of funding researchers at every stage of their career and have developed a robust grants program to support the best and brightest minds in the field. From engaging researchers early in their career to advancing the pace of innovative research, we are committed to helping fill the critical gaps innate to pediatric cancer investigation and support scientists dedicated to making important advancements that bring us one step closer to cures.

Neuroblastoma Children's Cancer Alliance UK

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London NW1 8DH
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Mail: info@ncca-uk.org
Website: www.ncca-uk.org

Silver Sponsor

NCCA UK (Neuroblastoma Children's Cancer Alliance UK) supports families affected by neuroblastoma. Its activities fall into three main areas: access to treatment, education and awareness and research funding. NCCA UK wants all families to have the best possible information and access to the best possible treatment for their child from diagnosis. Alongside this, NCCA UK facilitates the development of the next generation of neuroblastoma therapies. NCCA UK is a founding member of INBRACED (International Neuroblastoma Research and Collaboration for Effective Delivery), a global network of neuroblastoma charities, researchers and clinicians with the shared goal of improving outcomes.plication with the FDA during 2014. UT has received orphan drug designation for ch14.18 from the FDA and the EMA.

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Gala Dinner Sponsor

The mission of WGFRF is to promote advances in the field of oncology by shortening the cancer research timetable. The Foundation may provide grants for pilot research studies and educational efforts. Forbeck meetings are not to discuss published research, but to provide a forum for the cross fertilization of ideas, concepts and observations.

W-LAN Sponsor

GE Healthcare is one of Germany's leading supplier of medical-technical services and solutions. Furthermore, it is the only company operating in the fields of diagnostic imaging systems, pharmaceutical research and development as well as contrast agents all at the same time.

GENERAL INFORMATION

USEFUL INFORMATION

Banks, Credit Cards & Currency Exchange

The official currency in Germany is Euro. Currency exchange is possible at most banks and foreign exchange offices in the city.

Cash machines/ATMs can be found at airports, banks, hotels and throughout the city.
Major credit cards are accepted at hotels, restaurants, shops and cash machines.

Climate & Attire

In May, the weather in Cologne is usually warm and sunny with temperatures of approx. 18°C/64°F. We recommend to inform yourself about the upcoming weather conditions in the internet.

The conference attire is business casual.

Cologne

Cologne, Germany´s oldest city, is the location where the famous cathedral looks down upon innumerable cultural and historical treasures, world famous museums an active art scene. The world feels at home in Cologne, where people meet for a glass of Kölsch, the local beer, a chat or simply a laugh. Life in Cologne is uncomplicated and vivacious.

The city is easily accessible by air and has excellent local transportation facilities.

Enjoy the unique atmosphere of the Rhine River, the historical city center or the pulsing night life.
Herzlich Willkommen in Köln!

More information: www.cologne-tourism.com

Disclaimer/Liability

The ANR2014 Organizing Committee accepts no liability for any injuries and losses incurred by participants and/or accompanying people, nor loss of, or damage to any luggage and/or personal belongings.

Electricity

Electrical current in Germany is 230 Volt. Appliances designed to operate on a different number of Volt need a voltage converter and a plug adapter.

Hotels

For the ANR Congress 2014 we made prebookings at several excellent hotels that offer a reduced rate for ANR participants (incl. breakfast). Please contact the hotels directly by telephone or e-mail and refer to the respective booking subject when making your booking.
All hotels are well situated in an acceptable distance to the congress and have been inspected by the ANR2014 organizing committee.
The ANR Congress can be easily reached by foot or by using public transportation services.
For detailed information about the recommended hotels please take a look at the ANR Congress website under the following link:

www.anr2014.com/locations/hotels.htm

Smoking

The Congress Center is a smoke free area.

Smoking is prohibited in all the enclosed areas within the facility without exception. Please use designated smoking areas outside the building.

In Germany smoking is not allowed in all public areas and restaurants.

Special Needs

If not indicated upon online registration, participants and accompanying people are invited to advise the congress organization team about their special requirements. Please email at anr2014@welcome-gmbh.de. The entire congress area is easily accessible for people with special needs (PSN).

Time Zone

The time zone in Cologne is GMT + 1 hour. Daylight Saving Time is used in summer incl. the month of May.

Tipping

Since gratuity is included in the bill, tipping in Germany is an absolutely voluntary payment when visiting restaurants or bars, travelling by taxi or engaging in other services. However, if the service was pleasant, we recommend to leave a tip of around 5 - 10% of the total amount in order to show customer satisfaction.

Tourist Information

The congress staff at the registration counter will be available to give you some basic information about the city of Cologne.

For further information, please contact the official tourism office of Cologne at:

Address:
Kardinal-Höffner-Platz 1, 50667 Köln
(opposite the Cologne Cathedral)
Website: www.cologne-tourism.com
Email: info@koelntourismus.de
Phone: +49 (0) 221 346 43 0

Tramway

The Congress Center is well connected to public transportation. The entrance EAST is easily accessible by foot from two stations: “Köln Messe/ Deutz” and “Kölnmesse”, which is passed by tramways 3 and 4.

Every ANR2014 Congress participant will receive a tramway schedule in the congress bag.

The price for a one-way ticket is 1.90 €
(short haul for a maximum of 4 stops).

Prices for long haul tickets start at 2.40 €.
There are also daily and weekly tickets available.

For further information about the tramway schedule visit:
www.kvb-koeln.de/german/tarif/eng.html

Transportation from & to the Airport

The Airport Cologne/Bonn is located 16 km southeast of downtown Cologne. From the airport to the city, take the tramway S13 towards “Bahnhof Ehrenfeld” and exit at “Köln Messe/Deutz”. There you will find connecting trains and tramways. From train station “Köln Messe/Deutz” take tramway S13 in direction to Troisdorf Station. Tramways depart every 20 minutes during the day and the journey takes approx. 15 minutes. A one-way ticket costs 2.80 €.
Airport taxis are available outside the arrival platform. Prices may vary according to the time of the day (late-night surcharge). On average the cost is 27.00 € and a journey to downtown Cologne takes approx. 25 min. Please be aware that debit cards are not accepted and that only a few taxis accept credit cards. Please check with the driver in advance.

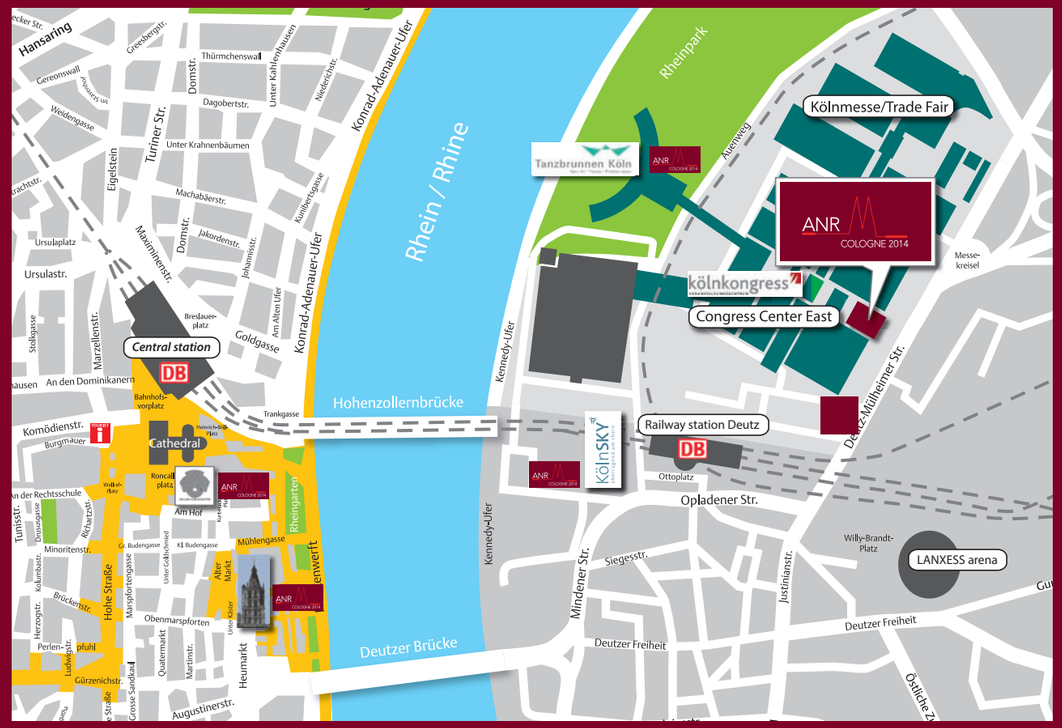
Please note that international flights usually arrive at Frankfurt International Airport or Düsseldorf International Airport.
For further information visit
www.frankfurt-airport.com or www.dus.com.

Travel Agency

The partner travel agency of the ANR Congress is “Travel Agency Knappe & Liese”. The agency will gladly assist you with the planning and organization of tours to various destinations within Germany and to neighboring countries. For further information please contact the agency at:

Address:
Kölner Strasse 159, 51149 Köln
Phone: +49 (0) 22 03 -18 11 12
Email: info@reiseagentur-liese.de

THE **CONGRESS**



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ANR Congress 2014

kölnkongress

from Tuesday, May 13th to Friday, May 16th, 2014
Congress Centre East Koelnmesse

Deutz-Mülheimer Straße 51. 50679 Köln
Public transportation station:
"Bahnhof Köln Messe/Deutz"

VIP Reception

KölnSKY

on Monday, May 12th, 2014 *
KölnSky 27th floor
Cologne Triangle

Ottoplatz 1 . 50679 Köln
Public transportation station: "Bahnhof Köln Messe/Deutz" or "Deutzer Freiheit"
* Invitation only

Welcome Reception

on Tuesday, May 13th, 2014
Historisches Rathaus (Historic Town Hall)

Rathausplatz 2 . 50667 Köln
Public transportation station: "Rathaus"

Philharmonic Concert

on Wednesday, May 14th, 2014
Kölner Philharmonie (Philharmonic Concert Hall)

Bischofsgartenstr. 1 . 50667 Köln
Public transportation station:
"Köln Hauptbahnhof / Cologne Main Station"

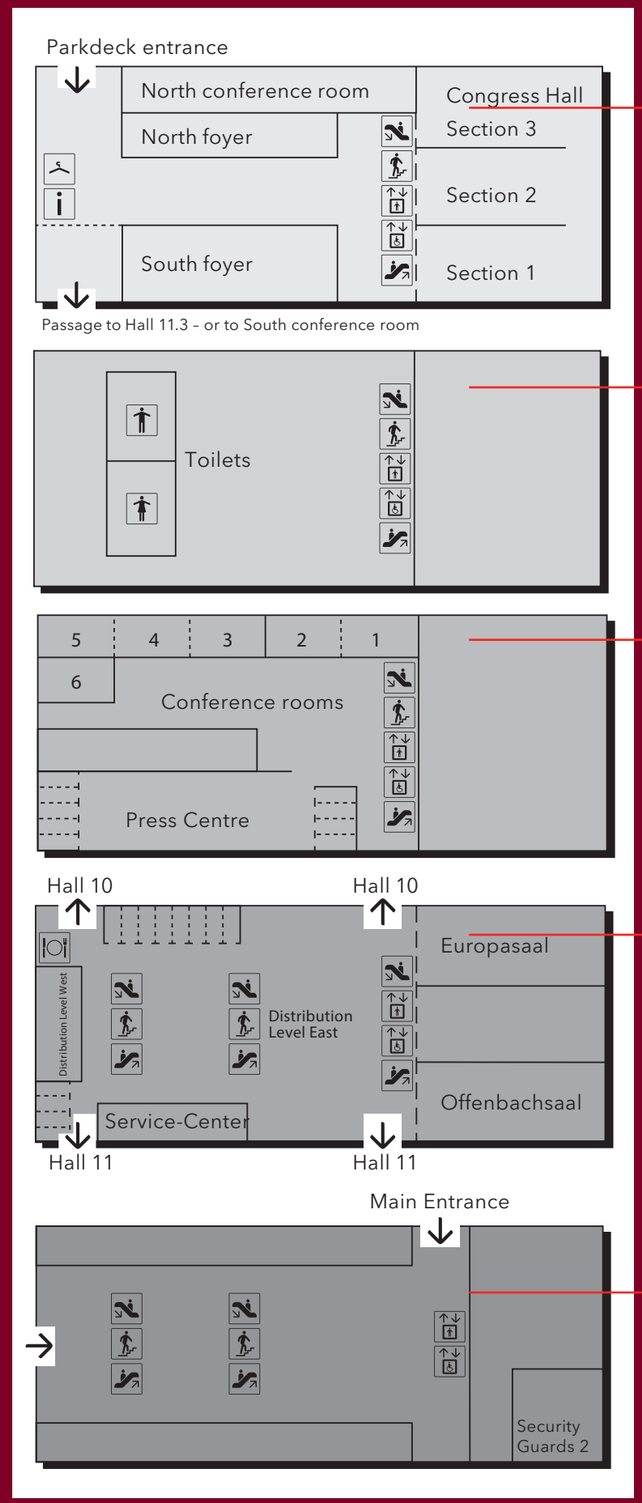
Gala Dinner Event

Tanzbrunnen Köln

on Thursday, May 15th, 2014
Theater am Tanzbrunnen & Rheinterrassen

Rheinparkweg 1. 50679 Köln
Public transportation station:
"Bahnhof KölnMesse/Deutz";
from there 5 - 7 minutes by foot

FIND YOUR WAY WITHIN THE CONGRESS CENTER EAST



kölnkongress

4th floor

Keynote and Plenary Sessions
Congress Saal Sec. 1/2

Poster Exhibition
Congress Saal Sec. 3
North and South Foyer

3rd floor

Toilets

2nd floor

Conference Rooms 1-5

Speakers Room:
Conference Room 6

1st floor

Parallel Sessions
Europasaal and Offenbachsaal

Industrial Exhibition

Catering Area for
Coffee Breaks and Lunches

Registration & Information Desk
(Wednesday to Friday)

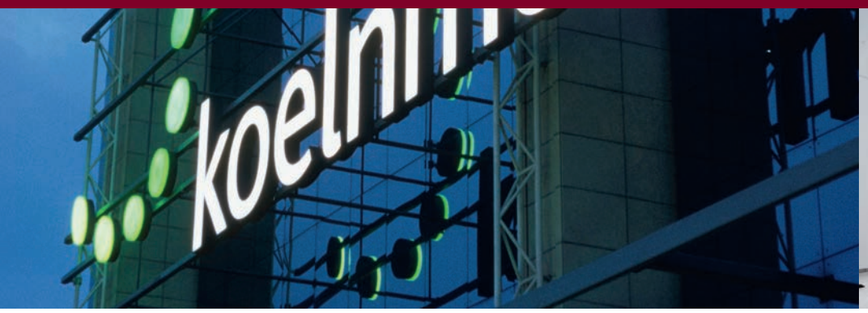
Media Desk

Groundfloor

Main Entrance

Registration & Information Desk
(Monday to Tuesday)

Wardrobe



Catering

Daily tea/coffee breaks and lunches are included in the registration fee. The catering area is located on the first floor of the congress center.

All ANR hotels offer complimentary breakfast within the agreed ANR rate.

Certificate of Attendance & CME

Upon request, a Certificate of Attendance will be issued to the ANR2014 congress participants on Friday, May 16th. Please visit the registration/information desk for further information and to collect your certificate.

Congress Organization

welcome Veranstaltungsgesellschaft mbH is the official organizer of the Advances in Neuroblastoma Research Congress (ANR 2014). welcome is a professional, full-service event agency providing comprehensive services for meetings, conferences and exhibitions as well as corporate events, incentives and incoming. Visit us at www.welcome-gmbh.de

Exhibition

The exhibition area is located on the first floor. For a detailed exhibitor list, please refer to page 32.

Internet

Free WLAN for congress participants is graciously sponsored by GE Healthcare. Log-in details are available at the registration counter.

GE Healthcare

Poster Exhibition

The poster exhibition can be found on the fourth floor of the congress center. Please follow the signs to find the areas for basic, translational and clinical poster presentations.

The poster exhibition is open to all conference participants during the official congress times from Wednesday to Friday.

Poster mounting time:
Tuesday, May 13th, 2014: 3:00 – 6:00 pm

Poster removal time:
Friday, May 16th, 2014: 5:00 – 6:00 pm

Registration

All ANR2014 participants and accompanying people are required to register at the registration desk to pick up their name badges, the information book and if booked, entrance tickets for the philharmonic concert and the gala dinner.

Congress participants are required to wear their identification badge in a visible place at all times. Entrance to meeting halls, poster and exhibition areas will not be permitted to any person without a congress badge. During social events, all participants and accompanying people are required to carry along their name badges as proof of identification.

Outstanding balances have to be settled by Tuesday May 13th 6:00 pm. Otherwise, the registration/reservation will forfeit. At the registration counter, we accept cash, MasterCard and Visa Card.

Registration and information desk opening hours:

Monday, May 12th, 2014:
3:00 pm – 6:00 pm, ground floor

Tuesday, May 13th, 2014:
8:00 am – 6:00 pm, ground floor

Wednesday, May 14th, 2014:
8:00 am – 7:00 pm, first floor

Thursday, May 15th, 2014:
8:00 am – 6:30 pm, first floor

Friday, May 16th, 2014:
8:00 am – 6:00 pm, first floor

Speakers

Time Limits
The time limits for a plenary session and a parallel session are 11-13 minutes and 8-10 minutes, respectively. The chairman/chairlady is advised to stop any talk then in order to allow enough time for the discussion of your presentation.

For a plenary session, 7-9 additional minutes are scheduled for discussion and for a parallel session 8-10 minutes.

Technical Equipment
The meeting rooms are equipped with laptop, beamer and a speaker's desk with microphone and laser pointer. Should you require special technical equipment, please contact the congress agency.

Slide Submission for Presentation
Speakers are required to submit their presentation in Microsoft PowerPoint (PPT) format. Please send your presentation to the congress agency until May 7th, 2014 or hand it over on a USB stick to the technical staff at the media desk on the first floor of the congress center (designated area) 1 hour prior to the beginning of the session at latest. There you can ensure that the slides project clearly and in the correct order.

Media Desk opening hours:

Monday, May 12th, 2014:
3:00 pm – 6:00 pm, first floor

Tuesday, May 13th, 2014:
8:00 am – 6:00 pm, first floor

Wednesday, May 14th, 2014:
8:00 am – 7:00 pm, first floor

Thursday, May 15th, 2014:
8:00 am – 6:30 pm, first floor

Friday, May 16th, 2014:
8:00 am – 6:00 pm, first floor

Speaker's Quiet Room
If you need a quiet place to review your presentation before submitting it, there is a speaker's room on the second floor (conference room No. 6). Please note that you are required to bring your own laptop. The speaker's room is open May 12th to May 16th, 2014 for the length of the congress.

Booth Number: # 01

APEIRON BIOLOGICS

biotech company – developing immunologic therapies against cancer – portfolio: five clinical projects and some preclinical approaches - lead project APN311 is a chimeric monoclonal antibody against the GD2 ganglioside

Contact:
APEIRON Biologics AG
Campus-Vienna-Biocenter 5
1030 Vienna
Austria

Contact person ANR Congress:
Mr. Hans Loibner
Phone: +43 (0) 1 86565 77
Fax: +43 (0) 1 86565 77-800
Mail: apeiron@apeiron-biologics.com
Website: www.apeiron-biologics.com

Booth Number: # 02

Agilent Technologies

leading supplier of life science systems - enable scientists to study complex biological processes and disease mechanisms - widespread Genomics product portfolio

Contact:
Agilent Technologies Deutschland GmbH
Herrenberger Str. 130
71034 Böblingen
Germany

Contact person ANR Congress:
Mrs. Kathrin Kiel
Phone: +49 (0) 7031 464-0
Fax: +49 (0) 7031 464-2020
Mail: kathrin_kiel@agilent.com
Website: www.agilent.de

Booth Number: # 03

United Therapeutics CORPORATION

leading supplier of life science systems - enable scientists to study complex biological processes and disease mechanisms - widespread Genomics product portfolio

Contact:
United Therapeutics Europe Ltd.
Unither House, Curfew Bell Road
Chertsey, Surrey KT169FG
United Kingdom

Contact person ANR Congress:
Mrs. Michelle Pernow
Phone: +44 (0) 1932 573800
Fax: +44 (0) 1932 571110
Mail: mpernow@unither.com
Website: www.unither.com

Booth Number: # 04

Baxter

global, diversified healthcare company - unique combination of expertise in medical devices, pharmaceuticals and biotechnology - products that save and sustain the lives of people with various diseases

Contact:
Baxter Deutschland GmbH
Edisonstraße 4
85716 Unterschleißheim
Germany

Contact person ANR Congress:
Mrs. Brigitte Huhn-Kiele
Phone: +49 (0) 89 317 010
Fax: +49 (0) 89 317 011 77
Mail: info@baxter.com
Website: www.baxter.de

Booth Number: # 05

inbraced
International Neuroblastoma Research & Collaboration for Effective Delivery

supports families affected by neuroblastoma - main areas: access to treatment, education and awareness and research funding - founding member of INBRACED

Contact:
Neuroblastoma Children's Cancer Alliance UK
5 Harmood Grove, London NW1 8DH
United Kingdom

Contact person ANR Congress:
Mrs. Claire Hislop
Phone: +1 (0) 20 7284 0800
Fax: +1 (0) 84 4884 6407
Mail: info@ncca-uk.org
Website: www.ncca-uk.org

Booth Number: # 06

PHARMORE
More Success with Pharmaceuticals

Xaluprine - mercaptopurine oral suspension - a fluid approach to acute lymphoblastic leukaemia. Xaluprine is exclusively distributed in Germany by Pharmore GmbH

Contact:
PHARMORE GmbH
Gildestraße 75
49479 Ibbenbüren
Germany

Contact person ANR Congress:
Mr. Michael Morys
Phone: +49 (0) 54 51 - 96 90 0
Fax: +49 (0) 54 51 - 96 90 925
Mail: service@pharmore.de
Website: www.pharmore.de and www.xaluprine.com

Booth Number: # 07

medac

pharmaceutical company specialised in the treatment of malignant diseases
- one of the leading manufacturers of oncology products in international markets
- engaged also in the field of diagnostics

Contact:
medac Gesellschaft für klinische Spezialpräparate mbH
Theaterstraße 6
22880 Wedel
Germany

Contact person ANR Congress:
Mr. Werner Köcher
Phone: +49 (0) 4103/ 8006-0
Fax: +49 (0) 4103/ 8006-100
Mail: contact@medac.de
Website: www.medac.de

Booth Number: # 08

Pierre Fabre Pharma ONKOLOGIE

focus in the areas of oncology, central nervous system, cardiovascular system, gynecology / urology and dermocosmetology - one of the main activities for many years is the treatment of cancer patients - oncology therapeutics Javlor®, Navelbine® Oral and Busilvex®

Contact:
Pierre Fabre Pharma GmbH
Jechtinger Straße 13
79111 Freiburg
Germany

Contact person ANR Congress:
Dr. Annette Zoeger
Phone: +49 (0) 7 61 - 4 52 61 - 0
Fax: +49 (0) 7 61 - 4 52 61 - 333
Mail: info.pharma@pierre-fabre.de
Website: www.pierre-fabre.de/pharma/

Booth Number: # 09

EUSAPharma
An International Division of Jazz Pharmaceuticals

focuses on in-licensing, developing and marketing late-stage oncology, oncology supportive care and critical care products - Veno-occlusive disease (VOD) - Defibrotide - Erwinase® for treatment of acute lymphoblastic leukaemia

Contact:
EUSA Pharma GmbH
Grillparzerstr. 18
81675 München
Germany

Contact person ANR Congress:
Mrs. Tanja Balzano
Phone: +49 (0) 89 411096060
Fax: +49 (0) 41109661
Mail: medinfo-de@eusapharma.com
Website: www.eusapharma.com

Booth Number: # 11

ANR CAIRNS 2016

ANR 2016: June 19-23rd
Cairns Convention Centre, Australia
outstanding science - wonderful destination
Get the first information

Contact:
ASN Events Pty Ltd
PO Box 200
Balnarring
Victoria, 3926 Australia

Contact person ANR Congress:
Mrs. Michelle Haber/ Maree Overall
Phone: +61 (0) 3 9988 8601
Mail: mhaber@ccia.unsw.edu.au / MO@asnevents.net.au
Website: www.anr2016.org

Booth Number: #10

Een actie voor Villa Joep!
Het fonds tegen neuroblastoom kinderkanker

connects the medical ambitions around neuroblastoma and is a resource of information for those involved - has established a Travel Grant for outstanding young neuroblastoma researchers to be able to attend (international) scientific conferences

Contact:
Villa Joep Foundation
Het Kleine Loo 414 G
2592 CK The Hague
The Netherlands

Contact person ANR Congress:
Mrs. Hera Lichtenbeld
Phone: +31 (0) 70 – 750 74 88
Mail: info@villajoep.nl
Website: www.villajoep.nl

Partner without an exhibition booth

NOVARTIS PHARMACEUTICALS

Contact:

Novartis Pharma GmbH
Roonstraße 25
90429 Nürnberg
Germany

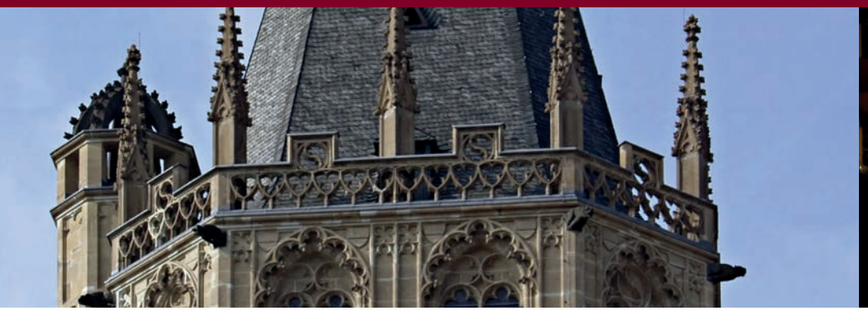
Phone: +49 (0) 911 - 273 - 0
Fax: +49 (0) 911 - 273 - 12653
Mail: novartis.online@novartis.com
Website: www.novartis.de

HEXAL

Hexal AG
Industriestraße 25
83607 Holzkirchen
Germany

Phone: + 49 (0) 8024-908-0
Fax: + 49 (0) 8024-908-1290
Mail: service@hexal.com
Website: www.hexal.de

THE **SOCIAL PROGRAM**



The social program is open to all registered participants and accompanying people and must be booked in advance, but no later than May 5th, 2014.

Welcome Reception

The Welcome Reception will take place at the Historic Town Hall of Cologne on Tuesday, May 13th from 7:00 – approx. 8:30 pm.

The program will include an opening speech by the mayoress of Cologne, Mrs. Angela Spitzig as well as welcome speeches by the president of ANR, Professor Andrew Pearson and by the ANR2014 Local Chairman, Professor Frank Berthold of the University Hospital of Cologne. This will be followed by a music performance by the famous Cologne Youth Choir St. Stephan. Complimentary finger food and beverages will be served during the evening.

The Historic Town Hall is located in the historic district of Cologne, where you can find a great variety of nice restaurants for a dinner after the Welcome Reception.

The reception is open to ANR2014 congress participants and accompanying people. Registration is necessary. Please bring your name badge.

Philharmonic Concert

A classical philharmonic concert will take place at the famous Philharmonic Hall of Cologne on Wednesday, May 14th at 8:00 pm. At that night, the guests will enjoy a performance of the Dresden Philharmonic Orchestra which is one of the leading German orchestras.

In case you booked a ticket for the concert with your online registration, you will receive it at the registration desk when registering for the congress.

Unfortunately, there are no more tickets available due to the fact that the concert is sold out.

Gala Dinner

The Gala Dinner will be held at the restaurant and event location "Tanzbrunnen & Rheinterrassen" on Thursday, May 15th from 7:30 pm until 0:30 am. The dinner venue is located directly at the banks of the Rhine River and after dinner, you can enjoy a relaxing lounge and party atmosphere with a spectacular view across the river on the historic city center and cathedral. Cologne has one of the most beautiful skylines throughout Europe.

The evening will feature a 3-course menu and a dessert buffet as well as a great variety of non-alcoholic and alcoholic beverages. Live entertainment will lead you throughout the evening.

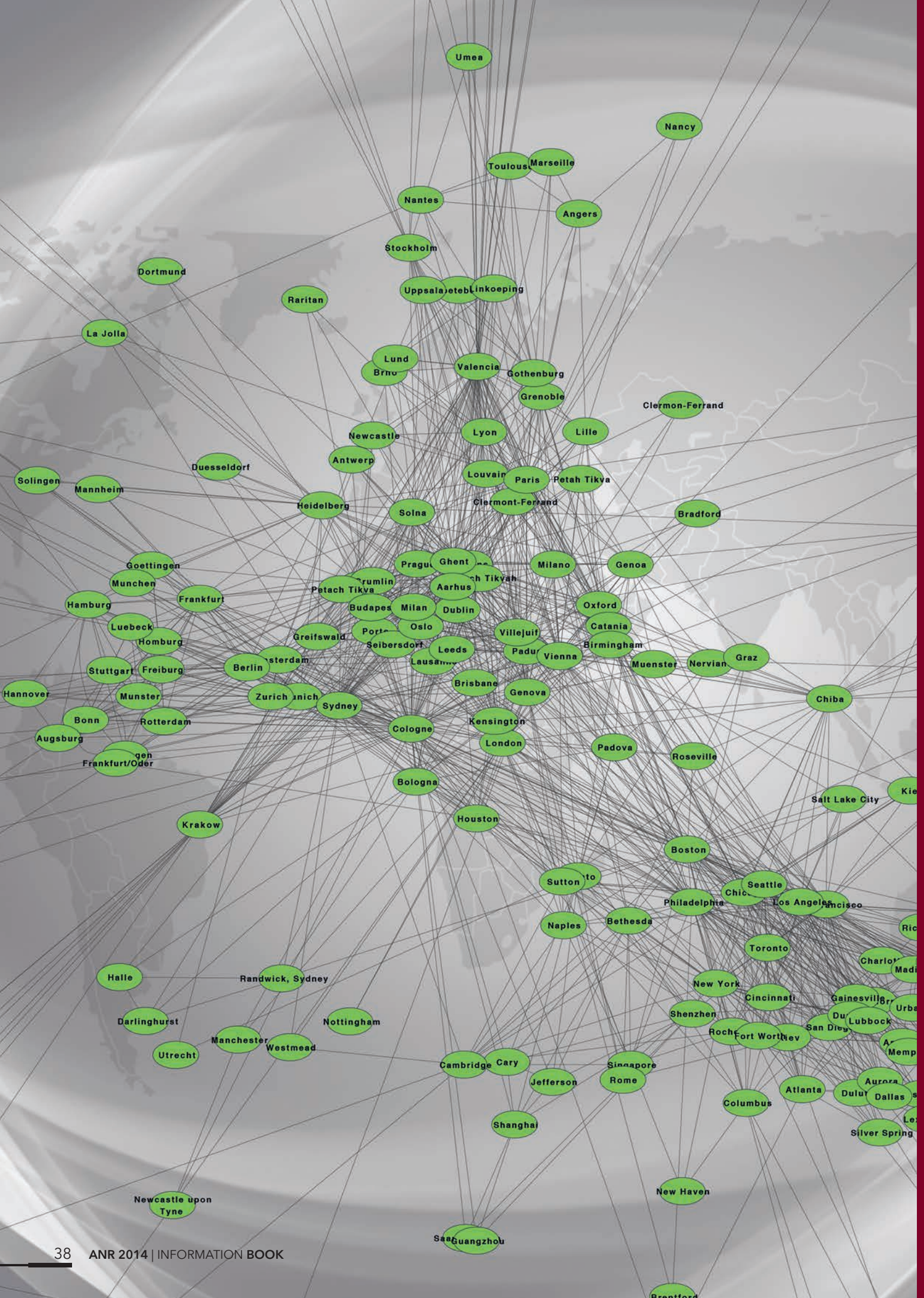
During the dinner, the best posters, the winners of the plenary session's prize competitions and the life time achievement award will be announced and awarded.

If not indicated upon online registration, participants and accompanying people are invited to advise the congress organization team about special food preferences (e.g. allergies, food intolerances, religious dietary and such like). Please email at anr2014@welcome-gmbh.de.

In case you booked a ticket for the gala dinner event with your online registration, you will receive a ticket at the registration desk when registering for the congress.

Thanks to the William Guy Forbeck Research Foundation sponsoring, several tickets are available at a reduced fee (25 €) for students and participants who qualified for the reduced registration fee.

WILLIAM GUY FORBECK
RESEARCH FOUNDATION
wgfrf.org
30 YEARS OF SCIENTIFIC COLLABORATION



SCIENTIFIC PROGRAM

TUESDAY, 13.05.2014
10:00 - 12:00
OFFENBACH SAAL

Workshop 1

Drug Discovery and Development for Neuroblastoma

Session Organizers: Michelle D. Garrett and Hubert N. Caron

Dr Michelle Garrett is Head of Biology in the Cancer Research UK Cancer Therapeutics Unit (CTU) at the ICR, London, UK. Her research specialises in the discovery and development of novel molecular targeted agents for the treatment of cancer.

Dr Garrett received her undergraduate biochemistry degree from the University of Leeds, UK, and then joined the ICR as a PhD student researching the Rho GTPase with Alan Hall. She then spent 8 years in the USA, three of these as a Lucille P. Markey Scholar at Yale University School of Medicine and then 5 years at Onyx Pharmaceuticals, California, USA, where she became a team leader involved in the development of cancer drugs targeting the cell division cycle. In 1999 Michelle returned to the ICR to take up a group leader position in the Cancer Research UK Cancer Therapeutics Unit (CTU). She was appointed Head of Biology for the CTU in 2013.

Hubert Caron is currently head of the paediatric oncology unit in the AMC, Amsterdam.
He is chair of the DCOG-NBL group and the DCOG-early trial group.
His main research interests are translational tumour biology and new drug development for children with solid tumours.

What is a 'good target'?
Jan Molenaar, Academic Medical Center (AMC), Amsterdam, The Netherlands

What is a 'good drug'?
Ian Collins/Michelle D. Garrett, Institute of Cancer Research, London, United Kingdom

How to match 'good drugs' with 'good NBL targets'?
Hubert N. Caron, Academic Medical Center (AMC), Amsterdam, The Netherlands

Design of Biomarker-driven Targeted Drug Trials
Lucas Moreno, Spanish National Cancer Research Center (CNIO), Madrid, Spain

Panel Discussion

TUESDAY, 13.05.2014
13:00 - 15:00
OFFENBACH SAAL

Workshop 2

Cancer Stem Cells and Differentiation in Neuroblastoma

Session Organizers: Frank Speleman and William Weiss

William A. Weiss MD, PhD is Professor of Neurology, Pediatrics, and Neurosurgery at UCSF. His lab has developed a number of models for neural cancers based on recapitulating cardinal genetic abnormalities in genetically egineered cells and in mice, and also studies developmental therapeutics focused on EGFR/PI3K/mTOR and Myc pathways.

Frank Speleman is currently holding a full time professorship at the Ghent University (Belgium). He obtained his Master in Biology in 1984 at the University of Antwerp (Belgium) and PhD in Genetics 1992 at Ghent University. He is supervisor of a research team of 20 members and supervisor of the laboratory of cytogenomics at the Ghent University Hospital. His research focuses on fundamental and translational aspects of neuroblastoma and T-ALL.

Neural Crest Development and the Epithelial to Mesenchymal Transition
Marianne Bronner, California Institute of Technology, Division of Biology, Pasadena, CA, United States

Uncovering Human Neural Crest Enhancers and its Implications for Human Neurocristopathies
Alvaro Rada-Iglesias, Center for Molecular Medicine Cologne, Section Developmental Genomics, Cologne, Germany

Study of Human Ips Cells in Relation to Neuroblastoma
Steven Roberts, Memorial Sloan Kettering Cancer Center, New York, United States

Modeling of NB from Human iPS Cells
Miller Huang, University of California, San Francisco, CA, United States

A miRNA ESC Signature Based Analyses Identifies Replicative Stress Gene Signature in Aggressive NB
Katleen De Preter, Center for Medical Genetics, Ghent University, Ghent, Belgium

The Pluripotent Phenotype of Neuroblastoma Cancer Stem Cells
Jason M. Shohet, Baylor College of Medicine, Houston, TX, United States

Neuroblastoma Include a Subset of Multidrug-Resistant Mesenchymal Cells Depending on NOTCH-PDGFRβ Signaling
Johan van Nes, Academic Medical Center, Amsterdam, The Netherlands

TUESDAY, 13.05.2014
15:30 - 17:30
OFFENBACH SAAL

Workshop 3

Epigenetics and Neuroblastoma

Session Organizers: Frank Westermann and David Jones

Frank Westermann is a physician and tumor geneticist, whose primary research interest is to elucidate the molecular mechanisms of NB development, spontaneous regression and malignant progression, including drug resistance and to develop more accurate risk prediction tools and new therapeutic concepts for high-risk NBs. He is head of the Neuroblastoma Genomics Research Group at the German Cancer Research Center (DKFZ) and the reference laboratory of the German Neuroblastoma Study Group in Heidelberg. As a principal investigator he has contributed to numerous national (e.g. NGFN2 and NGFN-plus) and international (EU EET Pipeline, ASSET) collaborative research projects on neuroblastoma genomics. He is currently coordinating the MYCNET, a systems biology research network on therapy resistance of MYC-driven neuroektodermal tumors.

David Jones is a biologist, whose primary research focus is the application of cutting-edge genomics techniques to identify new diagnostic, prognostic and therapeutic targets in the field of pediatric neurooncology. David completed his PhD in 2009, having worked in Peter Collins' lab in Cambridge investigating novel mechanisms of MAPK pathway activation. Since moving to the DKFZ in Heidelberg in 2010 to work with Peter Lichter and Stefan Pfister, he has played a major role in the ICGC PedBrain Tumor sequencing project, and been involved with several high-impact publications. He is now continuing to work on the ICGC PedBrain project, conducting integrative genomic and epigenomic analyses of medulloblastoma and high-grade glioma, as well as pursuing a number of interesting collaborations on other aspects of pediatric neurooncology.

Decoding the Regulatory Landscape of Medulloblastoma Using DNA Methylation Profiling
David Jones, German Cancer Research Center, Pediatric Neurooncology, Heidelberg, Germany

Understanding Pediatric Cancer Development by Studying Induced Pluripotency
Yasuhiro Yamada, Institute for Integrated Cell-Material Sciences, Center for iPSCell Research and Application, Kyoto University, Japan

MYCN, Gene Silencing and DNA Hypermethylation in Neuroblastoma
Frank Westermann, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany

EZH2 – Relieving Epigenetic Suppression in Neuroblastoma
Carol J. Thiele, National Cancer Institute, Pediatric Oncology Branch, Cell&Molecular Biology, Bethesda, MD, United States

TUESDAY, 13.05.2014
8:30 - 16:15
EUROPA SAAL

Neuroblastoma Update Course

Organizers and Chairpersons: Susan L. Cohn and Andrew D.J. Pearson

Neuroblastoma Genomics

8:30 – 9:00 **Neuroblastoma as a Copy Number Disease: Modeling Multiple Gene Dosage Effects to Explore Cooperative Drivers in Tumor Formation**
Frank Speleman, Ghent University Hospital, Ghent, Belgium

9:00 – 9:30 **Germline Variants that Influence Neuroblastoma Susceptibility and Phenotype**
Sharon Diskin, Children's Hospital of Philadelphia, Philadelphia, PA, United States

9:30 – 10:00 **Overview of the Prognostic Value of Genomic Aberrations and Copy Number Changes in Neuroblastoma**
Gudrun Schleiermacher, Institut Curie, Paris, France

Neuroblastoma Treatment Approaches

10:00 - 10:30 **Overview of the COG Treatment Approach for High-risk Disease**
Julie Park, Seattle Children's Hospital, University of Washington, Seattle, WA, United States

10:30 – 11:00 **Break**

11:00 – 11:30 **Overview of the SIOPEN Treatment Approach for High-risk Disease**
Dominique Valteau-Couanet, Institut Gustave Roussy, Villejuif, France

11:30 – 12:00 **Overview of GPOH Treatment Approach for Low- and Intermediate-risk Disease**
Frank Berthold, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany

12:00 – 12:30 **Overview on Therapeutic Possibilities for Neuroblastoma Patients in Countries with Limited Resources**
Akira Nakagawara, Chiba University School of Medicine, Chiba, Japan

12:30 – 13:30 **Lunch**

Biomarkers for High-risk Disease

13:30 – 14:00 **The Prognostic Value of MIBG Score in High-risk Neuroblastoma**
Greg Yanik, University of Michigan Medical Center, MI, United States

14:00 – 14:30 **Evaluating Blood and Bone Marrow Response Using Minimal Residual Disease (MRD) Molecular Markers**
Sue Burchill, University of Leeds, Leeds, United Kingdom

New Therapeutic Strategies

14:30 – 15:00 **Update of New Small Molecule Therapeutics**
Andrew D.J. Pearson, Royal Marsden Hospital NHS Trust, London, United Kingdom

15:00 – 15:15 **Break**

15:15 – 15:45 **Evaluating the Efficacy of Chimeric Antigen Receptors (CARs) in the Treatment of Neuroblastoma**
John Anderson, UCL Institute of Child Health / Great Ormond Street Hospital, London, United Kingdom

Late Effects

15:45 – 16:15 **Long-term Outcome and Late Sequelae of Children Diagnosed with Neuroblastoma**
Lisa Diller, Dana-Farber Cancer Institute, Boston, MA, United States

16:15 **Adjourn**

WEDNESDAY, 14.05.2014
8:30 - 8:45
CONGRESS SAAL

Welcome Address

Short addresses: Frank Berthold, Andrew D.J. Pearson

WEDNESDAY, 14.05.2014
8:45 - 9:30
CONGRESS SAAL

Keynote Lecture 1: Crest Development

Session Chairs: Akira Nakagawara, Tommy Martinsson

Gene Regulatory Events Controlling Neural Crest Stem Cell Development

Marianne Bronner, California Institute of Technology, Division of Biology, Pasadena, CA, United States

Dr. Marianne Bronner is the Ruddock Professor of Biology and Biological Engineering at the California Institute of Technology. Her laboratory studies the gene regulatory events underlying formation, cell lineage decisions, and migration of neural crest cells. Dr. Bronner received her Sc.B. from Brown University and her Ph.D. from Johns Hopkins University in biophysics. She is a Fellow of the American Academy of Arts and Sciences, past President of the Society for Developmental Biology and Editor-in-Chief of Developmental Biology.

Gene Regulatory Events Controlling Neural Crest Stem Cell Development

The neural crest is a population of multipotent, migratory progenitor cells that forms at the border of forming nervous system in vertebrate embryos. These cells then undergo an epithelial to mesenchymal transition (EMT) via which they migrate away from the central nervous system, following defined pathways. Finally, they populate numerous sites and differentiate into diverse cells types including sensory and autonomic neurons, melanocytes and much of the craniofacial skeleton. We are investigating the gene regulatory network of interacting transcriptional regulators and downstream effector genes that confers properties like multipotency and migratory capacity to nascent neural crest cells. We are testing network by systematically perturbing a subset of the transcription factors involved in early neural crest specification and examining the effect of these perturbations on likely downstream genes in order establish interrelationships. By isolating neural crest enhancers, we have identified additional inputs to the network and determined which interactions are direct. Moreover, these provide unique tools for imaging dynamic changes in gene regulation in living tissue and for isolating pure populations of cells for transcriptome profiling. The results suggest that a series of gene regulatory circuits are involved in inducing the migratory neural crest cell population, maintaining its stem cell properties for a time and finally leading to progressive differentiation. Perturbation of these regulatory events can lead to abnormal development, causing neural crest-derived birth defects and cancers like neuroblastoma.

WEDNESDAY, 14.05.2014
9:30 - 10:50
CONGRESS SAAL

Plenary Session 1

Basic Research I

Session Chairs: Shahab Asghardzadeh, Kenji Kadomatsu, Rogier Versteeg

PL001

Regulation of the Nuclear Hormone Receptor Family by MYCN-driven miRNAs Affects Differentiation and Survival in Neuroblastoma
Diogo Ribeiro, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden
ABSTRACTS, Page 104

PL002

MYCN Alters p53 Chromatin Binding and Modulates p53 Target Gene Activation via Direct MYCN-p53 Binding- A Novel Tumorigenic Mechanism
Jason M. Shohet, Baylor College of Medicine, Pediatrics, Houston, TX, United States
ABSTRACTS, Page 104

PL003

Identification and Pharmacological Inactivation of the MYCN Interactome as a Novel Treatment Approach for High-risk Neuroblastomas
Arturo Sala, Brunel University, London, United Kingdom ABSTRACTS, Page 104

PL004

The Landscape of Fusion Transcripts in Neuroblastoma
Fakhera Ikram, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, and Centre for Molecular Medicine Cologne (CMMC) and Cologne Center for Genomics (CCG), University of Cologne, Cologne, Germany
ABSTRACTS, Page 105

WEDNESDAY, 14.05.2014
11:20 - 12:40
CONGRESS SAAL

Plenary Session 2

Basic Research II and Translational Research I

Session Chairs: Carol J. Thiele, Matthias Fischer, Jason M. Shohet

PL005

The Long Intergenic Noncoding RNA LINC00340 is a Neuroblastoma Susceptibility Gene
Mike Russell, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States
ABSTRACTS, Page 105

PL006

Anticancer Compound CBL0137, that Simultaneously Suppresses NFkB and Activates p53, is Highly Effective at Treating Neuroblastoma in two Independent Mouse Models
Michelle Haber, Children's Cancer Institute Australia/Sydney, Experimental Therapeutics, Sydney, NSW, Australia
ABSTRACTS, Page 105

PL007

Whole Genome Sequencing of Relapse Neuroblastoma Identifies the RAS-MAPK Pathway as a Potential Therapeutic Target in Neuroblastoma
Thomas Eleveld, Academic Medical Center Amsterdam, Oncogenomics, Amsterdam, The Netherlands
ABSTRACTS, Page 106

PL008

Detection of PHOX2B and TH mRNA by RTqPCR in Peripheral Blood Stem Cell Harvests Predicts Relapse in Children with Stage 4 Neuroblastoma; a SIOPEN Study
Maria V. Corrias, Giannina Gaslini Institute, Genoa, Italy ABSTRACTS, Page 106

WEDNESDAY, 14.05.2014
13:45 - 15:15
OFFENBACH SAAL

Parallel Session A1

Basic Research/Oncogenesis I

Session Chairs: Darrel Yamashiro, Isabelle Janoueix-Lerosey

OR001
Activated SHP2 Synergizes MYCN in Neuroblastoma Tumorigenesis
Shinzhen Zhu, Mayo Clinic, Biochemistry and Molecular Biology, Rochester, MN, United States
ABSTRACTS, Page 110

OR002
The Neuroblastoma Oncogene LMO1: Mechanisms for Tumor Initiation and Progression
Derek Oldridge, Children's Hospital of Philadelphia, Philadelphia, PA, United States
ABSTRACTS, Page 110

OR003
ODZ3 Function in Neuroblastoma
Ksenija Drabek, Academic Medical Center, Oncogenomics, Amsterdam, The Netherlands
ABSTRACTS, Page 110

OR004
Role of MYCN Partners (MAX, MXD and MNT) in the Control of MYCN Oncogenicity
Emanuele Valli, University of Bologna, Pharmacy and Biotechnology, Bologna, Italy
ABSTRACTS, Page 111

OR005
Characterization of the Cre-conditional, MYCN-driven DBHiCre;LSL-MYCN Neuroblastoma Mouse Model
Kristina Althoff, University Children`s Hospital Essen, Department of Pediatric Oncology and Hematology, Essen, Germany
ABSTRACTS, Page 111

OR006
SOX11 is a Lineage-survival Oncogene in Neuroblastoma
Sara De Brouwer, Ghent University, Center for Medical Genetics, Ghent, Belgium
ABSTRACTS, Page 111

WEDNESDAY, 14.05.2014
13:45 - 15:15
EUROPA SAAL

Parallel Session B1

Translational Research/Preclinical Experimental Therapies I

Session Chairs: Jan Molenaar, Michael Hogarty

OR007
Drugging MYC Proteins through an Allosteric Transition in Aurora Kinase A
W. Clay Gustafson, University of California, San Francisco, Pediatric, Hematology and Oncology, San Francisco, CA, United States
ABSTRACTS, Page 112

OR008
A Cell-based High-throughput Screen Addressing 3'UTR-dependent Regulation of the MYCN Gene
Viktoryia Sidarovich, University of Trento, Center for Integrative Biology (CIBIO), Trento, Italy
ABSTRACTS, Page 112

OR009
Targeting of the MYCN Oncoprotein with c-MYC Inhibitors
Maria Arsenian-Henriksson, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden
ABSTRACTS, Page 112

OR010
Targeting the MYCN Pathway in Neuroblastoma with M606, a Novel Small Molecule Inhibitor with Clinical Potential
Murray Norris, Children's Cancer Institute Australia/Sydney, Sydney, NSW, Australia
ABSTRACTS, Page 113

OR011
N-Myc Protein Stability as a Therapeutic Target in MYCN Amplified Neuroblastoma
Anne Carstensen, University of Würzburg, Chair of Biochemistry and Molecular Biology, Würzburg, Germany
ABSTRACTS, Page 113

OR012
The Novel Small-Molecule MDM2 Inhibitor, RG7388, is Highly Effective Against Neuroblastoma in Vivo via P53-Mediated Apoptosis
Anna Lakoma, Baylor College of Medicine, Michael E DeBakey Department of Surgery/Division of Pediatric Surgery, Houston, TX, United States
ABSTRACTS, Page 113

WEDNESDAY, 14.05.2014
15:45 - 17:15
OFFENBACH SAAL

Parallel Session A2

Basic Research/Oncogenesis II

Session Chairs: Rani E. George, Jan van Nees

OR013
The RNA Aptamer Against Midkine Suppresses Neuroblastoma Xenograft Growth by Attenuating Midkine-Notch2 Pathway
Satoshi Kishida, Nagoya University, Graduate School of Medicine/Department of Biochemistry, Nagoya, Aichi, Japan ABSTRACTS, Page 114

OR014
The G-CSF/STAT3 Signaling Axis Regulates the Tumorigenic and Metastatic Potential of Neuroblastoma Cancer Stem Cells (Cscs)
Saurabh Agarwal, Baylor College of Medicine, Pediatrics, Houston, TX, United States ABSTRACTS, Page 114

OR015
Tumor-associated Macrophages (TAMs) Increase Neuroblastoma Proliferation and Growth through c-MYC Upregulation, an Effect Independent of IL6 Expression
Long Hung, Children's Hospital Los Angeles, Los Angeles, CA, United States ABSTRACTS, Page 114

OR016
The HBP1 Tumor Suppressor is a Druggable ALK Downregulated Gene Controlling MYCN Activity
Shana Claeys, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 114

OR017
Genomic Instability Model for Metastatic Neuroblastoma Carcinogenesis by Dictionary Learning Algorithm
Gian Paolo Tonini, Pediatric Research Institute, Fondazione Cittàdella Speranza, Neuroblastoma Laboratory, Padua, Italy ABSTRACTS, Page 115

OR018
miRNAHigh-throughput Functional Screening Identifies Several Potential Tumour Suppressive miRNAs in Neuroblastoma
Elena Afanasyeva, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany ABSTRACTS, Page 115

WEDNESDAY, 14.05.2014
15:45 - 17:15
EUROPA SAAL

Parallel Session B2

Translational Research/Preclinical Experimental Therapies II

Session Chairs: Alice L. Yu, Ruth Ladenstein

OR088
Emergence of New ALK Mutations at Relapse of Neuroblastoma
Gudrun Schleiermacher, Institut Curie, Paris, France ABSTRACTS, Page 116

OR019
Development of Chimeric Antigen Receptor (CAR) Cellular Therapies for Neuroblastoma
John Anderson, University College London, Institute of Child Health and Great Ormond Street Hospital, London, United Kingdom ABSTRACTS, Page 116

OR020
Targeting the PD1/PDL1 Immune Checkpoint Pathway in Neuroblastoma
Ferdousi Chowdhury, University of Southampton, Southampton, United Kingdom ABSTRACTS, Page 116

OR021
Chimeric Antibody c.8B6 to O-acetyl-GD2 Mediates the Same Efficient Anti-Neuroblastoma Effects than Therapeutic ch14.18 Antibody to GD2 without Induced Allodynia
Stéphane Birklé, INSERM, U. 892, Nantes, France ABSTRACTS, Page 117

OR022
Recombinant IL-21 and anti-CD4 Antibodies Cooperate in Syngeneic Neuroblastoma Immunotherapy and Mediate Long-lasting Immunity
Michela Croce, IRCCS AOU San Martino-IST, Genoa, Italy ABSTRACTS, Page 117

OR023
Anti-NLRR1 Monoclonal Antibody Inhibits Growth of Neuroblastoma Xenograft in Mice
Atsushi Takatori, Chiba Cancer Center Research Institute, Children's Cancer Research Center, Chiba, Japan ABSTRACTS, Page 117

WEDNESDAY, 14.05.2014
17:00 - 19:00
EUROPA SAAL, 4TH FLOOR

Poster viewing

17:30 - 18:30

Guided tours to selected posters of each category

Basic Research FOYER – NORTH
Session Chairs/Jury: Jo Vandesompele, Yael P. Mosse, Naoh Ikolkegaki

Translational Research FOYER - SOUTH
Session Chairs/Jury: Ina Oehme, Pieter Mestdagh, Murray Norris

Clinical Research CONGRESS SAAL
Session Chairs/Jury: Shakeel Modak, Weisong Cai, Holger Lode

THURSDAY, 15.05.2014
8:30 – 9:15
CONGRESS SAAL

Keynote Lecture 2: Epigenetics in Neuroblastoma

Session Chairs: Gudrun Schleiermacher, Johannes Schulte

Enhancer Hijacking - an old Mechanism of Oncogene Activation Revisited

Stefan M. Pfister, German Cancer Research Center, Department of Pediatric Hematology and Oncology, Division of Pediatric Neurooncology, Heidelberg, Germany

Stefan Pfister was appointed acting head of the Division Pediatric Neurooncology at the German Cancer Research Center (DKFZ) in 2012. Since 2014 he is professor for pediatric neurooncology at the DKFZ and heading the department permanently. Being a pediatrician by training, Pfister received his MD from Tübingen University, and his clinical education at Mannheim and Heidelberg University Hospitals. As a physician-scientist, he completed postdoctoral fellowships with Christopher Rudd at the Dana-Faber Cancer Institute/Harvard Medical School, and with Peter Lichter at the German Cancer Research Center, Division of Molecular Genetics. Pfister´s research focuses on the genetic and epigenetic characterization of childhood brain tumors by applying next-generation profiling methods and subsequently translating novel findings into a clinical context. For his translational neurooncology projects, Pfister received amongst others the German Cancer Award in 2012.

THURSDAY, 15.05.2014
9:20 - 11:00
CONGRESS SAAL

Plenary Session 3

Translational Research II and Clinical Research I

Session Chairs: John Maris, Rochelle Bagatell, Hubert N. Caron

PL009

The PLK-1 Inhibitor GSK461364 Has a Strong Antitumoral Activity in Preclinical Models of Neuroblastoma
Johannes H. Schulte, University Children`s Hospital Essen, Department of Pediatric Oncology and Hematology, Essen, Germany ABSTRACTS, Page 106

PL010

Evaluation of Clinical Response and Survival Following Long-term Infusion of anti-GD2 Antibody ch14.18/CHO in Combination with Subcutaneous Interleukin-2 in a Single Center Treatment Program in High-risk Neuroblastom
Ina Müller, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany ABSTRACTS, Page 107

PL011

A Randomized Clinical Trial of Cyclophosphamide and Prednisone with or without Intravenous Immuno-globulin (IVIG) for the Treatment of Neuroblastoma Associated Opsoclonus Myoclonus Ataxia Syndrome (OMA): A Children's Oncology Group Trial
Katherine K. Matthay, University of California, Pediatrics, San Francisco, CA, United States ABSTRACTS, Page 107

PL012

Influence of Surgical Excision on Survival of Patients with High Risk Neuroblastoma. Report from Study 1 of Siop Europe (Siopen)
Keith Holmes, SIOPEN, London, United Kingdom ABSTRACTS, Page 107

Why a Consortium of Neuroblastoma Foundations?
Susan Hay, Consortium of Neuroblastoma Foundations

THURSDAY, 15.05.2014
11:30 - 13:00
OFFENBACH SAAL

Parallel Session A3

Basic Research/ Oncogenesis III and Biological Models

Session Chairs: Meredith Irwin, Kathleen de Preter

OR024

MYCN Determines Entry into a Non-cycling State
Emma Bell, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany ABSTRACTS, Page 118

OR025

miR-183 Counteracts the MYCN Induced Transcriptional Activation of the MCM Complex in Neuroblastoma
Marco Lodrini, German Cancer Research Center, Clinical Cooperation Unit Pediatric Oncology, Heidelberg, Germany ABSTRACTS, Page 118

OR026

Identification of Trk-specific Signaling Events in Neuroblastoma Using Stable Isotope Labeling and Phosphoproteomics
Samuel L. Volchenboum, University of Chicago, Department of Pediatrics, Chicago, IL, United States ABSTRACTS, Page 118

OR027

Genes Mediating Bone Marrow Metastasis in Neuroblastoma
Jamie Fletcher, Children's Cancer Institute Australia, Sydney, NSW, Australia ABSTRACTS, Page 119

OR028

Neuroblastoma-derived Exosomes Communicate with Bone Marrow-derived Mesenchymal Stromal (BMMSC) Cells to Promote Neuroblastoma Cell Survival
Rie Nakata, Children's Hospital Los Angeles, University of Southern California, Pediatric, Hematology and Oncology, Los Angeles, CA, United States ABSTRACTS, Page 119

OR086

A Genome-wide Association Study (GWAS) Identifies Susceptibility Alleles within Neuroblastoma Oncogenes and Tumor Suppressors: An Explanation for the Paucity of Somatic Mutations?
John Maris, Children's Hospital of Philadelphia, Pediatric Oncology, Philadelphia, PA, United States ABSTRACTS, Page 119

THURSDAY, 15.05.2014
11:30 - 13:00
EUROPA SAAL

Parallel Session B3

Translational Research/Preclinical Experimental Therapies III

Session Chairs: Kelly Goldsmith, Tom van Maerken

OR029
MEK1/2 Inhibitors Significantly Reduce Tumor Proliferation and Growth in Neuroblastomas
Long Hung, Children's Hospital Los Angeles, Los Angeles, CA, United States
ABSTRACTS, Page 120

OR030
MYCN Expression in Neuroblastoma Induces Replicative Stress and Sensitizes Cells to PARP1 Inhibition
Kevin Petrie, Institute of Cancer Research, Sutton, United Kingdom
ABSTRACTS, Page 120

OR031
Dual ALK and CDK4/6 Pathway Inhibition Demonstrates On-target Synergy Against Neuroblastoma
Yael P. Mosse, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States
ABSTRACTS, Page 120

OR032
BET Protein InhibitorOTX015 Has Selective Anti-tumoral Activity in Preclinical Models of MYCN-amplified Neuroblastoma
Anton Henssen, University Children`s Hospital Essen, Department of Pediatric Oncology and Hematology, Essen, Germany
ABSTRACTS, Page 121

OR033
p53 Activation Enhances the Sensitivity of Neuroblastoma to mTOR Inhibitors
Eveline Barbieri, Baylor College of Medicine, Pediatrics, Houston, TX, United States
ABSTRACTS, Page 121

OR034
Wip1 Inhibition Provides a Novel Therapeutic Target in Neuroblastoma with Different p53 and MDM2 Status
Diana Treis, Karolinska Institutet, Department of Women's and Children's Health/Childhood Cancer Research Unit, Stockholm, Sweden
ABSTRACTS, Page 121

THURSDAY, 15.05.2014
13:45 - 15:30
OFFENBACH SAAL

Parallel Session A4

Basic Research/High Throughput Techniques

Session Chairs: Jan Koster, Hsueh-Fen Juan, Alexander Schramm

OR035
Massive Parallel Genomic Sequencing Reveals Distinct Mutation Patterns in Clinical Neuroblastoma Subgroups
Matthias Fischer, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology, Cologne, Germany
ABSTRACTS, Page 122

OR036
Identification of Recurrent Germline and Somatic Structural Variations (SVs) Influencing Tumorigenesis
Sharon Diskin, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States
ABSTRACTS, Page 123

OR037
RNA-Seq Provides Detailed Insights into the Neuroblastoma Transcriptome and is Suitable for Clinical Endpoint Prediction
Falk Hertwig, University Children's Hospital of Cologne, Cologne, Germany
ABSTRACTS, Page 123

OR038
Integrated Genomic Analyses Identifies Neural, Metabolic, and Inflammatory Subgroups of High-Risk Neuroblastoma with Clinical Significance
Shahab Asgharzadeh, Children's, Hospital Los Angeles, Los Angeles, CA, United States
ABSTRACTS, Page 123

OR039
High-Risk Neuroblastoma Genomic Plasticity Allows for Significant Clonal Evolution under the Selective Pressure of Chemotherapy
Derek Oldridge, Children's Hospital of Philadelphia, Philadelphia, PA, United States
ABSTRACTS, Page 124

OR040
Genomic Data Integration in High-Risk Neuroblastoma Patients Identifies BRIP1 as a Putative Oncogene on Chromosome 17q
Annelies Fieuw, Ghent University, Center for Medical Genetics, Ghent, Belgium
ABSTRACTS, Page 124

OR041
Whole Genome Screen to Identify Genes Targeting MYCN Driven Embryonal Tumors- Neuroblastoma (NB) Model
Carol J. Thiele, National Cancer Institute, Pediatric Oncology Branch, Bethesda, MD, United States
ABSTRACTS, Page 124

THURSDAY, 15.05.2014
13:45 - 15:30
EUROPA SAAL

Parallel Session B4

Translational Research/Preclinical Experimental Therapies IV and Minimal Residual Disease

Session Chairs: Robert Seeger, GodelieveTytgat, Susan Burchill

OR042
Parvovirus H-1 Induces Oncolytic Effects in Human Neuroblastoma Preclinical Evaluation in MYCN Amplified Xenograft-Bearing Rodent Models
Jeannine Lacroix, Heidelberg University Hospital, Pediatric, Hematology, Oncology and Immunology, Heidelberg, Germany ABSTRACTS, Page 125

OR043
Bone Marrow-Derived Mesenchymal Stromal Cells (BMMSC) and Tumor-associated Fibroblasts (TAF) Contribute to a Pro-tumorigenic Inflammatory Microenvironment that Promotes Drug Resistance in Neuroblastoma
Lucia Borriello, Children's Hospital Los Angeles, University of Southern California, Pediatric Hematology and Oncology, Los Angeles, CA, United States ABSTRACTS, Page 125

OR044
Synthesis of Para- and Meta 4-(Guanidinomethylphenoxyl)-butylmethansulfonate (pBBG / mBBG) and their Effects on Neuroblastoma Cells Compaired to mIBG and Busulfan
Gernot Bruchelt, Children's University Hospital Tuebingen, Tuebingen, Germany ABSTRACTS, Page 126

OR045
Temozolomide + irinotecan Followed by fenretinide/LXS + ketoconazole + vincristine is Active in Post-progressive Disease Neuroblastoma Xenografts
Lluis Lopez-Barcons, TexasTech University Health Sciences Center, Cancer Center and Department of Cell Biology and Biochemistry, Lubbock, TX, United States ABSTRACTS, Page 126

OR046
Defining Sensitivity Profiles for Bromodomain and Extra-Terminal (BET) Protein Inhibition
Robert Schnepp, Children's Hospital of Philadelphia, Philadelphia, PA, United States ABSTRACTS, Page 126

OR047
Minimal Residual Disease Detection in Autologous Stem Cell Grafts of High Risk Neuroblastoma Patients
Esther van Wezel, Sanquin Research, Experimental Immunohematology, Amsterdam, The Netherlands ABSTRACTS, Page 127

OR048
Bone Marrow Minimal Residual Disease after 2 Cycles of Immunotherapy was an Early Response Marker and the Strongest Independent Predictor of Survival for Patients with High-risk Metastatic Neuroblastoma Following Anti-GD2 Immunotherapy
Nai-Kong V. Cheung, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States ABSTRACTS, Page 127

THURSDAY, 15.05.2014
16:00 - 17:30
OFFENBACH SAAL

Parallel Session A5

Basic Research/Developmental Neuroblastoma Biology

Session Chairs: Garrett M. Brodeur, Per Kogner

OR049
Knock-in Mice with Activated Alk Display Prolonged Neurogenesis as a Predisposition Step to Neuroblastoma
Isabelle Janoueix-Lerosey, Institut Curie, Inserm U830, Paris, France ABSTRACTS, Page 128

OR050
Time-resolved Transcriptome Analysis of TH-MYCN Driven Hyperplastic Ganglia and Tumors Marks BRD3 as a Novel Candidate Oncogene
Anneleen Beckers, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 128

OR051
Genome Wide Analysis of Ascl1, Cdk Inhibitor and Retinoic Acid Induced Neuroblastoma Differentiation
Anna Philpott, University of Cambridge, Oncology, Cambridge, United Kingdom ABSTRACTS, Page 128

OR052
Role of the CHD5 Tumor Suppressor in Neuroblastoma Pathogenesis Using the Zebrafish Model
A. Thomas Look, Dana Farber Cancer Institute, Pediatric Oncology, Boston, MA, United States ABSTRACTS, Page 129

OR053
Transcription Factor Activating Protein 2 Beta (TFAP2B) Mediates Neuronal Differentiation and is a Prognostic Marker in Neuroblastoma
Fakhera Ikram, University Children's Hospital of Cologne, Cologne, Germany ABSTRACTS, Page 129

OR054
Integrative DNA Methylome and Transcriptome Analysis Reveals Suppression of Differentiation Programs in High-Risk Neuroblastoma
Kai-Oliver Henrich, German Cancer Research Center, Neuroblastoma Genomics, Heidelberg, Germany ABSTRACTS, Page 129

THURSDAY, 15.05.2014
16:00 - 17:30
EUROPA SAAL

Parallel Session B5

Clinical Research I

Session Chairs: Barbara Hero, Julie Park

OR074

Haploidentical Stem Cell Transplantation and Subsequent Immunotherapy with antiGD2 Antibody for Patients with Relapsed Metastatic Neuroblastoma
Peter Lang, University Children's Hospital Tuebingen, Hematology / Oncology, Tuebingen, Germany
ABSTRACTS, Page 130

OR075

Phase I Study of Haploidentical Natural Killer (NK) Cells plus Monoclonal Antibody 3F8 for Resistant High-Risk Neuroblastoma (HR-NB)
Shakeel Modak, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States
ABSTRACTS, Page 130

OR076

Influence of Extent of Resection on Survival in High Risk Neuroblastoma Patients: A Report from the COG A3973 Study
Daniel Von Allmen, Cincinnati Children's Hospital, Children's Oncology Group, Cincinnati, OH, United States
ABSTRACTS, Page 131

OR077

Likelihood of Bone Recurrence in Prior Sites of Metastasis in Patients with High-Risk Neuroblastoma
Alexei Polishchuk, University of California, San Francisco and UCSF Benioff Children's Hospital, Radiation Oncology, San Francisco, CA, United States
ABSTRACTS, Page 131

OR078

Feasibility of I^{131}-mIBG and Topotecan Therapy Followed by Consolidation with Busulfan, Melphalan and Autologous Stem Cell Transplantation for Refractory Metastatic Neuroblastoma
Dominique Valteau-Couanet, Institut Gustave Roussy, Paediatric Oncology, Villejuif, France
ABSTRACTS, Page 132

OR079

Is Additional Treatment Necessary for a Residual Tumor in Cases of Intermediate-Risk Neuroblastoma?
Tomoko Iehara, Kyoto Prefectural University of Medicine, Japan Neuroblastoma Study Group, Pediatrics, Kyoto, Japan
ABSTRACTS, Page 132

THURSDAY, 15.05.2014
17:30 - 18:30
4TH FLOOR

Poster Viewing

NOTICE

FRIDAY, 16.05.2014
8:30 - 10:15
EUROPA SAAL

Parallel Session A6

Basic Research, Translational Research, Clinical Research II

Session Chairs: Araz Marachelian , Thorsten Simon, Jean Michon

OR087
Selective Inhibition of CDK7 Targets Super-enhancer Driven Transcriptional Programsin MYCN-amplified Cells
Rani George, Dana Faber Cancer Institute, Pediatric Oncology, Boston, MA, United States
ABSTRACTS, Page 133

OR061
Apoptosis Induced by O-acetyl-GD2 Specific Monoclonal Antibodies Inhibit Neuroblastoma Growth
Denis Cochonneau, INSERM, U. 892, Nantes, France ABSTRACTS, Page 133

OR062
NKT Cells Turn Bad Inflammation into Good One via Selective Targeting of M2-like Macrophages in Neuroblastoma Microenvironment
Leonid Metelitsa, Baylor College of Medicine, Houston, TX, United States ABSTRACTS, Page 133

OR063
Tumor Infiltration of Regulatory T-cells and Effects of Anti-CTLA4 and Anti-PD1 Therapy in a Murine Model of Neuroblastoma
Shahab Asgharzaden, Children's Hospital Los Angeles, Los Angeles, CA, United States
ABSTRACTS, Page 134

OR064
Factors Associated with Recurrence and Length of Survival Following Relapse in UK Patients with High Risk Neuroblastoma
Nermine Basta, Newcastle University, Institute of Health and Society, Newcastle upon Tyne, United Kingdom ABSTRACTS, Page 134

OR065
Prognostic Factors for Response and Outcome on Phase I/II New Approaches to Neuroblastoma Therapy (NANT) Trials Utilizing the NANT Response Criteria (v1.0)
Araz Marachelian, Saban Research Institute, Children's Hospital Los Angeles and Keck School of Medicine of the University of Southern California, Pediatrics, Los Angeles, CA, United States ABSTRACTS, Page 134

OR066
Assessment of Primary Site Response in Children with High Risk Neuroblastoma: An International Multicenter Study
Rochelle Bagatell, The Children's Hospital of Philadelphia, Division of Oncology, Philadelphia, PA, United States ABSTRACTS, Page 135

FRIDAY, 16.05.2014
8:30 - 10:15
OFFENBACH SAAL

Parallel Session B6

Clinical Research III

Session Chairs: Susan Cohn, Bruno de Bernardi, Victoria Castel

OR067
Myeloablative Therapy (MAT) and Immunotherapy (IT) with ch14.18/CHO for High Risk Neuroblastoma: Update and News of Randomised Results from the HR-NBL1/SIOPEN Trial
Ruth Ladenstein, St. Anna Children's Hospital and Research Institute for the SIOP Europe Neuroblastoma Group, Paediatric Haematology/Oncology, Vienna, Austria ABSTRACTS, Page 136

OR068
Maintaining Outstanding Outcomes Using Response- and Biology-Based Therapy for Intermediate-Risk Neuroblastoma: A Report from the Children's Oncology Group study ANBL0531
Clare Twist, Stanford University, Palo Alto, CA, United States ABSTRACTS, Page 136

OR069
Phase I Study of the Aurora A Kinase Inhibitor MLN8237 with Irinotecan and Temozolomide for Patients with Relapsed or Refractory Neuroblastoma: A Report from the New Approaches to Neuroblastoma Therapy (NANT) Consortium
Steven DuBois, University of California San Francisco, School of Medicine, San Francisco, CA, United States
ABSTRACTS, Page 136

OR070
A Phase I Study of Vorinostat in Combination with Isotretinoin (RA) in Patients with Refractory/Recurrent Neuroblastoma (NB): A New Approaches to Neuroblastoma Therapy Consortium Trial
Julie Park, Seattle Children's Hospital/University of Washington, Pediatrics, Seattle, WA, United States
ABSTRACTS, Page 137

OR071
Generation and Administration of Autologous T cells Transduced with a 3rd Generation GD2 Chimeric Antigen Receptor for Patients with Relapsed or Refractory Neuroblastoma
Chrystal Louis, Baylor College of Medicine, Pediatric Oncology and Center for Cell and Gene Therapy, Houston, TX, United States ABSTRACTS, Page 137

OR072
Relapse in Patients with High-Risk Neuroblastoma (HR-NB) After Treatment with 3F8/GM-CSF+CIS-Retinoic Acid (3F8/GM+CRA) in First CR/VGPR: Patterns, Management and Long-term Outcome
Shakeel Modak, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States
ABSTRACTS, Page 137

OR073
Recurrent Neuroblastoma Metastatic to the Central Nervous System: Is it Curable?
Kim Kramer, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States
ABSTRACTS, Page 138

FRIDAY, 16.05.2014
11:00 - 12:30
EUROPA SAAL

Parallel Session A7

Translational Research/Molecular Markers

Session Chairs: Katherine Matthay, Gian Paolo Tonini

OR055

Assessment of Circulating microRNAs for Non-invasive Outcome Prediction of Neuroblastoma Patients
Fjoralba Zeka, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 138

OR056

Revised Risk Assessment and Treatment Stratification of Low- and Intermediate-risk Neuroblastoma Patients by Integrating Clinical and Molecular Prognostic Markers
Matthias Fischer, University Children's Hospital of Cologne, Department of Pediatric Oncology, Cologne, Germany ABSTRACTS, Page 138

OR057

Identification and Validation on a Six Hundred Neuroblastoma Patients' Dataset of a Novel Gene Signature Predicting Patients' Outcome and Measuring Tumor Hypoxia
Davide Cangelosi, Giannina Gaslini Institute, Laboratory of Molecular Biology, Genoa, Italy ABSTRACTS, Page 139

OR058

Developing a Responder Hypothesis for Therapeutic Stratification of Newly Diagnosed Patients with ALK-mutant NB
Yael P. Mosse, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States ABSTRACTS, Page 139

OR059

The Ornithine Decarboxylase G317A Polymorphism is Prognostic of Outcome in Primary Neuroblastoma and Differentially Affects Promoter Binding by the MYCN Oncogene
Murray Norris, Children's Cancer Institute Australia, Sydney, NSW, Australia ABSTRACTS, Page 140

OR060

Prevalence and Prognostic Impact of Alternative Lengthening of Telomeres (ALT) in 203 Neuroblastoma Tumors
Loretta Lau, University of Sydney, Sydney, NSW, Australia ABSTRACTS, Page 140

FRIDAY, 16.05.2014
11:00 - 12:30
OFFENBACH SAAL

Parallel Session B7

Clinical Research IV

Session Chairs: Angelika Eggert, Purna Kurkure, Wendy London

OR080

Clinical, Biological, and Prognostic Differences Based Upon Primary Tumor Site in Neuroblastoma: A Report from the International Neuroblastoma Risk Group (INRG) Project
Kieuhoa Vo, University of California, San Francisco and UCSF Benioff Children's Hospital, Pediatrics, San Francisco, CA, United States ABSTRACTS, Page 141

OR081

Prognostic Significance of Liver Metastases in Metastatic Neuroblastoma. A Study from the International Neuroblastoma Risk Group (INRG) Database
Daniel Morgenstern, Great Ormond Street Hospital, Haematology/Oncology, London, United Kingdom ABSTRACTS, Page 141

OR082

Stage 4N Neuroblastoma (Metastatic Disease Confined to Distant Lymph Nodes) Has a Better Outcome than Non-4N Stage 4 Disease: A Study from the International Neuroblastoma Risk Group (INRG) Database
Daniel Morgenstern, Great Ormond Street Hospital, Haematology/Oncology, London, United Kingdom ABSTRACTS, Page 141

OR083

Postponing Staging in Very Young Infants
Barbara Hero, University Children's Hospital Cologne, Department of Pediatric Oncology, Cologne, Germany ABSTRACTS, Page 142

OR084

Population-based Incidence and Survival of Neuroblastoma in Taiwan, 1979-2009
Meng-Yao Lu, National Taiwan University Hospital, Pediatric, Hematology and Oncology, Taipei, Taiwan ABSTRACTS, Page 142

OR085

Neuroblastoma Screening at One Year of Age Does Not Reduce Mortality and Stage 4 Incidence – Results after 12 Years of Follow-Up
Claudia Spix, University Medical Center Mainz, Institute for Medical Biometry, Epidemiology and Informatics (IMBEI), German Childhood Cancer Registry, Mainz, Germany ABSTRACTS, Page 142

FRIDAY, 16.05.2014
13:30 - 14:15
CONGRESS SAAL

Keynote Lecture 3: Cellular Immunology

Session Chairs: Nai-Kong V. Cheung, Stefan Nierkens

Cell Based Immunotherapies for Neuroblastoma: Opportunities and Challenges

Crystal L. Mackall, National Cancer Institute, Pediatric Oncology Branch, MD, United States

Crystal Mackall is Chief of the Pediatric Oncology Branch of the National Cancer Institute. She is an internationally recognized thought leader in the field of human immunology and T cell homeostasis and in addition, she leads a cutting edge clinical-translational program that seeks to bring recent progress in tumor immunotherapy to the problem of childhood cancer. Dr. Mackall is the co-leader of the StandUp2Cancer/St. Baldrick's/NCI Pediatric Dream Team and she is the recipient of numerous awards including the NIH Distinguished Clinical Teacher Award and several NCI Directors awards. She has authored over 130 scientific publications, is a member of the American Society of Clinical Investigation, and serves in numerous editorial and advisory positions.

Cell Based Immunotherapies for Neuroblastoma: Opportunities and Challenges

Exciting recent progress has been made in moving immune based therapy for cancer from a conceptual possibility to a therapeutic reality. Immune based therapies have become more potent, as evidenced by their capacity to induce regression of established tumors, and they have also become applicable to cancers beyond the originally narrow range of histologies classically considered as "immunogenic cancers". This raises a series of fundamental questions for pediatric oncologists regarding the potential to apply these new immunotherapies to pediatric tumors in general and neuroblastoma in particular. This lecture will discuss the current state of knowledge regarding the basis for natural T cells responses to cancer and will review the major mechanisms by which tumors evade natural immune responses, including discussions of regulatory T cells and myeloid derived suppressor cells. Several promising classes of emerging immunotherapeutics will be discussed including immune checkpoint inhibitors, tumor vaccines and adoptive T cell therapies using genetically engineered T cells. Finally, the current understanding of the immunobiology of neuroblastoma will be reviewed and possibilities for new immune based therapies for this disease will be proposed.

FRIDAY, 16.05.2014
14:20 - 15:40
CONGRESS SAAL

Plenary Session 4

Clinical Research II

Session Chairs: Lisa Diller, Godfrey Chan, Dominique Valteau-Couanet

PL013

Update of Outcome for High-Risk Neuroblastoma Treated on a Randomized Trial of Chimeric anti-GD2 Antibody (ch14.18) + GM-CSF / IL2 Immunotherapy in 1st Response: A Children's Oncology Group Study
Alice Yu, University of California in San Diego, Pediatrics, San Diego, CA, United States
ABSTRACTS, Page 108

PL014

Long-term Infusion of ch14.18/CHO Combined with s.c. interleukin-2 Applied in a Single Center Treatment Program Effectively Stimulates Anti-neuroblastoma Activity with Reduced Pain in High-risk Neuroblastoma Patients
Stefanie Endres, University Medicine Greifswald, Greifswald, Germany
ABSTRACTS, Page 108

PL015

Impact of Pre-immunotherapy Curie Scores on Survival Following Treatment with an Anti-GD2 Antibody (ch14.18) and Isotretinoin in High-risk Neuroblastoma
Katherine K. Matthay, University of California, Pediatrics, Hematology and Oncology, San Francisco, CA, United States
ABSTRACTS, Page 108

PL016

Treatment with Fenretinide (4-HPR)/Lym-X-Sorb™ (LXS) Oral Powder with Ketoconazole Increased Plasma Levels and had Anti-tumor Activity in Patients with High-Risk (HR) Recurrent or Resistant Neuroblastoma (NB): A NANT Study
Araz Marachelian, Children's Hospital Los Angeles Center for Cancer and Blood Diseases and Keck School of Medicine, University of Southern California, Center for Cancer and Blood Disease, Los Angeles, CA, United States
ABSTRACTS, Page 109

FRIDAY, 16.05.2014
15:40 - 16:10
CONGRESS SAAL

Life Time Achievement Award

Session Chairs: Michelle Haber, Andrew D.J. Pearson

16:10 - 16:20

Farewell

Session Chairs: Frank Berthold, Michelle Haber

16:20 - 17:00

Close with Coffee and Cake

17:00 - 18:00

Poster Removal

NOTICE

TUESDAY 18:00 - FRIDAY 17:00
4TH FLOOR
FOYER - NORTH

Basic Research

Basic Research: Oncogenesis

Basic Research

POB025 **GALNT2 Suppresses Malignant Phenotypes through Insulin-Like Growth Factor 1 Receptor and Predicts Favorable Prognosis in Neuroblastoma**
Min-Chuan Huang, National Taiwan University College of Medicine, Graduate Institute of Anatomy and Cell Biology, Taipei, Taiwan ABSTRACTS, Page 151

POB026 **The CDKN2/CDK4/CCND1 Genes are Altered by Various Mechanisms Including Copy Number and Point Mutations in Neuroblastoma**
Caroline Louis-Brennetot, Inserm U830 Institut Curie, Research Center, Paris, France ABSTRACTS, Page 151

POB027 **NCYM Promotes Production of Myc-nick of MYCN in Neuroblastoma**
Wataru Shoji, Chiba Cancer Center Research Institute, Chiba, Japan ABSTRACTS, Page 151

POB028 **Loss of the Promyelocytic Leukemia Protein (PML) in Neuroblastoma Promotes Angiogenesis and is a Marker of Recurrence in Localised Disease**
Paolo Salomoni, University College London, UCL Cancer Institute, London, United Kingdom ABSTRACTS, Page 152

POB029 **TRIM16 Inhibits Cell Growth through Direct Interaction and Modulation of TDP43 Protein Stability in Cancer Cells**
Belamy Cheung, Children's Cancer Institute Australia for Medical Research, Molecular Carcinogenesis Program, Sydney, NSW, Australia ABSTRACTS, Page 152

POB030 **Modulation of Mxi1 and Mxi0 Expression impacts N-Myc-Mediated Neuroblastoma Tumor Pathogenesis and Chemosensitivity**
Michael Armstrong, Duke University, Pediatrics, Durham, NC, United States ABSTRACTS, Page 152

POB031 **NCYM, a Cis-Antisense Gene of MYCN, is a De Novo Evolved Gene**
Yusuke Suenaga, Chiba Cancer Center Research Institute, Division of Biochemistry and Innovative Cancer Therapeutics and Children's Cancer Research Center, Chiba, Japan ABSTRACTS, Page 153

POB032 **Flotillin-1 Regulates Oncogenic Signaling in Neuroblastoma Cells through Receptor Endocytosis of Anaplastic Lymphoma Kinase**
Arata Tomiyama, National Cancer Center Research Institute, Division of Metastasis and Invasion Signaling, Tokyo, Japan ABSTRACTS, Page 153

POB033 **Analysis of the MYCN Amplicon in Neuroblastoma (NB) Cell Lines**
Tiangang Zhuang, Children's Hospital of Philadelphia, Philadelphia, PA, United States ABSTRACTS, Page 153

POB034 **Calreticulin Up-Regulates VEGF-A and VEGF-C Expression in Neuroblastoma Cell Lines**
Kaun-Hung Lin, National Taiwan University, Department of Life Science, Taipei, Taiwan ABSTRACTS, Page 153

POB035 **Neuroblastoma Contains Multidrug-Resistant Mesenchymal-Type Cells That Depend on NOTCH-PDGFRβ Signalling**
Tim Groningen, Academic Medical Center (AMC), Oncogenomics, Amsterdam, The Netherlands ABSTRACTS, Page 154

POB036 **Mutant ALK Controls the RET-ETV5 Signaling Axis in Neuroblastoma: Implications for Normal Development, ALK Driven Tumor Formation and Novel Therapeutic Strategies**
Irina Lambertz, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 154

POB037 **Replicative Stress Induces Copy Number Alterations in Neuroblastoma Cell Lines and Might Contribute to Tumor Evolution**
Guillaume de la Houssaye, Institut Curie, U830, Paris, France ABSTRACTS, Page 154

POB038 **Role of MYCN in Neuroblastoma Rb-E2F1 Network**
Andres Florez, German Cancer Research Center (DKFZ)-Bioquant, Division of Theoretical Systems Biology, Heidelberg, Germany ABSTRACTS, Page 155

POB039 **MYCN Safeguards its Upregulated Expression through Negatively Controlling its Upstream miRNAs**
Anneleen Beckers, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 155

POB040 **miR-129-3p Directly Binds and Regulates ALK Expression in Neuroblastoma**
Galina Feinberg-Gorenshtein, Molecular Oncology, Felsenstein Medical Research Center, Petah Tikva, Israel ABSTRACTS, Page 155

POB041 **Abrogation of ERK5 Activity Suppresses Neuroblastoma Growth and MYCN Expression Mediated by ALK (Anaplastic Lymphoma Kinase)**
Ganesh Umapathy, Umeå University, Department of Molecular Biology, Umeå, Sweden ABSTRACTS, Page 155

POB042 **The Oncofetal Protein IGF2BP1 Cooperates with MYCN within a Positive Feedback Loop in High-Risk Neuroblastoma**
Jessica Bell, Martin Luther University (Halle), Halle Saale, Germany ABSTRACTS, Page 156

POB043 **Putative Cross-Talk of the Two CXCL12 Receptors, CXCR4 and CXCR7, in Metastatic Dissemination of Human Neuroblastoma**
Julie Liberman, Pediatric Oncology Research Unit, Lausanne University Hospital (CHUV), Department of Pediatrics, Lausanne, Switzerland ABSTRACTS, Page 156

POB044 **MYCN, Proliferative Heterogeneity and Treatment Response in Neuroblastoma**
Tatjana Ryl, German Cancer Research Center (DKFZ), Theoretical Systems Biology, Heidelberg, Germany ABSTRACTS, Page 156

POB045 **CHD5 and MYCN Regulation Downstream of MAPK Pathways in Neuroblastoma**
Mayumi Higashi, Children's Hospital of Philadelphia Pediatrics-Oncology, Philadelphia, PA, United States ABSTRACTS, Page 157

POB046 **Aryl Hydrocarbon Receptor Suppresses Tumor Progression of Neuroblastoma**
Pei-Yi Wu, National Taiwan University, Taipei, Taiwan ABSTRACTS, Page 157

POB047 **No Evidence for Nucleotide Signatures of Tumor Viruses in Whole-Transcriptome and Whole-Genome Deep Sequencing Data of Neuroblastoma**
Sven-Eric Schelhorn, Max-Planck Institute for Informatics, Computational Biology and Applied Algorithmics, Saarbruecken, Germany ABSTRACTS, Page 157

POB048 **Neuropeptide Y Y5 Receptor in Neuroblastoma Chemoresistance**
Joanna Kitlinska, Georgetown University Medical Center, Washington DC, United States ABSTRACTS, Page 158

POB049 **MYCN-Dependent Expression of Sulfatase2 Regulates Neuroblastoma Cell Survival**
Valeria Solari, Children's Hospital Los Angeles, University of Southern California, Pediatric Hematology, Oncology, Los Angeles, CA, United States ABSTRACTS, Page 158

POB050 **Prostaglandin Signaling in Primary Neuroblastoma Reveals Novel Therapeutic Targets**
Anna Kock, Karolinska Institutet, Women's and Children's Health, Stockholm, Sweden ABSTRACTS, Page 158

Basic Research

POB051 **Heat-Shock Proteins as the Molecular Targets of Iron Chelators in Neuroblastoma Cells**
Viktoryia Sidarovich, University of Trento, Center for Integrative Biology (CIBIO), Trento, Italy
ABSTRACTS, Page 158

POB052 **Inhibition of ALDH Activity Influences the Stem Cell Properties of Neuroblastoma Cells**
Marjorie Flahaut, University Hospital CHUV, Paediatrics, Lausanne, Switzerland
ABSTRACTS, Page 159

POB053 **Lack of Correlation Between Telomerase Activity and Individual Components of the Telomerase Complex (hTR, hTERT, Dyskerin) in Neuroblastoma Cell Line Panel**
Rebecca Dagg, The Children's Hospital at Westmead / Discipline of Paediatric and Child Health, The University of Sydney, Westmead, NSW, Australia ABSTRACTS, Page 159

POB153 **Transcriptional Regulation of IGF-II mediated HIF2A Expression in Neuroblastoma**
Arash Hamidian, Lund University, Translational Cancer Research, Lund, Sweden
ABSTRACTS, Page 159

POB156 **Inactivation of SMC2 Shows a Synergistic Lethal Response in MYCN-amplified Neuroblastoma Cells**
Yuko Murakami-Tonami, Aichi Cancer Center Research Institute, Division of Molecular Oncology, Nagoya, Japan ABSTRACTS, Page 160

POB157 **Epigenetic Role of the MYCN/LSD1 Complex in Neuroblastoma**
Stefano Amente, University of Naples 'Federico II', Department of Biology, Naples, Italy
ABSTRACTS, Page 160

POB161 **Whole-genome Sequencing Analysis of Neuroblastoma's Clonal Evolution**
Léo Colmet Daage, Curie Institut, Paris, France ABSTRACTS, Page 160

POB165 **Modelling MYCN-dependent Apoptosis in Neuroblastoma**
Axel Kuehn, Systems Biology Ireland, Belfield, Ireland ABSTRACTS, Page 160

Basic Research: High Throughput Techniques

POB054 **Identification of Potential Synthetic Lethal MYCN Target Genes or Interactors with High MYCN Using High-Throughput RNAi Screening**
Sina Gogolin, German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany ABSTRACTS, Page 161

POB055 **Identification of Lead Organic Compounds Active against Stem Cell-Like Neuroblastoma Cells by High Throughput Screening**
Naohiko Ikegaki, University of Illinois at Chicago, Anatomy and Cell Biology, Chicago, IL, United States ABSTRACTS, Page 161

POB056 **Gene Expression Analysis of Neuroblastoma Tumors According to MKI and Differentiation Status: An Interactive International Neuroblastoma Risk Database (iINRGdb) Study**
Samuel Volchenboum, University of Chicago, Department of Pediatrics, Chicago, IL United States ABSTRACTS, Page 161

POB057 **The Neuroblastoma Translatome: Profiling Cancer-Related Translational Alterations in a Panel of Cell Lines**
Erik Dassi, University of Trento, Center for Integrative Biology (CIBIO), Laboratory of Translational Genomics, Trento, Italy ABSTRACTS, Page 162

POB058 **An Epigenetic Focused siRNA Screen Identifies Novel Potentially Druggable Targets That Inhibit Growth and Induce Differentiation in Neuroblastoma**
Veronica Veschi, National Institutes of Health, Bethesda, MD, United States ABSTRACTS, Page 162

POB059 **Exome Sequencing Suggests Candidate Genes Associated with Aggressiveness of Stage 4 Neuroblastoma Patients**
Roberta Gallesio, IRCCS AOU San Martino-IST, Genoa, Italy ABSTRACTS, Page 162

POB060 **Identifying Mutation Enriched Protein Networks in Cancers with Heterogeneous Mutation Spectra**
Frederik Roels, University Children's Hospital of Cologne, Department of Pediatric Oncology Cologne, Germany ABSTRACTS, Page 163

POB061 **Whole Genome Sequencing of Mitochondrial DNA in Low and High-Risk Neuroblastoma Patients**
Francesco Calabrese, University of Naples Federico II, CEINGE Biotecnologie Avanzate, Naples, Italy ABSTRACTS, Page 163

POB062 **Next-Generation Exome Sequencing Reveals Actionable Genetic Alterations in Relapsed Neuroblastomas: The Memorial Sloan-Kettering Cancer Center (Mskcc) Experience**
Shakeel Modak, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States ABSTRACTS, Page 163

POB063 **Single Cell Next-Generation RNA Sequencing Analysis in Neuroblastoma Tumor Initiating Cells for Identifying Novel Targets for Neuroblastoma**
Eiso Hiyama, Hiroshima University Hospital, Pediatric Surgery, Hiroshima, Japan
ABSTRACTS, Page 164

POB064 **Integrative Analysis Reveals Novel Subtypes of Neuroblastoma**
Marcus Klarqvist, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden ABSTRACTS, Page 164

POB065 **Active PI3K/mTORC1 Signaling Predicts Poor Outcome in Neuroblastoma**
Evan Santo, Academic Medical Center (AMC), Oncogenomics, Amsterdam, The Netherlands
ABSTRACTS, Page 164

POB067 **Neuroblastoma with Distinct Genomic Profiles Harbor Similar Mutational Patterns**
Guillaume de la Houssaye, Institut Curie, U830, Paris, France ABSTRACTS, Page 164

POB069 **Reduced DICER1 Functionality in Neuroblastoma through Reduced Expression Levels or Somatic Mutations**
Bram De Wilde, Ghent University, Center for Medical Genetics, Ghent, Belgium
ABSTRACTS, Page 165

POB070 **Genomic Anatomy of Chemotherapy-Resistant Neuroblastoma**
Yesenia Rojas, Texas Children's Cancer Center, Baylor College of Medicine, Surgery, Houston, TX United States ABSTRACTS, Page 165

POB071 **A Submicroscopic Constitutional 3p Deletion in a Neuroblastoma Patient Leads to Identification of CHL1 Gene as a Novel Tumor Suppressor Gene Candidate**
Annalisa Pezzolo, Giannina Gaslini Institute, Laboratory of Oncology, Genova, Italy
ABSTRACTS, Page 165

POB073 **Function-Based Genome-Wide Association Study Reveals cis- and trans- Modulators of BARD1 Expression are Associated with the Development of High-Risk Neuroblastoma**
Navin Pinto, The University of Chicago, Department of Pediatrics, Chicago, IL, United States ABSTRACTS, Page 166

Basic Research

POB074 **Integrating Cell-Based and Clinical Genome-Wide Studies to Identify Genetic Variants Contributing to Treatment Failure in Neuroblastoma Patients**
Navin Pinto, The University of Chicago, Department of Pediatrics, Chicago, IL, United States ABSTRACTS, Page 166

POB075 **Anaplastic Lymphoma Kinase Signalling in Model Systems, from Flies to Man**
Ruth Palmer, Umeå University, Department of Molecular Biology, Umeå, Sweden ABSTRACTS, Page 166

POB076 **Subtype-Specific Whole Exome Sequencing of 101 Neuroblastomas Identified Genetic Mutations Involved in Cancer-Related Pathways Particularly in Aggressive Subgroups**
Yuanyuan Li, Chiba Cancer Center Research Institute, Chiba, Japan ABSTRACTS, Page 167

POB077 **Focused Exome Sequencing Frequently Identifies Actionable Genomic Alterations in Neuroblastoma Samples**
B. Turpin, Cincinnati Children's Hospital Medical Center, Cancer and Blood Diseases Institute, Cincinnati, OH, United States ABSTRACTS, Page 167

POB078 **Next Generation Sequencing of Neuroblastomas Identified PPP3CB as a Novel Prognostic Marker of High-Risk Neuroblastoma**
Irina Shakhova, Chiba Cancer Center Research Institute, Chiba, Japan ABSTRACTS, Page 167

POB079 **Estimating of Copy Number Alteration in Neuroblastoma: Comparison of Exome Sequencing Data and SNP Microarrays**
Malin Östensson, Sahlgrenska Academy at University of Gothenburg, Department of Medical and Clinical genetics, Gothenburg, Sweden ABSTRACTS, Page 168

POB080 **Analysis for Neuroblastoma Tumors to Reveal Novel Target Using Next-Generation RNA Sequencing**
Mitsuteru Hiwatari, University of Tokyo, Pediatrics, Tokyo, Japan ABSTRACTS, Page 168

POB081 **Kinome Expression Profiling of Human Neuroblastoma Tumours Identifies Potential Drug-Targets for Ultra-High Risk Patients**
Roberta Russo, University of Naples Federico II, CEINGE Biotecnologie Avanzate, Naples, Italy ABSTRACTS, Page 168

POB082 **R2: A Public User-Friendly Website for Integrated Analysis of Expression Data and Associated Clinical Parameters in Neuroblastoma**
Jan Koster, Academic Medical Center (AMC), Oncogenomics, Amsterdam, The Netherlands ABSTRACTS, Page 168

POB083 **Integrative Genomic and Epigenomic Characterization of Stage 4S Neuroblastoma**
Sharon Diskin, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States ABSTRACTS, Page 169

POB084 **Gene Expression Studies on Disseminated NB Cells: The Long Way to Reliability**
Fikret Rifatbegovic, St. Anna Kinderkrebsforschung, Children's Cancer Research Institute (CCRI), Vienna, Austria ABSTRACTS, Page 169

POB085 **A Kinome-Wide RNAi Screen Identifies ALK as a Synthetic Lethal Target in the Interaction with HDAC8-Inhibitors**
Jing Shen, German Cancer Research Center (DKFZ), CCU Pediatric Oncology, Heidelberg, Germany ABSTRACTS, Page 169

POB086 **A Search for Genes Differentially Expressed in Neuroblastoma Using Next-Generation Sequencing and Spheroid Culture**
Shinya Ikematsu, Okinawa National College of Technology, Bioresources Engineering, Nago, Japan ABSTRACTS, Page 170

POB087 **Characterization of Anaplastic Lymphoma Kinase (ALK) Mutations in Neuroblastoma**
Bengt Hallberg, Umeå University, Umeå, Sweden ABSTRACTS, Page 170

POB154 **MicroRNA-Mediated Feed-Forward Regulation Driven by MYCN in Neuroblastoma**
Hsuan-Cheng Huang, National Yang-Ming University, Institute of Biomedical Informatics, Taipei, Taiwan ABSTRACTS, Page 170

POB155 **Single-cell Transcriptomics Reveals Mechanism of MYCN Promoting Cell Cycling**
Qin Zhang, German Cancer Research Center, Division of Theoretical Systems Biology, Heidelberg, Germany ABSTRACTS, Page 171

POB158 **Integrative Omics of MYCN Reveals Potential Novel Therapeutic Targets, including Modulation of Wnt Signalling**
David Duffy, Systems Biology Ireland, University College Dublin, Dublin, Ireland ABSTRACTS, Page 171

POB159 **Identification of Key Exploitable Vulnerabilities in Neuroblastoma by Integrative Functional Genomics Screening**
Dominik Bogen, Children's Cancer Research Institute, Vienna, Austria ABSTRACTS, Page 171

POB163 **Interaction Proteome of N-Myc in Neuroblastoma**
Melinda halasz, Systems Biology Ireland, University College Dublin, Dublin, Ireland ABSTRACTS, Page 172

POB166 **Unraveling TrkA Signaling in Neuroblastoma Using a Quantitative MS-based Approach**
Kristina Emdal, Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Proteomics Program, Copenhagen, Denmark ABSTRACTS, Page 172

Basic Research: Biological Models

POB088 **Hypoxia Promotes Transdifferention of Neuroblastoma Cells into Endothelial Cells through Epithelial-Mesenchymal Transition**
Annalisa Pezzolo, Giannina Gaslini Institute, Laboratory of Oncology, Genova, Italy ABSTRACTS, Page 172

POB089 **The Epigenetic Modifier CHAF1A Opposes Neuroblastoma Differentiation via Metabolic Reprogramming**
Eveline Barbieri, Baylor College of Medicine, Pediatrics, Houston, TX, United States ABSTRACTS, Page 173

POB090 **Modeling Human Neuroblastoma in Mice**
Noémie Braekeveldt, Lund University, Translational Cancer Research, Lund, Sweden ABSTRACTS, Page 173

POB091 **FOXP1 Inhibits Cell Growth and Attenuates Tumorigenicity of Neuroblastoma**
Sandra Ackermann, University Children's Hospital of Cologne, Department of Pediatric Oncology and Hematology and Center for Molecular Medicine Cologne (CMMC), Cologne, Germany ABSTRACTS, Page 173

POB092 **Profound Therapy Resistance in Neuroblastoma is Characterized by a Mitochondrial Phenotype**
Kelly Goldsmith, Emory University School of Medicine, Atlanta, GA, United States ABSTRACTS, Page 174

Basic Research

POB093 **BMCC1, a Multifunctional Scaffold Protein, Promotes Mitochondrial Apoptosis and Attenuates AKT Survival Signal after E2F1-Dependent Induction in Neuroblastoma**
Yasutoshi Tatsumi, Chiba Cancer Center Research Institute, Division of Biochemistry & Innovative Cancer Therapeutics, Chiba, Japan ABSTRACTS, Page 174

POB094 **MYCN Promotes Neuroblastoma Cell Migration by Regulating TRPM7 Channel and Kinase Activities**
Dana-Lynn Koomoa-Lange, University of Hawai at Hilo, Daniel K. Inouye College of Pharmacy, Hilo, HI, United States ABSTRACTS, Page 174

POB095 **miR-18a Inhibits Differentiation of MYCN-Amplified Neuroblastoma by Deregulation of Estrogen and NGF Signaling**
Ulrica Westermark, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden ABSTRACTS, Page 174

POB096 **The Neurodevelopmental Gene, COUP-TF1, Mediates Differentiation and Apoptosis to Affect Therapy Response and Survival in High-Risk Neuroblastoma (HR NB)**
Rachel Tanos, Emory University, Children's Healthcare of Atlanta, Atlanta, GA, United States ABSTRACTS, Page 175

POB097 **Hypoxic Preconditioning Promotes Invasion of Neuroblastoma Cells In Vivo and Enables the Metastasis of Adjacent Non-Hypoxic Cells**
Anne Herrmann, University of Liverpool, Institute of Integrative Biology, Cellular and Molecular Physiology, Liverpool, United Kingdom ABSTRACTS, Page 175

POB099 **Secretase Mediated Intramembrane Proteolysis of NLRR3 Leads to the Liberation of its Intracellular Domain: Its Role in Inducing Neuroblastoma Differentiation**
Jesmin Akter, Chiba Cancer Center Research Institute, Children's Cancer Research Center, Division of Biochemistry & Innovative Cancer Therapeutics, Chiba, Japan ABSTRACTS, Page 175

POB100 **Accumulation of Cytosolic Calcium Induces Necroptotic Cell Death in Human Neuroblastoma**
Motonari Nomura, Osaka Medical Center and Research Institute for Maternal and Child Health, Department of Pediatric Surgery, Osaka, Japan ABSTRACTS, Page 176

POB101 **BMCC1, a pro-apoptotic gene upregulated by E2F1 after DNA damage, facilitates the drug sensitivity in neuroblastoma**
Mohammad Islam, Chiba Cancer Center Research Institute, Div. of Innovative Cancer Therapeutics, Chiba, Japan ABSTRACTS, Page 176

POB102 **A Schwann Cell Expression-Profile Study - Normal, Benign and Neuroblastoma-Associated Schwann Cells Differ from F-Cells**
Tamara Weiss, St. Anna Kinderkrebsforschung, Children's Cancer Research Institute (CCRI), Vienna, Austria ABSTRACTS, Page 176

POB103 **Elucidating Molecular Mechanisms of Oncogene and Non-oncogene Addiction in Pediatric Neuroblastoma**
Gonzalo Lopez, Columbia University, Department of System Biology, New York, NY, United States ABSTRACTS, Page 176

POB104 **The Children's Oncology Group Cell Culture and Xenograft Repository Neuroblastoma Cell Lines and Patient-Derived Xenografts**
Tito Woodburn, Texas Tech University Health Sciences Center, Cancer Center, Lubbock, TX, Lubbock, TX, United States ABSTRACTS, Page 177

POB105 **The Inhibition of Hypoxia Inducible Factors Combined with Retinoic Acid Treatment Enhances Glial Differentiation of Neuroblastoma Cells**
Flora Cimmino, University of Naples Federico II, Department of Molecular Medicine and Medical Biotechnology, Naples, Italy ABSTRACTS, Page 177

POB106 **In Vivo Imaging of Human Orthotopic Neuroblastoma in Mice**
Daniel Bexell, Lund University, Molecular Medicine, Lund, Sweden ABSTRACTS, Page 178

POB107 **Investigation of ADCC Enhancement by NKT Cells Toward Neuroblastoma**
Naoko Mise, Chiba University Hospital, Medical Immunology, Pediatric Surgery, Chiba, Japan ABSTRACTS, Page 178

POB108 **Ultrasound Evaluation of Tumor Latency and Disease Progression in the Hemizygous TH-MYCN Transgenic Mice**
Yen-Lin Liu, Taipei Medical University Hospital, Pediatric Hematology/Oncology, Taipei, Taiwan ABSTRACTS, Page 178

POB109 **The Resistant Cancer Cell Line (RCCL) Collection**
Martin Michaelis, University of Kent, Centre for Molecular Processing and School of Biosciences, Canterbury, United Kingdom ABSTRACTS, Page 179

POB110 **A Developmental Approach to Analyze Neuroectodermal Childcancer in Matching Human Microenvironment in Vivo**
Isabell Hultman, Karolinska Institutet, Department of Women's and Children's Health, Stockholm, Sweden ABSTRACTS, Page 179

POB111 **Isolation and Characterisation of Neuroblastoma Cells from Bone Marrow Aspirates: A NCRI CCL CSG Neuroblastoma Group Study**
Sue Burchill, University of Leeds, Children's Cancer Research Group, Leeds, United Kingdom ABSTRACTS, Page 179

POB112 **A Comparison of 2D vs. 3D Culture Methods in Supporting Freshly Isolated Pediatric Neuroblastoma Cells**
Todd Jensen, University of Conneticut Health Center, Center For Vascular Biology, Farmington, CT United States ABSTRACTS, Page 179

POB113 **Live Cell Imaging Reveals Heterogeneous Proliferative Behaviour in Neuroblastoma**
Erika Kuchen, German Cancer Research Center (DKFZ), Theoretical Systems Biology, Heidelberg Germany ABSTRACTS, Page 180

POB114 **Hypoxia Regulated Proteins: Promising Targets for Neuroblastoma Treatment**
Flora Cimmino, University of Naples Federico II, Department of Molecular Medicine and Medical Biotechnology, Naples, Italy ABSTRACTS, Page 180

POB115 **Expression of the Monocyte Chemotactic Protein-1-Induced Protein 1 (MCPIP1) Decreases Human Neuroblastoma Cell Survival**
Elżbieta Boratyn, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Laboratory of Molecular Genetics and Virology, Krakow, Poland ABSTRACTS, Page 180

POB116 **The Modulation of Attachment, Survival and Differentiation of Neuroblastoma Cells by Nanofilms with Tunable Stiffness**
Nimrod Buchbinder, Institut for Innovation and Research in Biomedicine, University of Rouen, Laboratory of Microenvironment and Integrative Cell Renewing, Rouen, France ABSTRACTS, Page 180

POB160 **Screening of Plant-Derived Extracts for an Anti-Proliferative Effect on Neuroblastoma Cells and Associated Changes In Intracellular Calcium Levels**
Julia Moschny, Daniel K. Inouye College of Pharmacy, University of Hawai'i, Pharmaceutical Sciences, Hilo, HI, United States ABSTRACTS, Page 181

POB162 **ERK5 Positively Regulates HIF-1α activity in neuroblastoma SY5Y cells**
Fei Tan, Shengjing Hospital of China Medical University, Shenyang, China ABSTRACTS, Page 181

Basic Research: Developmental Neuroblastoma Biology

POB117 **Neuroblastoma in Dialog with its Stroma: NTRK1 is a Regulator of Cross-Talk with Schwann Cells**
Kristian Pajtler, University Hospital Essen, Pediatric Oncology, Essen, Germany
ABSTRACTS, Page 181

POB118 **MYC Proteins Promote Neuronal Differentiation by Controlling the Mode of Progenitor Cell Division**
Nikolay Zinin, Karolinska Institutet, Microbiology, Tumor and Cell Biology (MTC), Stockholm, Sweden
ABSTRACTS, Page 182

POB119 **From Human Embryonic Stem Cells to Sympathetic Neurons: A Model for Understanding Neuroblastoma Pathogenesis**
Jane Carr-Wilkinson, University of Sunderland and Newcastle University, North East Stem Cell Institute, Sunderland, United Kingdom
ABSTRACTS, Page 182

POB121 **Association of the MTHFR C677T Gene Polymorphism with Childhood Neuroblastoma Susceptibility and Progression**
N. Svergun, National Cancer Institute of the MPH of Ukraine, Kiev, Ukraine
ABSTRACTS, Page 182

POB122 **Neuroblastoma Oncogene Ortholog Lmo3 Cooperates with Hen2 on Induction of Aberrant Neurogenesis in Mice**
Eriko Isogai, Chiba Cancer Center Research Institute, Cancer Genomics, Chiba, Japan
ABSTRACTS, Page 183

POB123 **Elucidation of Mechanisms Underlying Fate Determination of Neuroblastoma in MYCN-Tg Mice**
Shoma Tsubota, Nagoya University, Graduate School of Medicine, Department of Biochemistry, Nagoya, Japan
ABSTRACTS, Page 183

POB124 **PBX1 is a Strong Favorable Prognostic Biomarker in Low-Risk and High-Risk Neuroblastoma and is Critical to Retinoid-mediated Differentiation**
Nilay Shah, Nationwide Children's Hospital, Center for Childhood Cancer and Blood Diseases, Columbus, OH, United States
ABSTRACTS, Page 183

POB125 **O-GlcNAcylation in Neuroblastoma**
Anoop Mayampurath, University of Chicago, Computation Institute, Chicago, IL, United States
ABSTRACTS, Page 184

POB126 **Mechanisms Involved in As2O3 and ATRA Induced Differentiation**
Alexandre Petit, University of Rouen, Laboratory MERCI EA 3829, Rouen, France
ABSTRACTS, Page 184

Basic Research: Epigenetics

POB129 **Silencing of CHD5 Expression by H3K27me3 in Human Neuroblastoma (NB)**
Mayumi Higashi, Children's Hospital of Philadelphia, Pediatrics-Oncology, Philadelphia, PA, United States
ABSTRACTS, Page 184

POB130 **Role of MicroRNAs in the Epigenetic Silencing of CHD5, a Tumor Suppressor in Neuroblastoma (NB)**
Koumudi Naraparaju, Children's Hospital of Philadelphia, Philadelphia, PA, United States
ABSTRACTS, Page 185

POB131 **DNA Hypomethylation Affects Cancer-Related Biological Functions and Genes Relevant in Neuroblastoma Pathogenesis**
Gemma Mayol, Hospital Sant Joan de Déu, Oncology, Barcelona, Spain
ABSTRACTS, Page 185

POB132 **CASZ1b Interacts with Chromatin to Suppress Tumor Cell Proliferation**
Zhihui Liu, National Cancer Institute, Bethesda, MD, United States
ABSTRACTS, Page 185

POB133 **Histone H3 Methyltransferase G9A Epigenetically Activates the Serine Synthesis Pathway to Sustain Cancer Cell Survival and Proliferation**
Han-Fei Ding, Georgia Regents University, Cancer Center, Augusta, GA, United States
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POB134 **GRHL1 Acts as Tumor Suppressor in Neuroblastoma and is Negatively Regulated by MYCN and HDAC3**
Johannes Fabian, German Cancer Research Center (DKFZ), Clinical Cooperation Unit Pediatric Oncology, Heidelberg, Germany
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POB135 **Epigenetic Modulation of MYCN-Driven Transactivation in Neuroblastoma Cell Lines and Tumors**
Daniel Dreidax, German Cancer Research Center (DKFZ), Neuroblastoma Genomics, Heidelberg, Germany
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POB136 **Histone Deacetylase 10 Promotes Autophagy-Mediated Cell Survival**
Ina Oehme, German Cancer Research Center (DKFZ), CCU Pediatric Oncology, Heidelberg, Germany
ABSTRACTS, Page 187

POB137 **CHD5 Forms a Novel Chromatin Remodeling Complex and Protein Interactions in Neuroblastoma (NB) Cell Lines**
Venkatadri Kolla, Children's Hospital of Philadelphia, Pediatrics-Oncology, Philadelphia, PA, United States
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POB138 **Genetic Alterations Associated with Neuroblastoma Cause EZH2 Mediated Epigenetic Dysregulation Which Can Be Reversed by Pharmacologic Targeting of EZH2**
Chunxi Wang, National Cancer Institute, Pediatric Oncology Branch, Bethesda, MD, United States
ABSTRACTS, Page 187

POB140 **Genome-Wide Methylation Analysis Unravels Differential DNA Methylation Patterns Between (Prognostic) Neuroblastoma Patient Subgroups**
Anneleen Decock, Ghent University, Center for Medical Genetics, Ghent, Belgium
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POB141 **Epigenetic Regulation of Transcription Factor Binding Limits Differentiation Potential**
Abraham Fong, Seattle Children's Hospital, Seattle, WA, United States
ABSTRACTS, Page 188

POB142 **Genome-Wide Methylation Analysis in Neuroblastoma**
Masafumi Seki, The University of Tokyo, Department of Pediatrics, Tokyo, Japan
ABSTRACTS, Page 188

POB143 **Extracellular miRNA Expression Profiling of Conditioned Media Derived from In Vitro Models of Multidrug Resistant Neuroblastoma**
Ross Conlon, Royal College of Surgeons in Ireland, Cancer Genetics Group, Department of Molecular and Cellular Therapeutics, Dublin, Ireland
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Translational Research: Preclinical Experimental Therapies

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POT033 **Inhibition of Exportin 1 (XPO1) Potently Suppresses Growth of Human Neuroblastoma Cell Lines**
Edward Attiyeh, Children's Hospital of Philadelphia, and the Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, United States
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POT034 **Optimizing Neuroblastoma-Specific CD171-Targiting CARs**
Annette Künkele, Seattle Children's Research Institute, BTCCCR, Seattle, WA, United States
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POT035 **Evaluation of Targeted Drugs in Combination with the MDM2 Antagonist RG738 in Neuroblastoma**
Alan Van Goethem, Ghent University, Center for Medical Genetics, Ghent, Belgium
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POT036 **Sensitization for chemotherapy-induced apoptosis by Smac mimetic LCL161 in Neuroblastoma is Independent of Ripoptosome-formation, NF-κB and TNF-α**
Georg Eschenburg, University Medical Center Hamburg-Eppendorf, Department of Paediatric Surgery Hamburg, Germany
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POT038 **Differential Expression of Uridine-Cytidine Kinases in Neuroblastoma. Implications for Development of a Targeted Therapeutic Approach**
André van Kuilenburg, Academic Medical Center (AMC), Genetic Metabolic Diseases, Amsterdam The Netherlands
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POT039 **Pre-Clinical Studies of the MDM2-p53 Antagonist RG7388 Alone and in Combination with Chemotherapy in Neuroblastoma**
Lindi Chen, Newcastle University, Northern Institute for Cancer Research, Newcastle upon Tyne, United Kingdom
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POT040 **Biodegradable Nanoparticles Loaded with a Rapidly Activatable SN38 Prodrug Effectively Inhibit Growth of Neuroblastoma (NB) Xenografts**
Radhika Iyer, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, PA, United States
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POT041 **Generation and Characterization of Ganglidiximab for Active Immunotherapy of Neuroblastoma**
Christin Eger, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald, Germany
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POT042 **Generation and Characterization of Two Expression Plasmids Encoding for Single Chain Variable Fragments of Ganglidiomab for Active Immunotherapy in Neuroblastoma**
Diana Brackrock, University Medicine Greifswald, Pediatric Hematology and Oncology, Greifswald Germany
ABSTRACTS, Page 205

POT043 **Sensitivity to CCNB1/cdk1 Complex Inhibition is Modulated by TP53 Mutational Status in Neuroblastoma Cell Lines**
Melanie Schwermer, University Hospital Essen, Children's Hospital, Essen, Germay
ABSTRACTS, Page 206

POT044 **Testing of SNS-032 in a Panel of Human Neuroblastoma Cell Lines with Acquired Resistance to a Broad Range of Drugs**
Martin Michaelis, University of Kent, Centre for Molecular Processing and School of Biosciences Canterbury, United Kingdom
ABSTRACTS, Page 206

POT045 **Role for Sirtuins in Chemoresistance in Neuroblastoma**
Branko Cuglievan, Miami Children's Hospital, General Pediatrics, Miami, FL, United States
ABSTRACTS, Page 206

POT046 **Interrelation Between PI3K, Akt, mTOR and P53 Signaling Routes in GD2 Ganglioside - Targeted Neuroblastoma Cell Lines**
Małgorzata Durbas, Jagiellonian University, Laboratory of Molecular Genetics and Virology, Krakow Poland
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POT047 **The Combination of 13-cis Retinoic Acid and Isorhamnetin Exerts Synergistic Anticancer Activity against Neuroblastoma Cell Lines**
Pamela Gatto, University of Trento, Centre for Integrative Biology (CIBIO), Trento, Italy
ABSTRACTS, Page 207

POT048 **Targeted Therapy by Smac Mimetic LCL161 Sensitizes Neuroblastoma for Chemotherapy-induced Apoptosis in a Drug Class-dependent Manner**
Georg Eschenburg, University Medical Center Hamburg-Eppendorf, Department of Paediatric Surgery Hamburg, Germany
ABSTRACTS, Page 207

POT049 **Synergistic Induction of Apoptosis by Dual PI3K/mTOR Inhibitor NVP-BEZ235 and Chloroquine in Neuroblastoma Cells via Mitochondrial-Lysosomal Amplification Loop**
Christian Seitz, Goethe University Frankfurt, Institute for Experimental Cancer Research in Pediatrics, Frankfurt am Main, Germany
ABSTRACTS, Page 208

POT050 **Targeted Nuclear Imaging and Radiotherapy with 68Ga-DOTA-TATE and 177Lu-DOTA-TATE on Neuroblastoma Preclinical Models**
Libo Zhang, The Hospital for Sick Children, Physiology and Experimental Medicine, Toronto Ontario, Canada
ABSTRACTS, Page 208

POT051 **Structurally Diverse MDM2-p53 Antagonists Act as Modulators of MDR-1 Function in Neuroblastoma**
Lindi Chen, Newcastle University, Northern Institute for cancer Research, Newcastle upon Tyne United Kingdom
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POT052 **HDAC8 Selective Inhibitors Exhibit Anti-Neuroblastoma Activity In Vitro and In Vivo and Enhance Retinoid Acid Mediated Differentiation**
Inga Rettig, German Cancer Research Center (DKFZ), CCU Pediatric Oncology, Heidelberg Germany
ABSTRACTS, Page 208

POT053 **GNF-4256 Enhances Chemotherapeutic Antitumor Efficacy in a Neuroblastoma (NB) Murine Xenograft Model**
Jamie Croucher, Children's Hospital of Philadelphia, Pediatrics, Philadelphia, PA, United States
ABSTRACTS, Page 209

POT054 **Preclinical Development of meta-[211At]astatobenzylguanidine ([211At]MABG) Targeted Radiotherapy for Neuroblastoma**
Vandana Batra, Children's Hospital of Philadelphia, Oncology, Philadelphia, PA, United States
ABSTRACTS, Page 209

POT055 **Targeting the Warburg Effect and Metabolic Plasticity in Neuroblastoma with FDA Approved Ritonavir and Metformin**
Surabhi Batra, Ann and Robert H. Lurie Children's Hospital of Chicago, Hematology/Oncology Chicago, IL, United States
ABSTRACTS, Page 209

POT056 **Hsp90 Inhibition is a Viable Therapeutic Strategy for High-Risk Neuroblastoma**
Elizabeth Tucker, The Institute of Cancer Research, Paediatric Tumour Biology Team, London United Kingdom
ABSTRACTS, Page 210

POT057 **The Histone Methyltransferase Activity of EZH2 in Neuroblastoma: Friend or Foe**
Laurel Bate-Eya, Academic Medical Center (AMC), Oncogenomics, Amsterdam, The Netherlands
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POT058 **Toll-Like Receptor 3 Agonist Augments 2-deoxyglucose-induced Suppression of Neuroblastoma Cell Growth**
Pei-Wen Wang, Kaohsiung Chang Gung Memorial Hospital, Nuclear and Internal Medicin Kaohsiung, Taiwan ABSTRACTS, Page 210

POT059 **Identification of Novel Candidate Compounds Targeting TrkB to Induce Apoptosis in Neuroblastoma: In Silico Screening Utilizing a Grid Computing Technology**
Yohko Nakamura, Chiba Cancer Center Research Institute, Chiba, Japan ABSTRACTS, Page 211

POT061 **Metabolic Characteristics of 13-cRA and Anti-tumor Activity of the 13-cis Retinoic Acid Metabolite 4-oxo-13-cis Retinoic Acid in Neuroblastoma**
Min Kang, Texas Tech University Health Sciences Center, Cell Biology and Biochemistry, Lubbock, TX United States ABSTRACTS, Page 211

POT062 **The Influence of Chelator- and Radiolabelling on the In Vivo and In Vitro Binding Characteristics of the Anti-GD2 Antibodies ch14.18, hu14.18K322A and ch14.18-Δch2**
Julia Schmitt, Eberhard Karls University Tuebingen, Werner Siemens Imaging Center, Department of Preclinical Imaging and Radiopharmacy, Tuebingen, Germany ABSTRACTS, Page 211

POT063 **New Synthetic Bismuth Zinc Compound Could Reduce Cisplatinuminduced Nephrotoxicity without Compromising the Anticancer Effect**
Godfrey Chi-Fung Chan, The University of Hong Kong, Paedaitrics and Adolescent Medicine, Hong Kong, China ABSTRACTS, Page 212

POT064 **The MRN Complex: A Potential Target to Fight against MYCN Amplified Neuroblastoma**
Francesca Sardina, University of Rome La Sapienza, Molecular Medicine, Rome, Italy ABSTRACTS, Page 212

POT065 **Noradrenaline Transporter Gene Transfer via Oncolytic Virotherapy Enhances 131I-MIBG uptake: A Novel Combination Viroradiotherapy Approach to High-Risk Neuroblastoma**
Keri Streby, Nationwide Children's Hospital, Columbus, OH, United States ABSTRACTS, Page 212

POT066 **Spermidine-Dependent Posttranslational Activation of Hypusinated eIF5A is Blocked by DHPS Inhibitor GC7 and Induces Rb-Mediated Inhibition of Neuroblastoma Tumor Growth**
Andrea Bandino, University of Hawaii at Hilo, College of Pharmacy, Pharmaceutical Sciences, Hilo HI, United States ABSTRACTS, Page 212

POT067 **Structure-Based Docking of Transcription Factor STAT3 to Withaferin A, a Natural Product That Impedes Neuroblastoma Tumor Cell Growth**
Lisette Yco, University of Hawaii at Hilo, College of Pharmacy, Pharmaceutical Sciences, Hilo, HI, United States ABSTRACTS, Page 213

POT068 **Resistance to Alisertib (MLN8237) in Xenograft Derived NeuroblastomaCcell lines is Mediated through Cell Cycle Arrest**
Stuart Cramer, University of Alabama at Birmingham, Pediatric Hematology and Oncology, Birmingham, AL, United States ABSTRACTS, Page 213

POT069 **Cytosolic Melanoma Differentiation-Associated Gene 5 functions to Complement Toll-Like Receptor 3 Agonist-Induced Neuroblastoma Cell Apoptosis**
Jiin-Haur Chuang, Kaohsiung Chang Gung Memorial Hospital, Surgery, Kaohsiung, Taiwan ABSTRACTS, Page 213

POT070 **SMARCA4/BRG1 is a New Epigenetic Target for Neuroblastoma Therapy**
Luz Jubierre, Vall d'Hebron Research Institute, Translational Research in Childhood Cancer, Barcelona, Spain ABSTRACTS, Page 214

POT071 **Changes in Phosphorylation of Aurora Kinases A, B, and C, and Expression of Natural Aurora A Substrates MYCN, P53, PHLDA1, in Neuroblastoma Cells Double Hit with an Anti-GD2 Ganglios de Antibody 14G2a and a Novel Aurora A Inhibitor MK-5108**
Irena Horwacik, The Jagiellonian University, Laboratory of Molecular Genetics and Virology, Kraków, Poland ABSTRACTS, Page 214

POT072 **Implications of the Down-Regulation of Stemness/Reprogramming Factor Expression by Ibuprofen and Biguanides**
Naohiko Ikegaki, University of Illinois at Chicago, Chicago, IL, United States ABSTRACTS, Page 214

POT073 **The Bcl-2 Selective Antagonist, ABT-199, Restores Apoptosis and Chemotherapy Response in High-Risk Neuroblastomas (HR NB)**
Rachel Tanos, Emory University, Children's Healthcare of Atlanta, Atlanta, GA, United States ABSTRACTS, Page 215

POT074 **Combined Therapeutic Effects of Irinotecan and Cyclophosphamide Chemotherapy in Neuroblastoma**
Weisong Cai, Shengjing Hospital, China Medical University, Oncology, Shenyang, Liaoning China ABSTRACTS, Page 215

POT075 **Taurolidine-induced Extrinsic and Intrinsic Induction of Apoptosis in Neuroblastoma**
Georg Eschenburg, University Medical Center Hamburg-Eppendorf, Department of Paediatric Surgery, Hamburg, Germany ABSTRACTS, Page 215

POT077 **Screening of Okinawan Natural Resources for Antitumor Activities Using Midkine as an Indicator**
Ari Zukeran, Okinawa National College of Technology, Bioresources Engineering Course, Creative System Engineering Advanced Course, Nago, Okinawa, Japan ABSTRACTS, Page 215

POT122 **The Effects of Hypoxia on BER Inhibitor-induced Radiosensitisation in Neuroblastoma Cells in Vitro**
Anthony McCluskey, University of Strathclyde, Strathclyde Institute of Pharmacy and Biomedical Sciences, Glasgow, United Kingdom ABSTRACTS, Page 216

POT125 **miRNA-deep Sequencing Identifies miR-10b-5p as the Most Abundant microRNA in Retinoic Acid Treated Neuroblastoma Cells**
Raseswari Turlapati, Martin Luther University, ZAMED, Cell and Molecular Biology, Halle, Germany ABSTRACTS, Page 216

POT128 **Development of Robust Pharmacodynamic Biomarker Assays for MYCN and the PI3K/AKT Pathway and Isolation of Bone Marrow-Resident Neuroblasts as a Substrate for Clinical Trials-Based Biomarker Analysis**
Jennifer Smith, The Institute of Cancer Research, Clinical PD Biomarker Group, London, United Kingdom ABSTRACTS, Page 216

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POT079 **The Entire Polyamine Gene Pathway is Coordinately Regulated by the MYCN Oncogene to Sustain Polyamine Levels and Support Neuroblastoma Progression**
Michelle Haber, Children's Cancer Institute Australia for Medical Research, Experimental Therapeutics, Sydney, NSW, Australia ABSTRACTS, Page 217

POT080 **Individual Patient Risk Stratification of High-Risk Neuroblastomas Using a Four-Gene Ratio Score Suited for Clinical Use**
Kristoffer von Stedingk, Lund University, Laboratory Medicine, Lund, Sweden ABSTRACTS, Page 217

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POT081 **An Extensible Data-Model for Neuroblastoma Information Management**
Massimiliano Izzo, Giannina Gaslini Institute, Genova, Italy ABSTRACTS, Page 217

POT082 **Segmental Chromosomal Aberrations of Disseminated Neuroblastoma Cells in Stage M Patients with and without Relapse**
M. Reza Abbasi, St. Anna Kinderkrebsforschung, Children's Cancer Research Institute (CCRI) Vienna, Austria ABSTRACTS, Page 217

POT083 **Characterization of Li-Fraumeni Syndrome Associated Germline TP53 Mutations R248W, R158H and E286K in Neuroblastoma**
Meredith Irwin, Hospital for Sick Children, Pediatrics (Hematology-Oncology), Toronto, Ontario, Canada ABSTRACTS, Page 218

POT084 **Genomic Background of Neuroblastomas with Intra-Tumor Heterogeneity of MYCN Amplification**
Inge Ambros, St. Anna Kinderkrebsforschung, Children's Cancer Research Institute (CCRI), Tumor Biology, Vienna, Austria ABSTRACTS, Page 218

POT085 **Targeted Analysis of TP53 Pathway SNPs in Neuroblastoma Patients**
Ali Rihani, Ghent University, Center for Medical Genetics, Ghent, Belgium ABSTRACTS, Page 218

POT086 **Primary Anatomic Location of Neuroblastomas Correlates with Genomic Profile: A Retrospective Study of 133 tumors**
Thomas Blanc, Necker-Enfants Malades Hospital, Pediatric Surgery, Paris, France ABSTRACTS, Page 219

POT087 **Deep microRNA Sequencing Reveals Downregulation of miR-29a in Neuroblastoma Central Nervous System Metastasis**
Nai-Kong Cheung, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States ABSTRACTS, Page 219

POT088 **Genome-Based Sub-Classification of Neuroblastoma: A Retrospective Study by Using 573 Neuroblastoma Samples Obtained in Japan**
Miki Ohira, Chiba Cancer Center Research Institute, Chiba, Japan ABSTRACTS, Page 219

POT089 **Study of Inflammatory Cells in Neuroblastoma**
Victor Zúñiga, Medical School, University of Valencia, Department of Pathology, Valencia Spain ABSTRACTS, Page 220

POT090 **Prognostic Role of Immunosuppressive Cell Molecular Markers in High-Risk Neurobalstoma Patients**
Maria Corrias, IRCCS Giannina Gasilini Institute, Laboratory of Oncology, Genoa, Italy ABSTRACTS, Page 220

POT091 **GSTP1 Gene Status in 11q Loss NB**
Ana P. Berbegall, Medical School and Medical Research Foundation INCLIVA, University of Valencia, Pathology, Valencia, Spain ABSTRACTS, Page 220

POT092 **Discovering Robust Survival signatures from Neuroblastoma mRNA Expression Profiles**
Alexander Schramm, University Hospital Essen, Children's Hospital, Essen, Germany ABSTRACTS, Page 221

POT093 **Establishment of Pharmacodynamic Biomarkers of MDM2-P53 Inhibitor Activity in Neuroblastoma Cells Using the Amnis Imagestream**
Elizabeth Gavens, Northern Institute of Cancer Research, Newcastle upon Tyne, United Kingdom ABSTRACTS, Page 221

POT094 **Clinical Characteristics and Outcome of Patients with Neuroblastoma Presenting Genomic Amplification of Loci other than MYCN**
Anne Guimier, Institut Curie, Pediatric Oncology, Paris, France ABSTRACTS, Page 221

POT095 **Genomic Profiling in Low and Intermediate Risk Neuroblastoma to Refine Treatment Stratification and Improve Patient Outcome - LINES: a SIOPEN Trial**
Gudrun Schleiermacher, Institut Curie, Department of Pediatric Oncology and U830 INSERM Paris, France ABSTRACTS, Page 222

POT097 **Chromothripsis in Neuroblastoma**
Susana Martín-Vañó, Medical School, University of Valencia, Pathology, Valencia, Spain ABSTRACTS, Page 222

POT099 **Prognostic Significance of Non-Cellular Extracellular Matrix Elements in Neuroblastic Tumors**
Irene Tadeo, Medical Research Foundation, INCLIVA, Department of Pathology, Medical School, University of Valencia, Valencia Spain ABSTRACTS, Page 222

POT100 **Telomere Biology in Neuroblastoma: Focusing on ATRX/DAXX Genes and Alternative-Strengthening of Telomere (ALT)**
Eiso Hiyama, Hiroshima University Hospital, Pediatric Surgery, Hiroshima, Japan ABSTRACTS, Page 223

POT101 **Genetic Variants in the Calcium-Sensing Receptor are Associated with Clinical Outcome of Neuroblastoma**
Laia Masvidal, St John of God Foundation, Developmental Tumor Biology Laboratory, Esplugues de Llobregat, Spain ABSTRACTS, Page 223

POT102 **Modulation of Radiation Biomarkers in Children with Neuroblastoma Treated with 131I-mIBG**
Erin Karski, University of California San Francisco, Pediatrics, San Francisco, CA, United States ABSTRACTS, Page 223

POT104 **Chemotherapy-Induced Upregulation of CHD5 Expression in Neuroblastoma is Associated with Patient Outcome: A Potential Marker for Early Assessment of Response to Treatment**
Gemma Mayol, Hospital Sant Joan de Déu, Oncology, Barcelona, Spain ABSTRACTS, Page 223

POT105 **The Role of FGFR4 in Neuroblastoma**
Sarah Whittle, Baylor College of Medicine, Pediatrics, Section of Hematology/Oncology, Houston, TX, United States ABSTRACTS, Page 224

POT106 **Paired Analysis of Primary Tumor and Metastatic Relapse of Localized Neuroblastoma Using FISH and aSNP**
Maite Blanquer-Maceiras,Medical Research Foundation INCLIVA, University of Valencia, Pathology, Valencia, Spain ABSTRACTS, Page 224

POT107 **Copy Number Variations of Chromosomal Regions are Associated with Unfavorable Outcome in Neuroblastoma Patients**
Alexander Druy, Regional Children's Hospital, Molecular Biology Laboratory, Ekaterinburg, Russian Federation ABSTRACTS, Page 224

POT108 **Characterization of Chromosomal Rearrangements Involving the Anaplastic Lymphoma Kinase (ALK) Gene in Neuroblastoma Tumors through Targeted Re-Sequencing**
Susanne Fransson, University of Gothenburg, Dept. Medical and Clinical Genetics, Gothenburg, Sweden ABSTRACTS, Page 225

POT109 **An 18-gene Myc Activity Signature for Neuroblastoma and Other Myc-Driven Cancers**
Michelle Henderson, Children's Cancer Institute Australia for Medical Research, Sydney, NSW Australia ABSTRACTS, Page 225

Translational Research

Translational Research: Minimal Residual Disease

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Clinical Research

Clinical Research: Diagnosis and Prognosis

POC001 **Computer-Assisted Curie Scoring of MIBG Scans for Neuroblastoma Patients**
Samuel Volchenboum, University of Chicago, Center for Research Informatics, Chicago, IL, United States
ABSTRACTS, Page 231

POC002 **Identification of Plasma Complement C3 as a Potential Biomarker for Neuroblastoma Using a Quantitative Proteomic Approach**
Belamy Cheung, Children's Cancer Institute Australia for Medical Research, Molecular Carcinogenesis, Program, Sydney, NSW, Australia
ABSTRACTS, Page 231

POC004 **Risk Stratification Using Clinical Factors, For Treatment of Neuroblastoma Patients in Developing Countries: A Study from the International Neuroblastoma (NB) Risk Group (INRG) Database**
Wendy London, Boston Children's Hospital / Dana-Farber Cancer Institute, Pediatrics, Boston, MA United States
ABSTRACTS, Page 231

POC005 **Immunocytological GD2 Expression on Neuroblastoma Cells in Bone Marrow is Overestimated and May Have Implications for α-GD2 Immunotherapy**
Roswitha Schumacher-Kuckelkorn, University Children's Hospital of Cologne, Pediatric Oncology and Hematology, Cologne, Germany
ABSTRACTS, Page 232

POC006 **The New Guideline from the International Neuroblastoma Risk Group (Inrg) Project Has Profound Effects on Clinical Trials Which Employed Image Defined Risk Factors**
Akihiro Yoneda, Osaka Medical Center and Research Institute for Maternal and Child Health, Pediatric Surgery, Izumi, Japan
ABSTRACTS, Page 232

POC007 **Characterisation and Evaluation of a GD2-Specific Peptide for Hybrid Imaging Using PET/MR**
Alexander Schramm, University Hospital Essen, Children's Hospital, Essen, Germany
ABSTRACTS, Page 232

POC008 **PET Imaging at Diagnosis as a Predictor of Gross Total Resection and Treatment Outcome in Neuroblastoma**
Wen-Ming Hsu, National Taiwan University Hospital, Pediatric Surgery, Taipei, Taiwan
ABSTRACTS, Page 233

POC009 **Diffusion-Weighted Imaging in Neuroblastoma and Ganglioneuroma - A Pilot Study**
Eline Deurloo, Academic Medical Center (AMC) , Radiology, Amsterdam, The Netherlands
ABSTRACTS, Page 232

POC010 **High-Risk Neuroblastoma Recurrence after High Dose Chemotherapy (HDC) and Autologous Stem Cell Transplantation (ASCT)**
Christelle Dupraz, Institute Gustave Roussy, Pediatric and Adolescent, Villejuif, France
ABSTRACTS, Page 233

POC011 **Characteristics of UK Patients with Relapsed, Intermediate Risk Unresectable, Non-MYCN Amplified Neuroblastoma: A Pilot Study**
Nermine Basta, Newcastle University, Institute of Health and Society, Newcastle upon Tyne United Kingdom
ABSTRACTS, Page 234

POC012 **Tumor Regression Rate in Neuroblastoma Stage 4S: Clinical, Biochemical and Radiological Parameters**
Kathelijne Kraal, Academic Medical Center (AMC), Pediatric-Oncology, Amsterdam, The Netherlands
ABSTRACTS, Page 234

POC013 **123I-MIBG scintigraphy and 18F-FDG-PET(-CT) imaging for diagnosing neuroblastoma: a Cochrane diagnostic test accuracy review**
Gitta Bleeker, Emma Kinderziekenhuis, Amsterdam Medical Center (AMC), Paediatric Oncology Amsterdam, The Netherlands
ABSTRACTS, Page 234

POC014 **Assessment of Bone and Bone Marrow Metastases on 123I-MIBG Scintigraphy versus MRI-STIR in Patients with Stage 4 Neuroblastoma**
Gitta Bleeker, Emma Kinderziekenhuis, Amsterdam Medical Center (AMC), Paediatric Oncology, Amsterdam, The Netherlands
ABSTRACTS, Page 235

POC015 **Neuroblastoma in Children with Sotos and Weaver Overgrowth Syndromes**
Diana Gomez Alcazar, University Children's Hospital of Cologne, Pediatric Oncology, Cologne, Germany
ABSTRACTS, Page 235

POC016 **The Sense and Sensibility of Philadelphia Score" in Stage 4S Neuroblastoma Patients**
Kathelijne Kraal, Academic Medical Center (AMC), Pediatric-Oncology, Amsterdam, The Netherlands
ABSTRACTS, Page 235

POC018 **Lungs Involvement in Patients with Neuroblastoma at Diagnosis and at Therapy Failure**
Aleksandra Wieczorek, Polish-American Institute of Pediatrics, Jagiellonian University Collegium Medicum Krakow Oncology and Hematology, Krakow, Poland
ABSTRACTS, Page 236

POC019 **Diagnostic and Prognostic Significance of Urinary Catecholamine Metabolites and n-myc Gene Amplification in Neuroblastoma**
Rachna Kapoor, All India Institute of Medical Sciences, New Delhi, Pediatric Surgery, New Delhi, India
ABSTRACTS, Page 236

POC020 **The Biological Characteristics and Treatment Results of Patients with Neuroblastoma in Belarus**
Inna Praliaskouskaya, Belarusian Center for Pediatric Oncology, Hematology and Immunology, Oncological-hematological Department # 2, Minsk, Belarus
ABSTRACTS, Page 236

POC021 **Is it Feasible to Improve Quality of Imaging Studies in Local Sites for NB Staging by Central Review Nowadays? : The Spanish NB Colllaborative Network.**
Adela Cañete, Polytechnic University Hospital La Fe, Oncology and Pediatrics, Valencia, Spain
ABSTRACTS, Page 236

POC022 **Definitions of Bone and Bone Marrow Metastases Used in Diagnostic Imaging in Patients with Neuroblastoma: A Systematic Review**
Gitta Bleeker, Emma Kinderziekenhuis, Amsterdam Medical Center (AMC), Paediatric Oncology Amsterdam, The Netherlands
ABSTRACTS, Page 237

POC023 **The Characteristics of Mediastinal Neuroblastoma in Childhood**
Ying Liu, The Forth Affiliated Hospital of China Medical University, Paediatrics, Shenyang, Liaoning, China
ABSTRACTS, Page 237

POC024 **Muscle Involvement in Neuroblastoma with Spontaneous Regression**
Olga Moser, University Hospital Bonn, Paediatric Haematology and Oncology, Bonn, Germany
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POC025 **Infant Stage4S Neuroblastoma with MYCN Amplification**
Youichi Haga, Toho University, Pediatrics, Tokyo, Japan ABSTRACTS, Page 238

Clinical Research: Experimental Therapies in Patients

POC026 **Accelerating Drug Development for Neuroblastoma: Summary of the New Drug Development Strategy Project Workshop from the Innovative Therapies for Children with Cancer (ITCC), European Network for Cancer Research in Children and Adolescents (ENCCA) and I**
Lucas Moreno, The Royal Marsden NHS Foundation Trust & Institute of Cancer Research, Paediatric Drug Development, Children and Young People's Unit, Sutton United Kingdom ABSTRACTS, Page 238

POC027 **Phase I Trial of Anti-GD2 Humanized 3F8 Monoclonal Antibody (MAb) Combined with Subcutaneous Interleukin-2 (scIL2) in Patients with Relapsed Neuroblastoma or Other GD2-Positive Solid Tumors**
Stephen Roberts ,Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States ABSTRACTS, Page 238

POC028 **A Phase I Study of Buthionine Sulfoximine (BSO) and Melphalan (L-PAM) with Autologous Stem Cell Support for Recurrent/Resistant High Risk Neuroblastoma: A New Approaches to Neuroblastoma Therapy Study**
Judith Villablanca, Saban Research Institute, Children's Hospital Los Angeles and Keck School of Medicine of the University of Southern California, Pediatrics, Los Angeles, CA, United States ABSTRACTS, Page 239

POC029 **Irinotecan and Temozolamide Chemotherapy as the Treatment of Progressive and Therapy Resistant Neuroblastoma - Experience of Polish Pediatric Solid Tumors Group**
Aleksandra Wieczorek, Polish-American Institute of Pediatrics, Jagiellonian University Collegium Medicum Krakow, Oncology and Hematology, Krakow, Poland ABSTRACTS, Page 239

POC030 **Parvovirus H-1 Induces Oncolytic Effects in Human Neuroblastoma - First Clinical Experience in a Compassionate Use Application to a Neuroblastoma Patient**
Jeannine Lacroix, Heidelberg University Hospital, Pediatric Hematology, Oncology, and Immunology, Heidelberg, Germany ABSTRACTS, Page 239

POC031 **Feasibility of Delayed Local Control Treatment in Patients with High Risk Neuroblastoma: Report of a Pilot study from the Japan Neuroblastoma Study Group (JNBSG)**
Hiroyuki Shichino, Nihon University School of Medicine / Japan Neurobalstoma Study Group, Department of Pediatrics, Tokyo, Japan ABSTRACTS, Page 240

POC077 **Pharmacokinetics & Pharmacogenetics of Isotretinoin in Indian Patients with High Risk Neuroblastoma and its Impact on Survival**
Purna Kurkure, Tata Memorial Hospital, Pediatric Oncology, Mumbai, India ABSTRACTS, Page 240

Clinical Research: Immunotherapy

POC032 **Phase I study of anti-GD2 humanized 3F8 (hu3F8) monoclonal antibody (MAb) plus granlocyte-macrophage colony-stimulating factor (GM-CSF) in patients with relapsed high-risk neuroblastoma (HR-NB)**
Brian Kushner, Memorial Sloan-Kettering Cancer Center, Pediatrics, New York, NY, United States ABSTRACTS, Page 241

POC033 **A Comprehensive Safety Trial of Chimeric Antibody 14.18 (ch14.18) with GM-CSF, IL-2 and Isotretinoin in High-Risk Neuroblastoma Patients Following Myeloablative Therapy: A Children's Oncology Group Study**
Mehmet Ozkaynak, New York Medical College, Pediatric Hematology/Oncology, Hawthorne, NY, United States ABSTRACTS, Page 241

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PL001

Regulation of the Nuclear Hormone Receptor Family by MYCN-Driven miRNAs Affects Differentiation and Survival in Neuroblastoma

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Background: Neuroblastoma is the most common extra-cranial solid tumor in children and arises from neural crest cells involved in the development of sympathetic nervous tissue. This childhood tumor is characterized by heterogeneity and amplification of the MYCN oncogene is highly associated with aggressive tumors and poor outcome. The aim of this study was to investigate the role of the MYCN-driven miR-17-92 cluster in neuroblastoma pathogenesis.

Methods: We have used in silico analysis of predicted NHRs targeted by the miR 17-92 cluster. we have also used TaqMan-based arrays and RT-PCR to evaluate expression levels of NHR family members in a neuroblastoma cell line stably expressing an inducible miR-17~92 cluster. Immunoblotting in MYCN or miR17-92-inducible cell lines as well as luciferase assays using 3'UTR reporters.

Results: In order to identify putative targets of miR-17-92 we performed in silico prediction analysis and found that miR-17-92 target sites are significantly enriched in the nuclear hormone receptor (NHR) super family, indicating a role for hormonal regulation in neuroblastoma tumorigenesis. Importantly, high expression of several of the NHRs correlated with increased event-free survival of neuroblastoma patients. By using non-MYCN amplified neuroblastoma cells with a tet-regulatable miR-17~92 we could verify a differentially expressed NHR profile in induced compared to uninduced cells. Interestingly, one of the most significantly dowregulated NHRs was the glucocorticoid receptor (GR). Using luciferase reporter assays containing the wildtype or mutated 3'UTR from the gene encoding GR we demonstrate that it is a direct target of miR-17~92. Importantly, both MYCN and miR17~92 downregulate GR in neuroblastoma cells. We further show that activation of GR signaling by dexamethasone induces differentiation markers and contributes to neural differentiation.

Conclusion: Taken together, our findings indicate an important role for miR-17-92 cluster in regulation of NHRs in neuroblastoma biology, with important implications for future therapeutic approaches in patients with MYCN-amplification.

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PL002

MYCN Alters p53 Chromatin Binding and Modulates p53 Target Gene Activation via Direct MYCN-p53 Binding- a Novel Tumorigenic Mechanism

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Background: Repression of p53 is essential for neuroblastoma tumorigenesis. Through integration of data from MYCN ChIP-Seq and p53 ChIP-Seq experiments with RNA-Seq based transcriptome analysis of p53 activation, we have identified a novel mechanism of MYCN-mediated inhibition of p53 function.

Methods: The MDM2 inhibitor Nutlin-3a was used to activate p53 transcription without genotoxic damage in a MYCN conditional cell line (Tet-On). Gene expression was evaluated by RNA-seq (Hi-Seq Platform) under all four conditions (i.e. MYCN-/Nutlin-, MYCN-/Nutlin+, MYCN+/Nutlin- and MYCN+/Nutlin+). MYCN ChIP-Seq and p53 ChIP-Seq data sets where used to evaluate MYCN and p53 binding sites in the promoters of regulated genes. Promoter occupancy and RNA-Seq gene expression data were validated with ChIP-qPCR (chromatin immunoprecipitation-qPCR) and q-PCR. Co-immunoprecipitation with MYCN-GST fusion constructs where used were used to evaluate MYCN-p53 interactions.

Results: We found 264 genes that were significantly modulated by p53 in all 4 sets (161 down and 103 up-regulated) (FDR < 0.001, FPKM ≥ 1). Gene Set Enrichment Analysis (GSEA) and gene functional annotation (http://david.abcc.ncifcrf.gov/) reveal that MYCN reduced expression of p53 pathway genes involved in apoptosis and tumor suppression and increased expression of cell cycle progression and mitosis pathways. Most of the genes directly activated by p53 (and repressed by MYCN) had both E-boxes and p53-binding sites closely associated in their promoters. Furthermore, ChIP-qPCR confirmed marked increase in promoter occupancy for both MYCN and p53 at these target genes. Co-immunoprecipitation with MYCN-GST constructs confirmed a strong binding interaction of the C-terminal domain of MYCN with the p53 protein.

Conclusion: RNA-Seq demonstrates that MCYN modulates p53 activity. We have discovered that MYCN directly binds to p53 and greatly enhances promoter occupancy at a subset of p53 target genes regulating apoptosis and cell growth. As increased binding is associated with decreased transcription, we propose that MYCN directly inhibits p53 transcriptional activity at these sites, perhaps preventing p53 phosphorylation or association with transcriptional co-activators.

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PL003

Identification and Pharmacological Inactivation of the MYCN Interactome as a Novel Treatment Approach for High-Risk Neuroblastomas

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Background: A major driver of the aggressive behaviour of high-risk neuroblastomas is the activation of the proto-oncogene and transcription factor MYCN. Small molecule inhibitors of transcription factors such as MYCN are very difficult to develop. We therefore planned to systematically identify the "druggable" genes interacting physically or functionally with MYCN crucial for its tumourigenic effects.

Methods: We used a genome-wide shRNA screen to identify genes synthetically lethal to MYCN (Figure 1). MYCN amplified and non-amplified cell lines were used in cell proliferation/death assays.

Results: We have recently shown that MYCN physically associates with EZH2 causing epigenetic repression of tumour suppressor genes in neuroblastoma cells. We hypothesized that the EZH2-specific inhibitor GSK126 could induce synthetic lethality in neuroblastoma cells with amplification of MYCN by re-activating tumour suppressor genes. Preliminary experiments support this hypothesis; GSK126 induced about 50% reduction of metabolic activity and death in MYCN amplified, but not in non-MYCN amplified cell lines. To find further genes whose inactivation is synthetically lethal to MYCN, we also used a global approach. We carried out a genome-wide shRNA screen and selected for further analysis AHCY, BLM, PKMYT1 and CKS1B because these genes are (a) directly regulated by MYCN and associated with poor prognosis of neuroblastoma patients and (b) their products are druggable by small molecule compounds. Cocktails of small molecule inhibitors of CKS1B, AHCY, BLM and PKMYT1 led to selective death of MYCN-amplified neuroblastoma cell lines.

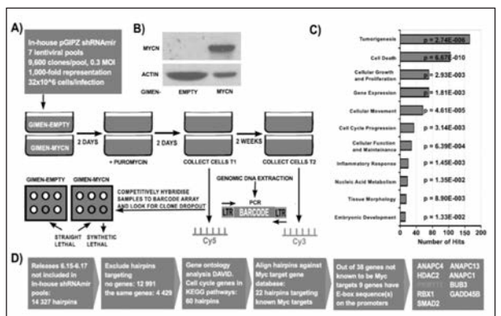

Figure 1. Whole-genome shRNA screening to identify genes synthetic lethal to MYCN. A) Schematic representation of the negative shRNA screen. Changes in the amount of an individual shRNA were determined by a competitive hybridization between PCR products, that included barcodes, labelled with Cy5 fluorescent dye at time point T1 and the Cy3 dye at time point T2. B) Western Blot showing expression of MYCN in GIMEN cells transfected with the MYCN expression vector and its isogenic control cell line transfected with empty pcDNA3 vector. C) Biological functions of the genes identified after the screening using the IPA software. Statistical significance of the enrichment of shRNAs in each Biological process is indicated by the p values. D) Further releases of the Open Biosystems library were used to identify shRNAs of potential MYCN target genes using a bioinformatic approach.

Conclusion: Our findings suggest that drugging the MYCN interactome is a promising avenue for the treatment of high-risk, MYCN-amplified neuroblastomas.

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PL004

The Landscape of Fusion Transcripts in Neuroblastoma

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Background: Chromosomal translocations and fusion genes have been underappreciated in solid tumors, including neuroblastoma. Massive parallel RNA-sequencing provides a tool to systematically discover expressed fusion transcripts resulting from genomic rearrangements.

Methods: We analysed 498 primary neuroblastomas by paired-end RNA-sequencing of 30.8 billion reads. We used two bioinformatic approaches to identify fusion transcripts in neuroblastoma and validated these by RT-PCR and Sanger sequencing.

Results: A total of 89 fusion transcripts were validated in 71 neuroblastomas. We found that 36 fusion transcripts were in-frame and might encode activating fusion proteins, while 48 fusion transcripts were out-of-frame. In five cases, the 5´-UTR of the 5´-fusion partner was coupled to the full reading frame of the 3´-fusion partner suggesting a juxtaposed promoter. Similarly to the heterogeneous mutation spectrum of neuroblastoma, revealed by genomic sequencing, we observed a heterogeneous spectrum of fusion transcripts, with FAM49A-NBAS occurring most frequently (4/89). Yet, several of the affected genes were previously reported to contribute to fusion transcripts in various cancer types including neuroblastoma, e.g. FOXR1. The genomic locations of the genes, suggested that 59 fusion transcripts resulted from intra-chromosomal and 30 transcripts from inter-chromosomal rearrangements. Thirty-six fusion partners were located on opposite strands implying inversion events. We observed that fusion partners were not randomly distributed across the genome, but occurred most frequently on chromosomes that are regularly affected by losses and gains in neuroblastoma, i.e. chromosome 2 (22.4%), 11 (8.9%) and 17 (17.4%). Finally, we noticed that the presence of fusion transcripts was associated with unfavorable prognostic markers such as stage 4, age >18 months, MYCN amplification and adverse patient outcome (p<0.001 each).

Conclusion: We here provide a comprehensive survey on fusion transcripts occurring in neuroblastoma, detected by RNA-sequencing. Spectrum of the involved genes is heterogeneous, several may represent passenger events and we hypothesize that individual fusion transcripts may contribute to neuroblastoma pathogenesis.

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PL005

The Long Intergenic Noncoding RNA LINC00340 is a Neuroblastoma Susceptibility Gene

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Background: Our genome-wide association study previously identified a cluster of SNPs on chromosome 6p22 that correlate with a significantly increased risk of developing of high-risk disease (p<1e-15). These polymorphisms fall within a long intergenic non-coding RNA (LINC00340) termed cancer-associated susceptibility candidate 15 (CASC15), which we hypothesize plays a critical role in the development of high-risk neuroblastoma through dysregulation of neuronal growth and differentiation pathways.

Methods: Illumina arrays were used to genotype 2101 neuroblastomas and 4202controls, genome-wide imputation was performed using IMPUTE2. CASC15 expression from patient samples (n=251) was obtained via Affymetrix HuEx1.0ST arrays; cell line expression was assessed by qRT-PCR. CASC15 isoform sequences were validated through via 5'/3' RACE, and subcellular localization was visualized using RNA-FISH. CASC15 depletion was achieved using siRNA and shRNA constructs, and cell viability and growth were measured using CellTiter-Glo and Xcelligence platforms. Gene expression analyses of neuroblastoma cell lines stably silenced for CASC15 was accomplished through hybridization to HTA2.0 arrays, followed by gene set enrichment and Ingenuity pathway analysis.

Results: Patients with high-risk neuroblastoma express significantly less CASC15 than do those with low-risk disease (p<0.0001), and CASC15 expression inversely correlates with overall survival (p<0.0001). CASC15 expression varies amongst neuroblastoma cell lines (n=21), and is predominantly nuclear. Depletion of CASC15 increases substrate-dependent adherent cellular growth, but has no effect on overall cell number or viability. In contrast, transient ectopic overexpression of CASC15 is not tolerated in multiple cell models. Cells with stable CASC15 depletion show overt morphological changes indicative of a reduced proneural phenotype. Gene set expression and pathway analyses confirm that these cells undergo a significant downregulation of neuronal markers, while upregulating cell adhesion molecules.

Conclusion: These data suggest CASC15 may be essential for proper neuronal development, and that loss of CASC15 leads to a poorly differentiated cell type, thereby contributing to the tumorigenesis of high-risk neuroblastoma.

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PL006

Anticancer Compound CBL0137, that Simultaneously Suppresses NFkB and Activates p53, is Highly Effective at Treating Neuroblastoma in Two Independent Mouse Models

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Background: CBL0137 is a carbazole-based anti-cancer agent with a unique mechanism of action. It is an indirect inhibitor of the chromatin remodeling complex FACT (Facilitates Chromatin Transcription). Inhibition of FACT by CBL0137 modulates the activity of several transcription factors involved in cancer: NF-kB and HSF1 are suppressed, while p53 is activated (Science Transl. Med, 2011). We have examined FACT expression in neuroblastoma as well as the efficacy of CBL0137 in preclinical models of this disease.

Methods: Expression profiles of 650 primary untreated neuroblastomas were analyzed for the expression of two FACT subunits, SSRP1 and SPT16, and related to clinical outcome. Colony-forming assays were used to study the effect of CBL0137, either alone or combined with chemotherapeutic drugs in human MYCN-amplified neuroblastoma cell lines. Cohorts of homozygous TH-MYCN mice with small palpable tumours, or BE(2)-C xenografted nude mice, were treated with CBL0137, alone or combined with chemotherapeutic drugs.

Results: High levels of two FACT subunits were associated with MYCN amplification, and were strongly predictive of poor neuroblastoma outcome ($p<0.0001$). As a single agent, CBL0137 administered iv had remarkable anti-tumour activity in both mouse models. CBL0137 synergized strongly with a variety of chemotherapeutic agents, including vincristine, cisplatin, etoposide and cyclophosphamide, both in vitro in colony forming assays and in vivo, in TH-MYCN tumour-bearing mice. Most dramatic results were observed when CBL0137 was combined with cyclophosphamide/topotecan, a highly active therapy for relapsed neuroblastoma that is currently in clinical trial for newly diagnosed patients. Cylophosphamide/topotecan plus CBL0137 resulted in cure of 90% of tumour-bearing MYCN-transgenic mice and a doubling of the lifespan of BE(2)-C xenografted nude mice. CBL0137 also synergized strongly with the alternate relapse backbone, irinotecan/temozolomide.

Conclusion: These results are superior to any combination chemotherapy regimens we have tested in these models, and a Phase I COG trial of CBL0137 in refractory pediatric cancer patients is currently being planned.

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PL007

Whole Genome Sequencing of Relapse Neuroblastoma Identifies the RAS-MAPK Pathway as a Potential Therapeutic Target in Neuroblastoma

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Background: Neuroblastoma relapses are very aggressive and no curative regimen is known for these refractory tumors.

Methods: We performed whole genome sequencing for six triplets of primary neuroblastoma tumors, corresponding relapses and normal tissue.

Results: The relapses consistently show more aberrations than the primary tumors. Some mutations are conserved from primary tumor to relapse, while others that are observed in the primary tumor are not present in the relapse. This indicates that clonal evolution occurs and that secondary mutations take place in the formation of neuroblastoma relapses., Analysis revealed several relapse specific events which have also been reported in primary neuroblastoma tumors in low frequency. Two relapses show strong enrichment for point mutations in ALK and PTPN11. Three other tumors show de novo structural aberrations in NF1 (homozygous deletion), ALK (amplification)and BRAF (truncated protein). All these genes affect the RAS-MAPK signaling pathway which suggests involvement of this pathway in neuroblastoma tumor evolution. This hypothesis is strengthened by WGS of a panel of 25 neuroblastoma cell lines which are mainly derived from pretreated or relapsed neuroblastoma tumors. A high frequency of mutations in genes affecting RAS-MAPK signaling is found and the presence of these mutations in a cell line is associated with sensitivity to the MEK inhibitor GSK1120212., Currently we are validating the relevance of these mutations in neuroblastoma cell lines. For the ALK mutation and the NF1 deletion we could indeed show activation of the RAS-MAPK pathway which is accompanied by an increase in colony forming capacity in soft agar.

Conclusion: We found a high frequency of mutations that affect the RAS-MAPK pathway in relapsed neuroblastoma tumors, which suggests involvement of this pathway in the aggressive phenotype of neuroblastoma relapses. This warrants further validation of the RAS-MAPK pathway as a potential therapeutic target in relapsed neuroblastoma.

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PL008

Detection of PHOX2B and TH mRNA by RTqPCR in Peripheral Blood Stem Cell Harvests Predicts Relapse in Children with Stage 4 Neuroblastoma; a SIOPEN Study.

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Background: The purpose of this study was to evaluate the predictive power of paired-like homeobox 2b (PHOX2B), tyrosine hydroxylase (TH) and doublecortin (DCX) mRNAs detected by reverse transcriptase quantitative polymerase chain reaction (RTqPCR) in peripheral blood stem cell harvests (PBSC) from children with stage 4 neuroblastoma entered into the European trial HR-NBL-1/SIOPEN (www.SIOPEN-R-NET.org).

Methods: RTqPCR was performed for PHOX2B, TH and DCX mRNA in PBSC (n=314). Samples were collected and analysed according to standard operating procedures. Quality control was maintained across reference laboratories by the bi-annual analysis of blind control samples (Viprey VF, Corrias MV, Kagedal B, et al. Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET. Eur J Cancer 2007;43:341-50).

Results: When mRNA is considered a binary variable the detection of TH or PHOX2B mRNA in PBSC is predictive of relapse-free survival (RFS; log rank test $p=0.04$ and $p=0.02$ respectively) and overall survival (OS; $p=0.05$ and $p=0.0001$ respectively), with children in which TH or PHOX2B mRNAs are detected having a worse outcome than those in which these mRNAs are not detected. The presence of TH or PHOX2B mRNA predicted RFS of 38% and 21% respectively at 3 years, compared to that of 47% and 49% in children where these mRNAs were not detected. In contrast the detection of DCX mRNA was not predictive of RFS (log rank test $p=0.26$) or OS (log rank test $p=0.48$). In a multivariable cox regression model, treating mRNAs as continuous variables, only PHOX2B mRNA was predictive of RFS ($p=0.03$) and overall survival ($p<0.0001$).

Conclusion: PHOX2B and TH mRNA detected by RTqPCR in PBSC from children with stage 4 neuroblastoma predicts RFS and OS. The presence of DCX mRNA was not predictive and does not warrant inclusion in future studies.

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PL009

The PLK-1 Inhibitor GSK461364 Has a Strong Antitumoral Activity in Preclinical Models of Neuroblastoma

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Background: PLK-1 is a serine/threonine (ser/thr) kinase involved in cell cycle regulation at the G2/M transition, and PLK-1 is overexpressed in high risk neuroblastoma. Recently, we and others demonstrated that PLK-1 is a potential drug target in neuroblastoma, and reported antitumoral actvity of the PLK-1 inhibitors BI2536 and BI6727 in neuroblastoma preclinical models. We here analyzed the antitumoral activity of ATP-competitive PLK-1 inhibitor GSK461364.

Methods: We analyzed the effect of GSK461364 on 6 established neuroblastoma (NB) cell lines. We performed cell viability analysis (MTT), cell cycle profiling and analyzed markers for apoptosis and proliferation after 72h-treatment at 10pM – 1µM GSK461364 or IC50, IC80 and 5x IC50, respectively. In vivo efficacy of GSK461364 was assessed in SKNAS xenografts, treatment was started after tumor volume reached 200µl.

Results: Treatment of neuroblastoma cells with GSK461364 resulted in decreased cell viability. The concentrations of 50% inhibition (IC50) ranged between 7 and 20 nM in MYCN single copy as well as in MYCN-amplified cell lines. Treatment resulted in massive induction of apoptosis and reduced proliferation. GSK461364 treatment also resulted in an increase in the percentage of cells in G2/M phase, as determined by flow cytometry. In vivo treatment with GSK461364, significantly decreased tumor burden after 26 days, induced significant tumor regression and significantly prolonged survival as compared to vehicle-treated mice. Of note, after discontinuation of treatment, relapse and fatal tumor progression was observed in all analyzed mice.

Conclusion: These preclinical findings highlight the promise of PLK-1 inhibitors as novel agents for neuroblastomas and serve as rationale to move forward with early phase clinical trials in children. We are currently analyzing which other compound may synergize with PLK-1 inhibitors to prevent relapse after discontinuation of treatment.

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PL010

Evaluation of Clinical Response and Survival Following Long-Term Infusion of Anti-GD2 Antibody ch14.18/CHO in Combination with Subcutaneous Interleukin-2 in a Single Center Treatment Program in High-Risk Neuroblastom

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Background: Immunotherapy with monoclonal anti-GD_2 antibody (mAb) ch14.18 in combination with cytokines prolonged survival in high risk neuroblastoma (NB) patients. We piloted a less toxic treatment using long-term infusion (LTI) of ch14.18/CHO in combination with subcutaneous (s.c.) interleukin-2 (IL-2) and report initial clinical response and survival.

Methods: 53 high-risk NB patients received up to 6 cycles of $6x10^6$ IU/m^2 s.c. IL-2 (d1-5; 8-12), continuous infusion of 100 mg/m^2 ch14.18/CHO (d8-17) and 160 mg/m^2 oral 13-cis-RA (d19-32). We established validated PCR-based methods to analyze KIR, FCGR2A (H131R), -3A (V158F) and -3B (NA1/NA2) polymorphisms which are associated with response to some mAbs. Clinical response was assessed by mIBG, MRI/CT, bone marrow- and catecholamine- analysis before, after 2/3 and after 5/6 cycles of immunotherapy in patients with measurable disease.

Results: Response rates were 41.7 % in mIBG (15/36), 31.8 % MRI/CT (7/22), 28.6 % bone marrow- (6/21) and 38.1 % in catecholamine- (8/21) measurements. External review of mIBG responses of 28 patients confirmed a 32.1% response rate (9/28). The overall response following INRG guide lines was determined at 30% (12/40). The event-free-survival rate in the entire cohort was 32.4 % (observation 0.1 - 3.2 years, mean: 1.6 years) and an overall-survival rate of 66.8% (observation 0.3 - 3.9 years, mean: 3.1 years). Interestingly, patients with KIR/KIRL mismatch and high affinity FCGR alleles showed a trend towards longer event-free survival ($P = 0.08$), which supports NK cell mediated antibody dependent cytotoxicity as the mechanism of action.

Conclusion: The use of ch14.18/CHO in the LTI setting in combination with s.c. IL-2 and 13-cis-RA shows clinical activity in NB patients. Furthermore, analysis of KIR/KIRL-mismatch and FCGR-polymorphisms by genotyping may predict clinical outcome. This single center treatment program was supported by SIOPEN and is subject to prospective analysis in a SIOPEN Phase II study (EudraCT: 2009-018077-31).

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PL011

A Randomized Clinical Trial of Cyclophosphamide and Prednisone with or without Intravenous Immunoglobulin (IVIG) for the Treatment of Neuroblastoma Associated Opsoclonus Myoclonus Ataxia Syndrome (OMA): A Children's Oncology Group Trial

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Background: To determine if cyclophosphamide and prednisone (CP) is an effective treatment of OMA and if the addition of IVIG improves the response to cyclophosphamide and prednisone. , Background: OMA, an immunologically mediated paraneoplastic syndrome, affects 2-3% of children with neuroblastoma. Most children have low-stage neuroblastoma and survive their tumor but are handicapped by neurological sequelae. Steroid immunosuppression has been the established treatment for this disorder with other immunosuppressants reported as effective in case reports or case series. We report here preliminary data on the only randomized clinical trial for this disorder.

Methods: Children age ≤8 years, newly diagnosed with neuroblastoma associated OMA, were randomized to receive six monthly treatments of IVIG (1 gm/kg) with CP (Rx1) or six monthly treatments of CP alone (Rx2). Children with intermediate or high risk neuroblastoma that required chemotherapy for their tumor received stage-specific chemotherapy instead of cyclophosphamide. The best overall OMA response was selected from evaluations at 2 months, six months and one year using a standardized OMA response scale evaluating stance, gate, arm and leg function, opsoclonus, and mood/behavior. Patients who crossed over from Rx2 to Rx1 were considered OMA non-responders.

Results: 53 eligible subjects were enrolled, 26 on Rx1 and 27 on Rx2. Thirty-three of 52 evaluable patients responded to therapy, 21 on Rx1 (81%) and 12 on Rx2 (46%)($p=0.0096$). Eleven patients in Rx2 crossed over to Rx1 and were automatically considered non-responders. Eighteen subjects had an OMA relapse requiring further treatment. One subject died of infection after high-dose chemotherapy and autologous stem cell recue for high risk neuroblastoma. The 3-year disease (OMA) free survival is 60.9±7.9%.

Conclusion: The addition of IVIG to CP significantly improved the short-term response over CP alone Follow up is ongoing to determine the long-term disease (OMA) free survival rate and the long-term neurological outcome of OMA.

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PL012

Influence of Surgical Excision on Survival of Patients with High Risk Neuroblastoma. Report from Study 1 of Siop Europe (Siopen)

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Background: Purpose: To evaluate the influence of operation on the survival of patients with High-Risk Neuroblastoma: (INSS stages 2, 3, 4, 4s with MYCN amplification and INSS stage 4 age > 12 months.

Methods: All patients were offered operation after an intensive platinum based induction protocol. There-after patients received myeloablative therapy, stem cell rescue, radiation to the primary site and minimal residual disease therapy with 13 cis-retinoic acid. A small number in this cohort received immunotherapy. The result of surgical excision was defined as: macroscopic complete (CE), macroscopic incomplete, (IE) or not attempted (0E). Data were collected on death or complication

related to operation, overall and event free survival. Survival was related to the completeness of surgical excision.

Results: 1324 operation data sets were analysed. Operation related mortality was 0.5%, morbidity 10%. (CE) was achieved in 76%, (IE) in 23% and 2% were inoperable (0E). 5 year Event Free Survival (EFS) for all patients was 38% (OS 44%). 5 year EFS for 1002 patients with (CE) was 38% (OS 44%) compared with 27% (OS 36%) for patients with (IE) and (0E) combined (EFS p=0.001; OS p=0.013). 5 year EFS for 895 Stage 4 patients with (CE) was 33% (OS 40%) compared with 24% (OS 33%) for patients with (IE) and (0E) combined (EFS p=0.006; OS p= 0.049). 5 year EFS was 43% (OS 42%) for patients who did not suffer operative complications compared with 39% (OS 37%) for those who did (EFS p=0.030; OS p=0.010).

Conclusion: Macroscopic completesurgical excision of High-Risk Neuroblastoma is safe and confers a survival advantage to patients treated with intensive multi-modality therapy.

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PL013

Update of Outcome for High-Risk Neuroblastoma Treated on a Randomized Trial of chimeric Anti-GD2 Antibody (ch14.18) + GM-CSF / IL2 Immunotherapy in 1st Response: A Children's Oncology Group Study

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Background: Anti-GD2 mAb ch14.18 +cytokines has shown efficacy in a randomized phase III study, COG ANBL0032. Newly diagnosed high-risk NB patients who achieved a CR or PR to induction therapy and received stem cell transplant were randomized to isotretinoin x 6 cycles or isotretinoin x 6 with 5 concomitant cycles of ch14.18+ GM-CSF or IL2 (immunotherapy). In 2009, an interim analysis of the initial 226 randomized patients showed that event-free survival (EFS) and overall survival (OS) were significantly higher for patients randomized to immunotherapy (p=0.01, and 0.02, respectively). The interim monitoring boundary for large early benefit of immunotherapy was met and randomization halted.

Methods: An intent-to-treat comparison of randomized treatment groups was performed using a 2-sided log rank test for EFS and OS. The outcome data for the randomized patients were updated to within one year of June 30, 2012.

Results: One of the 226 randomized patients was subsequently deemed ineligible, leaving 225 analyzed herein. Four patients crossed over from the isotretinoin arm to receive immunotherapy after randomization was halted, and were censored at the start of antibody therapy. The median follow-up time for patients alive without an event is 5.5 years. The updated EFS (± standard error) for immunotherapy was 67±4%(2-year) and 59±5%(4-year) versus 51±5%(2-year) and 48±5%(4-year) for isotretinoin alone (p= 0.11). The updated OS was significantly better for immunotherapy (2-year:83±4%; 4-year:74±4%) than isotretinoin alone (2-year:76±4%; 4-year:59±5%)(p=0.02). For stage 4 patients (N=180), EFS was 64±5%(2-year) / 54±5%(4-year) versus 45±5%(2-year)/44±5%(4-year)(p=0.1); OS was 83±4%(2-year)/72±5%(4-year) versus 75±5%(2-year)/56±5%(4-year)(p=0.02) for immunotherapy and for isotretinoin, respectively. For 25 patients who were non-randomly assigned to immunotherapy for biopsy-proven residual disease, 4-year EFS and OS were 32±9% and 53±11%. Peak anti-α-Gal antibody levels were higher for patients with allergic reactions than those without (p=0.03 one-sided).

Conclusion: Immunotherapy with mAb ch14.18 and cytokines following frontline therapy and autologous stem cell transplant significantly improves survival of patients with high-risk neuroblastoma but late relapses are observed.

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PL014

Long-Term Infusion of ch14.18/CHO Combined with s.c. Interleukin-2 Applied in a Single Center Treatment Program Effectively Stimulates Anti-Neuroblastoma Activity with Reduced Pain in High-Risk Neuroblastoma Patients

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Background: Immunotherapy with anti-GD2 mAb ch14.18/CHO combined with interleukin-2 (IL-2) is a treatment option for high-risk neuroblastoma (NB) patients. We developed a 10 day long-term infusion (LTI) of ch14.18/CHO in combination with subcutaneous (s.c.) IL-2 in order to improve the toxicity profile.

Methods: 53 NB patients received up to 6 cycles of $6x10^6$ IU/m^2 s.c. IL-2 (d1-5; 8-12), continuous infusion of 100 mg/m^2 ch14.18/CHO (d8-17) and 160 mg/m^2 oral 13-cis-RA (d19-32). Usage of i.v.-morphine to treat neuropathic pain was analyzed over time aiming for a pain free treatment schedule as determined by established pediatric pain scoring systems. Immunomodulation was investigated by flow cytometry of immune cells, analysis of cytokine profiles and functional assays (complement-dependent cytotoxicity (CDC); antibody-dependent cellular cytotoxicity (ADCC) and whole blood assay (WBT)). Levels of ch14.18/CHO antibody were determined by validated ELISA.

Results: LTI of ch14.18/CHO translated into a decreasing degree of i.v. morphine usage allowing for treatment in the outpatient setting. The expansion of cytotoxic natural killer cells (3 fold) and T-lymphocytes (2 fold) was observed. At the same time an increase of granulocytes and regulatory T-cells was noted. Increased levels of IL-2, IL-6, IL-8 and particularly IFNγ suggests a pro-inflammatory immune response., The average serum level of ch14.18/CHO was 12.48 ± 0.93 µg/ml at the end of antibody infusion. Serum, cells and blood of patients showed activity against neuroblastoma cells in functional assays (CDC, ADCC, WBT). Interestingly, the determination of antibody levels and functional parameters before subsequent treatment cycles indicate persistent anti-neuroblastoma activity measurable for the entire treatment period of 6-7 months.

Conclusion: LTI of ch14.18/CHO combined with s.c. IL-2 showed an interesting toxicity and activity profile resulting in long lasting anti-neuroblastoma effects. This single center treatment program was supported by SIOPEN and is subject of evaluation in a prospective SIOPEN Phase II study (EudraCT: 2009-018077-31).

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PL015

Impact of Pre-immunotherapy Curie Scores on Survival Following Treatment with an Anti-GD2 Antibody (ch14.18) and Isotretinoin in High-risk Neuroblastoma

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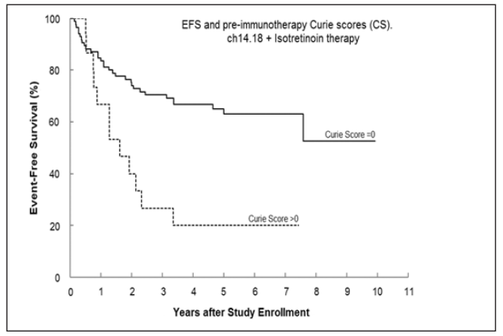
Background: Immunotherapy with anti-GD2 antibody (ch14.18) plus isotretinoin (RA) is associated with improved outcome in patients with high risk neuroblastoma. We now examine the impact of Curie scores (CS) as a predictor of outcome following treatment with RA±ch14.18.

Methods: 197 patients with high risk neuroblastoma enrolled on COG ANBL0032 were examined. Eligible patients were in complete (CR) or partial (PR) remission following induction and consolidation therapy, and had a history of prior MIBG avid disease. Patients were randomized to receive RA(n=97) or ch14.18+RA(n=100). Curie scores were determined from pre-immunotherapy (baseline) MIBG scans, with event-free survival (EFS) determined from time of enrollment.

Results: There was a significant outcome difference according to baseline CS [CS=0 (n=167) vs CS>0 (n=30)], with 3-year EFS 59.2±3.9% vs 33.3±8.6%,p=<0.01. For patients randomized to the ch14.18+RA arm, outcomes were significantly higher for patients with a baseline CS=0 compared to those with a CS>0 (3-year EFS: 70.5±5.0% vs 26.7±11.4%,p<0.001).



For patients randomized to the RA arm, the outcome comparisons were not significant based upon baseline CS (CS=0 vs CS>0), with 3-year EFS 47.5±5.6% vs 40.0±12.6%,p=0.22. For the entire cohort, patients with a CS=0 at baseline faired significantly better when treated with ch14.18+RA than RA, with 3-year EFS 70.5±5.0% vs 47.5±5.6%,p=0.02. There was no survival advantage for ch14.18+RA therapy when baseline CS>0 [3-year EFS: 26.7% (ch14.18+RA) vs 40.0% (RA),p=0.93].

Conclusion: ch14.18+RA is highly effective in patients with CS=0. In contrast, ch14.18+RA may be insufficient therapy for patients with a CS>0, and novel therapeutic strategies should be considered post-immunotherapy for such patients.

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PL016

Treatment with Fenretinide (4-HPR)/Lym-X-Sorb™(LXS) Oral Powder with Ketoconazole Increased Plasma Levels and had Anti-tumor Activity in Patients with High-Risk (HR) Recurrent or Resistant Neuroblastoma (NB): A New Approaches to Neuroblastoma Therapy Study

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Background: Fenretinide (4-HPR) is a cytotoxic retinoid with preclinical activity in neuroblastoma. Previously, 4-HPR/LXS® oral powder obtained higher plasma levels than a capsule formulation but exhibited a PK plateau at higher doses. Two expansion cohorts were undertaken to determine the effect of 1) an unrestricted diet on 4-HPR plasma levels and, 2) if ketoconazole, an inhibitor of 4-HPR metabolism, could increase 4-HPR plasma levels if given concurrently.

Methods: Eligible patients had high-risk neuroblastoma with recurrent or refractory disease, including patients in complete response (CR) after relapse. Treatment was 7 days of 4-HPR at 1500 mg/m^2/day, divided TID, every 3 weeks, in Cohort One (C1), with the addition of 7 days of concurrent ketoconazole at 6 mg/kg/day for Cohort Two (C2).

Results: C1 and C2 accrued 23 (3 never treated) and 22 patients (3 too early), respectively, with 14 and 15 patients, respectively, evaluable for full PK's. There were no DLT's. Grade 3 toxicities included rash, elevated triglycerides, lymphopenia, diarrhea, transient transaminase elevation. 39 patients received median 3 (range: <1-27) courses. In Course 1, median (Q_{Lower}-Q_{Upper}) Day 7, peak 4-HPR plasma micromolar levels were 18.3 (9.6- 21.5) for C2 and 12.8 (9.9-15.8) for C1 (p=0.21); in Course 2 these were 18.4 (13.9-24.8) for C2 and 11.7 (7.9-16.4) for C1 (p=0.025, Wilcoxon test comparison)., Of 16 patients in C1 and 18 patients in C2 with evaluable disease at baseline, 2 patients had complete, 1 partial , and 1 mixed response (MIBG CR), 14 stable disease, 12 progressive disease; 4 did not complete 1st course. Median progression-free survival (PFS) was 4.1 (1.35-36.9+) months.

Conclusion: Ketoconazole given concurrently with 4-HPR/LXS® is well-tolerated and increased 4-HPR plasma levels. Objective responses and encouraging PFS in this heavily pre-treated patient population support evaluation of 4-HPR/LXS® with ketoconazole in future upfront trials of high-risk neuroblastoma.

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PARALLEL SESSIONS

A1: Basic Research/Oncogenesis I

OR001

Activated SHP2 Synergizes MYCN in Neuroblastoma Tumorigenesis

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Background: Neuroblastoma, an embryonic tumor of peripheral sympathetic nervous system (PSNS), accounts for 10% of all childhood cancer deaths. We recently developed a robust zebrafish model of neuroblastoma and demonstrated that activated ALK synergizes with MYCN by inhibiting a developmentally-timed apoptotic response that is otherwise induced by MYCN. Genomic studies show that PTPN11 (encoding SHP2) is the second most frequently mutated genes in high-risk neuroblastoma. We have now used our zebrafish model to ask whether mutationally activated SHP2 functions as an oncogene in neuroblastoma.

Methods: We developed a transgenic line in which ptpn11^{E69K} is overexpressed in the PSNS under control of the dopamine-beta- hydroxylase promoter.

Results: Ptpn11^{E69K} synergized with MYCN to accelerate the onset of neuroblastoma in the interrenal gland (IRG), the zebrafish analogue of the adrenal medulla. Tumors began to appear at 15 weeks in transgenic fish overexpressing MYCN alone, whereas latency was shortened to 7 weeks and the penetrance was increased more than four-fold in transgenic fish coexpressing MYCN and ptpn11^{E69K}. Coexpression of ptpn11^{E69K} and MYCN also induced ganglioneuroma in the sympathetic ganglia, an outcome that has not been observed in transgenic fish overexpressing MYCN and activated ALK. In addition, coexpression of ptpn11^{E69K} and MYCN induced adrenal neuroblastic hyperplasiain the IRG of all the transgenic fish at 5 weeks of age, a phenotype that has only been observed in about 50% of transgenic fish overexpressing activated ALK and MYCN or MYCN alone.

Conclusion: These results suggest that activated SHP2 can collaborate with MYCN in neuroblastoma tumorigenesis via mechanisms different from those of activated ALK. Thus, zebrafish appears to provide a robust model system for functional genomic analysis and for the investigation of the mechanisms and pathways underlying genetic alterations identified in integrative genomic studies.

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OR002

The Neuroblastoma Oncogene LMO1: Mechanisms for Tumor Initiation and Progression

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Background: SNPs at LMO1, a transcriptional co-regulator, are robustly associated with high-risk neuroblastoma. We sought to define the molecular pathogenesis linking common DNA variants at LMO1 to neuroblastoma susceptibility and oncogenic addiction.

Methods: To discover causal DNA variation, we prioritized imputed SNPs by disease association, transcription factor motif disruption, conservation, DNase hypersensitivity, and then performed allelic-imbalance and reporter assays. To assess the contribution of LMO1 expression to tumorigenesis we compared MYCN, LMO1, or double MYCN-LMO1 transgenic zebrafish. To identify LMO1 regulatory networks, we performed expression profiling and LMO1 knockdown in neuroblastoma cell lines.

Results: SNP rs2168101 G>T had the most significant association with neuroblastoma ($OR_{homozygous\ 0.55}$, $p=6x10^{-21}$) and the minor (protective) TATA allele ablates a highly conserved GATA binding site within a sizeable domain of p300-marked enhancers. The G allele was associated with LMO1 expression in cell lines ($p=0.047$) and tumors heterozygous at this SNP showed allelic imbalance favoring the G allele ($p<0.0005$). In minimal promoter luciferase assays, the G allele demonstrated 10-100 fold higher activity than the T allele. In MYCN zebrafish, co-expression of LMO1 reduced tumor latency (11 to 5 weeks), increased penetrance (15% to 80%), and increased sympathoadrenal hyperplasia in the interrenal gland (homologous to the human adrenal gland, $p<0.0001$). LMO1 targeted shRNAs in Kelly (MYCN addicted) and SHSY5Y (mesenchymal) cells resulted in complete protein depletion, profound cytotoxicity, and a highly specific reversion of the transcriptomic signature defining their respective molecular subclasses.

Conclusion: A common SNP in an intronic super-enhancer of LMO1 promotes GATA binding and acts in cis to increase LMO1 expression, resulting in sympathoadrenal hyperplasia and tumorigenesis. In tumor cells, LMO1 co-regulates transcription factors critical to maintaining the malignant phenotype. Defining the mechanisms by which LMO1 overexpression contributes as a master regulator of signatures that define different subtypes of high-risk neuroblastoma should pinpoint essential pathways for the development of novel targeted therapeutics.

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OR003

ODZ3 Function in Neuroblastoma

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Background: We recently showed by whole genome sequencing of a series of neuroblastoma that the ODZ3 gene is relatively frequently inactivated by small deletions or point mutations. Low ODZ3 mRNA expression was associated with a poor prognosis. ODZ3 belongs to family of highly conserved type II transmembrane proteins (ODZ1-4) with as most prominent site of expression the developing and adult central nervous system. Functional studies have shown that ODZ proteins can stimulate neurite outgrowth and might play a role in axon guidance. So far nothing is known about ODZ3 function in NB and NB neuritogenesis.

Methods: ODZ3 expression was knocked down in SY5Y and SKNBE neuroblastoma cells by inducible shRNA lentiviral silencing system. Inducible transient transfection or lentiviral-mediated transduction was used to over-express HA-tagged ODZ3 constructs in SKNBE cells.

Results: Over-expression of ODZ3 in neuroblastoma cell line SKNBE induced cell differentiation. The ODZ3 protein was expressed in cellular extensions and the cytoplasm. Western-blot analysis showed N-terminal peptides of ODZ3 that could correspond to intermediate processing products and the ODZ3-intracellular domain (ODZ3-IC). Induced expression of ODZ3-IC resulted in nuclear staining, suggesting that this domain may play a role in transcription regulation. Silencing of ODZ3 in neuroblastoma SY5Y and SKNBE cells induced morphological changes. Both cell lines normally respond to retinoic acid by formation of neurite extensions, but silencing of ODZ3 conferred RA-resistance to these cells. While over-expression of ODZ3 thus induced differentiation, silencing conferred resistance to RA-mediated differentiation. These data are in line with a tumor-suppressive role of ODZ3.

Conclusion: Taken together these data show involvement of the ODZ3 gene in regulation of neuroblastoma cells differentiation. Our data suggest that the ODZ3 protein can be proteolytically processed leading to release of the intracellular domain that translocates to the nucleus. Further studies are aiming at the identification of the downstream signaling pathways and function of ODZ3.

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OR004

Role of MYCN Partners (MAX, MXD and MNT) in the Control of MYCN Oncogenicity

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Background: Over 25% of cases of neuroblastoma are characterized by MYCN oncogene amplification. However, many patients without MYCN amplification also have a poor clinical outcome. Importantly, the transcriptional function of MYCN is critically dependent on its partner, MAX, with which MYCN forms a translational active heterodimer. Furthermore, variations in expression levels of other factors involved in the MYCN network (MXD and MNT) can also influence MYCN function. We therefore explored how expression levels of MYCN partners can affect MYCN function and significantly influence the biology of neuroblastoma cells.

Methods: Expression profiles of 251 primary untreated neuroblastomas were analyzed for the expression of MAX, MXD and MNT. Neuroblastoma cell lines were generated to stably overexpress FLAG-tagged MAX, MXD or MNT in different MYCN expression contexts, or alternatively expressing specific shRNAs targeting these genes. Cell clones were assayed for growth rate, differentiation, apoptosis, motility/migration and adhesion.

Results: Low mRNA levels of all MYCN partner genes were predictive of poor neuroblastoma outcome. Following adjustment for MYCN and stage in multivariate analysis, these genes retained independent prognostic significance. The overexpression of MAX, MXD or MNT in MYCN amplified cell lines (SK-N-BE and IMR32) inhibited cell proliferation and migration rate, and induced the expression of specific neuronal differentiation markers. In contrast, MAX, MXD or MNT silencing in cells with low MYCN expression SH-SY-5Y and SHEP) significantly increased cell proliferation and migration rates.

Conclusion: Expression levels of MAX, MXD and MNT strongly impact the proliferation, migration and differentiation rates of neuroblastoma cells. The results demonstrate that the oncogenic potential of MYCN is strongly influenced by the intracellular levels of its partners and that expression levels of these factors may be key determinants of clinical outcome. These findings highlight the possibility of influencing the oncogenicity of MYCN by modulating the intracellular levels of the MAX, MXD or MNT partners.

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OR005

Characterization of the Cre-Conditional, MYCN-Driven DBHiCre; LSL-MYCN Neuroblastoma Mouse Model

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Background: Transgenic mouse models are valuable tools for preclinical studies. We recently established a new, Cre-conditional, MYCN-driven neuroblastoma mouse model by introducing the CAG-LSL-MYCN-IRES-Luciferase vector (LSL-MYCN) into the ROSA26 locus, and crossbreeding these mice with DBH-iCre mice to target MYCN expression to the neural crest. The DBHiCre;LSL-MYCN mice develop neuroblastomas at high incidence. We here report further in-depth characterization of the DBHiCre;LSL-MYCN neuroblastoma mouse model.

Methods: Tumors were harvested, DNA and RNA was prepared for further analysis. DNA was analyzed by aCGH, RNA was analyzed by Affymetrix microarrays. Data was analyzed using arrayCGHbase, R statistical language and gene set enrichement analysis (GSEA). A cell line was established using standard cell culture procedures.

Results: Murine MYCN-driven neuroblastomas were characterized by genomic aberrations syntenic to human neuroblastomas, including 17q gain. In addition, the Rosa26 locus harbouring the transgene was further amplified. Transcriptomes showed patterns of canonical MYCN-related mRNA and miRNA signatures, as revealed by GSEA. The conformity with human neuroblastoma was further supported by the observation that genes, differentially expressed between high and low risk human neuroblastomas, either with or without inclusion of tumors with MYCN amplification, were significantly altered in LSL-MYCN;Dbh-iCre tumors compared to normal adrenals (Kolmogorov-Shmirnov test, $p < 0.001$). To facilitate in vitro analysis, as well as serial transplantation of isogenic tumors into recipient mice, a cell line was established from a tumor derived from a DBHiCre;LSL-MYCN mouse. Further analysis of this cell line revealed high MYCN expression and sensitivity to the MYCN inhibitor JQ1.

Conclusion: High throughput analysis further supported predominance of MYCN signaling in the DBHiCre;LSL-MYCN neuroblastoma model, and cross species analysis revealed that tumors resembled human high risk, MYCN amplified neuroblastoma.

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OR006

SOX11 is a Lineage-Survival Oncogene in Neuroblastoma

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Background: Neuroblastoma (NB) is a cancer of the developing sympatho-adrenergic nervous system accounting for 15% of childhood cancer deaths. Amplification of MYCN is the most frequent oncogenic event, occurring in approximately half of the high-risk tumors. Recently two other amplified oncogenes, ALK and LIN28B, have been identified in NB, albeit at much lower frequencies.

Results: In this study we report a focal high-level amplification at distal 2p of SOX11, which encodes an SRY-related HMG-box transcription factor, and is the only protein-coding gene within the amplification. Recurrent gain was observed in 36% of NB tumours. SOX11 expression levels are significantly positively correlated with SOX11 DNA copy-number levels. Moreover, we demonstrate significant negative correlation between SOX11 expression and overall survival, indicating that SOX11 contributes to tumor aggressiveness. Interestingly, SOX11 is implicated in survival and differentiation programs of the sympatho-adrenergic lineage, and in NB its expression is significantly positively correlated with genes involved in neuronal development and migration. These findings are in line with our data showing that SOX11 is part of a miRNA-mRNA regulatory network, including the master regulator for sympathetic neuronal differentiation, PHOX2B. Also, SOX11 expression levels are high in NB tumors and cell lines as compared to other tumor entities. Taken together, these observations fit with the dependency hypothesis that SOX11 is a lineage-survival oncogene in NB.
To test this hypothesis, we performed in vitro analysis, showing strong effects on colony formation, anchorage-independent cell growth, invasion capacity and differentiation following SOX11 perturbation.

Conclusion: Using gene expression profiling and subsequent data mining we established a SOX11-driven oncogenic transcriptome in NB cells. Finally, in vivo studies are ongoing including mouse xenografts of SOX11 overexpressing SH-EP cells to assess tumorigenicity. Also, we are injecting a dbh promoter-driven SOX11 construct into MYCN transgenic zebrafish to determine if forced SOX11 expression in the sympatho-adrenergic cells accelerates NB onset.

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B1:
Translational Research/Preclinical Experimental Therapies I

OR007

Drugging MYC Proteins Through an Allosteric Transition in Aurora Kinase A

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Background: MYC genes contribute to a range of cancers including neuroblastoma, where amplification of MYCN confers a particularly poor prognosis. Proteolytic degradation of MYCN protein is regulated in part by a kinase-independent function of Aurora Kinase A. Current inhibitors of Aurora Kinase A are optimized to block kinase activity rather than this MYCN stabilizing scaffolding function.

Methods: Putative conformation disrupting inhibitors were genertated by standard organic chemical techniques and chemical moitiies analyzed for potential conformation disrupting activities. Western blotting, flow cytometry, and co-immunopreciptiation was performed as previously described. Purified Aurora A protein was generated and purified from bacteria as described. Crystallization conditions were identified from sparse matrix screens, with X-ray diffraction conducted using synchrotron radiation at UCSF affiliated beam-lines.

Results: We describe a class of inhibitors designed to profoundly disrupt the native conformation of Aurora A and cause degradation of MYCN protein across MYCN-expressing neuroblastoma cell lines. Lead conformation disrupting inhibitors potently inhibit Aurora A, destabilize MYCN protein, and disrupt MYCN/Aurora Kinase A complexes. Lead compound has effects on cell cycle consistent with complete MYCN blockade. Comparison of co-crystal structures with structure-activity relationships across multiple inhibitors and chemotypes, coupled with mechanistic studies, cellular cytotoxicity, and biochemical assays, delineates an Aurora A conformation-specific effect on proteolytic degradation of MYCN, rather than simple nanomolar-level inhibition of Aurora Kinase A kinase activity.

Conclusion: This new class of inhibitors, which disrupts stabilizing interactions between Aurora A and MYCN through a uniquely potent allosteric mechanism, represents candidate agents to target MYCN-driven neuroblastoma and potentially other tumors driven by MYCN/MYC.

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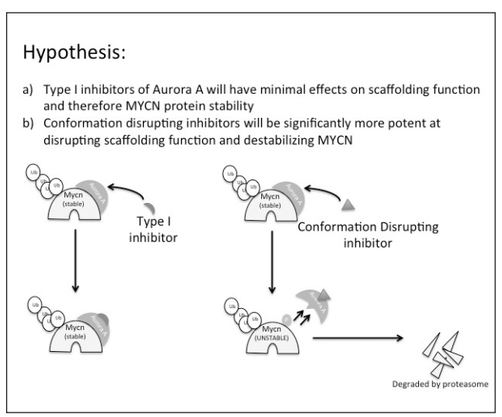

OR008

A Cell-Based High-Throughput Screen Addressing 3'UTR-Dependent Regulation of the MYCN Gene

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Background: Both transcriptional and post-transcriptional regulation has a profound impact on genes expression. However, commonly adopted screening assays focus on transcriptional regulation, being essentially aimed at the identification of promoter-targeting molecules. As a result, post-transcriptional mechanisms are largely uncovered. Here we describe the development and validation of a cell-based assay aimed to investigate the role of a mRNA 3' untranslated region (3'UTR) in the modulation of the fate of its mRNA, and to identify compounds able to affect it. Neuroblastoma, the most common extracranial solid tumor of infancy, was used as a biological model and the MYCN oncogene, whose amplification strongly predicts adverse outcome of neuroblastoma, as a target gene.

Methods: Luciferase reporter constructs with the MYCN 3'UTR were generated and stably integrated in the CHP134 neuroblastoma cell line. After validation, the developed cell-based reporter assay was used to screen a 2000 compound library including about 1000 FDA-approved drugs. Molecules affecting luciferase activity were checked for reproducibility and counter-screened for promoter effects and cytotoxic activity, resulting in selection of four upregulating molecules as truly dependent on the MYCN 3'UTR.

Results: Three of the compounds belong to the anthracycline class, while the forth one is the anti-mycotic compound ciclopirox olamine. The effect of the latter on the endogenous MYCN protein was confirmed, thus validating the approach.

Conclusion: We propose this cell-based reporter gene assay as a valuable tool to screen chemical libraries for compounds modulating posttranscriptional control processes. Identification of such compounds could potentially result in development of clinically relevant therapeutics for various diseases including neuroblastoma.

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OR009

Targeting of the MYCN Oncoprotein with c-MYC Inhibitors

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Background: The members of the MYC family are the most frequently deregulated oncogenes in human cancer and are correlated with aggressive disease and poorly differentiated tumors. In patients with MYCN-amplified neuroblastoma, targeting MYCN represents a promising therapeutic approach. We have demonstrated that the small molecule 10058-F4, previously shown to bind and inhibit c-MYC, is sufficient to induce neuronal differentiation of MYCN-amplified neuroblastoma cell lines in vitro and to cause anti-tumorigenic effects in neuroblastoma tumor models with MYCN overexpression (Zirath et al, PNAS 100, 10258-10263, 2013).

Methods: We have purified His-tagged MYC proteins from bacteria followed by circular dichrosim (CD) to verify folding. Binding to small molecules have been performed using Biacore. We have used Western blot for proteins levels, apoptosis and differenatioation assays as well as Oil Red O stainings of lipids.

Results: Here, we analyzed the direct binding of 10058-F4 and a selection of other small molecule c-MYC inhibitors to the bHLHZip domain of MYCN as well as their ability to induce apoptosis, neurite outgrowth and lipid accumulation. We confirmed that the c-MYC binding molecules tested could also bind to MYCN. The 10058-F4 analog #474 had the highest affinity to c-MYC and MYCN and was together with #764 also the most potent molecule in inducing apoptosis in MYCN-amplified cells. Interestingly, the structurally unrelated compound 10074-G5 showed a higher affinity to MYCN than to c-MYC and was together with 10058-F4 the most efficient compound at inducing neuronal differentiation and lipid accumulation.

Conclusion: Our data suggests that MYCN-targeting molecules could be a promising strategy for development of future therapies against MYCN-amplified neuroblastoma.

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OR010

Targeting the MYCN Pathway in Neuroblastoma with M606, a Novel Small Molecule Inhibitor with Clinical Potential

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Background: The possibility of delivering highly effective therapies targeting the MYCN oncogenic pathway is an area of research of great interest, particularly for children with aggressive MYCN amplified neuroblastoma. We have used high throughput cell-based chemical library screening to isolate putative inhibitors of MYCN with clinical potential.

Methods: A 34,000 diverse chemical library of small molecules was screened using a cell-based assay and MYCN reporter construct. PCR, cytotoxicity assays, Westerns, siRNA knockdown, metabolic analysis, and signal transduction pathway analyses (Attagene) were used to characterize the most promising hit compound, M606.

Results: M606 was identified as a potent MYCN inhibitor following primary library screening and subsequent filtering and focussed library screening. M606 dramatically reduced MYCN protein levels and activity in human MYCN-amplified neuroblastoma cells, as well as c-myc protein levels in myc-overexpressing tumour cells. Attagene signal transduction pathway analysis demonstrated that M606 inhibited Myc mediated transcription in a dose-dependent manner, and led to 50-100 fold induction of hypoxia inducible factor alpha (HIF1A). Knockdown of both MYCN/MYC and HIF1A with siRNAs showed that these two events were independent of each other. HIF1A upregulation by M606 results from inhibition of HIF1A prolyl hydroxylase leading to subsequent HIF1A protein stabilization. Metabolic analysis indicated that in common with another prolyl hydroxylase inhibitor, desferrioxamine, M606 demonstrates iron chelating activity, and the effects of M606 on both MYCN/Myc and HIF1A levels are reversible by the addition of iron. However, M606 is far more potent and less toxic than desferrioxamine, and moreover is able to inhibit the progression of tumours in MYCN transgenic mice.

Conclusion: M606 represents a novel small molecule that inhibits MYCN and MYC activity at both the transcriptional and translational level. This promising compound appears to have clinical potential for targeting MYCN/MYC overexpressing tumours.

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OR011

N-Myc Protein Stability as a Therapeutic Target in MYCN Amplified Neuroblastoma

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Background: Amplification of the N-Myc encoding proto-oncogene MYCN, is a driver mutation in a subset of human neuroendocrine tumors, including neuroblastoma. For human neuroblastoma patients the MYCN amplification is a prognostic marker and is linked to aggressive tumor types and a poor prognosis. Up to date no small molecule inhibitors targeting N-Myc are clinically available, but we showed that two inhibitors of Aurora-A kinase activity, MLN8054 and MLN8237, specifically decrease N-Myc protein levels in vitro, in human neuroblastoma cell lines, and in vivo, in an N-Myc-driven neuroblastoma mouse model. Aurora-A interacts with and stabilizes N-Myc in MYCN amplified neuroblastoma cells irrespective of its kinase activity. MLN8054 and MLN8237 act on N-Myc stability, because they do not only inhibit kinase activity, but also induce a conformational change of Aurora-A. However the exact mechanism of Aurora-A mediated N-Myc stabilization is still unclear.

Methods: Therefore we performed a proteomic analysis of N-Myc complexes and identified previously unknown N-Myc interactors possibly playing a role in Aurora-A mediated stabilization of N-Myc. One group of possible candidates are deubiquitinating enzymes (DUBs) that could be recruited by Aurora-A and remove the ubiquitin chain targeting N-Myc to the proteasome. For this reason we utilized a human DUB siRNA library to screen for N-Myc regulating DUBs in addition to the proteomic approach.

Results: We identified five DUBs playing a role in N-Myc protein stability, including Usp7. Usp7 was also identified as a new N-Myc interaction partner in the proteomic analysis. We have validated the interaction of N-Myc and Usp7, as well as the Usp7 dependent regulation of N-Myc via Usp7 knockdown. Moreover we were able to show that N-Myc protein levels increase upon overexpression of exogenous Usp7.

Conclusion: Currently we are further investigating how Usp7 knockdown affects proliferation and differentiation aiming to identify a new therapeutic strategy targeting MYCN amplified neuroblastoma.

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OR012

The Novel Small-Molecule MDM2 Inhibitor, RG7388, is Highly Effective against Neuroblastoma In Vivo via p53-Mediated Apoptosis

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Background: Novel less toxic therapeutic approaches to neuroblastoma therapy are urgently needed to improve survival and reduce treatment related side effects. As neuroblastoma is nearly uniformily p53 wild-type, research efforts have focused on activating the innate p-53 mediated apoptotic mechanisms, specifically via inhibition of MDM2, the primary inhibitor of p53. Here we demonstrate the efficacy of this new class of MDM2 inhibitors against p53 wild-type neuroblastoma in vitro and in vivo orthotopic models of neuroblastoma.

Methods: Human neuroblastoma cell lines, NGP, SH-SY5Y, LAN5 and LAN5 si-p53 (p53 silenced by shRNA), were utilized for in vitro and in vivo studies. Cell viability and apoptosis with RG7388 was measured by MTS assay and flow cytometric annexin assay, respectively. In vivo effect of RG7388 was assessed in an orthotopic mouse model of neuroblastoma (80 female NCr nude mice). The mice were randomly divided into a control group which received vehicle and a treatment group which received RG7388 once daily for 14 days (25mg/kg for NGP and SH-SY5Y, 35mg/kg for LAN5 and LAN5 si-p53). At five weeks post-implantation, all mice were sacrificed.

Results: Using the MTS assay and annexin apoptosis assay, we found robust decrease in cell proliferation and increase in apoptosis in response to RG7388. The p53 silenced cell line, LAN5 si-p53, was found to be resistant to RG7388 with no decrease in cell proliferation and no increase in apoptosis. In vivo, all mice tolerated RG7388 well without morbidity. RG7388 significantly inhibited tumor growth by 59% in NGP ($p=0.003$), 67% in SH-SY5Y ($p=0.006$), and 75% in LAN5 ($p=0.0019$) xenograft tumors. However, RG7388 had no inhibitory effect on LAN5 si-p53 xenograft tumors compared to vehicle treatment ($p=0.57$).

Conclusion: The MDM2 small molecule inhibitor RG7388 significantly inhibits p53 wild-type neuroblastoma tumor growth but has no effect on p53-silenced xenografts in the orthotopic mouse model. Our studies suggest RG7388 inhibits tumor growth by p53-mediated apoptosis.

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A2:
Basic Research/Oncogenesis II

OR013

The RNA Aptamer against Midkine Suppresses Neuroblastoma Xenograft Growth by Attenuating Midkine-Notch2 Pathway

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Background: Midkine (MK) is a growth factor highly expressed in various cancers including neuroblastoma (NB). The plasma MK level is the reliable poor prognosis factor of NB. In terms of the molecular mechanism for the MK function in NB, we reported in ANR2012 that Notch2 would mediate its signaling as a receptor. MK-Notch2 axis should play a pivotal role in NB tumorigenesis, and can be an effective target of molecular therapy.

Methods: In order to target MK, we generated an RNA aptamer against MK. An RNA aptamer is considered as a nucleic acid analog to antibodies. It consists of short (20-80-mer) RNA, and forms unique 3-dimensional structure to specifically recognize the target molecule. As the tool for molecular therapy, it is said that RNA aptamers are superior to antibodies in terms of immunogenicity, productivity, applicability to chemical modification, and cost. We established the RNA aptamer Apt-1, which specifically binds to MK.

Results: Apt-1 blocked both the binding of MK to NB cell surface in vitro and the soft agar colony formation. Because the inhibitory effect of Apt-1 in soft agar assay was cancelled by the addition of recombinant MK protein, Apt-1 should specifically neutralize MK. Finally, the intratumor injection of Apt-1 to engrafted subcutaneous tumors efficiently suppressed the tumor growth in vivo. Interestingly, the Notch2-HES1 pathway was attenuated in those Apt-1-treated tumor tissues. This result supports the previous idea that MK-Notch2 pathway is involved in NB tumorigenesis.

Conclusion: The antitumor effect of Apt-1 strongly suggested that MK could be a potent target of NB therapy and also that the RNA aptamer Apt-1 could be a useful tool.

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OR014

The G-CSF/STAT3 Signaling Axis Regulates the Tumorigenic and Metastatic Potential of Neuroblastoma Cancer Stem Cells (CSCs)

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Background: We recently characterized a novel cancer stem cell-like cellular subpopulation within neuroblastoma based on expression of the receptor for Granulocyte Colony-Stimulating Factor (G-CSF) (PMID:23687340). This highly tumorigenic, self-renewing subpopulation of tumor cells reflects a pre-migratory early neural crest phenotype consistent with the developmental origins of neuroblastoma. STAT3 activation is the primary consequence of G-CSFr ligation in granulocytic precursors. Here we demonstrate in vivo that G-CSF/STAT3 signaling promotestumor growth and metastasis of both human and murine neuroblastoma.

Methods: G-CSF receptor + and - subpopulations were isolated by FACS from cell lines, xenografts and TH-MYCN transgenic tumors. Colony formation, drug sensitivity, metastasis and proliferation were evaluated in response to G-CSF administration or STAT3 inhibition. STAT3 target genes were evaluated by qPCR and chromatin immunoprecipitation (ChIP-qPCR). Bone marrow metastasis was determined by MYCN-qPCR.

Results: In vivo, G-CSF-induced STAT3 phosphorylation/activation stimulates multiple STAT3 target genes including those with anti-apoptotic and growth-promoting functions. G-CSF promoted colony formation from single receptor+ cells with no effect on receptor- cells. Furthermore, Chip-qPCR confirmed CSF3R (encoding the receptor) as a direct transcriptional target of STAT3, defining a positive feedback mechanism maintaining G-CSF/STAT3 signaling. In vivo, STAT3 inhibition significantly inhibited tumor growth and metastasis in orthotopic xenografts in nude mice. In immunocompetent G-CSF knockout mice (CSF-/-), exogenous G-CSF increased tumor growth and increased the incidence of bone marrow metastasis of murine allografts. STAT3 inhibitors (e.g. Stattic) reversed these effects both in vitro and in vivo.

Conclusion: G-CSF/STAT3 signaling within the CSC-like G-CSFr+ subpopulation has potent pro-tumorigenic effects in both human and murine models of neuroblastoma. Beside the direct growth-promoting effect of G-CSF on the G-CSFr+ neuroblastoma initiating cells, our data suggest that this cytokine may also stimulate G-CSFr+ immune or stromal cells and indirectly promote tumor growth and metastasis. Our data suggest blockade of G-CSF signaling through STAT3 inhibition should be considered for therapeutic testing.

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OR015

Tumor-associated Macrophages (TAMs) Increase Neuroblastoma Proliferation and Growth through c-MYC Upregulation, an Effect Independent of IL6 Expression

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Background: Our group has demonstrated the significance of inflammation-related genes in poor outcome of children diagnosed with metastatic MYCN non-amplified neuroblastoma (NBL-NA). We investigated the effect of TAMs on neuroblastoma growth using a novel murine model.

Methods: A NBL-NA murine model driven by SV40's Large T-antigen (NBL-Tag) was characterized for expression of immune-related genes at various stages of tumor development. Immune cells frequency and intracellular IL6 levels were analyzed using flow-cytometry.

Results: NBL-Tag tumor growth coincided with IL6 levels becoming detectable and increasing in blood. Gene expression analysis of NBL-Tag adrenal glands in mice at pre-tumor development ages (4-12 weeks old) showed age-dependent increase in expression of IL6 and CCL2 compared to wild-type controls demonstrating early monocyte chemotaxis and establishment of a pro-inflammatory niche. Flow cytometry analyses of tumors showed high expression of IL6, and infiltration by IL6-producing TAMs. In vitro co-culture of macrophages from wild type mice increased proliferation of NBL-Tag cell line by average of 50% over its basal rate ($p<0.001$). While this effect coincided with two-fold increase in IL6 protein level, the proliferative changes were only partially reversed by blocking IL6, suggesting involvement of other tumor-promoting factors. IL6 genetic ablation studies were conducted to generate NBL-Tag/IL6$^{-/-}$ mice, which convincingly demonstrated IL6 was not required for tumor growth and development. Macrophages from IL6$^{-/-}$ mice increased in vitro proliferation of tumor to similar levels as those from wild-type mice, while IHC staining of NBL-Tag/IL6$^{-/-}$ tumors revealed similar level of TAM infiltration. Protein and gene expression studies showed increase in phospho-STAT3 levels and upregulation of MYC (aka c-MYC) independent of IL6.

Conclusion: Recruitment of TAMs occurs early in pathogenesis of neuroblastoma in NBL-Tag mice. Neuroblastoma and TAMs cross-talk leads to tumor cell proliferation in vivo and in vitro through upregulation of pSTAT3 and MYC and independent of IL6 activity.

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OR016

The HBP1 Tumor Suppressor is a Druggable ALK Downregulated Gene Controlling MYCN Activity

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Background: ALK is an important druggable target in ALK mutant neuroblastoma (NB). In this study, we sought novel vulnerable nodes downstream of ALK for combinatorial therapy.

Results: First, we identified a 79-gene signature that recapitulates the transcriptional response upon ALK inhibition based on transcriptome profiling of ALK wild type, ALK^{F1174L} or ALK^{R1275Q} mutant and ALK amplified NB cell lines following ALK inhibition using NVP-TAE684, LDK-378, X-396 and Crizotinib. This signature was validated in primary tumor samples and in the SK-N-AS cell line with regulated expression of ALK wild type, ALK^{F1174L} and ALK^{R1275Q}. The majority of the mutant ALK dependent downstream signaling components were implicated in MAPK/ERK, PI3K/AKT/mTOR, MYC/MYCN and neuronal differentiation signaling. Bioinformatic analysis using the iRegulon Cytoscape plugin (iregulon.aertslab.org) resulted in the identification of the transcriptional repressor HBP1 as a hub gene, acting downstream of ALK. HPB1 is a known negative regulator of MYC/MYCN activity. Using MYCN and miR-17-92 inducible SHEP modelsystems, we established a MYCN/miR-17-92/HBP1 positive feedback regulatory loop in which MYCN represses the negative control of HBP1 through upregulation of miR-17-92 which targets the HBP1 3'UTR. Next, we showed that stable HBP1 overexpression inhibits growth and induces apoptosis of NB cells. Finally, we explored effects of the green tea compound epigallocatechin gallate (EGCG) on HBP1 upregulation in NB cells. EGCG treatment induced HBP1 expression and had a strong negative effect on cell growth and survival. EGCG/ALK inhibitor combination treatment in a panel of cell lines showed clear additive effects.

Conclusion: In conclusion, we identified HBP1 as a novel important ALK downregulated gene controlling MYCN activity, further stressing the important interconnection between oncogenic MYCN and ALK signaling. EGCG upregulates HBP1 levels and is a strong candidate for further in vivo testing for additive or synergistic effects with ALK inhibitors or MYCN targeting compounds such as JQ1.

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OR017

Genomic Instability Model for Metastatic Neuroblastoma Carcinogenesis by Dictionary Learning Algorithm

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Background: Metastatic neuroblastoma (mNB) occurs both in infants (stage 4s) and in children (stage 4). Stage 4 tumors show several structural copy number aberrations (CNAs) that are less frequent in stage 4s. How these aberrations occur is still unclear although genomic instability play a critical role in the process.

Methods: We deciphered the NB carcinogenesis by aCGH of 190 mNBs using the dictionary learning method (DLm). In DLm, the aCGH signal was approximated by a linear weighted combination of the atoms, which are the elements of a learned dictionary. We used a DLm that grouped the relevant alterations in elementary patterns and sorted them according to how many times such patters occurred in the data. This is a built-in property of DLm and provides a compact way of analyzing the co-occurrent alterations.

Results: We propose a genomic instability progressive (GIP) model of carcinogenesis for mNB. The GIP model suggests a common ancestor of both mNB because most atoms at first level share common chromosome aberrations (2p, 7 and 17q gain). However, the events are slightly different between mNB stages: chromosome losses in stage 4s and 4 occurs at different levels probably associated with diverse tumor aggressiveness in the two mNBs (Figure). We also found significant ($p = 0.025$) association between CNAs and age for stage 4, only.

Conclusion: We show that whole/partial chromosome gain is an early common step in carcinogenesis of all mNB suggesting that initial event is a cell cycle deregulation leading to chromosome endoreplication or non-disjunction. On the contrary, chromosome losses are late events associated with tumor aggressiveness.

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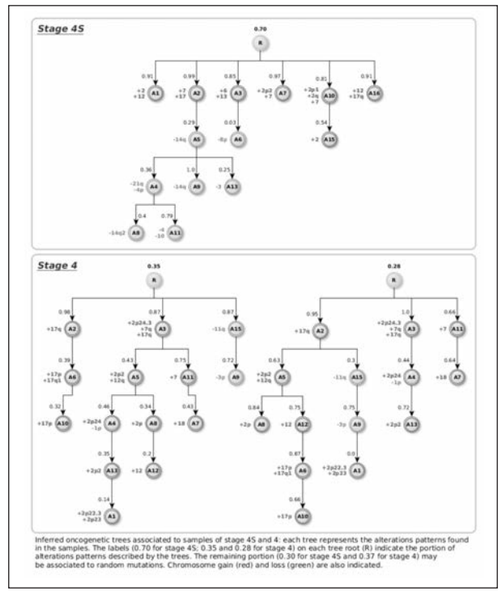

Inferred oncogenetic trees associated to samples of stage 4S and 4: each tree represents the alterations patterns found in the samples. The labels (0.70 for stage 4S; 0.35 and 0.28 for stage 4) on each tree root (R) indicate the portion of alterations patterns described by the trees. The remaining portion (0.30 for stage 4S and 0.37 for stage 4) may be associated to random mutations. Chromosome gain (red) and loss (green) are also indicated.

OR018

miRNA High-Throughput Functional Screening Identifies Several Potential Tumour Suppressive miRNAs in Neuroblastoma

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Background: Most of the functional studies of miRNAs in neuroblastoma have been based on the miRNA expression data (i.e. from RT-qPCR, array platforms or small RNA library sequencing) and have been so far aimed at single miRNA species.

Methods: We utilized genome wide miRNA functional screening with the gain-of-function (with miRNA mimics library) and the loss-of-function (with miRNA inhibitors library) approaches in order to identify miRNAs which play a role in neuroblastoma pathogenesis.

Results: Phenotypic miRNA high-throughput functional screening was performed with MYCN-inducible SH-SY-5Y and the data were analysed first for putative synthetically lethal miRNAs. However, only few candidates, which fit this criterion, were retrieved. Therefore, the potential of miRNA screening was not confined to the concept of synthetic lethality, because new tumor suppressive miRNAs might be identified as well. Using these criteria, we selected 180 miRNA species from the original large scale miRNA screening. The selected miRNAs were mapped to the human genome. We retrieved five miRNAs from 1p, which showed striking growth inhibitory potential in neuroblastoma cell lines. This set included well-known mir-34a. Remarkably, the remaining four candidates have not yet been functionally characterized in neuroblastoma. Moreover, dozens of growth inhibitory and growth promoting miRNAs were mapped to two largest miRNA clusters in the human genome, C19MC at chromosome 19 and 14q32 miRNA cluster.

Conclusion: Our results provide an update of neuroblastoma miRNAome, with several new tumor suppressive and oncogenic miRNAs. miRNA-based therapeutics, which compensate missing tumour suppressive miRNAs or neutralizing oncogenic miRNAs identified in our screen, may be a feasible neuroblastoma therapy.

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B2:
Translational Research/ Preclinical Experimental Therapies II

OR088

Emergence of New ALK Mutations at Relapse of Neuroblastoma

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Background: The ALK receptor tyrosine kinase is activated by point mutations in neuroblastoma and constitutes a potent therapeutic target in this disease.

Methods: To evaluate the role of ALK mutations in neuroblastoma progression or relapse, we searched for ALK mutations in a large series of 54 paired diagnostic–relapse neuroblastoma samples using Sanger sequencing. When an ALK mutation was observed in one sample, deep sequencing was used to seek for a minor mutated component in the other sample.

Results: Among the paired samples, all 9 ALK-mutated cases at diagnosis demonstrated the same mutation at relapse by Sanger sequencing. Nevertheless, in one case, the mutation was detected in only one of several nodules at relapse. In contrast, in 5 cases, the mutation appeared relapse-specific. Among these, 4 cases could be further investigated by deep sequencing. In two cases no evidence of the mutation was observed at diagnosis. In one case, the mutation occurring at relapse could be identified at a sub-clonal level at diagnosis, whereas in another case, two different mutations resulting in identical amino acid changes could be detected at diagnosis and relapse. Further evidence of clonal evolution of ALK-mutated cells was provided by the observation of a minor ALK-mutated cell population in a tumour from which a cell line with ALK mutation in all cells was derived.

Conclusion: These results indicate that, in neuroblastoma, ALK mutations newly detected at the time of relapse can be present in a subclone at the time of diagnosis with subsequent clonal expansion at relapse. With the advent of targeted therapy using ALK inhibitors, precise knowledge of the ALK status is mandatory. Our observation of a significant spatio-temporal variation of ALK mutations is of utmost importance in clinical practice, highlighting the potential of NGS and the importance of serial samplings for therapeutic decisions.

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OR019

Development of Chimeric Antigen Receptor (CAR) Cellular Therapies for Neuroblastoma

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Background: Antibodies targeting GD2 have shown promise as immunotherapeutics for neuroblastoma. Chimeric antigen receptors combine the specificity of monoclonal antibodies with potent T cells activatory signals. Clinical studies using adoptive transfer of second generation CAR-expressing autologous T cells targeting CD19 have demonstrated clinical responses in relapsed leukaemia patients. Early experience in neuroblastoma, using first generation CAR targeting GD2, demonstrated good tolerability.

Methods: We have developed a platform of reagents for translation into clinical studies by screening single chain variable fragments (scFv) for affinity to neuroblastoma surface antigens GD2, O-acetyl GD2, ALK and B7H3. ScFv are screened for specific binding to cells engineered to express the target neuroblastoma antigens. High affinity binders are cloned into human antibody chains or into viral vectors for expression of CARs. The CARs are co-expressed with a novel sort-suicide gene RQR8. CARs are transduced into conventional alpha beta T lymphocytes or into expanded gamma delta T lymphoctyes, for evaluation of relative killing of neuroblastoma cells.

Results: The second generation GD2-CAR has completed its preclinical evaluation and is expected to enter phase I in 2014. ALK and B7H3 binding scFv have been derived from a combination of techniques; genetic immunizations in rats, protein immunization in mice, and derivation from existing hybridomas. O-acetyl GD2 scFv have been derived from an existing antibody. The neuroblastoma antigen specific scFvs show specific killing of antigen positive target cells in both antibody and CAR formats.

Conclusion: Immunotherapy of neuroblastoma may be enhanced through targeting of new antigens with less off target toxicity than has been experienced with anti-GD2 antibodies. Our development of new antibodies and CARs against alternate target antigens will pave the way for their evaluation in clinical studies.

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OR020

Targeting the PD1/PDL1 Immune Checkpoint Pathway in Neuroblastoma

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Background: Recent immunotherapy strategies in adult cancers have focussed on tumour immune evasion mechanisms and the role of programmed death-1 (PD-1) and its ligand (PD-L1). PD-1 is an immune-checkpoint receptor, expressed on activated T-cells following an immune response, which binds PD-L1 to enable a controlled suppression of the normal immune response. This PD1:PD-L1 pathway has an important protective role to prevent autoimmunity and healthy tissue damage. However, several tumours have exploited this pathway, and express PD-L1 on their cell surface, enabling them to suppress anti-tumour T-cell responses. Clinical trials involving adults with melanoma and non-small-cell lung cancer have shown promising results with monoclonal antibodies (mAb) targeting either PD-1 or PD-L1 to block their interaction. These studies showed response rates were higher in patients whose tumours were PD-L1 positive. The aim of this work was to investigate PD-L1 expression in high-risk neuroblastoma (NB) patients, and with the use of pre-clinical NB murine models assess the therapeutic effect of PD-1 mAb.

Methods: Pre-clinical in vivo assessmentwas performed using the syngeneic NXS2 neuroblastoma model, treated with PD-1 antibody, given with and without a peptide vaccine. Tumour progression and survival was monitored, and tumour microenvironment was assessed using flow cytometry., Expression of PD-L1 in human paediatric tumours was evaluated using immunohistochemical staining. 5 tumour types were assessed: neuroblastoma, osteosarcoma, Ewings sarcoma, embryonal rhabdomyosarcoma and alveolar rhabdomyosarcoma. Primary and metastatic neuroblastoma samples, pre- and post-chemotherapy, were examined.

Results: Anti-PD-1 mAb showed potent efficacy in pre-clinical murine models, and was associated with a marked CD8 lymphocyte infiltration in treated tumours. Strong membranous expression of PD-L1 was found in the majority of neuroblastoma tumours examined, including high risk tumours.

Conclusion: High-risk NB express high levels of PD-L1, and along with the results of our pre-clinical model, support the need to further explore the progression of PD-1 mAb therapy in NB clinical trials.

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OR021

Chimeric Antibody c.8B6 to O-acetyl-GD2 Mediates the Same Efficient Anti-Neuroblastoma Effects than Therapeutic ch14.18 Antibody to GD2 without Induced Allodynia

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Background: Ganglioside GD2, a sialic acid containing glycosphingolipid, is a neuroblastoma-associated antigen and anti-GD2 antibodies, such as the mouse-human chimeric ch14.18 IgG1, have become a proven therapy for GD2-positive tumor. Anti-GD2 IgG1 antibodies mediate lysis tumor cells via antibody-dependant cellular cytotoxicity (ADCC) and complement-dependant cytotoxicity (CDC). However, anti-Anti-GD2 immunotherapy with ch14.18 is, however, associated with severe side effects, such as pain and neuropathies. The adverse effects are attributed to GD2-expression on human peripheral nerve fibers.

Methods: We established the mouse-human chimeric antibody c.8B6 specific to OAcGD2 in order to reduce potential immunogenicity in patients and to fill the need for a selective agent that can kill neuroblastoma cells without inducing adverse neurological side effects caused by anti-GD2 antibody immunotherapy. We further analyzed some of its functional properties compared with anti-GD2 ch14.18 therapeutic antibody.

Results: With the exception of allodynic activity, we found that antibody c.8B6 shares the same anti-neuroblastoma attributes as therapeutic ch14.18 anti-GD2 mAb when tested in cell-based assays and in vivo in an animal model.

Conclusion: The absence of OAcGD2 expression of nerve fibers and the lack of allodynic properties of c.8B6–which are believed to play a major role in mediating anti-GD2 mAb dose-limiting side effects–provide an important rationale for the in vivo administration of c.8B6 to patients with high-grade neuroblastoma.

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OR022

Recombinant IL-21 and Anti-CD4 Antibodies Cooperate in Syngeneic Neuroblastoma Immunotherapy and Mediate Long-lasting Immunity

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Background: IL-21 is an immune enhancing cytokine produced by T helper cells that showed promising results in pre-clinical and clinical cancer immunotherapy. We previously observed that the administration of anti-CD4 cell-depleting antibody strongly enhanced the anti-tumor effects of an IL-21-engineered neuroblastoma (NB) cell vaccine. Here we studied the therapeutic effects of a combination of recombinant (r)IL-21 and anti-CD4 monoclonal antibodies (mAb) in a disseminated syngeneic NB model.

Methods: rIL-21 therapy at 0.5 or 1 μg/dose was administered for 5 times with or without anti-CD4 mAb in syngeneic mice bearing disseminated NB. The percentage of tumor-free survival of treated mice was evaluated. In vivo depletion studies were performed using anti-CD8 or irrelevant antibodies.

Results: Subcutaneous rIL-21 had a limited effect on NB development. However, co-administration of rIL-21 at the two dose levels and a cell-depleting anti-CD4 mAb allowed to cure 28% and 70% of mice, respectively. Moreover, combination immunotherapy by anti-CD4 mAb and rIL-21 was effective even beginning 7 days (instead of 2 days) post NB induction allowing a significant increase in survival time and the cure of 30% of mice. Anti-CD4 mAb efficiently depleted CD4+CD25^{high} Treg cells, but alone had limited impact on NB. Combination immunotherapy induced a strong CD8+ CTL response, which was required for tumor eradication and for long-lasting immunity. CD4+ T cells repopulating mice after combination immunotherapy were required for long lasting immunity to NB antigens, as indicated by CD4+ T cell depletion and re-challenge experiments.

Conclusion: Our data support a role for regulatory CD4+ T cells in a syngeneic NB model and suggest that rIL-21 combined with CD4-T cell depletion reprograms CD4+ T cells from immune regulatory to an anti-tumor memory phenotype. These data open new perspectives for the use of IL-21-based immunotherapy in conjunction with transient CD4+ T cell depletion in human stage 4 NB.

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OR023

Anti-NLRR1 Monoclonal Antibody Inhibits Growth of Neuroblastoma Xenograft in Mice

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Background: The genes contributing to neuroblastoma (NB) aggressiveness under MYCN regulation are not well defined. We previously reported that neuronal leucine-rich repeat protein (NLRR) 1, a member of NLRR family, is a direct target of MYCN and upregulated in various adult cancers as well as aggressive NB. NLRR1 acts as a key regulator of cell proliferation in both NB and embryonic development. Therefore, we generated anti-NLRR1 monoclonal antibodies (mAbs) and obtained candidate antibodies with growth inhibitory effect in NB cells. In the present study, we identified the functional domains of NLRR1 responsible for promoting growth signals and examined the efficacy of NLRR1 mAb in NB xenograft model.

Methods: Growth inhibition assays using NB cells were performed to select mAbs with potent inhibitory effect. Tumor growth inhibition and mAb localization were assessed in xenograft model using NLRR1 stably expressing NB cells. Deletion mutants of NLRR1 were used to identify responsible domains for its function. Antibody-binding sites were determined by NLRR1 deletion mutants and epitope mapping using a peptide array.

Results: Inhibition of NLRR1 with mAb treatment reduced tumor cell proliferation and potentiated the efficacy of EGFR inhibition. Even at relatively low dose of mAb (100 μg, i.p., twice a week), NLRR1 mAb treatment significantly reduced NLRR1 expressing NB tumor growth. Deletion mutants of NLRR1 demonstrated that the upregulated cell growth by NLRR1 was impaired by the deletion of a fibronectin type III (FNIII) domain. Of note, deletion of FNIII domain diminished the mAb binding to cells and mAb epitope sites has been confirmed within the FNIII domain of NLRR1.

Conclusion: We propose here that NLRR1 is a novel molecular target for treating particular cancers including NB and that its function to regulate growth signals is dependent on its extracellular domain which can be a target for antibody-based therapy of aggressive NB.

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A3:
Basic Research/Oncogenesis III and Biological Models

OR024

MYCN Determines Entry into a Non-Cycling State

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Background: MYC is thought to act as a transcriptional amplifier, globally up-regulating the expression of active genes. Elevated MYCN expression in neuroblastoma increases cell proliferation and sensitises cells to apoptosis. In order to understand how MYCN influences cell fate and checkpoint decisions we have profiled gene expression in synchronised cells during each phase of the cell cycle.

Methods: MYCN amplified IMR5/75 cells with a tetracycline inducible MYCN shRNA vector were synchronised using a thymidine block. We examined cell cycle progression by flow cytometry, Western blotting and RNA-seq. An eGFP-E2F reporter construct was used to determine levels of E2F activity throughout the cell cycle.

Results: After release from G1:S arrest, MYCN+ cells progressed synchronously through the cell cycle. A smaller proportion of synchronised cells were observed after MYCN knock-down, due to an increase in non-cycling cells. The non-cycling state was defined by cells having a G1 complement of DNA, low levels of protein expression and low E2F activity. Western blotting revealed that expression of CDK4, a direct MYCN target gene, is elevated in all phases of the cell cycle in MYCN+ compared to MYCN- cells. CDK4 phosphorylates RB at several sites including serine 780. RB (phospho ser 780) was elevated in MYCN+ cells during G2. Preliminary RNA-seq data, normalised to cell number with ERCC spike-in controls, indicate that cell cycle stage strongly affects levels of gene transcription. RNA-seq data will form the basis of a mathematical model of how MYCN influences cell fate. Candidate gene expression will be analysed on a panel of 709 primary neuroblastomas.

Conclusion: MYCN prevents entrance into a non-cycling state through increased CDK4 activity during G2 phase. Transcriptional amplification observed with MYC family members may be due to an increased level of cycling cells.

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OR025

miR-183 Counteracts the MYCN Induced Transcriptional Activation of the MCM Complex in Neuroblastoma

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Background: MYCN is a master regulator controlling many processes necessary for tumor cell proliferation and survival. On a molecular level, MYCN stimulates both the initiation of DNA replication and the transition from G_1- to S-phase by transcriptional activation of the minichromosome maintenance (MCM) complex.

Methods: To unravel the role of epigenetically regulated microRNAs in MYCN amplified neuroblastoma cells, profiling studies were performed. Upstream regulatory mechanisms were investigated by (Re)-ChIP qRT-PCR, downstream signaling pathways by label-free proteome analysis.

Results: Histone deacetylase (HDAC) inhibitor treatment most strongly induced miR-183. Enforced miR-183 expression inhibited proliferation and triggered apoptosis. The mechanism of miR-183 induction was found to contribute to the cell death phenotype induced by HDAC inhibitors. Experiments to identify the HDAC(s) involved in miR-183 transcriptional regulation showed that HDAC2 depletion induced miR-183. HDAC2 overexpression reduced miR-183 levels and counteracted the induction caused by HDAC2 depletion or HDAC inhibitor treatment. MYCN was found to recruit HDAC2 in the same complexes to the miR-183 promoter, and HDAC2 depletion enhanced promoter-associated histone H4 pan-acetylation, suggesting epigenetic changes preceded transcriptional activation. Label-free mass spectrometry of miR-183 over-expressing MYCN-amplified neuroblastoma cells revealed 85 differentially expressed proteins, including all six members of the MCM complex, MCM2-7, which were identified as strongly repressed proteins. Annotation category enrichment analysis of all regulated proteins revealed a 14-fold enrichment in the protein module category "minichromosome maintenance", and different microRNA target programs predicted several MCM proteins as direct miR-183 targets.

Conclusion: These data indicate that MYCN and HDAC2 jointly repress the tumor suppressive properties of the miR-183 signaling network in neuroblastoma and suggest that the MCM complex presents a critical node in this network.

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OR026

Identification of Trk-Specific Signaling Events in Neuroblastoma Using Stable Isotope Labeling and Phosphoproteomics

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Background: Neuroblastoma (NBL) aggressiveness is correlated with age, clinical stage, and MYCN amplification. Clues to the biological pathways responsible for disease heterogeneity may lie in understanding the behavior of two highly homologous neurotrophin receptors, TrkA and TrkB. High expression levels of TrkA and TrkB are associated with favorable or poor outcomes, respectively. Despite divergent clinical behaviors, gene expression analysis of a model SY5Y neuroblastoma cell line stably transfected with TrkA and TrkB revealed surprisingly similar global gene expression behavior. Additionally, a proteomic study using 2D-gel electrophoresis with MALDI MS noted only a few differential proteins between activated TrkB (22) and TrkA (9) in SY5Y cell lines. We hypothesize that phosphoproteomic analysis will detect signaling pathway variations that could explain the dynamic differences in clinical behavior associated with TrkA and TrkB expression.

Methods: Using TrkA/TrkB transfected cell lines activated by NGF/BDNF, respectively, and inhibited by lestaurtinib, we identified phosphorylated targets of TrkA and TrkB signaling. Untreated SY5Y-TrkB samples were compared to BDNF, NGF and BDNF+Inh-treated samples using SILAC labeling followed by phosphopeptide enrichment and LC-MS/MS analysis. Spectra were searched and proteins quantified using MaxQuant.

Results: Analysis revealed 83 proteins higher in the phosphoenriched fraction in SY5Y-TrkB BDNF samples. A similar analysis of SY5Y-TrkA samples (none, BDNF, NGF, and NGF+Inhibitor-treated) revealed 62 differentially expressed proteins in SY5Y-TrkA NGF compared to other samples. The data suggest an enrichment of microtubule related proteins as a result of TrkB signaling and an enrichment of neuromuscular related proteins as a result of TrkA signaling. Western blot-based validation studies are ongoing.

Conclusion: This is the first proteomics study defining expression differences between TrkA and TrkB activated neuroblastoma cells and is now being extended to MYCN-amplified cell lines. This work sheds light on how these similar receptors can effect such divergent outcomes and enrich our understanding of the molecular pathways that account for NBL heterogeneity.

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OR027

Genes Mediating Bone Marrow Metastasis in Neuroblastoma

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Background: Bone marrow is the most frequent site for neuroblastoma metastasis and the most common site of relapse. This study aims to identify and validate genes contributing to neuroblastoma metastasis to this site.

Methods: Bioluminescent human neuroblastoma cells were injected intravenously into immunodeficient mice, with metastases arising in the adrenal medulla, liver and bone marrow. Tumour cell populations were isolated from bone marrow and engrafted into secondary recipients to determine the subpopulation tropism for bone marrow. Real-time PCR and gene expression microarray analyses were used to identify genes differentially expressed between bone marrow selective and non-selective populations. Genes were validated using transwell migration assays with bone marrow stromal cells (BMSCs) as a chemoattractant and in intravenous and orthotopic xenograft mouse models. Protein expression or activity was inhibited using shRNA or small molecule inhibitors.

Results: Neuroblastoma cell populations were selected in vivo for tissue tropism. Populations with high tropism for bone marrow had elevated expression of a gene signature including the chemokine receptor CXCR4 and regulators and effectors of the small GTPases Rac1 and CDC42. Small molecule inhibitors of each of these components reduced or abolished neuroblastoma cell migration toward BMSCs in vitro. CXCR4 levels had a modest effect on bone marrow tropism in the intravenous xenograft model without altering the primary tumour growth rate in an orthotopic (adrenal) xenograft model. Other components of the gene expression signature are currently under investigation.

Conclusion: In vivo selection methods can be used to identify genes contributing to bone marrow metastasis in neuroblastoma. CXCR4 and the small GTPases Rac1 and CDC42 and their effectors may contribute to metastasis to this site.

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OR028

Neuroblastoma-derived Exosomes Communicate with Bone Marrow – derived Mesenchymal Stromal (BMMSC) Cells to Promote Neuroblastoma Cell Survival

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Background: Exosomes are endosome-derived microvesicles secreted by cells that play a role in cell-cell communication. Here we have explored whether exosomes produced by neuroblastoma (NB) cells contributed to their ability to communicate with BMMSC that we have previously shown to promote NB cell survival and metastasis in the bone marrow microenvironment (Sohara et al., Cancer Research 2005; Ara et al., Cancer Research 2013).

Methods: Exosomes [identified as 30-100 nm microvesicles rich in tetraspanins and heat shock protein (hsp)-90] were isolated in 8 human and 1 murine NB cell lines, and analyzed by proteomics and added to BMMSC in culture.

Results: We observed that exposure of BMMSC to NB-derived exosomes activated Akt/PKB and stimulated the production of several pro-tumorigenic cytokines and chemokines, such as interleukin (IL)-6, IL-8, vascular endothelial cell growth factor (VEGF) and monocyte chemotactic protein (MCP)-1, that promoted NB survival and drug resistance but not of anti-tumorigenic cytokines like interferon g and tumor necrosis a. The analysis of the protein content of exosomes derived from 3 NB cell lines (CHLA-255, SK-N-BE[2] and NB-19) revealed the presence of 102 proteins present in exosomes from all 3 cell lines. Whereas most proteins identified were enzymes (metabolic 14%; synthetic 11%), structural proteins (17%), ribosomal proteins (8%) and chaperones (8%), only 2 proteins with a regulatory function in inflammation were identified. These included 1) high motility group box-1 (HMGB-1), a nuclear protein with a chromatin regulatory function that when secreted is a regulator of inflammation and 2) midkine, a heparin-binding growth factor expressed by NB cells and ligand for Alk that promotes survival and drug resistance.

Conclusion: Our data thus provide evidence, for the first time in neuroblastoma, that NB cells use exosomes to communicate with the tumor microenvironment and to stimulate the production of cytokines and chemokines that are favorable to their survival.

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OR086

A Genome-Wide Association Study (GWAS) Identifies Susceptibility Alleles within Neuroblastoma Oncogenes and Tumor Suppressors: An Explanation for the Paucity of Somatic Mutations?

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Background: A neuroblastoma GWAS has identified several bona fide susceptibility alleles, accounting for approximately 10% of disease heritability.

Methods: Illumina single nucleotide polymorphism (SNP) arrays were used to genotype lymphocyte-derived DNA from 3982 neuroblastoma cases and 10,201 control children without cancer. Genome-wide genotype imputation was performed using IMPUTE2 and association testing performed using logistic regression after case:control matching by multidimensional scaling. Significant associations were replicated in at least two additional case series, and candidate genes genetically manipulated in cell lines and animal models.

Results: Thousands of highly associated SNPs clustering to 11 separate gene loci have been discovered (CASC15, BARD1, LMO1, NBPF23, HACE1, LIN28B, RSRC1, TP53, DUSP12, DDX4 and HSD17B12), and top SNPs replicated at each locus (discovery P_{range} $1.4x10^{-7}$-$6.9x10^{-16}$). Strikingly, for each of the 7 loci studied to date, robust functional evidence for the gene behaving as a tumor suppressor (CASC15, HACE1, TP53) or oncogene (BARD1, LMO1, LIN28B, DUSP12) has been generated. This is in contrast to our somatic tumor sequencing efforts where recurrent mutations are restricted to a small number of known genes (MYCN, ALK, ATRX). Depletion and overexpression of each gene product in cell and animal models show major effects on neuroblast proliferation and survival, consistent with classic tumor suppressor and oncogenes activated via mutational events.

Conclusion: An unbiased GWAS scan has identified multiple germline polymorphic variants in genes critical to sympathetic nervous system development and the genesis of neuroblastoma. Most, if not all, of these genes play critical roles in the maintenance of established neuroblastoma oncogenic networks and are providing unanticipated insights into new therapeutic strategies. Embryonal tumors may show a low somatic mutation burden due to the potency of cooperating germline variants on malignant transformation and sustenance.

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B3: Translational Research/ Preclinical Experimental Therapies III

OR029

MEK1/2 Inhibitors Significantly Reduce Tumor Proliferation and Growth in Neuroblastomas

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Background: MAPK pathway plays a role in the tumor microenvironment and several genes in this pathway are among recurrent mutations found in neuroblastomas, We have shown that co-culture of macrophages with neuroblastomas increased tumor proliferation in vitro an effect that we hypothesized could be blocked by MAPK inhibitors.

Methods: NBL-Tag mice (MYCN non-amplified murine model) and two derived cell-lines (NBT2,NBT-Luc) and three human neuroblastomas cell-lines were used in Brdu incorporation or in vivo tumor growth models.

Results: Trametinib (MEK1/2 inhibitor) demonstrated the highest anti-proliferative activity compared to other kinase inhibitors and effectively blocked cell cycle arrest in G1 phase in NBT2 and human cell-lines. These anti-proliferative effects could not be rescued by co-culturing tumor cells with murine or human macrophages. NBL-Tag mice tumor growth was significantly impaired with daily oral administration of trametinib starting at 10 weeks of age (prior to tumor being visible by MRI) compared to controls (volumes at 17 weeks: 1424 vs. 43 mm3, $p<0.001$, respectively). Treatment of 15 week-old NBL-Tag mice (visible tumor by MRI) with tramatenib after chemotherapy administration (5-days of Cytoxan+Topotecan) also significantly impaired regrowth (volume four weeks post-chemo, 491 vs. 42 mm3, $p=0.037$).

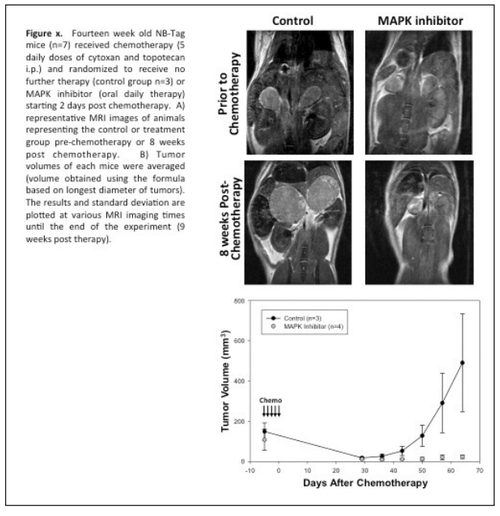

Figure x. Fourteen week old NB-Tag mice (n=7) received chemotherapy (5 daily doses of cytoxan and topotecan i.p.) and randomized to receive no further therapy (control group n=3) or MAPK inhibitor (oral daily therapy) starting 2 days post chemotherapy. A) representative MRI images of animals representing the control or treatment group pre-chemotherapy or 8 weeks post chemotherapy. B) Tumor volumes of each mice were averaged (volume obtained using the formula based on longest diameter of tumors). The results and standard deviation are plotted at various MRI imaging times until the end of the experiment (9 weeks post therapy).

Subcutaneous murine models also showed similar results. Tumors or macrophages collected from mice treated with trametinib showed effective block in phosphorylation of ERK. Transgenic animals that grew despite trametinib treatment showed strong STAT3 phosphorylation suggesting this pathway as an escape mechanism.

Conclusion: Our results provide strong evidence that trametinib could be a novel drug that effects neuroblastoma and its microenvironment.

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OR030

MYCN Expression in Neuroblastoma Induces Replicative Stress and Sensitizes Cells to PARP1 Inhibition

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Background: The MYC family of proto-oncogenes, including neural-specific MYCN, encode transcription factors that are master regulators of multiple processes involved in cell growth and metabolism and are widely implicated in tumourigenesis Mapping the MYCN interactome in high-risk MYCN-amplified (MNA) neuroblastoma (NB) will lead to a better understanding of the diverse roles played by this oncoprotein and the identification of novel druggable targets.

Methods: To identify novel MYCN-interacting proteins in NB, we performed immunoprecipitation/mass spectroscopy (IP-MS). We also performed a small molecule inhibitor (SMI) screen directed against SH-EP NB cells (lacking endogenously expressed MYCN) stably expressing wild-type MYCN or stabilized protein. Candidate drugs were tested pre-clinically in the Th-MYCN genetically engineered mouse model (GEMM) of NB.

Results: MYCN was found by IP-MS to associate with components of the DNA pre-replication complex and localize to sites of DNA synthesis. Overexpression of MYCN led to DNA damage as measured by an increase in γH2AX foci. MYCN was also found to interact with DNA damage repair pathway factors including poly (ADP-ribose) polymerase 1 (PARP1), knockdown of which impaired growth in cells expressing MYCN. Consistent with these results, an SMI screen revealed that MYCN overexpression or stabilized protein conferred sensitivity to agents that induce DNA damage or target repair factors such as PARP1, including the clinical inhibitor Olaparib. Treatment of Th-MYCN transgenic mice with Olaparib, either as a single agent or in combination with Temozolomide, extended survival, blocked tumour growth and induced tumour-specific γH2AX foci.

Conclusion: We have identified novel non-transcriptional roles for MYCN in the initiation of DNA replication and compensatory recruitment of DNA repair factors, including PARP1. Therapeutic inhibition of PARP1, either alone or in combination with other agents including those that target components of the DNA damage response such as the checkpoint kinase CHK1, should be considered for clinical evaluation in high-risk MNA neuroblastoma.

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OR031

Dual ALK and CDK4/6 Pathway Inhibition Demonstrates On-target Synergy Against Neuroblastoma

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Background: Activated ALK is a validated therapeutic target, and identifying strategies to overcome primary resistance will be critical to improve clinical responses. We hypothesized that simultaneous targeting of ALK and additional oncogenic networks would improve efficacy.

Methods: We performed a synergy screen combining targeted compounds (n=14) and standard-of-care agents (n=8) in neuroblastoma cell lines (n=14). We investigated the combination of LDK378 and LEE011 on in vitro proliferation, cell cycle, viability, caspase activation, and the Cyclin D/CDK4/CDK6/RB and pALK signaling networks in cell lines with representative ALK status. Transport inhibitor studies were performed as well as in vivo trials comparing LDK378, LEE011, and the combination, with pharmacokinetics to evaluate for drug-drug interactions.

Results: Pairwise combination screening for in vitro cell viability identified synergistic interactions of LDK378 (ALKi), with LEE011 (CDK4/6i). In both mutant and wild-type ALK cell lines, there was synergy at clinically relevant high effect sizes. Combination therapy increased dose dependent abrogation of pALK and pRb, inhibition of proliferation, apoptosis and decreased cell viability. LDK378 did not show significant cellular accumulation after P-gp, BCRP, and MRP2 inhibition; LEE011 showed modest accumulation with MRP2 inhibition. In NB1691 (ALK wild-type) and SHSY5Y (ALK-F1174L) xenografts, combination therapy significantly prolonged survival compared to either drug alone ($P<0.0001$), with complete tumor regression using murine doses that achieved plasma drug exposures comparable to the adult recommended doses for LEE011 and LDK378. Combination therapy increased tumor LEE011 and LDK378 maximum concentration and area under curve 2-3 fold over monotherapy, but plasma concentrations were unaltered.

Conclusion: Dual ALK and CDK4/6 inhibition demonstrates on-target in vitro synergy and in vivo activity with augmented abrogation of respective molecular targets, resulting in inhibition of proliferation and cell death. While mechanisms for altered intratumoral drug distribution require further investigation, these data support the development of a clinical trial with eligibility not restricted to cases with ALK mutations.

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OR032

BET Protein Inhibitor OTX015 Has Selective Anti-tumoral Activity in Preclinical Models of MYCN- Amplified Neuroblastoma

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Background: Neuroblastomas harboring MYCN amplifications are highly lethal tumors. They are often resistant to standard chemotherapy, yet the development of targeted therapies has been hampered by a lack of compounds targeting MYCN. We and others have recently discovered that targeting BET bromodomain proteins, especially BRD4, disrupts epigenetic regulation of MYCN and its targets in neuroblastoma. OTX015, a new BET protein inhibitor, is the first lead into clinical phase I/II trials and has shown promising pharmacological properties in adults. Here, we investigate the preclinical efficacy of OTX015 in MYCN-amplified neuroblastoma.

Methods: We tested in vitro OTX015 efficacy in 6 established neuroblastoma (NB) cell lines. We performed cell cycle profiling and analyzed markers for apoptosis and proliferation after 72h-treatment at 500 nM OTX015. The effect of OTX015 on MYCN expression and global MYCN-associated transcriptional activity was assessed by quantitative real time PCR and gene expression microarray profiling, respectively. In vivo efficacy of orally OTX015 was assessed in IMR5 xenografts, a N-MYC driven NB model, using diffent treatment schedules (50mg/kg/day, 100mg/kg/day and 50mg/kg/bidaily).

Results: Treatment of MYCN-amplified neuroblastoma cells with OTX015 resulted in decreased cell viability, induction of apoptosis and reduced proliferation. Concentrations of 50% inhibition (IC_{50}) ranged between 50nM and 500nM. OTX015 treatment also resulted in an increase in the percentage of cells in G1 phase. This corresponded with the downregulation of MYCN mRNA and protein levels and MYCN-associated transcriptional activity. Interestigly, MYCN amplified cell lines were most sensitive to OTX015 treatment. In contrast, no effect was observed with OTX15 on normal cells. In vivo treatment with OTX015, significantly decreased tumor burden after 4 weeks and prolonged survival respect to vehicle-treated mice.

Conclusion: These preclinical findings highlight the promise of BET bromodomain inhibitors as novel agents for MYCN-driven neuroblastomas and serve as rationale move forward with early phase clinical trials for children with these highly lethal tumors.

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OR033

p53 Activation Enhances the Sensitivity of Neuroblastoma to mTOR Inhibitors

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Background: mTORC1 inhibitors show promise as anticancer therapies in neuroblastoma (NB), however no molecular alterations predictive of response have been identified. Furthermore, combination therapies need to be explored to improve their clinical efficacy. p53 and mTOR signaling machineries can cross-talk and coordinately regulate cell growth, proliferation, and death. Given the clinical implications of these observations, we decided to determine whether p53 affects the response to mTORC1 inhibition.

Methods: Using an orthotopic xenograft model we investigated the molecular and anti-tumor effects of temsirolimus, a potent and specific mTORC1 inhibitor, in neuroblastoma expressing decreased wtp53 or mtp53. We then evaluated growth and apoptosis of NB lines as well as NB xenografts in the setting of combined therapy with temsirolimus and RG7388, a potent and selective second generation MDM2 inhibitor currently in clinical development. Lastly, we assessed tumor re-growth after temsirolimus or combined temsirolimus and RG7388 treatments.

Results: In vitro and in vivo silencing of p53 significantly increases mTOR activity. In vivo studies with p53 knockdown and mutant lines show that a functional p53 is required for the anti-tumor activity of temsirolimus in NB. Furthermore, mTORC1 inhibition by temsirolimus significantly blocks NB tumor growth. However, it does not elicit an apoptotic response, and tumors rapidly re-grow within two weeks after treatment. Conversely, reactivation of p53 by MDM2 inhibition in wtp53 NB xenografts: 1) strongly ($p<0.01$) enhances the anti-tumor response of temsirolimus, 2) induces a significant apoptotic response, and 3) effectively delays tumor re-growth after treatment completion.

Conclusion: These data support the hypothesis that p53 status is critical for temsirolimus-mediated anti-tumor activity and propose a novel therapeutic strategy combining MDM2 and mTOR inhibition for relapsed and de novo neuroblastoma harboring wtp53.

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OR034

Wip1 Inhibition Provides a Novel Therapeutic Target in Neuroblastoma with Different p53 and MDM2 Status

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Background: The p53-MDM2-p38 circuit is important for cell-cycle regulation and DNA-damage repair in neuroblastoma, providing a significant therapeutic avenue. Wip1, a serine/threonine phosphatase of the PP2C family, negatively regulates this circuit. Wip1 is overexpressed in several cancers, and gain of Wip1 at 17q is a common genetic aberration and marker of poor prognosis in neuroblastoma. Therefore,

Wip1 has been proposed as a therapeutic target in neuroblastoma.
Methods: Eleven human neuroblastoma cell lines including p53-mutant and -wildtype, as well as MDM2-amplified/-wildtype cell lines were exposed to substances targeting the p53-MDM2-p38 circuit. Wip1-expressing MCF-7 breast cancer cells were also tested. Specifically, we compared the p53-MDM2 interaction inhibitor RITA, the MDM2 antagonist Nutlin-3 and the most potent out of three Wip1 inhibitors. Cell viability was then estimated with a colorimetric formazan-based assay. In addition, we examined Wip1 expression by qPCR, and performed transfection experiments to evaluate the effect of Wip1 knockdown by shRNA in neuroblastoma cell lines.

Results: Among the substances compared, the Wip1 inhibitor was most potent in cytotoxic/cytostatic effect in nine out of eleven neuroblastoma cell lines and in MCF-7. In the remaining, RITA was most potent. In seven neuroblastoma cell lines, sub-micromolar IC50 values were calculated for the Wip1 inhibitor. Median IC50 values were 0.80 μM for the Wip1 inhibitor (0.43–1.3 μM), 2.3 μM for RITA (0.17–119 μM), and 5.4 μM for Nutlin-3 (0.47–14 μM). Dose-response curves were generally steeper for the Wip1 inhibitor. Our wide range of neuroblastoma cell lines expressed various mRNA levels of Wip1. Knock-down of Wip1 yielded reduced neuroblastoma cell viability.

Conclusion: Wip1, regulating p53-MDM2-p38, is a promising therapeutic target in neuroblastoma. Among drugs targeting the p53-MDM2-p38 circuit, the Wip1 inhibitor showed superior cytotoxic/cytostatic potency for most neuroblastoma cell lines, making it interesting for additional evaluation. Further molecular characterization and in vivo testing are ongoing.

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A4: Basic Research/ High Throughput Techniques

OR035

Massive Parallel Genomic Sequencing Reveals Distinct Mutation Patterns in Clinical Neuroblastoma Subgroups

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Background: The genetic etiology of neuroblastoma has remained largely enigmatic to date. Analysis of mutation spectra of clinical subgroups may provide insights into the pathogenesis of the diverse biological neuroblastoma phenotypes.

Methods: In our ongoing collaborative study, we thus far analyzed 158 primary neuroblastomas comprising all stages of the disease (stage 1, n=12; stage 2, n=23; stage 3, n=24; stage 4, n=82; stage 4S, n=17) by whole-exome (n=128) or whole-genome (n=30) massive parallel sequencing. Ninety samples were obtained from high-risk patients, 48 of which were MYCN-amplified.

Results: In line with previous reports, we observed low mutation frequencies (16.7 mutations/tumor) and low recurrence of mutated genes in the entire cohort. Mutation rates, however, differed markedly between clinical subgroups. The lowest mutation frequency was detected in stage 4S neuroblastoma (3.6 mutations/tumor), and continuously increased from stage 1 to stage 4 (6.8, 8.7, 9.7 and 25.1 mutations/tumor, respectively). Within the high-risk group, we observed that MYCN-amplified tumors had significantly fewer mutations than non-amplified tumors (11.0 vs 37.9 mutations/tumor, P<0.001). Similarly, we found substantial differences between neuroblastoma subgroups when the correlation of mutation frequencies and patient age at diagnosis was examined. Mutation rates significantly increased with patient age in the entire cohort, in the MYCN-amplified subgroup, and in the non-high risk subgroup (r=0.62, r=0.66, and r=0.55, respectively). By contrast, the mutation frequency was not correlated with patient age in MYCN non-amplified high-risk neuroblastomas (r=0.234, P=0.134). Moreover, Cox regression analysis indicated an association between mutation rate and poor survival in the cohort without MYCN-amplification (P=0.017), but not in the MYCN-amplified group (P=0.683). Finally, whole-genome sequencing revealed chromothripsis in 3/30 samples, all of which represented MYCN non-amplified high-risk tumors.

Conclusion: Mutation rates of clinical neuroblastoma subgroups differ markedly, pointing towards distinct genetic etiologies and tumor evolution processes underlying the diverse biological neuroblastoma phenotypes. Subgroup-related analyses of the pattern of mutated genes are currently underway to elucidate the genetic etiology of neuroblastoma.

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OR036

Identification of Recurrent Germline and Somatic Structural Variations (SVs) Influencing Tumorigenesis

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Background: Structural variations (SVs) including translocations, inversions, deletions, duplications, and other complex events can occur in germline DNA or be acquired somatically in tumors. Recurrent SVs affecting known genes are likely to be functional and may elucidate novel tumor suppressors or oncogenes in neuroblastoma.

Methods: Whole-genome sequencing (WGS) of 187 matched tumor-normal pairs from high-risk patients is being performed in the TARGET project by Complete Genomics. Lymphocyte-derived DNA from 7,500 neuroblastoma cases and 15,000 controls is being genotyped using Illumina SNP arrays in a neuroblastoma GWAS. Somatic SVs are detected using WGS; germline SVs are identified using both SNP array data and WGS of normal DNA. Validation of SVs is accomplished with Sanger sequencing and functional studies are performed using cell line models.

Results: To date, we have analyzed WGS data from 85 matched tumor-normal pairs. Among 2,458 high-confidence somatic SVs, we observed recurrent focal deletions in ALK (n=2), ZFHX3 (n=2), and DIAPH2 (n=2), in addition to ATRX, ARID1B, and CDKN2A reported previously. 167 interchromosomal translocations disrupting known genes were detected (average:1.97 per tumor, range:0-9). Notably, 12.9% of tumors (11/85) harbored breakpoints within SHANK2 at 11q13. SHANK2 breakpoints were validated by Sanger sequencing and decreased SHANK2 expression was associated with poor survival ($P=6.3x10^{-5}$), suggesting SHANK2, known to regulate neuronal differentiation, may function as a tumor suppressor. We next analyzed rare (<1%) germline CNVs in 2,083 cases and 6,146 controls and identified a 550-Kb deletion at 16p11.2 associated with neuroblastoma (cases:0.43%; controls:0.04%; P=4.0x10-4; OR:8.9, 95% CI:2.2-41.3). The deletion was confirmed by WGS and the association replicated in 1,167 cases and 56,752 published controls (P=0.003; OR:7.8, 95% CI:2.3-23.6). Additional recurrent SVs are being validated.

Conclusion: A plethora of SVs exist in neuroblastoma, and unlike somatic point mutations, many are recurrent. Ongoing studies will determine the biological relevance of SHANK2 and other genes recurrently altered by SVs in neuroblastoma.

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OR037

RNA-Seq Provides Detailed Insights into the Neuroblastoma Transcriptome and is Suitable for Clinical Endpoint Prediction

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Background: Considering the power of RNA deep sequencing (RNA-Seq) in characterizing cancer transcriptomes, it is tempting to speculate that RNA-Seq-based classifiers may improve the performance of gene expression prediction models as compared to microarrays. Here, we aimed at evaluating this hypothesis by systematically comparing RNA-Seq and microarray-based classifiers for clinical endpoint prediction.

Methods: We characterized gene expression profiles of 498 primary neuroblastomas (stage 1, n=120, stage 2, n=77, stage 3, n=63, stage 4, n=184, stage 4S, n=54) by paired-end RNA-Seq and 44K microarrays. The cohort was randomly divided into training and validation sets; six independent analysis teams generated 360 models to predict six different endpoints (EFS and OS in the entire cohort, EFS and OS of high-risk patients, extreme clinical courses, and sex of the patient). The effects of a range of variables on the prediction performances were analyzed.

Results: Analysis of 30.8 billion sequencing reads revealed expression of 50,640 genes, 222,227 transcripts and 511,936 exon-junctions, 251,806 of which were newly discovered. The transcribed part of the genome covered 316 megabases, including 39,052 novel exons in regions previously considered to be untranscribed. The performance of predictive models was heavily dependent on clinical endpoints, with top performances observed in predicting patient sex (Matthews correlation coefficient [MCC], 0.97) and extreme clinical courses (MCC, 0.85), while models predicting EFS and OS of high-risk patients performed worst (MCC, 0.11 and 0.09, respectively). Additional factors significantly influencing performance metrics were the analysis team, and model size. By contrast, the choice of the gene expression platform (RNA-Seq vs microarray), feature level (gene vs transcript vs exon junction level), and transcriptome database (RefSeq vs AceView) had no significant impact on the performance of predictive classifiers.

Conclusion: While RNA-Seq-based expression profiles provide unprecedented insights into the neuroblastoma transcriptome, RNA-Seq and microarray-based classifiers perform similarly in predicting clinical endpoints.

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OR038

Integrated Genomic Analyses Identifies Neural, Metabolic, and Inflammatory Subgroups of High-Risk Neuroblastoma with Clinical Significance

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Background: To better understand the biology of neuroblastoma, the TARGET project conducted high-dimensional profiling studies.

Methods: 366 cases (282 high-risk [HR], 84 low/intermediate risk [LR]) were profiled for gene expression using Human Exon microarrays (HuEx). For 138 cases, U133A+B microarray data were available. A German cohort of 416 Agilent microarray data (135-HR, 281-LR) was used as validation cohort. 225 cases (88 with HuEx profiles) underwent DNA methylation profiling using HumanMethylation450 BeadChip.

Results: Three molecular subgroups of HR neuroblastoma were identified and labeled as Neural (25%), Inflammatory (33%), or Metabolic (42%) based on NMF clustering. A 428-gene HuEx signature was developed to predict subgroup membership for samples analyzed by U133 and Agilent microarray platforms. Expression

of PHGDH was highest in the Metabolic subgroup suggesting utilization of serine and glycine metabolism, while inflammation-related genes were enriched in the Inflammatory subgroup. Furthermore, approximately 75% of MYCN-amplified tumors were identified as Metabolic, a pattern consistent across the platforms. The Neural subgroup closely clustered with low/intermediate risk group and showed highest expression of NTRK1. Pathway enrichment studies revealed deregulation of the MAPK pathway as a prominent feature across all 3 HR subgroups. Overall survival (OS) for the Neural subgroup was significantly higher than the Inflammatory or Metabolic subgroups in the HuEx high-risk cohort (OS=47% vs. 30%, and 23%; p=0.004, respectively), in the German high-risk validation cohort (OS=52% vs. 20%, and 35%; p=0.09, respectively), and the German low/intermediate cohort (OS=100% vs. 89%, and 75%; p<0.001, respectively). Initial analysis of methylation profiles used to estimate leukocyte infiltration revealed significantly higher infiltration levels in the Inflammatory subgroup compared to other subgroups (p=0.01). Ongoing analyses show trend in improving prognostication when combining HuEx-based subgroups with DNA methylation clusters, but only in the Neural and Inflammatory subgroups.

Conclusion: Integration of genome-wide gene expression and methylation data identifies clinically relevant molecular subsets of high-risk neuroblastomas.

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OR039

High-Risk Neuroblastoma Genomic Plasticity Allows for Significant Clonal Evolution under the Selective Pressure of Chemotherapy

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Background: Tumor relapse occurs in 60% of patients treated for high-risk neuroblastoma. We sought to characterize the differences between primary and relapse tumor genomes in order to gain a better understanding of the molecular mechanisms of tumor relapse that may yield therapeutic insights.

Methods: The neuroblastoma TARGET project performed whole-genome deep sequencing (Complete Genomics) on 9 neuroblastoma trios, consisting of primary tumor, relapse tumor, and normal blood samples from the same patient. Trio identity was confirmed by direct comparison of germline SNP genotypes from sequencing data. Relapse-specific somatic mutations were called using high-stringency thresholds on read and alignment quality and prioritized based on functional prediction in silico and overlap with the COSMIC cancer mutation database.

Results: Relapse tumors contained approximately 38% more somatic mutations on average than their corresponding primary tumor (range -8% to +175%). While a large proportion of high-confidence somatic mutations present in primary tumors were preserved in the paired relapse tumors, a substantial fraction of point mutations and chromosome or chromosome arm-level copy number rearrangements were not preserved, indicating significant genomic plasticity. There were no recurrent mutations unique to the relapse samples, although two cases had new lesions in the MAPK pathway (activating HRAS mutation and an inactivating NF1 mutation). Activating ALK, KRAS, and NRAS mutations and MYCN amplification present in primary tumors were also observed at relapse, indicating that potent oncogenic drivers may be preserved in relapsed disease.

Conclusion: Neuroblastoma tumor evolution under the selective pressure of chemotherapy is characterized by significant genomic plasticity. Relapse tumor genomes are often quite different from the diagnostic tumor genome, further emphasizing the need to access relapse tissue for mutational profiling efforts that will impact therapy decisions. RNASeq and DNA methylation studies on these samples currently underway through TARGET may yield additional insights into the differences between primary and relapsed disease.

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OR040

Genomic Data Integration in High-Risk Neuroblastoma Patients Identifies BRIP1 as a Putative Oncogene on Chromosome 17q

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Background: Neuroblastoma is an aggressive neural crest derived childhood tumor with poor survival rates for the high stage cases. Thus far three bona fide oncogenic driver genes have been identified in neuroblastoma: MYCN, amplified in roughly half of all aggressive neuroblastoma cases, ALK mutations or amplifications in 10% of cases and rare LIN28B amplifications. Importantly, for three major recurrent DNA copy number alterations, i.e. 1p and 11q deletions and 17q gains, no convincing driver genes have been identified so far despite intensive research. More specifically, in high-risk tumors without MYCN amplification, our insights into the molecular pathogenesis remains very limited and novel targets for molecular therapy are needed.

Methods: Therefore, in the present study we aimed to contribute to the identification of neuroblastoma candidate driver genes in the high-risk neuroblastoma subgroups by applying the CONEXIC algorithm (copy number and expression in cancer) to a unique and extensive data set of 211 primary neuroblastoma tumor samples, consisting of combined mRNA and miRNA expression data and DNA copy number data.

Results: In total, 144 unique candidate driver genes were identified, including several known cancer-related genes such as MYCN, BRIP1, TGFBR2 and hsa-miR-10a. Further prioritization of this gene list was done using a scoring system taking into account several (publically available) data sets, including recent large-scale sequencing studies and cancer-related databases. BRIP1 (BRCA1 interacting protein, C-terminal helicase 1), located on 17q23.2, was identified as the top-ranked candidate oncogenic neuroblastoma driver.

Conclusion: BRIP1 is dynamically upregulated during TH-MYCN driven tumor formation in mice as studied in hyperplastic ganglia and tumors. BRIP1 is involved in DNA repair and of particular interest, given that yet another pathway component, BARD1, was recently identified as a neuroblastoma susceptibility gene. In vitro and in vivo zebrafish assays are performed in order to functionally validate the putative role of BRIP1 as oncogene.

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OR041

Whole Genome Screen to Identify Genes Targeting MYCN Driven Embryonal Tumors- Neuroblastoma (NB) Model

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Background: Since MYCN is a driver of neuroblastoma tumorigenesis, we sought to identify regulators of MYCN transcription by performing a whole genome screen for regulators of MCYN promoter activity and NB cell viability.

Methods: The readout system comprised a plasmid containing 1.3kb MYCN promoter fused to luciferase and integrated into the genome of NGP NB cells. A clone, NGP-MYCNpluc, was selected based on the ability of ATRA and HDAC inhibitors to decrease luciferase activity to a similar extent as endogenous MYCN mRNA levels. After assay optimization, we tested siRNAs targeting 11,000 genes using 3 siRNAs/gene. This library encompassed the druggable genome and most transcription factors. Using a 384-well format, siRNAs were reverse transfected in duplicate into NGP-MYCNpluc cells, cultured for 3 days and analyzed for luciferase activity and viability using assayONE-Glo and CellTiter-Glo assays.

Results: A robust statistical measure of median absolute deviation (MAD) was used to standardize siRNA activities in the screen. This identified 36 "high-confidence" genes in which all 3 siRNAs significantly decreased NGP-MYCNpluc luciferase activity by two MAD z-scores, and 49 genes which significantlydecreaseded cell viability. Most drugs associated with these essential viability genes have shown activity in NB cells (bortizamab, gemcitabine/paclitaxel, erlotinib/gemcitabine, UCN-01 and BI 2536). Several MYCNpluc hits don\'t affect a control CMVpluc reporter, suggesting specificity to the MYCN reporter. A subset of specific hits also caused decreases in cell viability that were less than the decreases in MYCN promoter activity, consistent with the known findings that decreases in MYCN decrease cell viability. Low-throughput secondary screens are being utilized for assay confirmation and mechanistic evaluation in additional NB cell lines.

Conclusion: A 23,000 unique gene set is currently under evaluation and more elegant informatics tools will be utilized to illuminate "actives" within the data. This study has the potential to identify regulatory networks for MYCN and NB cell viability and identify novel therapeutically tractable targets.

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B4: Translational Research/ Preclinical Experimental Therapies IV and Minimal Residual Disease

OR042

Parvovirus H-1 Induces Oncolytic Effects in Human Neuroblastoma Preclinical Evaluation in MYCN Amplified Xenograft-Bearing Rodent Models

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Background: The oncolytic parvovirus H-1 (H-1PV) is currently under clinical investigation in a phase I/IIa trial in adult glioblastoma patients, which confirmed clinical safety of intratumoral virus application without reaching dose-limiting toxicity. The oncoselectivity of H-1PV viral transduction and the capacity of efficient virus replication and toxic effects of H-1PV has been recently reported for neuroblastoma (NB) cell lines (n=12). These data implicate the possible application of H-1PV to NB treatment.

Methods: The therapeutic efficacy of intra-tumoral H-1PV infection in vivo was determined in immundeficient rat models bearing xenografts established from two MYCN amplified NB cell lines, IMR-32 and SK-N-BE(2)-c. 2 x 10^6 cells were subcutaneously implanted in nude rats and established tumors were treated with a single intratumoral H-1PV injection. Animals either received 10^9 p. f. u. of wild type H-1PV (treatment group), or an equivalent dose of empty, UV-inactivated capsids (control group). The results were confirmed in a subcutaneous SK-N-BE(2)-c xenograft mouse model. Clinical condition of the tumor-bearing animals and their response to treatment were subsequently monitored.

Results: No relevant virus-induced toxicity was observed in immunodeficient rodents, including the rat which is the natural host of H-1PV. H-1PV significantly repressed tumor growth ($p < 0.001$) and significantly prolonged survival ($p < 0.001$) in all three animal models – irrespective from the host animal species and the cell line implanted. In a comparative study performed in the mouse model, a single intratumoral parvovirus injection was significantly more efficient than continuous doxorubicin treatment. Intratumoral H-1PV treatment induced complete long-term remissions of up to one year in several animals.

Conclusion: In different high-risk neuroblastoma animal models, a single intratumoral injection of H-1PV induced highly significant treatment response. The present data provide pre-clinical evidence that H-1PV is a promising oncolytic virus that warrants further clinical investigations for virotherapeutic effects in neuroblastoma patients.

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OR043

Bone Marrow-Derived Mesenchymal Stromal Cells (BMMSC) and Tumor-Associated Fibroblasts (TAF) Contribute to a Pro-Tumorigenic Inflammatory Microenvironment That Promotes Drug Resistance in Neuroblastoma

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Background: Therapeutic resistance is the major cause of failure to eradicate neu-

roblastoma (NB). The observation that the detection of circulating NB cells in patients in remission (minimal residual disease) predicts relapse, suggests that the bone marrow is a sanctuary protecting NB cells from therapeutic injury.

Methods: $CD90^+$,$CD44^+$,$CD105^+$,$CD34^-$ human BMMSC were isolated from bone marrow of healthy volunteers and NB patients. NB cells grown in the presence of conditional medium (CM) from co-culture of BMMSC and NB cells were exposed to VP-16 and examined for viability by CytoGlow (Promega) assay.

Results: Exposure of CHLA-255 and SK-N-SH human NB cells to this conditioned medium (CM) increased their resistance to etoposide (IC50 = 0.73 and 0.60 uMol/L respectively, $p < 0.0001$) and activated signal transduction and activator of transcription (STAT)-1 and -3 and extracellular signal regulated kinase (ERK1/2). The sensitivity of NB cells to etoposide in the presence of this CM was restored, when NB cells were pre-treated with a JAK2/STAT3 kinase inhibitor (Ruxolitinib; 1 μM) in combination with a MEK inhibitor (Trametinib; 1μM) whereas alone, each inhibitor had no effect. In vivo, co-injection of NB cells and BMMSC in NOD/SCID mice resulted in a significant increase in tumor formation and growth. We also isolated from a primary human NB tumor, $CD90^+$,$CD44^+$,$CD105^+$,$CD34^-$ cells that share many properties of BMMSC including their production of IL-6 and VEGF and ability to promote resistance to etoposide. Like BMMSC, these cells expressed fibroblast-associated protein α (FAPa), a marker of tumor-associated fibroblasts (TAF).

Conclusion: Our data provide a new insight into a mechanism of drug resistance in NB by demonstrating that MSC in the bone marrow and in primary tumors (as TAF) are a source of chemokines and cytokines that promote NB cell survival. They suggest that blocking pathways activated in NB cells by these cytokines/chemokines can restore drug-sensitivity.

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OR044

Synthesis of Para- and Meta 4-(Guanidinomethylphenoxyl)-Butylmethansulfonate (pBBG / mBBG) and their Effects on Neuroblastoma Cells Compaired to mIBG and Busulfan

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Background: Radio-labeled mIBG (meta-Iodobenzylguanidine) is widely used for diagnosis and therapy of neuroblastoma. Busulfan (in combination with melphalan) proofed to be very effective against neuroblastoma.The aim of our project was to synthesize a hybrid molecule of butylmethansulfonate, the alkylating group of busulfan, and benzylguanidine (pBBG / mBBG). It was investigated whether the new substances combine properties of mIBG (specific uptake and influence of glucose metabolism) and anti-neuroblastoma effects of busulfan.

Methods: pBBG and mBBG were synthesized according to the method described in [1].Effects of both substances were analyzed on four neuroblastoma cell lines (SK-N-SH; SiMa; Kelly; LS) compared to unlabeled mIBG and to busulfan (MTT-proliferation assay; LDH release assay; influence on glucose metabolism; competitive uptake studies using radiolabeled noradrenaline).

Results: pBBG as well as mBBG retained properties of mIBG: Competitive uptake studies showed that the incorporation of noradrenaline was effectively blocked by an excess of pBBG / mBBG, whereas busulfan was without any effect. Furthermore, increase of glucose consumption and lactate production, known as a consequence of mIBG treatment [2], were observed during incubation with pBBG / mBBG, but not with busulfan. Proliferation / vitality of cells was stronger influenced by BBGs than by busulfan (pBBG>mBBG>busulfan>mIBG; MTT-test). Similarly, in cytotoxic assays (LDH-release) pBBG and mBBG were much more effective than busulfan and mIBG.

Conclusion: The synthesized chimeric substances pBBG and mBBG combine properties of mIBG (specific transport into neuroblastoma cells; stimulation of glycolysis) and anti-neuroblastoma effects of busulfan. We conclude that they could be useful for a more effective therapy of neuroblastoma by combining the features of an alkylating drug and a more specific uptake into the target cells. [1]: Th. Hampel. Thesis (2013). Institute of Organic Chemistry and Biochemistry, Albert-Ludwigs University of Freiburg, Germany. [2]: C.Loesberg et al. Impaired mitochondrial respiration and stimulated glycolysis by meta-iodobenzylguanidine (MIBG). Int. J. Cancer 46: 276-281 (1990).

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OR045

Temozolomide + irinotecan Followed by fenretinide/LXS + ketoconazole + vincristine is Active in Post-progressive Disease Neuroblastoma Xenografts

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Background: Chemotherapy for recurrent/refractory high-risk neuroblastoma commonly employs cyclophosphamide (cyclo) + topotecan (topo), or temozolomide (TMZ) + irinotecan (irino). Fenretinide (4-HPR) achieved multiple complete responses in a phase I clinical trial in recurrent neuroblastoma when formulated as 4-HPR/LXS oral powder; 4-HPR plasma levels (clinically) and associated anti-tumor activity (pre-clinically) are increased by P450 inhibitor, ketoconazole (keto). Vincristine (VCR) significantly increased 4-HPR/keto activity in neuroblastoma xenografts. We sought to identify an optimal re-induction chemotherapy combination and test the activity of 4-HPR/keto/VCR against minimal residual disease (MRD).

Methods: Multidrug-resistant, human neuroblastomas cell lines (CHLA-79, CHLA-171, FU-NB-2006 and CHLA-136) and patient-derived xenograft (COG-N-426), all from patients with progressive disease were xenografted into nu/nu mice. Drugs: TMZ (25 mg/kg, p.o.), irino (7.5 mg/kg, i.v.), cyclo (22 mg/kg, i.p.) and topo (0.4 mg/kg, i.p.), were all given q.d. x 5 = 1 cycle, every 21 days; 4-HPR/LXS (180 mg 4-HPR/kg, p.o.), keto (38 mg/kg, p.o.) were given q.d. x 5/week; VCR (0.125 mg/kg, i.v.) was given twice weekly, alternating weeks.

Results: Event-free survival of mice at 250 days after 3 cycles of therapy for CHLA-79 was: 5/5 (100%) for TMZ+irino vs. 0/5 (0%) for cyclo+topo ($P<0.002$); for CHLA-171 xenografts: 2/6 (33%) for TMZ+irino vs. 0/6 (0%) for cyclo+topo ($P<0.03$). CHLA-136 xenograft responses and event-free survival were higher ($P<0.005$) with TMZ+irino (105.0 ± 14.94 days) vs. cyclo+topo (67.0 ± 20.9 days). TMZ+irino activity was greater than either single agent ($P<0.03$). We tested one cycle of TMZ+irino followed by 4-HPR+keto+VCR: in FU-NB-2006, survival at 100 days was 4/7 in CR (57%) with 4-HPR+keto+VCR vs. 0/7 (0%) with no further treatment ($P<0.0002$). In COG-N-426, survival at 100 days with 4-HPR+keto+VCR was 2/10 slowly progressing (20%) vs. 0/9 (0%) with no further treatment ($P<0.01$). The combination regimens were well-tolerated.

Conclusion: TMZ+irino was a more effective re-induction regimen than cyclo+topo for recurrent/refractory neuroblastoma xenografts. 4-HPR+keto+VCR was active against MRD after TMZ+irino. A phase I study of 4-HPR/LXS oral powder + keto + VCR is ongoing in the South Plains Oncology Consortium (www.SPONC.org).

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OR046

Defining Sensitivity Profiles for Bromodomain and Extra-Terminal (BET) Protein Inhibition

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Background: MYCN amplification is the most frequent somatically acquired genomic alteration in neuroblastoma and is a potent oncogenic driver. Recently, several groups have shown that inhibiting the epigenetic functions of BET proteins resulted in silencing of MYC family protein expression, demonstrating therapeutic efficacy in preclinical cancer models. We hypothesized that BET inhibitors (BETi) would show anti-tumor activity in preclinical neuroblastoma models and that reliable biomarkers of activity could be discovered.

Methods: GlaxoSmithKline BETi GSK726 and GSK762 were used for in vitro and in vivo studies, respectively. We analyzed cell cycle by flow cytometry and apoptosis by cleaved PARP immunoblotting. GSK762 efficacy was tested in subcutaneous xenograft and genetically engineered neuroblastoma mouse models. For biomarker discovery, gene expression data from neuroblastoma cell lines were analyzed.

Results: Neuroblastoma cell lines were differentially sensitive to BETi, with sensitive cell lines demonstrating G1 arrest, apoptosis and delayed in vivo tumor growth. Importantly, MYCN amplification status did not fully account for BETi sensitivity. Thus, we identified additional biomarkers by examining baseline gene expression data, comparing sensitive (N=6; $IC_{50}<128$ nM) and resistant (N=4; $IC_{50}>940$ nM) neuroblastoma cell lines. Univariate analysis (FDR < 0.25) revealed 6 genes differentially expressed between sensitive and resistant cell lines. While all 6 genes predicted BETi sensitivity in the MYCN-amplified subset, only DACH1 expression predicted sensitivity irrespective of MYCN amplification status ($p=7.92x10^{-7}$). DACH1 levels were heterogeneously expressed in primary neuroblastoma tumors, with high expression correlated with poor outcome ($p=1.74x10^{-5}$). Depletion of DACH1 in SKNFI via shRNA conferred increased resistance to BET inhibition therapy.

Conclusion: BET inhibitors demonstrated significant anti-tumor activity in a subset of neuroblastoma models. As MYCN amplification status alone did not fully explain BETi sensitivity, we are determining whether DACH1 expression serves as a biomarker for sensitivity to BETi and whether DACH1 itself influences BETi sensitivity.

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OR047

Minimal Residual Disease Detection in Autologous Stem Cell Grafts of High Risk Neuroblastoma Patients

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Background: The presence of minimal residual disease (MRD) detected by real-time quantitative PCR (qPCR) in autologous stem cell grafts in high risk neuroblastoma remains controversial. In this retrospective multicenter study, autologous stem cell grafts of a large cohort were studied by using a panel of RNA markers.

Methods: In total, 17 BM, 76 PBSC and 47 CD34 selected grafts from 140 high risk neuroblastoma patients, who received autologous stem cell transplantation as first line treatment, were retrospectively collected at 2 Dutch and 12 German centers between 1986 and 2012. qPCR was performed by using six neuroblastoma specific markers: PHOX2B, TH, DDC, GAP43, CHRNA3 and DBH. The prognostic impact of tumor contaminated grafts and clinical response were assessed using Kaplan-Meier curves, log-rank tests and multivariate Cox analysis. Correlation between harvest contamination and BM MRD positivity was done using Fisher's exact test.

Results: BM grafts were most often contaminated (6/17). In PBSC 9/76 and in CD34 selected grafts 6/47 samples were contaminated. Graft contamination was not significantly correlated with an unfavarouble outcome (5-years EFS of 24.5% versus 44.6% , p=0.08). Contamination of the graft was significantly associated with BM MRD (qPCR or immunocytology) positivity at time of harvest. In multivariate Cox analysis only BM disease at time of harvest was significantly associated with survival.

Conclusion: BM grafts were more often contaminated than PBSC or CD34 selected grafts. Tumor contamination of the harvest was significantly correlated with BM positivity at time of harvest and BM positivity at time of harvest was associated with a poor survival. So the autologous graft reflects BM remission status, which is significantly correlated with prognosis.

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OR048

Bone Marrow Minimal Residual Disease after 2 Cycles of Immunotherapy was an Early Response Marker and the Strongest Independent Predictor of Survival for Patients with High-risk Metastatic Neuroblastoma Following Anti-GD2 Immunotherapy

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Background: Immunotherapy using anti-GD2 antibody is now a standard of care for children with high-risk metastatic neuroblastoma. Identifying predictive markers of response and survival should help optimize therpeutic strategies.

Methods: Prognostic markers were analyzed in 343 patients treated at Memorial Sloan-Kettering Cancer Center under 4 protocols (NCI-V90-0023, NCT00002634, NCT00002560, and NCT00072358). All patients had stage 4 disease, diagnosed at ≥18 m at diagnosis and/or with MYCN amplification. They were treated with anti-GD2 antibody mouse 3F8 ± granulocyte-macrophage colony-stimulating factor (GM-CSF) ± cis-retinoic acid. The three disease groups were first remission (n=169), primary refractory (n=105), and ≥ second remission (n=69). Immunotherapy cycles were given every 1-3 months over 2 years if human anti-mouse antibody (HAMA) response was negative. Progression-free survival (PFS) and overall survival (OS) were estimated by Kaplan-Meier method; prognostic variables were tested using log-rank test and Cox regression analysis. Bone marrow minimal residual disease (MRD) before 3F8 (pre-MRD) and post-second 3F8 cycle (post-MRD) was measured by quantitative reverse transcription-PCR (qRT-PCR) using a 4-marker panel (B4GALNT1, CCND1, ISL1, and PHOX2B).

Results: For PFS, post-MRD and disease group (reflecting remission status) were significant independent prognostic factors. HAMA, disease group, post-MRD, FCGR2A polymorphism, and missing killer immunoglobulin-like receptor (KIR) ligand were all independently predictive of OS. Moreover, there was statistically significant interaction between FCGR2A and disease group, as well as between post-MRD and disease group; FCGR2A being strongest among primary refractory patients, while post-MRD was strongest among 1st remission group.

Conclusion: Marrow MRD determined after two cycles of immunotherapy was the strongest and most consistent predictor of outcome, followed by remission status before immunotherapy.

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Endpoint	Prognostic Variable	Hazard ratio	Lower bound	Upper bound	p-value
PFS	Post-MRD (positive *vs* negative)	4.23	3.14	5.70	<0.001
	1st remission *vs* primary refractory	0.68	0.49	0.95	0.023
	2nd remission *vs* primary refractory	1.24	0.85	1.81	0.270
OS	HAMA (positive *vs* negative)	0.41	0.28	0.59	<0.001
	Post-MRD (positive *vs* negative)	3.06	1.65	5.69	<0.001
	FCGR2A ("HR or RR" *vs* "HH")	0.31	0.16	0.60	0.001
	Missing KIR ligand (favorable *vs* unfavorable)	0.61	0.42	0.87	0.006
	1st remission *vs* primary refractory	0.17	0.07	0.39	<0.001
	2nd remission *vs* primary refractory	0.52	0.22	1.21	0.129

A5: Basic Research/ Developmental Neuroblastoma Biology

OR049

Knock-In Mice with Activated Alk Display Prolonged Neurogenesis as a Predisposition Step to Neuroblastoma

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Background: Neuroblastoma, an embryonal neoplasm of the sympathetic nervous system (SNS), is one of the most frequent and aggressive pediatric cancer. Activating mutations of the ALK (Anaplastic Lymphoma Kinase) gene, encoding a tyrosine kinase receptor that belongs to the insulin-receptor superfamily, have been identified in both sporadic and familial cases.

Methods: To decipher ALK function in neuroblastoma predisposition and oncogenesis, we have characterized knock-in (KI) mice bearing the two most frequent mutations observed in neuroblastoma patients.

Results: A significant enlargement of sympathetic ganglia is observed in AlkF1178L mice from embryonic to adult stages and is associated with an increased proliferation of sympathetic neuroblasts at birth. Whereas neither AlkR1279Q nor AlkF1178L mice spontaneously develop tumors, TH-MYCN/KI Alk mice present neuroblastomas with much higher penetrance and shorter latency than TH-MYCN mice. Our data demonstrate that the oncogenic potential of the F1178L mutation is higher than the one of the R1279Q mutation in vivo. Furthermore, we show that tumors expressing the R1279Q mutation are sensitive to ALK inhibition upon crizotinib treatment. Most MYCN/Alk tumors exhibit signs of neuronal differentiation and a dual expression of adrenergic and cholinergic markers. Moreover, we identify Ret and Vip as targets of activated Alk in murine MYCN/Alk tumors and human neuroblastoma cell lines.

Conclusion: These findings provide new insights into the role of the ALK gene in development and pathogenesis of the SNS and indicate that these mice represent relevant Alk-dependent neuroblastoma models.

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OR050

Time-Resolved Transcriptome Analysis of TH-MYCN Driven Hyperplastic Ganglia and Tumors Marks BRD3 as a Novel Candidate Oncogene

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Background: Time-resolved transcriptome analysis of a transgenic mouse model has been shown useful to study the molecular events associated with tumor progression, and to identify novel therapeutic candidates. However, broader knowledge of the ongoing biological processes and the contribution of miRNAs in this network of molecular events is lacking.

Methods: Therefore, we performed a time-resolved transcriptome analysis for both mRNA and miRNA genes, of hyperplastic lesions, at one and two weeks of age, and tumors derived from the TH-MYCN transgenic mouse model.

Results: Analysis of known oncogenic and suppressive mRNA/miRNA genes involved in neuroblastoma showed the expected dynamic regulation during tumor development, validating our dataset and data-analysis strategy. Upon assessment of the dynamic expression profiles of the 513 Cancer Gene Census genes, 21 upregulated CGC genes were identified, mainly involved in chromatin remodeling and DNA repair. BRD3 was identified as top-ranked gene in this analysis. Like BRD4, BRD3 belongs to the family of a highly conserved class of epigenome readers: the BET family of bromodomain-containing proteins that bind to acetylated lysine residues in histones, recruit chromatin-modifying enzymes to target promoters, and function as co-activators or co-repressors in a context-dependent manner. The present observation warrants further investigation into the role of BRD3 in neuroblastoma. Further analysis of the time-resolved transcriptome data identified several hubs in the regulatory network specifically expressed in neuroblastoma, according to the Cancel Cell Line Encyclopedia, marking these genes as putative therapeutic targets. Finally, we integrated the top-ranked differentially expressed protein coding genes and miRNAs into a global perturbed regulatory network.

Conclusion: In conclusion, time-resolved transcriptome analysis reveals a strong enrichment of upregulated chromatin modifiers and DNA repair genes, thus offering novel entry points for therapeutic intervention. Moreover, the overexpressed chromatin remodeling and DNA repair genes mark a stem cell-like phenotype, which may explain the refractory behavior of neuroblastoma tumors to chemotherapy.

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OR051

Genome Wide Analysis of Ascl1, Cdk Inhibitor and Retinoic Acid Induced Neuroblastoma Differentiation

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Background: Neuroblastoma (NB) is an infant cancer affecting the noradrenergic (NA) sympathetic ganglia. NB cells resemble undifferentiated precursors suggesting they may be locked in a pro-proliferative progenitor state hence NB differentiation may be beneficial to patients. NB cells differentiate in response to retinoic acid (RA) treatment and Cyclin dependent kinase (Cdk) inhibition. Mechanistically, Cdk regulation of pro-neural protein phosphostatus has been shown to drive differentiation and Ascl1 is the master pro-neural regulator expressed in precursor cells of the NA lineage. We hypothesised Cdk inhibition, Ascl1 de-phosphorylation and RA treatment target common pathways required for NB differentiation.

Methods: We generated stable, inducible WT and S-A mutant (where all putative Cdk phosphorylation sites have been mutated) Ascl1 expressing SHSY5Y cells and analysed the effect of Ascl1 over-expression on NB differentiation, comparing it to RA treatment and Cdk inhibition. Chromatin immunoprecipitation and genome wide DNA sequencing was used to determine direct Ascl1 transcriptional targets while RNA sequencing characterized the transcriptome of RA or Cdk inhibitor treated and Ascl1 over-expressing NB cells.

Results: WT and S-A Ascl1 over-expression resulted in growth arrest and differentiation of SHSY5Y cells with S-A cells presenting as morphologically more mature than their WT counterparts. Genome wide characterization of Ascl1 transcriptional targets identified enhanced promoter enrichment for S-A relative to WT protein. We are currently comparing the transcriptome of Ascl1 expressing SHSY5Y cells to that of retinoic acid (RA) and Cdk inhibitor differentiated NB cells to determine essential regulators of NB differentiation.

Conclusion: We have expanded our knowledge of Ascl1 transcriptional targets in NB cells as well as the key involvement of this transcription factor and its phosphorylation status in NB differentiation. Furthermore, our data should identify transcriptional profiles of NB differentiated cells for three differentiation protocols and determine converging pathways which we are exploring as druggable targets for novel and RA combination therapies.

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OR052

Role of the CHD5 Tumor Suppressor in Neuroblastoma Pathogenesis Using the Zebrafish Model

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Background: Chromosome band 1p36 deletions are found in about 35% of primary neuroblastoma (NB) samples and in 70%-80% of high-risk tumors with MYCN gene amplification. However, the role of 1p36 loss in NB pathogenesis remains elusive. CHD5 (chromodomain, helicase and DNA-binding protein 5) has emerged as a candidate haploinsufficient tumor suppressor gene on chromosome 1p36. CHD5 is a key component of the NURD complex, which is recruited to super-enhancers and is required to repress the expression of pluripotency genes when progenitor cells are induced to differentiate.

Methods: To assess the contribution of CHD5 loss to NB tumorigenesis in vivo, we generated loss-of-function CHD5 mutant zebrafish lines by genome editing using Zinc Finger Nuclease (ZFN) and Transcription Activator-Like Effector Nuclease (TALEN) strategies.

Results: Fish homozygous for disruption of CD5 coding sequences are viable and fertile. Using a panel of lineage markers for the peripheral sympathetic nervous system (PSNS), we found expansion of cells committed to PSNS development in the CHD5 mutant fish. Increased sympathoadrenal cells were observed in the superior cervical ganglia and in the interrenal gland (IRG, the zebrafish equivalent of the human adrenal gland) of CHD5 mutants. However, CHD5 mutant fish did not develop NB or other tumors. To test the ability of CHD5 loss to synergize with MYCN in transformation, CHD5 mutant lines were intercrossed with dβh:MYCN transgenic zebrafish. We observed increased penetrance and marked acceleration of the onset of NB tumors in CHD5 haploinsufficient fish. Tumors arose from 6 weeks of age in the dβh:MYCN; CHD5+/- fish, compared to more than 12 weeks in dβh:MYCN; CHD5+/+ fish.

Conclusion: These in vivo studies implicate CHD5 as at least one of the important 1p36 haploinsufficient tumor suppressors in NB pathogenesis, providing an ideal zebrafish model system to investigate mechanisms through which CHD5 loss synergizes with MYCN overexpression in NB pathogenesis.

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OR053

Transcription Factor Activating Protein 2 beta (TFAP2B) Mediates Neuronal Differentiation and Is a Prognostic Marker in Neuroblastoma

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Background: Induction of tumor cell differentiation by retinoic acid is an important part of current neuroblastoma treatment protocols. The molecular mechanisms underlying differentiation processes in neuroblastoma, however, are still poorly understood. Transcription factors of the AP-2 family (TFAP2) play important roles in embryonic differentiation and development. We here aimed to investigate the role of TFAP2B in neuroblastoma pathogenesis and differentiation.

Methods: The association of TFAP2B expression with prognostic markers and outcome was analyzed in 649 primary neuroblastomas using microarray data. Methylation of CpG sites related to TFAP2B was investigated by using Illumina 450K arrays in 105 primary neuroblastomas. To evaluate the functional relevance of TFAP2B in neuroblastoma, TFAP2B was re-expressed in IMR-32 and SH-EP neuroblastoma cells, and knocked down in SH-SY5Y and BE(2)-C neuroblastoma cells.

Results: Low expression of TFAP2B was associated with adverse patient outcome ($p<0.001$) and unfavorable prognostic markers, e.g. stage 4 disease, age >18 months, MYCN amplification, and unfavourable gene expression-based classification ($p<0.001$ each). In addition, low TFAP2B expression was strongly correlated with methylation of promoter regions of the TFAP2B gene in high-risk neuroblastoma. In IMR-32 cells, demethylation with 5-Aza-2´-deoxycytidine induced TFAP2B expression. Tetracycline inducible re-expression of TFAP2B in IMR-32 and SH-EP cells significantly impaired proliferation and led to G1-arrest ($p<0.01$ each). Morphological signs of neuronal differentiation and senescence were observed after TFAP2B induction in IMR-32 and SH-EP cells, respectively. Knock-down of TFAP2B by lentiviral transduction of specific shRNAs effectively abrogated retinoic acid induced neuronal differentiation of SH-SY5Y and BE(2)-C cells as indicated by microscopic examination and lack of up regulation of neuron related genes such as microtubule associated protein 2 (MAP2), neurofilament middle chain (NEFM) and synaptophysin (SYP).

Conclusion: Our data suggest that TFAP2B expression is silenced in high-risk neuroblastoma by promoter methylation, and that TFAP2B expression might be critical for retinoic acid induced differentiation in neuroblastoma.

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OR054

Integrative DNA Methylome and Transcriptome Analysis Reveals Suppression of Differentiation Programs in High-Risk Neuroblastoma

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Background: Neuroblastoma pathogenesis is associated with chromosomal deletions, amplified MYCN and a limited number of additional recurrent genetic events. However, the extraordinary clinical heterogeneity seen in neuroblastoma patients can only partially be explained by genetic alterations identified to date. Deregulation of cancer-relevant genes by DNA methylation has been shown to substantially contribute to carcinogenesis and understanding these mechanisms holds great promise for neuroblastoma diagnosis and therapy.

Methods: Global DNA methylation and trancriptome profiles of 105 primary neuroblastomas and two 5-Aza-2\'-deoxycytidine (DAC)-treated neuroblastoma cell lines were generated using Illumina 450k methylation and Agilent 4x44k arrays, respectively. Unsupervised hierarchical clustering was performed on the 1,000 most variable CpG probes. To identify genes potentially regulated by DNA methylation, maximally selected log-rank statistics were used.

Results: Unsupervised clustering of 105 neuroblastoma patients based on global DNA methylation identified subgroups of divergent biology with respect to clinicobiological variables, survival and MYCN activity. Transcriptome integration identified genes differentially methylated and expressed between high- and low-risk patients. Negative correlation between DNA methylation and gene expression was associated with 5'-regulatory regions, whereas positive correlation was seen for gene body methylation. Genes downregulated and hypermethylated in high-risk patients are

enriched for neuronal functions and have been previously shown to be mutated in primary neuroblastomas to a substantial fraction. DAC treatment induced their significant demethylation and reexpression in neuroblastoma cell lines.

Conclusion: The strong association of global DNA methylation with clinicobiological parameters and patient survival indicates that aberrant DNA methylation is a hallmark of neuroblastoma biology and illustrates its potential to stratify and reveal biological differences between neuroblastoma subtypes. Downregulation of genes with neuronal functions in high-risk tumors suggests that aberrant methylation contributes to neuroblastoma dedifferentiation. A subset of these genes is targeted independently by mutation, indicating that genetic and epigenetic events cooperate to impair neuroblastoma suppressive functions.

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B5: Clinical Research I

OR074

Haploidentical Stem Cell Transplantation and Subsequent Immunotherapy with antiGD2 Antibody for Patients with Relapsed Metastatic Neuroblastoma

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Background: Pediatric patients with relapsed metastatic neuroblastomas have a poor prognosis and additional therapeutic strategies are needed. We present an ongoing SIOPEN phase I/II-trial with subsequent immunotherapy with an anti-GD2mAb (CH14.18/CHO) after HLA mismatched, haploidentical stem cell transplantation (SCT).

Methods: T- and B-cell depleted stem cells from parental donors were used in combination with melphalan 140mg/m^2, thiotepa 10mg/kg, fludarabin 160mg/m^2 and ATG-F. Infusions with CH14.18/CHOmAb were started on day 60-180 posttransplant: 6 cycles with 20mg/m^2/day x 5; in cycles 4-6, 1x10^6 units/m² IL2 were given additionally. 22 patients with 1st or 2nd metastatic relapse were enrolled up to now.

Results: During antibody infusions, endogenous secretion of Interleukin 2 was increased (928U/ml prior vs. 1690U/ml post, p<0.001), which resulted in significantly increased numbers of activated CD69+ Natural Killer (NK) cells (3 vs. 13% p<0.01). In all investigated patients, effective ADCC and complement mediated (CDC) anti-tumor effects against neuroblastoma cells were detectable in-vitro (69% specific lysis, E:T-ratio=20:1, BATDA-release). 16/22 patients finished the protocol so far and were evaluable after 6 cycles, 6 patients dropped out during therapy. 6/22 patients could maintain a CR, 7 patients improved their partial remission and 8 (36%) patients progressed during therapy (according to whole body MRI and/or MIBG scan and bone marrow aspirate), 1 patient died from HHV6 encephalitis. Thus, success of treatment defined as stable disease or improvement was shown in 60%. Overall/progression free survival at 2 years was 60% and 40% (median follow up: 500 days). Side effects were: pain, fever and CRP elevation in all patients; SIRS, n=4; seizures, n=3; transient graft versus host disease (GvHD) II, n=1; persistent vomiting, n=1.

Conclusion: Conclusions: CH14.18 infusions after haploidentical stem cell transplantation appear to be feasible without increased risk of inducing GvHD. Preliminary results of our ongoing study suggest an anti tumor effect of the new, donor-derived immune system in-vitro and in-vivo.

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OR075

Phase I Study of Haploidentical Natural Killer (NK) Cells plus Monoclonal Antibody 3F8 for Resistant High-Risk Neuroblastoma (HR-NB)

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Background: KIR and HLA genotypes define NK activity and are key prognostic markers in 3F8-treated patients. Haploidentical NK-cells can be optimized for anti-NB cytotoxicity by selecting NK donors: from licensed NK-cells responding to "missing self (MS)" or from unlicensed NK-cells responding to "missing ligand (ML)". We previously (ANR meeting 2012) reported preliminary data on our phase I study of haploidentical NK-cells plus 3F8 for the treatment of resistant high-risk NB (www.clinicaltrials.gov NCT00877110). Having almost completed patient accrual, we present an update.

Methods: The primary objective was to determine the maximum tolerated NK-cell dose (MTD). Secondary objectives included assessing anti-NB activity and its relationship to KIR/HLA genotypes, NK function, and chimerism. For the first cycle, patients received a lymphodepleting regimen of high-dose cyclophosphamide, topotecan and vincristine (days 1-3) prior to infusion (day 5) of NK-cells isolated from donor leukophereses using a process of CD3-depletion (to <2x10^4 CD3+ cells/kg) followed by CD56-enrichment. For subsequent cycles (≤3), conditioning was reduced to cyclophosphamide alone. 3F8 (20mg/m^2/day) was administered on days 8-12.

Results: 20 patients received 23 cycles: 1, 9, 5 and 5 at dose-level 0(<1x10^6 CD56+cells/kg), I(1-4.99x10^6), II (5-9.99x10^6) and III(10-30x10^6) respectively. 7, 7 and 6 donors had ML, ML+MS and neither respectively. MTD has not yet been reached. One patient had DLT: grade 4 hypertension and vomiting. The only other >grade 2 possibly-related toxicity was transient hepatic transaminitis. Neither GvHD nor myeloablation was observed. Responses by INSS (n=19) were: 3 complete remission (n=3), 13 stable disease (7/13 had improved MIBG scores [median-4]), and 3 progressive disease (all donors without ML or MS).

Conclusion: Results from this first-in-human trial of NK-cells plus antibody against solid tumors establish its safety following cytoreduction, and efficacy for some patients with high-risk NB. Future plans include reducing conditioning to permit multiple NK-cell infusions, expanding NK-cell ex vivo, incorporating humanized 3F8 and further exploring the role of ML and/or MS.

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OR076

Influence of Extent of Resection on Survival in High Risk Neuroblastoma Patients: A Report from the COG A3973 Study

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Background: The role of primary tumor resection in high risk neuroblastoma patients has been controversial, with numerous contradictory reports in the literature. To determine the effect of extent of primary tumor resection on local recurrence-free survival (LRFS), event-free survival (EFS), and overall survival (OS), we analyzed data from the Children's Oncology Group (COG) A3973 high risk neuroblastoma study.

Methods: A3973 surgery central review data were available for 243 (50%) of 486 eligible patients on the study. Twenty-three additional patients were excluded: 21 who had surgery on a non-primary site, one who was determined ineligible for A3973 after surgery, and one who died on study (deemed unevaluable). Data for the remaining 220 patients were analyzed, with stratification according to extent of primary tumor resection (≥90% vs <90%). Identical radiotherapy to the primary site was prescribed for all patients regardless of extent of resection and consisted of 21.6 Gy (1.8 Gy per daily fraction) to the post-induction chemotherapy, pre-operative tumor volume. Kaplan-Meier analysis was used to estimate OS, EFS, and LRFS probabilities, and the log-rank test was used to assess the statistical significance of differences between stratified patient subgroups.

Results: Primary tumor resection of ≥90% was achieved in 154 (70%) patients, and resection of <90% was achieved in 66 (30%). LRFS, EFS, and OS at 5 years for all 220 patients were 87.3%±3.5%, 43.5%±3.7%, and 54.9%±3.7%, respectively. Survival stratified by extent of resection is presented in the table.

Survival (% at 5 years)	≥90% resection	<90% resection	*P* value
LRFS	91±6	77±9	0.0014
EFS	46±4	38±7	0.0430
OS	57±4	49±7	0.27

Conclusion: This study shows the feasibility of ≥90% resection in high risk neuroblastoma patients. Further, ≥90% resection positively correlated with both event-free survival and local control. These data provide support for ≥90% resection of the primary tumor in high risk patients.

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OR077

Likelihood of Bone Recurrence in Prior Sites of Metastasis in Patients with High-Risk Neuroblastoma

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Background: Despite recent improvements in outcomes, 40% of children with high-risk neuroblastoma will relapse. Whether recurrences are at new sites or sites of original disease may guide decision-making during initial therapy.

Methods: Patients identified at first metastatic relapse of high-risk neuroblastoma were included in this retrospective cohort study if they satisfied all of the following criteria: disease involving MIBG-avid metastatic sites at diagnosis and 1st relapse; complete or partial response with no more than one residual MIBG-avid site prior to 1st relapse; no total body irradiation or therapy with ^{131}I-MIBG prior to 1st relapse. Anatomically defined metastatic sites were tracked from diagnosis through first relapse to determine tendency of disease to recur at previously involved vs. uninvolved sites, and to assess whether this pattern was influenced by site irradiation.

Results: Of a total of 158 MIBG-avid metastatic sites identified among 43 patients at first relapse, 130 (82.3%) overlapped anatomically with the set of 522 sites present at diagnosis. This distribution was similar for bone sites but patterns of relapse were more varied for the smaller subset of soft tissue metastases. Among all sites, only 3/19 previously irradiated metastatic sites (15.8%) recurred as compared to 127/503 unirradiated sites (25.2%).

Conclusion: Metastatic bone relapse in neuroblastoma usually occurs at sites of previous disease involvement. Radiation appears to reduce the risk of relapse at metastatic sites. The large number of bony metastatic sites present at diagnosis precludes treatment with external beam radiation, and suggests a role for systemic treatment with a targeted radiopharmaceutical, such as ^{131}I-MIBG.

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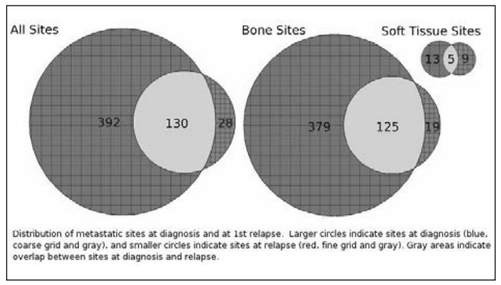

Distribution of metastatic sites at diagnosis and at 1st relapse. Larger circles indicate sites at diagnosis (blue, coarse grid and gray), and smaller circles indicate sites at relapse (red, fine grid and gray). Gray areas indicate overlap between sites at diagnosis and relapse.

OR078

Feasibility of I^{131}-mIBG and Topotecan Therapy Followed by Consolidation with Busulfan, Melphalan and Autologous Stem Cell Transplantation for Refractory Metastatic Neuroblastoma

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Background: ^{131}I-metaiodobenzylguanidine (mIBG) produces more than 30% response rate in refractory neuroblastoma and could be used to improve remission status prior to myeloablative chemotherapy. The aim of our study was to evaluate the safety of ^{131}I-mIBG therapy with Topotecan followed by busulfan and melphalan(BuMel) with autologous stem cell transplantation (ASCT) in patients with refractory metastatic neuroblastoma.

Methods: In this retrospective analysis, toxicity data from patients with refractory neuroblastoma enrolled in the MIITOP protocol followed by BuMel were assessed. During MIITOP, patients received an activity of 12 Mci/kg of ^{131}I-mIBG combined with topotecan. In vivo dosimetry was used to calculate a second activity of ^{131}I-mIBG to be given with topotecan on day 21 to deliver a total whole-body dose of 4 Gy. ASCT was performed on day 32. After MIITOP, patients without progressive disease could receive BuMel consisting of IV busulfan on days -7 to -3 (0.8-1.2mg/kg according to body weight strata), melphalan (140 mg /m^2) on day -2 with ASCT on Day 0. Toxicity was assessed after MIITOP and after Bu-Mel.

Results: Seven patients completed MIITOP followed by BuMel/ASCT (median interval 11 weeks after MIITOP). Immediate tolerance of MIITOP was good with grade 3 non-hematologic toxicity limited to two patients (fever of unknown origin (FUO)). Two patients developed late complications: 1 grade 4 adrenal insufficiency and 1 grade 2 hypothyroidy. After BuMel, two patients developed bacterial sepsis and five FUO. Grade 3 and 4 mucositis occurred in five patients. One patient developed grade 4 sinusoidal obstructive syndrome that recovered on day 32. Median duration of neutropenia and thrombocytopenia was 12 and 36 days respectively .One patient had a persistent thrombocytopenia 140 days post ASCT. At the end of treatment, there were one complete remission, five stable diseases and one progressive disease.

Conclusion: BuMel can be safely administered 11 weeks after MIITOP therapy (^{131}I-mIBG up to 24 mCi/kg with topotecan) in refractory metastatic neuroblastoma. The impact on survival of this treatment combination should be evaluated in a phase II trial.

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OR079

Is Additional Treatment Necessary for a Residual Tumor in Cases of Intermediate-Risk Neuroblastoma?

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Background: It remains unclear whether a residual tumor mass following surgical resection influences the prognosis.

Methods: We retrospectively reviewed 20 patients with intermediate-risk tumors treated at our institution between 1993 and 2012. We examined their clinical course and prognosis after treatment, to elucidate whether additional treatment is required for residual tumors in the next intermediate study of JNBSG.

Results: The patient ages ranged from zero days to seven years old, and the mean observation period was 13.5 years. The five-year overall survival rate was 94.4%. Thirteen patients had stage 3 disease and seven cases had stage 4 disease. Nine cases showed intra-spinal extension.Seventeen of the 20 patients received chemotherapy. Twelve patients had a residual tumor mass at the completion of therapy, and eight showed intra-spinal extension.Five of these 12 cases showed an MIBG uptake at the end of treatment, but the uptake disappeared during the follow-up period.Except for one patient who died due to treatment complications, the rest are all alive, and nine are alive with a residual mass.We performed a biopsy or subtotal resection for the residual mass in four of the nine cases, but the residual tissue had differentiated into a ganglioneuroma or changed to necrotic tissue in all four cases. For the three cases with neurological symptoms at the end of treatment, some slight neurological symptoms still remained during the follow-up.Five cases with an intra-spinal mass eventually presented with new symptoms, such as scoliosis or hydronephrosis.

Conclusion: The presence of a residual mass at the end of treatment did not influence the patients' prognosis. Therefore, an invasive radical surgical resection and additional treatment should be avoided during the next intermediate study of the JNBSG. Cases with a residual intra-spinal mass therefore also require a long-term follow-up to assess the neurological prognosis.

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A6: Basic Research, Translational Research, Clinical Research II

OR087

Selective inhibition of CDK7 targets super-enhancer driven transcriptional programs in MYCN-amplified cells

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Background: Oncogenic MYC family transcription factors act as universal amplifiers of the existing gene expression program in many cancer cells, thus reducing rate-limiting constraints on growth and proliferation. Here we exploit MYCN-driven global transcriptional amplification to specifically target MYCN-deregulated neuroblastoma (NB) cells by inhibiting CDK7, a cyclin-dependent kinase with major roles in transcriptional initiation and elongation.

Methods: We used CDK7-IN-1, a new, selective, first-in-class covalent inhibitor of CDK7, and determined the effects of CDK7 inhibition on MYCN expression and global transcriptional activity.

Results: NB cells expressing high levels of MYCN were 10 times more sensitive to CDK7 inhibition than normal cells or NB cells not driven by amplified MYCN. CDK7-IN-1 was more active than its reversible (non-covalent) analogue and other CDK inhibitors. Cytotoxicity in treated MYCN-amplified NB cells resulted from G2 arrest and apoptosis. We observed a disproportional decrease in transcriptional initiation and elongation as well as downregulation of MYCN and MYCN-associated transcriptional programs in MYCN-amplified NB cells. In MYCN-amplified cells, CDK7 inhibition led to preferential loss of histone H3K27 acetylation at super-enhancers with consequent inhibition of super-enhancer-associated gene expression. CDK7-IN-1 also significantly slowed tumor growth in a xenograft model of MYCN-amplified NB (median growth, 56.8% vs. 100% for vehicle-treated mice, $P < 0.05$; n=6 per group). Mice remained free of toxicity over 4 weeks of CDK7-IN-1 treatment, suggesting that a therapeutic window may exist for NB cells with high MYCN expression.

Conclusion: In conclusion, we show for the first time that selective suppression of MYCN expression and MYCN-associated transcriptional activity can be achieved through CDK7 inhibition, with associated antitumor effects in high-risk NB. We demonstrate that the preferential sensitivity of MYCN-amplified cells to CDK7 inhibition is due to the presence of super-enhancers that regulate key oncogenic drivers including MYCN and are exquisitely sensitive to perturbation.

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OR061

Apoptosis Induced by O-acetyl-GD2 Specific Monoclonal Antibodies Inhibit Neuroblastoma Growth

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Background: We have previously generated the mouse monoclonal antibody (mAb) 8B6 that is specific for O-acetyl-GD2 ganglioside (OAcGD2) with no cross-reaction to GD2. Like GD2, OAcGD2 is over-expressed by GD2-positive neuroblastoma (NB) cells but importantly it is not found on human peripheral nerves. These properties provide a distinct advantage to mAb against OAcGD2 for selectively targeting NB and suggest that OcGD2 mAbs have the potential to be less toxic than anti-GD2 therapeutic antibodies. Here, we investigated the mechanisms by wich mAb 8B6 kills NB cells.

Methods: Immunological toxicities and apoptotic activities of mAb 8B6 was studied in vitro using the mouse NXS2 and the human IMR5 NB cells lines. The Apoptotic signalling pathway triggered by mAb 8B6 was investigated by Western blot analysis. In vivo anti-NB activity of mAb was examined in NOD-SCID mice bearing established IMR5 NB tumors.

Results: We found that in addition to immunological cytotoxicty mediated by complement and immune effector cells such as NK cells , mAb 8B6 directly inhibits NB cell proliferation in vitro by inducing NB cell apoptosis. Antibody 8B6 activates p38 phosphorylation and the mictochondrial apopotic signalling patway in NB cells. These apoptotic properties demonstrated in vitro are also efficient to inhibit NB tumor growth in NOD-SCID mice that are characterized by a functionnal deficit in NK cells and by the absence of circulating complement.

Conclusion: Our results suggest that mAb triggers NB cell apoptosis that lead to a significant inhibition of NB tumor growth in vitro and in vivo. Therefore, our results provided new biological function of OAcGD2 in tumor cell biology, but also may be helpful in optimizing immunotherapy with anti-GD2 mAbs for NB treatment.

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OR062

NKT Cells Turn Bad Inflammation into Good One via Selective Targeting of M2-like Macrophages in Neuroblastoma Microenvironment

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Background: CD1d-reactive Vα24-invariant NKT cells (NKTs) are associated with good outcome in neuroblastoma (NB). However, the mechanism of NKT-cell antitumor activity in NB and other CD1d-negative tumors has remained enigmatic. We reported that NKTs co-localized with CD1d-positive tumor-associated macrophages (TAMs) in primary NB. TAMs comprise M1-like and M2-like subsets but only CD163^{high} M2-like TAMs predict metastatic disease and are a part of a recently discovered inflammatory signature that serves as an independent prognostic factor of poor outcome in NB patients.

Methods: To examine whether NKTs differentially recognize macrophage subsets, we generated M2 (CD163^{high}HLA-DR^{low}) and M1 (CD163^{low}HLA-DR^{high}) macrophages after culture of human monocytes with M-CSF and GM-CSF, respectively. We also used a humanized NB model in NSG mice to study the effect of NKT-cell therapy on macrophage polarization in the tumor microenvironment.

Results: We found that upon direct contact with antigen-pulsed macrophages, NKTs selectively killed M2 cells in a CD1d-dependent manner. Although there was no difference in the level of CD1d expression between M2 and M1 macrophages, the former selectively expressed CD204, a scavenger receptor responsible for cellular uptake and CD1d loading of NKT ligands. Anti-CD204 blocking mAb strongly inhibited NKT-cell cytotoxicity against M2 macrophages. We also found that antigen-activated NKTs could in a cell contact independent manner polarize M2- into M1-like macrophages with downregulation of CD163 and IL-10 expression. Neutralization of GM-CSF strongly inhibited M1-polarizing activity of NKT-cell supernatant. Adoptive transfer of human NKTs resulted in a significant reduction of M2 TAMs in NB xenografts in humanized NSG mice that correlated with anti-metastatic activity.

Conclusion: Our results reveal that NKTs mediate antitumor activity via CD204-dependent killing and GM-CSF-dependent M1-like polarization of M2-like TAMs, providing the mechanistic basis for therapeutic control of tumor-supportive inflammation in NB and other solid tumors.

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OR063

Tumor Infiltration of Regulatory T-cells and Effects of Anti-CTLA4 and Anti-PD1 Therapy in a Murine Model of Neuroblastoma

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Background: The tumor microenvironment is important in the prognosis of neuroblastoma. The role of regulatory T-cells (T-regs) in neuroblastoma is unclear, so we hypothesized that T-regs play an important role in neuroblastoma development and immunotherapy targeted to T-regs could modify the natural course of a murine model of neuroblastoma driven by SV40 large T-antigen (NBL-Tag).

Methods: CD4 T-cell infiltration and inflammation-related gene expression were assessed by immunohistochemistry (IHC), and RT-PCR. T-regs ($CD3\varepsilon^+CD4^+CD25^+FOXP3^+$) were assessed by flow-cytometry. A cell-line from NBL-Tag implanted subcutaneously in syngeneic mice was used for immunotherapy experiments using anti-CTLA-4 and anti-PD-1 antibodies (or isotype) in low and high tumor burden scenarios.

Results: CD4 expression in tumors of ≥12-weeks old NBL-Tag mice (time tumor is visible by MRI) was increased compared with wildtype (WT) adrenals ($p<0.01$) or with tumors of younger mice ($p=0.03$). TGF-β expression, a critical driver of T-reg differentiation, was also increased in these tumors compared with WT- adrenals ($p<0.001$). Twenty-percent of $CD4^+$ cells in NBL-Tag tumors (n=17) were T-regs by flow cytometry. There were also significantly more T-regs compared to WT in relevant para-aortic lymph nodes (mean=9.4% of $CD4^+$ in NBL-Tag vs 0.4% in WT, $p<0.05$). In subcutaneous models, T-regs also infiltrated syngeneic tumors (mean=58.8% of CD4+, n=7 mice). Combined anti-CTLA-4 and anti-PD-1 therapy was not effective in well-established tumors (p=NS) but completely prevented tumor formation in a minimal residual disease (MRD) model. Animals in the antibody treated group failed to form any tumors while 100% of controls formed tumors by 6 weeks post-implantation ($p<0.0001$, n=5 per group).

Conclusion: T-regs are present in NBL-Tag tumors and treatment with anti-CTLA-4 and anti-PD-1 prevented formation of neuroblastoma tumors in a MRD model. Our data provides pre-clinical evidence that Ipilimumab and Nivolumab, recently approved melanoma drugs, may be useful in treating neuroblastoma in children with minimal residual disease.

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OR064

Factors Associated with Recurrence and Length of Survival Following Relapse in UK Patients with High Risk Neuroblastoma

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Background: Despite therapeutic advances for high risk (HR) neuroblastoma patients, survival following relapse remains poor. This pilot study investigated clinical and biological factors associated with recurrence and survival length following relapse in patients with HR disease in the UK.

Methods: All cases of relapsed HR neuroblastoma diagnosed during 1990-2010 were identified from 4 UK Children's Cancer and Leukaemia Group centres. High risk disease = stage 4 >1 year of age or MYCN amplified (MNA) localised (stage 2 & 3) or MNA infant (<12 months).

Results: 159 cases of relapsed HR neuroblastoma were identified. The median age at diagnosis was 3 years (0-19), 96% were stage 4, 45/111 (41%) were MNA, 35/54 (65%) 1p deleted, 120/125 (96%) had unfavourable International Neuroblastoma Pathology Classification histology and 78% relapsed within 2 years of diagnosis. The median progression free survival was 14.4 months (inter-quartile range (IQR) 9.2-21.2). 13% patients relapsed at primary site. Table 1 shows the different treatments received at relapse. , (IMAGE), The median overall survival was 21.8 months (IQR) 13.5–33.5) and the median post relapse overall survival (PROS) time was 4.4 months (IQR 1.9–11.1). 5-year PROS was 12.6% (95% CI 8.2%-17.9%). MNA disease had worse PROS compared with non-MNA cases (Hazard ratio (HR) = 2.0, 95% CI 1.34-3.02; P=0.001). The median PROS for MNA disease was 2.9 months (95% CI 1.9–4.3) versus 7.9 months for non-MNA disease (95% CI 5.9–10.9).

Conclusion: This study confirms that MNA relapsed HR neuroblastoma has worse survival, and 80% of HR relapses occur within 2 years from diagnosis. Analysis of early phase clinical trial data should be stratified by MYCN status, and more patients need to be recruited to early phase studies.

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Table 1: Treatments received at relapse.

Treatment at relapse	Number of cases (N = 132)	Percent
Second line Chemotherapy	55	41.7
mIBG therapy	7	5.3
Palliative radiotherapy	22	16.7
Other (ex. Surgery or radiotherapy)	9	6.8
Supportive care	17	12.9
Combination of treatments	16	12.1
Phase I or II trials	6	4.6

OR065

Prognostic Factors for Response and Outcome on Phase I/II New Approaches to Neuroblastoma Therapy (NANT) Trials Utilizing the NANT Response Criteria (v1.0)

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Background: The NANT Response Criteria (NRC) were developed to define response in recurrent/refractory neuroblastoma patients, who commonly have bone and bone marrow (BM) metastases not assessed by RECIST criteria. This is the first analysis of prognostic factors for response and outcome in a large phase I/II neuroblastoma trials series using consistent eligibility and response criteria.

Methods: A retrospective review was performed of 283 NANT enrollments (217 unique patients) from 3/1/2000 to 10/9/2009 on 13 trials. MIBG therapy trials (5/13) required MIBG-avid sites at entry. Three components of response defined overall response: CT/MRI (RECIST criteria), MIBG (Curie scoring), and BM (morphology). Overall response analyses used all enrollments; EFS/OS analyses used patient's enrollment on first trial. Central review was performed of scans and BM slides for responders and all enrollment MIBG scans. Statistical methods included Pearson Chi-square tests, Fisher's exact tests, logrank tests, and Kaplan-Meyer plots.

Results: Median follow-up was 4.3 years. Disease sites for all enrollments were CT/MRI (50%), MIBG (92% all trials; 86% non-MIBG trials), and BM (48%). Patients without measurable CT/MRI lesions at enrollment were more likely to have overall CR/PR ($p=0.028$). Non-measurable CT/MRI correlated with higher EFS ($p=0.020$) and OS ($p=0.006$). Negative BM (non-MIBG trials) correlated with OS ($p=0.008$). No MIBG sites (non-MIBG trials) correlated with EFS ($p=0.013$) and OS ($p=0.011$). Curie score did not correlate with EFS/OS. Overall CR/PR correlated with OS ($p=<0.001$) but not EFS ($p=0.15$) from time of response. Increasing age at diagnosis ($p=0.007$) correlated with OS. MYCN correlated with EFS ($p=0.020$) and OS ($p=0.031$). Sex and tumor stage were not significant.

Conclusion: Patient characteristics at diagnosis and study enrollment may be prognostic for outcome of recurrent/refractory neuroblastoma, and facilitate optimal Phase I/II trial design to achieve homogenous patient populations and use of EFS/OS endpoints. Response defined by NANT Response Criteria is correlated with survival.

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OR066

Assessment of Primary Site Response in Children with High Risk Neuroblastoma: An International Multicenter Study

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Background: Per International Neuroblastoma Response Criteria (INRC), partial response (PR) in a primary tumor is defined as >50% reduction in volume. Measurement of the longest diameter of a tumor is used in RECIST criteria. We compared the operating characteristics of one- vs. three-dimensional assessments of primary tumor response.

Methods: Newly diagnosed patients with high-risk neuroblastoma treated at seven centers were included if three tumor measurements [antero-posterior(AP), transverse, cranio-caudal(CC)] were recorded at least twice prior to resection. A PR in one dimension (1D) was defined as >30% reduction in longest diameter. A PR in three dimensions (3D) was defined as >50% reduction in volume (AP x transverse x CC x $\pi/6$). Patients were categorized by prognostic factors including age, stage, MYCN status, and histology. Survival plots, life tables, log-rank and chi-squared tests were used to compare survival and tumor resectability in responders vs. non-responders by 1D or 3D measurements. Cox proportional hazard models were fit to determine prognostic strength of response measures.

Results: Five-year overall survival for all 248 patients was 51.6%±5.9%. Tumor diameter reduction >30% was observed in 150 patients, volume reduction >50% in 196, and volume reduction >65% in 174. While volume reduction >50% was sensitive for detection of response (76.5% responders by this criterion), it was a poor predictor of outcome (specificity 17%). Sensitivity of maximum diameter reduction was 55%, however it too poorly predicted survival (specificity 30.5%). Cox models showed that no response measure was independently predictive of outcome when compared to covariates MYCN status and stage, and none accurately predicted extent of tumor resection.

Conclusion: None of the primary tumor response measures evaluated is a superior predictor of outcome or resectability in high-risk neuroblastoma. A single measurement is easier to perform, therefore comparison of single largest diameters is recommended for assessment of primary tumor response in the revised INRC.

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B6: Clinical Research III

OR067

Myeloablative Therapy (MAT) and Immunotherapy (IT) with ch14.18/CHO for High Risk Neuroblastoma: Update and News of Randomised Results from the HR-NBL1/SIOPEN Trial

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Background: The HR-NBL1/SIOPEN trial randomised 2 essential treatment concepts: Randomisation R1 investigated BUMEL superiority whilst randomisation R2 tested the benefits of adding subcutaneous interleukin 2 (scIL2) to ch14.18/CHO-mAB immunotherapy (IT).

Methods: After Rapid Cojec induction patients (pts) were randomised in R1 (296 BuMel, 302 CEM) till 09/2010. Median follow up is 6.2 years. Eligibility included complete bone marrow remission and ≤3, but improved mIBG positive spots. Local control included surgery and radiotherapy of 21 Gy. R2 was initiated in 2009 aiming at 400pts receiving ch14.18/CHOmAB as 8-hour infusion with 20mg/m² over 5 days and 13 cis RA over a total of 5 IT cycles. The schedule requires high dose morphine to control for neuropathic pain. R2 addressed a scIL2 question, using a dose of 6x10E6/m²/day over 5 days twice in a weekly interval, given in week 2 in parallel with ch14.18/CHOmAb.

Results: The superiority of BuMel in EFS and OS over CEM (3-years EFS&OS 50%/61% vs. 38%/52%; p<0.001) is maintained with a significantly lower relapse and progression rate with BuMel (48% vs. 58%) as major factor. Severe toxicity rates (ICU, toxic deaths) are below 10%, but are higher for CEM (p=0.012). Hence the MAT toxicity profile favours BuMel in spite of a VOD rate of 24% (grade 3: 4%) vs. 10% in CEM (Grade3: 1%). In August 2013, R2 reached the target and the randomisation is currently suspended with last patient out in 01/2014. The R2 population undisclosed for treatment arms shows currently a 2 year EFS/OS of 56%/68%. The scIL2 arm carries a significantly higher toxicity burden related to IL2 associated side effects like fever and capillary leak with a number of pts in the IL2 arm stopping treatment early.

Conclusion: BuMel is maintained as SIOPEN standard treatment whilst disclosed and detailed R2 results are expected for ANR.
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OR068

Maintaining Outstanding Outcomes Using Response- and Biology-Based Therapy for Intermediate-Risk Neuroblastoma: A Report from the Children's Oncology Group Study ANBL0531

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Background: This Phase III prospective reduction of therapy study used tumor biologic features to determine the minimal treatment needed to achieve excellent outcomes in patients with intermediate risk (IR) neuroblastoma (NB).

Methods: Between 10/8/2007 and 6/30/2011, 464 patients enrolled; 401 had evaluable IR NB. Eligibility included newly diagnosed single copy MYCN NB and: age <12 years with stage 2A/B <50% resected or stage 3 with favorable biology, age <365 days with stage 3 or 4, and all patients <365 days with stage 4S, including those too ill to undergo biopsy. Toddlers age 365-<547 days with favorable biology stage 4 NB and age 365-<547 days with stage 3 and unfavorable histology (UH) received isotretinoin in addition to chemotherapy. Risk-stratification and therapy assignment utilized age, INSS stage, INPC, MYCN, and tumor ploidy. Therapy reduction was prescribed for tumors without loss of heterozygosity (LOH) at 1p36 and/or 11q23. Therapy included 2-8 courses of chemotherapy +/- surgery. Treatment endpoint for localized favorable biology tumors was partial response.

Results: The 3-year EFS/OS for all IR patients was 83±3%/95±2%. The presence of 1pLOH and/or unbalanced 11qLOH was detected in 18% of IR tumors. To date, there were no deaths due to disease in patients with localized favorable biology tumors. Among 124 stage 4 patients <365 days, 3-year EFS/OS for favorable biology (n=60) was 90±5%/95±4%; for unfavorable biology (diploid and/or UH, irrespective of LOH, n=24) was 63±16%/76±13% and 65±14%/95±7% for tumors with LOH (n=20). Among 9 stage 4 toddlers there were 3 relapses; 3-year OS 100%. Eight of 46 patients with stage 4S NB died; 5 were due to complications of hepatomegaly.

Conclusion: Further reduction of therapy and prospective refinement of treatment using genomic stratification achieved excellent survival in the majority of IR NB patients. Some unfavorable biology IR tumors may benefit from novel treatment strategies.

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OR069

Phase I Study of the Aurora A Kinase Inhibitor MLN8237 with Irinotecan and Temozolomide for Patients with Relapsed or Refractory Neuroblastoma: A Report from the New Approaches to Neuroblastoma Therapy (NANT) Consortium

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Background: The Aurora A kinase inhibitor MLN8237 has preclinical activity in neuroblastoma and additive effects when combined with irinotecan/temozolomide. The aims of this ongoing phase 1 trial are to: determine the recommended phase 2 dose of MLN8237 with irinotecan/temozolomide; assess antitumor activity; and describe pharmacokinetic parameters.

Methods: Patients 1-30 years old with relapsed or refractory high-risk neuroblastoma, measurable or evaluable disease, and adequate organ function are eligible. All patients receive irinotecan (50 mg/m²/dose IV) and temozolomide (100 mg/m²/dose orally) on Days 1-5 of 21-day courses. Patients receive MLN8237 orally daily on Days 1-7. Using a rolling 6 design, dose escalation started at 60% of the single-agent pediatric dose of MLN8237 (45 mg/m²/day = dose level 1), then 60 mg/m2/day (level 2), then 80 mg/m²/day (level 3).

Results: Twenty-one patients have enrolled to date. Two of 6 patients at dose level 1 had first-course dose-limiting toxicity [DLT; G3 mucositis, anorexia, and dehydration and prolonged G4 neutropenia and thrombocytopenia in 1 patient; prolonged G3 neutropenia in 1 patient]. Two of 6 patients had G3 diarrhea in course 1 that were not DLTs. A protocol amendment added mandatory myeloid growth factor starting on Day 8 and oral cephalosporin diarrhea prophylaxis on Days -1 to +8. Six additional patients were enrolled at dose level 1 with the required supportive care; none had first-course DLT. Six patients were treated at dose level 2 with the required supportive care, one with first-course DLT (prolonged G4 neutropenia). The first 18 patients have received a median of 4 (range 1-18) courses. Evaluation of dose level 3 continues. Irinotecan and MLN8237 pharmacokinetic analyses and central review of response are ongoing.

Conclusion: When administered with prophylactic myeloid growth factor and cephalosporin diarrhea prophylaxis, MLN8237 is tolerable at doses of at least 60 mg/m²/day for 7 days in combination with irinotecan/temozolomide.

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OR070

A Phase I Study of Vorinostat in Combination with Isotretinoin (RA) in Patients with Refractory/Recurrent Neuroblastoma (NB): New Approaches to Neuroblastoma Therapy Consortium Trial

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Background: Vorinostat combined with retinoids produces antitumor effects in preclinical studies of neuroblastoma. Higher systemic exposure of vorinostat than previously achieved in pediatric Phase 1 trials is necessary for in vivo peripheral blood mononuclear cell histone acetylation and cytotoxic activity. We conducted a phase I trial to determine the feasibility and toxicity of escalating the vorinostat dose beyond the single agent pediatric maximal tolerated dose (MTD) (230mg/m²) by studying an interrupted schedule combined with RA in children with refractory NB.

Methods: RA (80mg/m²/dose bid PO) was administered on days 1-14 in combination with vorinostat daily dosing on days 1-4 and 8-11 of a 28-day course. Vorinostat dose was escalated (180, 230, 300, 360 and 430mg/m²/day; 800mg maximum absolute dose) following a 3+3 design +6 at expanded MTD. Patients received suspension formulation during course 1.

Results: 29 patients (16 male), median age 10.3 (range: 3.3 – 19.4) years, enrolled of which 28 are evaluable for toxicity and 23 had measurable or evaluable disease for response assessment. The median number of cycles completed was 2 (range 1-12) with a total of 100 cycles delivered. DLT occurred in 4 patients: Grade 3 cheilitis and pain (180mg/m²), Grade 3 pain (300mg/m², C2), Grade 3 creatinine increase and Grade 3 rash (430mg/m²). Grade 3 toxicities related to study therapy occurring in >1 patient included neutropenia (n=8), thrombocytopenia (n=6), pain (n=2), increased ALT (n=2). No Grade 4 toxicities were observed. The recommended Phase 2 dose was 430mg/m2. No objective responses were observed but 6 patients received ≥4 cycles (median 10, range 4-12). Vorinostat and RA pharmacokinetic and histone acetylation analyses will be discussed.

Conclusion: Vorinostat in combination with RA is tolerable on an interrupted schedule up to 430mg/m². Prolonged stable disease observed in 26% of evaluable patients supports further study in patients with neuroblastoma.

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OR071

Generation and Administration of Autologous T cells Transduced with a 3rd Generation GD2 Chimeric Antigen Receptor for Patients with Relapsed or Refractory Neuroblastoma

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Background: Administration of T cells modified with a 1st generation chimeric antigen receptor (CAR) targeting GD2 has been safe and can produce clinical responses, including prolonged complete remissions. Although responses were more frequent, and progression free survival was longer in patients in whom we were able to detect CAR-T cells for more than 6 weeks after infusion, these cells were only present at very low frequency in peripheral blood. Increasing the in vivo proliferation of GD2 T cells after adoptive transfer may lead to improved T cell to tumor cell ratios, increasing anti-tumor activity and improving patient survival. We therefore incorporated the OX40 and CD28 co-stimulatory endodomains into our GD2-CAR construct in the hope that these co-stimulatory endodomains would enhance in vivo expansion and persistence of CAR T-cells in patients with relapsed/refractory neuroblastoma.

Methods: This is a Phase I, dose-escalating, safety trial administering 3rd generation GD2-CAR T cells to patients with relapsed/refractory neuroblastoma.

Results: We have manufactured 4 autologous 3rd generation GD2-CAR T cell products (patient ages: 4-23 years). Starting with a median of 20x10⁶ PBMCs, we obtained a median of 450x10⁶ CAR⁺ T cells (range: 240x10⁶ to 480x10⁶, 18-fold expansion) after 10+/-3 days of culture. The transduction efficiency was 79+/-5% by FACS. Phenotypically, the T cell lines were 27+/-11% CD4⁺ and 67+/-10% CD8⁺, with 8+/-2% central memory (CD45RO⁺CD62L⁺CCR7⁺), and 12+/-10% naive (CD45RA⁺CCR7⁺) cells in the final products. 51Cr release assays showed the T cell lines produced 70+/-7% and 55+/-8% lysis of GD2⁺ targets (LAN-1 and CHLA255, respectively) at a 20:1 effector:target ratio. One patient has been treated, so far without significant toxicity. Clinical and immunological results are pending.

Conclusion: Generation of autologous 3rd generation CAR T cells for the treatment of high-risk neuroblastoma appears feasible and the safety and anti-tumor activityof the approach are now being assessed.

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OR072

Relapse in Patients with High-Risk Neuroblastoma (HR-NB) After Treatment with 3F8/GM-CSF+CIS-Retinoic Acid (3F8/GM+CRA) in First CR/VGPR: Patterns, Management and Long-term Outcome

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Background: Anti-GD2 immunotherapy is now standard of care for HR-NB. However, prognosis after first relapse post-immunotherapy is deemed dismal; patients are often enrolled on early phase studies without curative intent.

Methods: HR-NB patients relapsing post-3F8/GM+CRA in first CR/VGPR (NCT00072358) underwent the following reinduction strategies: (1) Isolated CNS relapse (CNS-R): multimodality therapy including intra-Ommaya radioimmunotherapy (MM-RIT) (J Neurooncol 97:409). (2) disseminated (D-R) or focal soft-tissue relapse (FS-R): High-dose chemotherapy using active agents as previously described (Cancer 119: 665; Bone Marrow Transplant 48:642; Pediatr Blood Cancer 56: 403; Eur J Cancer 47: 84; Cancer 116: 3054) ±surgery (±IORT)±radiation, (3) focal bone relapse (FB-R): Low-dose chemotherapy (J Clin Oncol 24:5271) ± radiation.

Patients achieving second CR/VGPR were retreated with 3F8/GM+CRA ±anti-GD2/GD3 humoral vaccine (ClinCanRes, in press). Progression-free (PFS) and overall survival (OS) analyses were performed (Kaplan-Maier method) and prognostic variables compared (log-rank test).

Results: Of 145 consecutive patients treated with 3F8/GM+CRA in first CR/VGPR, 64 (44%) relapsed (median follow-up 60 months from starting 3F8): 17 CNS-R, 24 D-R, 12 FS-R, 11 FB-R (Table). 29% continued in remission 5 years after relapse. Adverse prognostic factors for survival post-relapse included failure to achieve second CR/VGPR, D-R as opposed to other relapse patterns, and early (<6mo from starting 3F8) relapse ($p<0.05$ for each), but not MYCN amplification ($p=0.4$).

Conclusion: Long-term PFS and OS are possible in HR-NB patients post-relapse if CR/VGPR can be achieved, especially when relapse is focal. Agents with proven anti-NB activity should be used at first relapse rather than experimental approaches.

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Relapse Pattern	N	Mean (± SD) time to relapse (mo)	# pts receiving reinduction regimen	# pts achieving 2nd CR	# pts retreated with 3-GM CRA	# pts in continuous remission (median f/u in mo)	OS post-relapse: median± SE (mo)	% ±SE PF at 2 y from relapse	% ±SE PF at 5 y from relapse	%±SE alive at 2y from relapse	% ±SE alive at 5y from relapse
CNS-R	17	8.0± 5.4	13 (MM-R2T+M); 4 (others=O)	14	14	9 (59.5)	82±8 (M); 5±2.3 (O)	77±12(M); 0 (O)	58±15(M); 0 (O)	100 (M); 25±22 (O)	68±13(M); 0 (O)
D-R	24	15.7± 15.1	19	9	9	1 (15.3)	17.1 ± 5.6	2 ± 3	0	37 ± 1	10 ± 7
FS-R	12	17.9± 12.1	8	9	6	3 (58.9)	30.5 ± 19	25 ± 13	25 ± 13	75 ± 13	32 ± 14
FB-R	11	14.8± 8.4	9	7	7	6 (44.4)	55.5 ± 9	54 ± 15	54 ± 15	73 ± 13	62 ± 15
Overall	64	13.9± 11.9	49	39	36	19 (55.7)	30.5 ± 5	31 ± 6	29 ± 6	62 ± 8	29 ± 7

OR073

Recurrent Neuroblastoma Metastatic to the Central Nervous System: Is it curable?

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Background: CNS NB is challenging to cure (median survival < 6 months). Intraventricular compartmental radioimmunotherapy (cRIT) using ^{131}I-3F8 or ^{131}I-8H9 has been used to eradicate NB cells in cerebrospinal fluid (CSF) space. We now summarize the clinical outcome of all cases of CNS NB at MSKCC since 2003.

Methods: Patients had radiographic and/or pathologic confirmation of CNS NB. Patients underwent MSKCC temozolamide/irinotecan based CNS salvage regimen incorporating cRIT as described (gr 1) plus systemic immunotherapy using 3F8 + GMCSF. Non-regimen treatments used other therapies +/- RIT (gr 2). Disease evaluation included serial MR brain/spine, MIBG, CT, and bone marrows.

Results: Of 83 patients with CNS NB, cRIT was possible in 56 (67%), 42 (51%) following salvage regimen (gr 1), 33% presenting with multiple parenchymal masses +/- leptomeningeal disease. In gr 1, 26/42 (62%) patients are alive and well, mean OS 82.6 months, including 5/14 (35%) with leptomeningeal disease or multiple parenchymal masses; 3/42 (7%) deaths were due to non-NB complications. OS for gr 2 patients was 15% (mean 21 months). The only long term survivors among gr 2 patients were 7 patients who received cRIT (mean OS 42.8 mon). Overall, 47 patients (56%) died of NB involving the CNS only (N=12, 25%), systemic only (N=14, 30%), CNS and systemic (N=16, 34%) or toxicity (N=5, 11%). Treatment related toxicity included CNS hemorrhage (1) and pulmonary insufficiency during chemotherapy (1). Deaths in long term survivors included infection (1), pulmonary fibrosis (1), and AML (1).

Conclusion: Two-thirds of patients with CNS NB are able to complete aggressive CNS salvage regimen including cRIT; long term remissions are achieved in >60% and more commonly observed in patients with unifocal CNS metastasis. A survival advantage was seen for patients treated with cRIT even in the absence of CNS salvage regimen.

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A7: Translational Research/ Molecular Markers

OR055

Assessment of Circulating microRNAs for Non-Invasive Outcome Prediction of Neuroblastoma Patients

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Background: Accurate assessment of prognosis of children with neuroblastoma is believed to contribute to more effective cancer therapy and increased survival rates. The presence of cell free microRNAs in body fluids offers the possibility to develop a fast and non-invasive risk-classification method. In this study we evaluated serum miRNA expression for overall improvement of pre-treatment risk-prediction and identification of ultra high-risk patients.

Methods: Ninety serum samples from patients with contrasting clinical outcome (high-risk deceased patients, high-risk survivors and low-risk survivors), were screened for 1805 human mature miRNAs using RT-qPCR technology.

Results: Large and numerous differences were observed between low-risk survivors and high-risk deceased patients; more than 200 significantly differentially expressed miRNAs were identified (Mann-Whitney test, $P < 0.05$). Prediction analysis of Microarrays (PAM) followed by 10x10 cross validation and estimation of misclassification error (MCR) resulted in risk class prediction with 94.7% accuracy. Comparing high-risk survivors and high-risk deceased patients revealed a handful of differentially expressed miRNAs (Mann-Whitney test, $P < 0.05$). Assessment of high-risk expression data by 10x10 cross-validation PAM and MCR-estimate revealed 71.7% prediction accuracy. Many miRNAs, reported as relevant to neuroblastoma development and biology, were identified amongst the differentially expressed miRNAs (e.g. miR-137, miR-9-3p, miR-542-5p, miR-10a, miR-10b…). Two additional independent and large high-risk serum cohorts (n=100) are being profiled in order to increase the statistical power and to select for more robust prognostic miRNAs to improve ultra-high-risk prediction accuracy.

Conclusion: We could clearly document the presence of differentially expressed circulating miRNAs associated with patient outcome. Given the positive results on the first set of serum samples, we expect to establish a useful classifier upon ongoing profiling of 2 additional high-risk cohorts.

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OR056

Revised Risk Assessment and Treatment Stratification of Low- and Intermediate-Risk Neuroblastoma Patients by Integrating Clinical and Molecular Prognostic Markers

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Background: Precise risk estimation is essential to avoid under- and overtreatment of neuroblastoma (NB) patients. Previously, we have demonstrated that gene expression signatures derived from NB tumors are highly accurate in predicting clinical outcome. We therefore aimed at improving current NB risk stratification systems by integrating established prognostic markers and gene expression signatures.

Methods: We generated microarray-based gene expression profiles of 709 primary NBs. Classification models were built using a training set of 75 tumors with contrasting courses of disease. Subsequent validation was performed in a test set (n=634) using Kaplan-Meier estimates and multivariate Cox regression analyses.

Results: The best-performing classifier (SVM_th10) considered 139 genes (194 probes) and predicted patient outcome with an accuracy of 0.95 (sensitivity 0.93, specificity 0.97) in the validation cohort. The highest potential clinical value of the classifier was observed in current low- and intermediate risk patients (LR and IR, respectively). In these subgroups, the classifier significantly distinguished patients with diverging outcome (LR: 5-year OS 0.99±0.01 vs 0.76±0.11; IR: 5-year OS 1.0 vs 0.70±0.09; both $p<0.001$). In multivariate Cox regression models for non-high risk (non-HR) patients, the classifier outperformed risk assessment of the current German trial NB2004 (EFS: hazard ratio 5.07, 95% confidence interval 3.20-8.02; OS: hazard ratio 25.54, 95% confidence interval 8.40-77.66; both $p<0.001$). Based on these findings, we developed a revised risk stratification system for non-HR NB patients by integrating established prognostic markers and the SVM_th10 classifier. According to this system, we newly identified patient subgroups with poor outcome (5-year EFS 18.5±7.8%), for whom we propose intensified treatment, and patient subgroups with beneficial outcome (5-year EFS 87.4±5.3%), who may benefit from treatment de-escalation.

Conclusion: We propose a novel risk assessment and treatment stratification system for non-HR NB patients that integrates established prognostic markers and a gene expression-based classifier. We are aiming to implement this risk estimation system in the upcoming prospective clinical trial NB2013 LR/IR.

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OR057

Identification and Validation on a Six Hundred Neuroblastoma Patients' Dataset of a Novel Gene Signature Predicting Patients' Outcome and Measuring Tumor Hypoxia

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Background: Cancer patient's outcome is written, in part, in the tumor microenvironment described by gene expression profile. Neuroblastoma tumor hypoxia is related to tumor aggressiveness and can be measured by gene expression. We report on a new neuroblastoma hypoxia-based gene signature designed for outcome prediction validated on a 636 neuroblastoma patients' dataset.

Methods: Signature design and bioinformatic analysis is based on Attribute Driven Incremental Discretization of continuous variables, Logic Learning Machine models for classification and feature selection. Gene expression and clinical data of 636 patients, accessible in the R2:microarray analysis visualization platform (http://r2.amc.nl) as separate datasets, were merged and normalized in a single dataset.

Results: We defined a new 7 genes signature (NB-hop) measuring tumor hypoxia but tailored to patient's outcome prediction. Multivariate Cox analysis demonstrated that NB-hop was an independent risk factor for NB patients. NB-hop was highly accurate (87%) in predicting NB patients' outcome. The outcome prediction attribute did not detract from the ability of NB-hop to measure tissue hypoxia, as shown by immunohistochemistry of tumor slides with the hypoxia markers CA or VEGF and by the Hypoxia Gene Set Enrichment analysis in poor outcome tumors. Unsupervised K-means clustering divided the patients in high (174) and low (462) tumor hypoxia. Kaplan-Meier analysis showed a significant low probability of survival in patients with highly hypoxic tumors ($P< 0.00001$). Selected groups of high risk patients were further stratified by NB-hop in statistically significant groups. Outcome prediction analysis identified a homogeneous poor outcome group defined by Stage 4, age > 12 months, MYCN non amplified, NB-hop high, that could be a prototype of the Ultra High Risk category.

Conclusion: Studies on 636 tumor specimens demonstrated that NB-hop signature predict very accurately neuroblastoma patients' outcome and tumor hypoxia. NB-hop is an independent risk factor that identify poor outcome patients benefitting from hypoxia targeted therapies.

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OR058

Developing a Responder Hypothesis for Therapeutic Stratification of Newly Diagnosed Patients with ALK-Mutant NB

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Background: ALK activation in neuroblastoma is complex, and inhibition of this mutant tyrosine kinase domain (TKD) is more challenging than fusion-proteins present in lymphomas and lung cancers. To maximize clinical benefit, we sought to define the spectrum of ALK mutations in a large representative case series and discover structure-function relationships that allow for the development of robust biomarkers for sensitivity and resistance to ALK inhibitors.

Methods: We analyzed the spectrum of ALK DNA alterations at diagnosis in samples from 1596 patients. We defined their functional consequences and sought to identify the optimal inhibitor for direct ALK kinase inhibition. We integrated genomic profiling, biochemical and functional approaches to develop an algorithm for predicting, which newly emerging ALK mutations are drivers, and which ALK inhibitors are effective at inhibiting different ALK variants.

Results: We identified putative mutations distributed over 22 positions within the TKD; these occurred across all clinical risk-groups and were more commonly observed in older patients. The presence of an ALK aberration was predictive of reduced event-free ($p<0.0001$) and overall survival ($p=0.0002$). Biochemical analyses revealed that only half of the variants were strongly activating, data that correlated with oncogenic potential in focus formation assays. Most activating mutations occur in the activation loop or aC helix and disrupt autoinhibitory interactions crucial for stabilizing the inactive conformation. Certain mutations affect these interactions indirectly, and/or alter stability so that the active conformation becomes selectively stabilized. Analysis of the ATP-binding and in vitro crizotinib inhibition characteristics for the spectrum of ALK variants studied indicated that crizotinib may have utility for neuroblastoma driven by mutations at 5 positions, whereas the remaining activated variants are likely to share the resistance displayed by the F1174L mutation.

Conclusion: These data allow us to formulate molecular therapeutic stratification recommendations, which will be important as ALK inhibitors for neuroblastoma are integrated into upfront clinical trials.

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OR059

The Ornithine Decarboxylase G317A Polymorphism is Prognostic of Outcome in Primary Neuroblastoma and Differentially Affects Promoter Binding by the MYCN Oncogene

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Background: Polyamines are highly regulated essential cations that are elevated in rapidly proliferating tissues. We have previously shown that ornithine decarboxylase(ODC1), rate-limiting for polyamine synthesis, is an oncogenic MYCN target and an independent prognostic marker in neuroblastoma (Cancer Res, 2008). An international NANT Phase I trial for refractory neuroblastoma, based on ODC1 inhibition has recently opened. We have now examined the prognostic significance of a single nucleotide polymorphism (SNP) within the ODC1 promoter.

Methods: 839 primary neuroblastomas, and 161 lung cancer samples were genotyped for the G317A (rs2302615) promoter SNP by qPCR. The effect of G317A on MYCN/MAX regulation of ODC1 was examined by E-box mutational analyses, EMSA binding and luciferase reporter assays.

Results: Of the neuroblastoma cohort, 507 patients (60.4%) were homozygous GG, 271 (32.3%) heterozygous, and 61 (7.3%) homozygous AA (mutant) for the G317A SNP. In MYCN amplified neuroblastoma (n=142; 17%), the GG genotype strongly predicted poorer outcome ($p<0.005$), whilst in non-amplified patients (n=695; 83%), the presence of one or two G alleles was associated with worse outcome ($P=0.025$). These results contrast with previous findings where the AA genotype was associated with worse colon cancer outcome (Zell et al, Clin Cancer Res, 2009), a finding we confirmed in our lung cancer cohort ($P=0.017$). MYCN/MAX binding to the E-box closest to the G317A SNP was 3-5 fold stronger in the presence of the G-, rather than, A-allele. This G-allele/E-box element, which we showed by mutation analysis to be most critical for MYCN-mediated ODC1 transcription, was also 2-fold more efficient in driving luciferase transcription in MYCN-inducible cells.

Conclusion: The results demonstrate the functional importance of the ODC1 G317A promoter polymorphism in contributing to neuroblastoma clinical outcome. The association between ODC1 genotype and mRNA expression levels is currently being determined in neuroblastoma and lung cancer cohorts, to elucidate possible tissue-specific influences of the ODC1 SNP.

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OR060

Prevalence and Prognostic Impact of Alternative Lengthening of Telomeres (ALT) in 203 Neuroblastoma Tumors

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Background: Unlimited proliferation of cancer cells requires the activation of one of two telomere maintenance mechanisms: telomerase or homologous recombination-based ALT.

Methods: A novel telomere qPCR-based method was used to detect C-circles (extrachromosomal telomeric circular DNA) as a marker of ALT activity in 203 neuroblastoma (NB) tumors (117 Australian and 86 Children's Oncology Group patients).

Results: There were no significant differences in various characteristics and outcome between the Australian and COG patients, and all high-risk patients received myeloablative therapy. ALT was detected in 24% (36/151) of all high-risk NB, 36% (36/100) of MYCN non-amplified high-risk, and <6% (3/52) of non-high-risk NB. All ALT+ samples were MYCN non-amplified. Whole genome sequencing results were available in 24 high-risk COG samples from a published study (Pugh et al, 2013): ATRX and DAXX aberrations (ALT-associated genes) were present in 6 and 1 of the 12 ALT+ tumors, respectively, while none of the 12 ALT- samples harbored ATRX/DAAX mutations. ALT+ NB had significantly higher telomeric content than ALT- tumors ($P<0.0001$), consistent with the ALT phenotype of long telomeres. Age at diagnosis was significantly older for ALT+ than ALT- NB (median 4.4 vs. 1.8y; $P<0.001$). High-risk/ALT+ tumors (n=36) (all MYCN non-amplified) had a similarly poor survival as the MYCN-amplified tumors (n=51) (5-yr EFS: 31% vs. 26%; 5-yr OS: 36% vs. 29%). Of interest, the remaining high-risk tumors (n=64) (ALT-/MYCN non-amplified) had the best survival (5-yr EFS & OS: 51% & 59%; $P<0.0003$). Furthermore, ALT+ patients succumbed to NB significantly later than ALT- patients (18% vs. 75% of deaths occurred by 2 years from diagnosis; $P<0.0001$).

Conclusion: 1. ALT and MYCN amplification are mutually exclusive. ALT status predicts survival and disease course in high-risk NB. Lack of ALT and MYCN amplification (40% of high-risk) confers a good prognostic subgroup (survival 60%).

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B7: Clinical Research IV

OR080

Clinical, Biological, and Prognostic Differences Based Upon Primary Tumor Site in Neuroblastoma: A Report from the International Neuroblastoma Risk Group (INRG) Project

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Background: Neuroblastoma (NB) is a heterogeneous malignancy arising along the sympathetic nervous system. The impact of primary tumor site in driving the heterogeneity of NB remains unclear.

Methods: Children <21 years of age diagnosed with neuroblastoma or ganglioneuroblastoma between 1990-2002 with an assigned primary tumor site were identified from the INRG database. Data were compared between primary sites regarding clinical features (age, tumor type, stage); biologic features (MYCN status, histology, serum ferritin and LDH, segmental chromosomal aberrations); and event-free survival (EFS).

Results: The distribution of primary tumor site among 8,369 children was: 47.4% adrenal; 23.8% abdominal/retroperitoneal; 15.1% thoracic; 3.0% pelvic; 2.7% neck; and 7.9% other. All evaluated clinical and biologic variables differed statistically by primary site. The most discrepant features (>10% difference between groups) were stage 4 disease, MYCN amplification, elevated ferritin, elevated LDH, and segmental chromosomal aberrations, all more frequent in adrenal vs. non-adrenal tumors ($p<0.001$). Patients with thoracic tumors had higher proportions of favorable results for these same variables plus histology compared to non-thoracic tumors (>10% difference; $p<0.001$). EFS was significantly different according to primary site (5-year EFS +/- standard error): adrenal, 56 +/- 0.8%; abdominal/retroperitoneal, 64 +/- 1.1%; other, 63 +/- 2.2%; pelvic, 81 +/- 2.6%; neck, 79 +/- 2.8%; and thoracic, 80 +/- 1.2%. After controlling for differences in age, MYCN, and stage, patients with adrenal tumors remained at increased risk for event (HR=1.13 compared with non-adrenal; 95% CI 1.03-1.23; $p=0.008$) and thoracic tumors remained at decreased risk for event (HR=0.79 compared with non-thoracic; 95% CI 0.67-0.92; $p=0.003$).

Conclusion: Clinical and biologic features show important differences by primary site. Independent of these differences, adrenal and thoracic sites are associated with inferior and superior EFS, respectively.

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OR081

Prognostic Significance of Liver Metastases in Metastatic Neuroblastoma. A study from the International Neuroblastoma Risk Group (INRG) database

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Background: Metastatic disease is an important predictor of poor outcome in neuroblastoma. However, specific organ involvement is not part of current risk stratification systems. Although the prognostic significance of liver metastases has been explored in infants, less is known about its importance or biomarker associations in older children.

Methods: Retrospective analysis of data from INRG database for stage 4 patients diagnosed 1990–2002, excluding those with missing metastatic data. Survival was estimated using Kaplan-Meier methodology, compared using log-rank tests.

Results: 2647 INSS stage 4 patients were identified. 401 (15%) had liver metastases. Liver metastases were more common in infants (27% of those aged ≤18 months v 9.5% for >18 months; $P<0.0001$). Children aged >18 months with liver metastases had significantly worse outcomes than those without (5-yr EFS 15%±3% v 26%±1%, OS 16%±3% v 35%±1%; $P<0.0001$). Similar results were seen in infants 12-18 months (EFS 37%±8% v 55%±3%; $P=0.04$), but liver involvement did not influence outcome in infants <12 months (EFS 69%±4% v 73%±2%; NS). In children >18 months, presence of liver metastases was significantly associated with MYCN amplification, adrenal primary tumours and lung metastases. No other differences in tumour biological characteristics were identified. Subgroup analysis based on MYCN status confirmed that presence of liver metastases remained significantly associated with poorer outcomes. Analysis based on initial treatment demonstrated that while liver metastases were associated with worse outcome in those treated without stem cell transplant (SCT) (EFS 12%±4% v 21%±2%; $P<0.0001$), there was no difference for those treated with SCT (EFS 31%±2% v 27%±7%; NS).

Conclusion: The presence of liver metastases in stage 4 neuroblastoma patients aged >12 months is associated with worse outcome, which may be partly explained by an association with MYCN amplification. Increased intensity provided by myeloablative therapy and SCT in more recent protocols may have particular benefit for those with liver metastases.

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OR082

Stage 4N Neuroblastoma (Metastatic Disease Confined to Distant Lymph Nodes) Has a Better Outcome Than Non-4N Stage 4 Disease: A Study from the International Neuroblastoma Risk Group (INRG) Database

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Background: The presence of metastatic disease is one of the most powerful predictors of poor outcome in patients with neuroblastoma. However, the pattern of metastatic spread is not part of current risk stratification systems. Small case series have suggested that patients with metastatic neuroblastoma limited to distant lymph nodes (4N disease) may have improved outcomes.

Methods: Retrospective analysis of data from INRG database for patients diagnosed 1990–2002. 4N patients were compared to the remaining stage 4 patients (non-4N), excluding those with missing/inconsistent metastatic site data.

Results: 2250 INSS stage 4 patients with complete data were identified, of whom 146 (6.5%) had 4N disease. Patients with 4N disease had significantly better outcomes than those with non-4N stage 4 disease (EFS 77%±4% v 35%±1%; OS 85% v 42%±1%; $P<0.0001$). 4N patients were more likely to be younger ($P<0.0001$) and have tumors with favorable characteristics, including absence of MYCN amplification (89% v 69%, $P<0.0001$), lower mean mitosis karyorrhexis index ($P=0.0011$), differentiating grade (21% v 8%, $P=0.006$) and favorable International Neuroblastoma Pathologic Classification (63% v 26%, $P<0.0001$). Insufficient cytogenetic data prevented analysis of associations between particular segmental chromosomal abnormalities and 4N/non-4N patterns of disease. In a multivariable analysis, 4N disease remained a significant predictor of outcome (hazard ratio for non-4N v 4N 3.40 for EFS, 3.69 for OS). Within subgroups defined by age at

diagnosis and tumor MYCN status, 4N pattern was significantly associated with improved outcomes.

Conclusion: 4N represents a subgroup with better outcome than other patients with metastatic disease. These findings suggest that the biology and treatment response of 4N tumors differ from other stage 4 tumors, and that less intensive therapy should be considered for this cohort. Future exploration of biological factors (chromosomal aberrations, mRNA profiles and host factors) that determine the pattern of metastatic spread is warranted.

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OR083

Postponing Staging in Very Young Infants

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Background: In young asymptomatic infants with suspected neuroblastoma, we recommend to postpone staging investigations needing sedation until the age of three months. Here we report first results of this strategy.

Methods: Data of patients with neuroblastoma suspected until the age of 60 days were analysed. For asymptomatic infants, guidelines recommended ultrasound at suspicion of neuroblastoma, and mIBG scintigraphy, bone marrow aspiration, and biopsy at the age of three months or at progression.

Results: Between 2000 and 2012, 326 patients with (suspected) neuroblastoma were registered (stage 4: n=6, 1.8%; stage 4S: n=86, 26.4%, localized n=234, 71.7%). Neuroblastoma was suspected prenatally in 77 patients and postnatally at the median age of 20 days (1 – 60 days). , At diagnosis, 72 symptomatic patients were treated with first-line chemotherapy (34/234 localized, 14.5%; 3/6 stage 4, 35/86 stage 4S, 40.7%). Symptoms were related to hepatomegaly (n=36), intraspinal involvement (n=14), or compression of the airways (n=11), the urinary tract (n=7) or vessels (n=4). Within one month after diagnosis, additional 150 patients underwent resection (n=91), biopsy (n=40) or mIBG-scintigraphy (n=19). , In 104 asymptomatic patients, extended staging and biopsy were postponed (16 stage 4S, 88 localized). Until the age of three months, progression was seen in 21 patients with local growth of primary (n=11), evolution of liver metastases in patients with localized disease (n=7), or symptomatic progression of liver metastases (n=2). In one additional patient staging at clinical progression revealed stage 4 disease. At the scheduled time for extended staging, regression was seen in 44/104 patients (42.3%, 4/16 stage 4S; 10/88 localized). Of those, 37 patients never underwent tumor biopsy. , Altogether, MYCN was assessed in 266 patients, 12 tumors were amplified (4.5%). Eighteen of 326 patients died (3-year-OS 0.95.±0.01), 16 tumor-related. Of the cohort with postponed extended staging, two patients had MYCN amplification, assessed at progression. One of them died.

Conclusion: From these first results we conclude that the strategy to postpone elaborate staging investigations until the age of three months is safe, although unfavourable molecular markers are rarely found in this group of patients.

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OR084

Population-Based Incidence and Survival of Neuroblastoma in Taiwan, 1979-2009

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Background: The clinical course of neuroblastoma (NB) is heterogeneous and dependent on age and, probably, on race/ethnicity. This study aims to characterize the epidemiological features of NB in Taiwan.

Methods: Taiwan Cancer Registry is a population-based database established in 1979. All cancers from hospitals with 50 or more beds were registered and validated. Cases of NB or ganglioneuroblastoma diagnosed at age 0–30 years during 1979–2009 were analyzed.

Results: A total of 590 cases were included for analysis. The median age at diagnosis was 940 days (2y6m$^+$), which was 1y1m$^+$ older than that observed in the INRG cohort (Moroz et al., 2011). The age-standardized incidence rate had risen dramatically from 0.02 to 0.36 per million per year, which trend remained increasing after the implementation of National Health Insurance, a universal coverage program, in 1995 (rate of increment, 5.8% [1.4%–10.4%] per 3-year interval after 1995 among age 0–14 years). The 5-year relative survival rate was better in the youngest age group and the latest treatment era: 81.4% for 0–12 months (n = 181), 69.2% for 12–18 months (n = 30; P = 0.08), and 42.5% for >18 months (n = 379; P < 0.0001); and was 43.0% for cases diagnosed during 1985–1994, 56.7% during 1995–2001 (P = 0.0034), and 58% during 2002–2009 (P = 0.0001). Univariate Cox proportional hazard modeling revealed a continuous relationship between hazard ratio and age cutoffs, which curve peaked before 1 year, reached a plateau at 1y6m$^+$, and then gradually decreased, with the best cutoff falling at the interval between 242 and 560 days (7m$^+$–1y6m$^+$, all had HR≥4.00 and P < 10^{-10}; cutoff with maximal HR, 261 days [8.5 months], HR = 7.79, P = 5.24×10^{-13}).

Conclusion: In contrast to the other study groups, NB in Taiwan had an older age at diagnosis and a younger age cutoff with maximal outcome difference. The survival rate showed significant improvement over treatment periods.

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OR085

Neuroblastoma Screening at One Year of Age Does Not Reduce Mortality and Stage 4 Incidence - Results after 12 Years of Follow-Up

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Background: Neuroblastoma screening at one year of age has been abandoned because of its inability to reduce the incidence of metastatic disease and preliminary mortality data (N Engl J Med 2002;346:1047). This study reports the screening effects in Germany 12 years after the end of the program.

Methods: Children born between 1994 and 1999 were offered urine screening for neuroblastoma at approximately one year of age in a study area, but not in a control area. Population based incidences and mortalities were compared at a data lock of September 2013.

Results: The study and control area were comparable prior to screening (births 1990-1993) with respect to mortality and incidence. 1,483,925 children underwent neuroblastoma screening (61% compliance). 155 cases were detected by screening, mostly in their 2nd year of life; 81 false negatives were diagnosed until the 6thyear. The overall incidence in 2nd-6th year in the screening participants was 15.7 compared to 8.2 (per 100000) in the control area. Among the cases detected by screening, there were more localized cases compared to the2nd year in the control area (9.6 vs. 1.2), but no difference in stage 4 incidence (1.3 vs. 1.1) or the incidence of "stage 4 or MYCN amplified" (1.6 vs. 1.7). The false negatives up to age 6th year did not differ from control area cases in the 3rd-6th year regarding stage distribution or incidence (stage 4: 3.3 vs. 3.5). The cumulative mortality of cases diagnosed at ages 2nd-6th year was similar (screening vs. control area: 3.5 and 3.8). Mortality was lower among participants (3.1) compared to non-participants (4.1) in the screening area.

Conclusion: Neuroblastoma screening at one year of age does not reduce mortality or stage 4 incidence, but results in overdiagnosis of localized disease. Screening participants may represent a more health-conscious subcohort with a more favorable outcome.

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POSTER EXHIBITION

BASIC RESEARCH

Basic Research: Oncogenesis

POB001

Modeling MYCN-Amplified Neuroblastoma Using Human Induced Pluripotent Stem Cells

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Background: Although neuroblastoma is derived from multipotent neural crest (NC) cells, the precise progenitor cell of neuroblastoma has not been defined. Neuroblastoma frequently arises in the adrenal medulla or in paraspinal ganglia, suggesting an origin from bipotential sympathoadrenal cells and/or the sympathoadrenal progenitors. Amplification of the oncogene MYCN, a marker of risk, can block differentiation pathways at stages that are vulnerable to transformation. Therefore, we hypothesize that the cell of origin for MYCN-amplified neuroblastoma may be distinct from MYCN non-amplified neuroblastoma.

Methods: We analyzed a panel of MYCN-amplified and non-amplified neuroblastoma cell lines for markers of immature NC and sympathoadrenal cells. To determine whether identified markers inform the cell of origin for neuroblastoma, we are developing protocols to differentiate human induced pluripotent stem (iPS) cells toward immature NC and sympathoadrenal cells. We transduced iPS cells with doxycycline-inducible MYCN and analyzed the ability of MYCN to block NC differentiation and promote proliferation of NC cells.

Results: Analysis of MYCN-amplified and non-amplified neuroblastoma cell lines by qPCR showed decreased levels of NC markers, even compared to human iPS cells. In contrast, markers for sympathoadrenal cells were upregulated specifically in MYCN-amplified neuroblastoma lines compared to iPS cells. Ectopic expression of MYCN in iPS cells blocked differentiation towards the NC. At the NC stage, endogenous MYCN expression was downregulated compared to iPS cells, whereas exogenous mis-expression of MYCN promoted proliferation.

Conclusion: Since over-expression of MYCN blocked the differentiation of iPS cells, our results: suggest that amplification of MYCN in neuroblastoma occurs after embryonic cells have committed to NC lineage. This order of events is supported by the fact MYCN is down-regulated at the NC stage, as compared to iPS cells; and that mis-expression of MYCN increased proliferation in NC cells. Our Results also suggest that MYCN amplified neuroblastoma likely arise from the sympathoadrenal lineage.

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POB002

NCYM Maintains Stemness via the Induction of OCT4 Expression in Neuroblastoma

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Background: Neuroblastoma (NB) is one of the most common solid tumors in children. MYCN amplification is frequently observed in unfavorable NBs and MYCN have critical roles in the maintenance of stemness in NB cells as well as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. Recently, we reported that NCYM, a cis-antisense gene of MYCN, encodes protein product evolutionally conserved only in the taxonomic group containing humans and chimpanzees. NCYM stabilizes MYCN via inhibition of GSK3β in human NBs. However, the precise functions of NCYM to obtain the stemness of NB are still unclear. Here we show that NCYM-MYCN network regulates symmetric cell division via the induction of OCT4 in human NB cells.

Methods: The mRNA and protein were obtained from NB cell lines and primary NBs. Those expressions were examined by quantitative RT-PCR and Western blot. Transcriptional activation was investigated by luciferase reporter and chromatin immunoprecipitation analysis. Cellar invasion was evaluated by invasion assay. Cell spheres were counted under the microscope. Symmetric cell division was observed by immunoflorescence.

Results: Overexpression of NCYM induces several stemness-related genes such as OCT4, SOX2, NANOG and LIN28, but not c-MYC and KLF4. Quantitative RT-PCR analysis reveals that the expression levels of NCYM are significantly correlated with the expression levels of OCT4 in 93 primary neuroblastomas. We then focused on the regulation of OCT4 expression by NCYM. Knockdown of NCYM decreases the expression of OCT4 and the recruitment of MYCN onto OCT4 promoter, resulting in the downregualtion of OCT4 expression. NCYM reduction also inhibits stem cell properties such as cell sphere formation and invasion in human NB cells. Additionally, NCYM increases the percentage of symmetric cell division in human NB cells.

Conclusion: Collectively, our Results suggest that NCYM controls stem cell properties of NB through the induction of OCT4 expression.

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POB003

p19-INK4d Inhibits Neuroblastoma Cell Growth, Induces Differentiation and is Hypermethylated and Downregulated in MYCN-amplified Neuroblastomas

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Background: Uncontrolled cell cycle entry, resulting from deregulated CDK-RB-E2F pathway, is a crucial determinant of neuroblastoma (NB) cell malignancy. Here we identify NB suppressive functions of the CDK inhibitor p19-INK4d and uncover mechanisms of its repression in high-risk NB patients.

Methods: Customized oligonucleotide microarrays were used to determine p19-INK4d expression in primary NBs. DNA methylation was assessed using 450K methylation array and Massarray analyses. Tetracycline-inducible ectopic expression of proteins (p19-INK4d, NTRK1, MYCN) and shRNA (MYCN) was used for functional testing in NB cell lines.

Results: In a set of 478 primary NBs, reduced p19-INK4d expression was associated with poor event-free and overall survival and NB risk factors, including amplified MYCN. High MYCN repressed p19-INK4d mRNA and protein in different NB cell models with conditional MYCN expression.450K methylation array and Massarray analyses of 105 primary NBs uncovered a differentially methylated region within p19-INK4d. Hypermethylation of this region was associated with reduced p19-INK4d expression. In accordance, endogenous p19-INK4d expression was activated in NB cell lines upontreatment with the demethylating agent 2-deoxy-5-azacytidine. Ectopic p19-INK4d expression decreased viability, clonogenicity and the capacity for anchorage-independent growth of NB cells, and shifted the cell cycle towards the G1/0 phase. p19-INK4d also induced neurite-like processes and markers of neuronal differentiation. Moreover, NB cell differentiation, induced by all-trans retinoic acid or NGF-NTRK1-signaling, activated p19-INK4d expression.

Conclusion: Our findings pinpoint p19-INK4d as a NB suppressor and provide evidence for MYCN-mediated repression and epigenetic silencing of p19-INK4d by DNA hypermethylation in high-risk NBs.

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POB004

Wild-Type ALK, and Activating Mutations ALK-R1275Q and ALK-F1174L Initiate Tumor Formation in Murine Neural Crest Progenitor Cells via Upregulation of c-Myc

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Background: The anaplastic lymphoma kinase gene (ALK) is overexpressed, mutated or amplified in most neuroblastoma (NB). The ALK-F1174L mutation contributes to NB tumorigenesis in transgenic mouse models, and cooperates with MYCN in the oncogenic process. However, the precise role of ALK activating mutations or ALK-wt overexpression in NB tumor initiation needs further clarification.

Methods: Human ALK-wt and the most frequent mutations ALK-F1174L, and ALK-R1275Q were stably expressed in murine neural crest progenitor cells (NCPC), MONC-1 or JoMa1, immortalized with v-Myc or Tamoxifen inducible c-MYC-ERT, respectively, and further implanted subcutaneously or orthotopically (adrenal gland) in nude mice.

Results: While orthotopic implantations of MONC-1 parental cells in nude mice generated various tumor types, such as NB, osteo/chondrosarcoma, and undifferentiated tumors, due to v-Myc oncogenic activity, MONC-1-ALK-F1174L cells only produced undifferentiated tumors. Furthermore, ALK-wt, ALK-F1174L, or ALK-R1275Q expression in JoMa1 cells, in absence of exogenous c-MYC-ERT activity, was sufficient to initiate highly aggressive undifferentiated tumor formation after subcutaneous or orthotopic implantations. Interestingly, orthotopic JoMa1-ALK tumors or their derived cell lines upregulated c-Myc endogenous expression, resulting from ALK activation, and both ALK and c-Myc activity were necessary to confer in vitro clonogenic capacity of tumor-derived cell lines.

Conclusion: This is the first demonstration of the in vivo oncogenic activity of ALK-wt and the ALK-R1275Q activating mutation in NCPC. Moreover, ALK-wt, ALK-F1174L, and ALK-R1275Q were sufficient to induce the formation and maintenance of highly undifferentiated neural crest cell-derived tumors, but not to drive NB tumor development. Our data in addition suggest that activated ALK cooperate with c-Myc to confer tumorigenic properties to NCPC in vitro and in vivo.

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POB005

BRD4 as a Target Gene for Treatment of MYCN Amplified Neuroblastoma

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Background: The BRD4 inhibitor JQ1 inhibits growth and induces apoptosis in a number of MYC overexpressing cancer types. This study aims to assess the efficacy of JQ1 in neuroblastoma cell lines and to define the mechanism of action.

Methods: A panel of neuroblastoma cell lines was screened for JQ1 sensitivity by using Alamar blue viability assays, soft agar assays and FACS live/dead and cell cycle staining. MYCN mRNA and protein levels were estimated by quantitative PCR and western blotting. A tetracycline-inducible MYCN shRNA expression system (IMR5/75 MYCN shRNA) and BRD4 siRNA were used to investigate the relationship between MYCN and BRD4. Global transcript expression was profiled by RNA-seq.

Results: Viability screens showed that MYCN-amplified neuroblastoma cell lines are generally more sensitive to JQ1 treatment than non-amplified cell lines. Reduced viability was mirrored by the absence of anchorage-independent growth and an increase of G1 fraction after JQ1 treatment. MYCN protein levels were reduced in 4/6 MYCN-amplified cell lines cell lines, however this did not correlate with sensitivity. BRD4 knockdown in IMR5/75 cells did not affect MYCN levels but reduced Cyclin D1 expression. Global expression analysis of IMR5/75 cells emphasizes the importance of TP53-related signaling pathways in the response to JQ1 treatment. Additionally, DNA damage repair genes were up-regulated in early time points, 6 and 12 hours after JQ1 treatment. A combination of doxorubicin and JQ1 synergistically reduced cell viability.

Conclusion: JQ1 treatment promotes a G1 arrest and almost completely suppresses anchorage-independent growth. MYCN down-regulation is not the central mechanism of action mediating JQ1-induced proliferation inhibition.

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POB006

Inhibition of the Wnt/PCP Signaling Pathway is a Novel Therapeutic Target in High-Risk Neuroblastoma?

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Background: The non-canonical Wnt/planar cell polarity (Wnt/PCP) pathway regulates cytoskeletal organization, migration and neuritogenesis. Signaling is characterized by activation of the GTPases Rho and Rac, and the downstream Rho-associated protein kinases (ROCK1 and ROCK2). Genetic analyses of neuroblastoma tumors have revealed several mutations and aberrations in the regulators Rho/Rac, implicating significant defects in neuritogenesis in neuroblastoma development. The aim of this study was to further characterize the Wnt/PCP signaling and the therapeutic effects of ROCK inhibition in neuroblastoma.

Methods: Cytotoxic activity of ROCK inhibitors was studied in cell viability assays. Morphology, differentiation and invasion were studied with microscopy. Molecular mechanisms were characterized using cell- and molecular biology techniques including Western blot, Real-Time quantitative PCR (Q-PCR), siRNAknockdown and organotypic culturing. In vivo studies in mice were carried out to validate the therapeutic effects and toxicity.

Results: Several mediators in the Wnt/PCP pathway were differentially expressed in investigated human cell lines. In neuroblastoma patient samples ROCK2 was vastly expressed, both in the cytoplasm and in the nucleus. Using compounds blocking ROCK1 and ROCK2 activity it was revealed that the ROCK2 inhibitor HA-1077 effectively repressed proliferation and reduced cell viability in neuroblastoma. Additionally, HA-1077 inhibited cell migration and induced differentiation through initiating neurite outgrowth. Furthermore, HA-1077 treatment affected the expression of several upstream Wnt/PCP mediators as Prickle-1, downregulated proliferation markers as cyclin D1 and induced apoptosis. The effects produced by HA-1077 were mimicked by ROCK2 knockdown. Finally, HA-1077 significantly reduced the growth of established neuroblastoma xenografts in nude mice.

Conclusion: These Results provide insights in the biological understanding of the Wnt/PCP signaling in neuroblastoma, and suggest that Wnt/PCP plays a significant role in neuroblastoma development and further that inhibition of this signaling pathway in general and ROCK in particular is a promising new therapeutic target for high-risk neuroblastoma.

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POB007

The ALK R1275Q and F1174L Mutations Display Different Effects on Survival and Oncogenesis

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Background: The ALK gene encodes a tyrosine kinase receptor preferentially expressed in the central and peripheral nervous systems. ALK mutations have been identified in both familial and sporadic neuroblastoma cases with different spectra: whereas three hotspots of mutations (F1245, R1275 and F1174) have been described in sporadic cases, no germline mutation affecting the F1245 and F1174 residues has been reported in neuroblastoma families. Recently, a syndromic presentation associating congenital neuroblastoma with severe encephalopathy and abnormal shape of the brainstem has been described in two sporadic cases harbouring de novo germline F1174V and F1245V ALK mutations.

Methods: In order to get insights into the role of the ALK R1275Q and F1174L mutations in development and oncogenesis, we developed and characterized Knock-in (KI) mice targeting the corresponding residues in the mouse Alk receptor.

Results: For both mutations, heterozygous (HT) mice had a normal appearance and were fertile. Heterozygous KI Alk^{F1178L} mice did not reproduce the severe neurological disorders, i.e. breathing and feeding difficulties observed in patients with de novo germline activating ALK mutations. Matings of HT yielded the expected proportions of WT, HT and homozygous (HM) animals at birth. Strikingly, whereas HM KI Alk^{R1279Q} mice enjoyed a peaceful life, a high post-natal lethality was noticed for the Alk^{F1178L}HM. Evaluation of basic physiological functions 12 hours after birth is ongoing. Furthermore, in a MYCN transgenic context, we demonstrate that the F1178L mutation displaysa higher oncogenic potential than the R1279Q mutation.

Conclusion: Overall, our data demonstrate that the F1174L mutation leads to an higher activation of the ALK receptor compared with the R1275Q mutation with subsequent effects on survival and oncogenesis.

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POB008

Aldehyde Dehydrogenase 1A2 Expression Correlates with Cancer Stem Cell Properties in Neuroblastoma

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Background: Neuroblastoma is an aggressive tumor characterized by its heterogeneity ranging from spontaneous regression to malignant progression. Despite the incorporation of 13-cis-retinoic acid (13-cRA) in maintenance therapy, over 50% of high-risk neuroblastoma patients have experienced a tumor relapse. As in most cancers, neuroblastoma recurrence is primarily driven by chemoresistant cancer stem cells (CSCs). Previous studies have identified neuroblastoma CSCs as spheres, side population cells, and cell-surface marker-positive cells based on the markers associated with stem cell populations. Although aldehyde dehydrogenases (ALDHs), which catalyzed the oxidation of aldehydes to carboxylates and comprised 19 isoforms in human cells, was used as a CSC-marker in many types of cancers, it remained to be characterized in neuroblastoma.

Methods: Neuroblastoma cells established from 3 different high-risk patients were analyzed. Neuroblastoma CSCs were isolated as spheres grown in sphere medium with a non-adherent dish.ALDH activity was determined using Aldefluor kit.ALDH shRNAs and cDNAs were expressed in BE(2)-C cells and analyzed for CSC properties including cell proliferation, sphere formation, colony formation, and xenograft tumor formation activities. The correlation of ALDH1A2 expression with overall survival probabilities of neuroblastoma patients was analyzed by R2 using the dataset Tumor Neuroblastoma–Versteeg–88–MAS5.0–u133p2.

Results: Although high ALDH activity was enriched in spheres compared to parental cells, the most abundantly expressed ALDH isoform varied between different neuroblastoma cells. Among 3 ALDH isoforms consistently induced in spheres, ALDH1A2 knockdown most severely impaired the sphere and colony formation activities. Reciprocally, increased expression of ALDH1A2 enhanced these activities in neuroblastoma cells. ALDH1A2 expression was further correlated with the differentiation of neuroblastoma xenografts, the resistance to 13-cRA, and the overall survival of neuroblastoma patients.

Conclusion: Present results suggest that ALDH1A2 expression correlates with cancer stem cell properties in neuroblastoma.

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POB009

The MYCN/ miR-26a-5p/ LIN28B Regulatory Axis Controls MYCN-Driven LIN28B Upregulation in Neuroblastoma

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Background: The RNA binding protein LIN28B is an essential regulator of stem cell self-renewal and has been identified as a bona fide oncogene in neuroblastoma. LIN28B is known to enhance MYCN expression through downregulation of let-7 microRNAs (miRNAs). As part of a broader study of dynamic miRNA and mRNA regulation during neuroblastoma development, we observed unexpected dynamic upregulation of LIN28B in MYCN-driven hyperplasia and tumors in mice. Hence, we hypothesized that MYCN and LIN28B are involved in a positive feedback loop through one or more miRNAs that are acting as regulatory switches between the hubs in this regulatory network.

Methods: In order to fully explore this putative network, we experimentally studied all possible LIN28B-miRNA interactions through an unbiased LIN28B 3'UTR-miRNA library screen for a total of 470 miRNAs.

Results: This LIN28B 3'UTR-miRNA library screen identified 30 miRNAs potentially targeting LIN28B. Subsequently, integrated analyses with miRNA expression profiles of a large cohort of primary neuroblastoma tumors, yielded miR-26a-5p as top candidate miRNA for LIN28B targeting in neuroblastoma. Importantly, miR-26a-5p is directly downregulated by MYCN in the MYCN-inducible MYCN3 cell line, thus providing strong evidence for MYCN-driven LIN28B upregulation. In vivo assessment using xenograft experiments with miR-26a-5p modulated neuroblastoma cell lines is currently ongoing.

Conclusion: The discovery of this MYCN/ miR-26a-5p/ LIN28B regulatory axis marks LIN28B as an important effector of the MYCN oncogenic phenotype and underlines once more the importance of MYCN-regulated miRNAs in establishing the MYCN-driven oncogenic process. Consequently, LIN28B can be regarded as a prominent therapeutic target for MYCN-driven neuroblastoma tumors. Finally, given the role of both MYCN and LIN28B as bona fide stem cell markers, these novel findings are of broader significance for normal development and cancer biology.

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POB010

A LIN28B/RAN/AURKA Signaling Network Promotes Neuroblastoma Tumorigenesis

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Background: Genome-wide association studies have elucidated the genetic basis of neuroblastoma, uncovering BARD1 and LMO1, among other genes, as oncogenic drivers in neuroblastoma subsets. Recently, we and others showed germline variation in LIN28B, a master regulator of the let-7 microRNA family, promotes susceptibility to neuroblastoma (Nat. Gen., 2012). We hypothesized that LIN28B is a major neuroblastoma oncogene and sought to define its oncogenic mechanisms.

Methods: We performed pathway analyses (Ingenuity) to discover molecular pathways associated with LIN28B expression.We used siRNA, shRNA and microRNA mimetics to genetically manipulate transcripts of interest in neuroblastoma cells, and then measured effects on downstream signaling, protein-protein interactions, and proliferation.

Results: Expression analysis of 221 high-risk primary neuroblastomas showed a strong positive correlation between LIN28B and RAN, a member of the Ras family that promotes AURKA activation, and other RAN-associated proteins. Additionally, recurrent somatic copy number gain of 12q24, where RAN is located, was observed in 15% of neuroblastomas and associated with increased RAN expression. LIN28B and RAN RNA and protein levels were positively correlated in neuroblastoma cell lines (p=0.02). LIN28B depletion resulted in decreased RAN levels, and this was also seen with let-7 overexpression. LIN28B/let-7 promoted RAN protein expression in part by influencing RANBP2, which binds RAN. We next showed that LIN28B promoted both activated AURKA and total AURKA and we demonstrated that AURKA is a let-7 target. siRNA-mediated RAN depletion resulted in decreased neuroblastoma cell proliferation, and exogenous RAN expression rescued the effects of LIN28B depletion.

Conclusion: LIN28B promotes the expression of RAN and AURKA, influencing a signaling network that drives neuroblastoma oncogenesis. A fuller understanding of LIN28B-influenced pathways provides a foundation for designing strategies to target this oncogene.

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POB011

Neural Crest Stem Cell Expression Signature Identifies Role for KLF5 in Neuroblastoma

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Background: Neuroblastoma (NB) is a sympathetic nervous system tumour derived from neural crest stem cells (NCSC). Thus, we hypothesized that genes that are differentially expressed between NCSC and differentiated sympathetic neurons may be involved in the molecular pathogenesis of NB and/or NB stem cell properties. Our top candidate, the transcription factor Kruppel-Like Factor 5 (KLF5), regulates cell growth, differentiation and apoptosis. KLF5 promotes or inhibits these functions in a context-dependent manner but has not been studied in neuronal models.

Methods: Dorsal root ganglia from rat embryos (E14.5) were FACS sorted for neural crest stem cell markers CD49d (α_4-integrin) and CD271 (p75NGFR). Differential gene expression was evaluated by Affymetrix microarray (Rat 230 2.0) and validated by qRT-PCR. Kaplan-Meier survival curves were produced in two online databases.

Results: 143 genes were differentially expressed between NCSC and differentiated populations, 27 of which were also detected in a panel of NB cell lines. For 12 of these genes the level of expression in patient tumours correlates with patient survival. KLF5 levels were lower in NCSC as compared to differentiated cells, and low expression in tumours correlates with worse overall survival. In cells with MYCN amplification or high c-myc KLF5 undergoes SUMOylation. The subcellular localization of SUMOylated KLF5 is nuclear, whereas unmodified KLF5 is cytoplasmic. Lentiviral shRNA knockdown of KLF5 in NB cells with SUMOylated KLF5 **Results:** in increased proliferation.

Conclusion: A NCSC gene expression signature identified KLF5 as a regulator of NB cell proliferation that is correlated with its SUMOylation. Future studies will determine the role of KLF5 and its modification by SUMO on retinoic acid-induced differentiation as well as determine the downstream targets required for its tumour suppressor activities. Additional studies are underway to functionally characterize other candidates for roles in NB.

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POB013

PA2G4 Promotes Neuroblastoma Oncogenesis Through Direct Binding and Modulation of MYCN Protein Levels

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Background: Proliferation-associated protein 2G4 (PA2G4) is a cell-cycle regulated protein capable of interacting with DNA, RNA and protein. There are two protein isoforms of PA2G4 (p42 & p48). The long isoform has an oncogenic function, whereas the short isoform acts as a tumour suppressor. However, the role of PA2G4 in neuroblastoma and the link between PA2G4 and MYCN is unknown. Here we identify PA2G4 as a novel MYCN protein binding partner, with a role in neuroblastoma oncogenesis.

Methods: We used a panel of human neuroblastoma cell lines and patient tumour samples to analyse the expression of PA2G4 by real-time PCR and Western blotting. The binding of PA2G4 protein to MYCN protein was examined by co-immunoprecipitation assays. We used siRNA, shRNA and overexpression of PA2G4 plasmid DNA to study the protein-protein interaction of MYCN and PA2G4. Colony and neurite formations, cell proliferation assays, flow cytometry, and transwell migration assays were used to study the phenotypic changes of PA2G4 on neuroblastoma.

Results: We identified PA2G4 as a binding partner for the MYCN NH2-terminal domain using pull-downs in cells overexpressing individual MYCN protein domains. We confirmed that MYCN protein directly binds to PA2G4 protein in neuroblastoma cells. High expression of PA2G4 was a strong clinical indicator of poor survival and positively correlated with MYCN expression in neuroblastoma patients. We found higher PA2G4 protein expression in MYCN or c-MYC amplified neuroblastoma cell lines, compared with MYCN non-amplified cells. Down-regulation of the long isoform of PA2G4, p48, decreased MYCN protein levels, promoted neurite formation, and, reduced cell proliferation and colony formation. Overexpression of PA2G4 increased MYCN protein levels and cell growth in neuroblastoma cells.

Conclusion: PA2G4 is a novel MYCN binding partner and positive regulator of oncogenesis in neuroblastoma.

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POB014

Novel 1p Tumor Suppressor DMAP1 Regulates MYCN/ATM/p53 Pathway

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Background: Deletions in chromosome 1p are frequently found in unfavorable neuroblastomas (NBs) and are correlated with MYCN amplification; however, it remains to be elucidated how the 1p loss contributes to MYCN-related oncogenic processes in NB. In this study, we identified the role of Dnmt1-associated protein 1 (DMAP1), coded on chromosome 1p34 and included MYC/MYCN/Max-complex (Cell, 143: p313–324, 2010), in the processes.

Methods: Gene knockdown and over-expression were performed by lentiviral systems and retrovirus systems in NB cell lines and fibroblasts, respectively. Microarray-based comparative genomic hybridization was performed as described previously (Tomioka N et al., Oncogene, 2008).

Results: MYCN transduction in MYCN-single NB cells accelerated Doxorubicin (Doxo)-induced apoptotic cell death; MYCN is implicated in DMAP1 protein stabilization and ATM phosphorylation in these situations. DMAP1 knockdown attenuated MYCN-dependent ATM phosphorylation and NB cell apoptosis. Intriguingly, DMAP1 induced ATM-phosphorylation and ATM-focus formation in the presence of Doxo. By DMAP1 expression in NB and fibroblasts, p53 was activated in an ATM-dependent manner and p53-downstream pro-apoptotic Bcl-2 family molecules, BAX and Noxa, were induced at the mRNA level, resulting in p53-induced apoptotic death. By wet lab- and in silico-expression analysis, we found that low-level expression of DMAP1 related to poor prognosis, unfavorable histology, and 1p LOH of primary NB samples, suggesting that the locus is under selective pressure of MYCN amplification.

Conclusion: Together, DMAP1 appears to be a new candidate for a 1p tumor suppressor and its reduction contributes to NB tumorigenesis via inhibition of MYCN-related ATM/p53 pathway activation.

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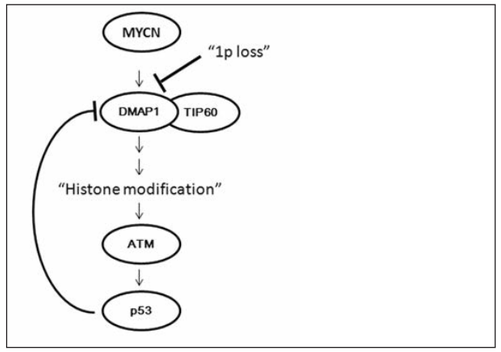

POB015

GALNT14 as a Novel Candidate Gene for Neuroblastoma Predisposition

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Background: Although several genes have been identified as involved in neuroblastoma (NB) predisposition and aggressiveness, further genes should be implicated in participating to the overall risk of developing this pediatric cancer. In an attempt to discover additional NB predisposing genes, we carried out whole-exome sequencing on two affected cousins and two unlinked healthy relatives from a large family with hereditary NB.

Methods: Germ-line DNA samples were sequenced in paired-end mode on the Illumina HiSeq 2000. After quality control of sequencing data by FastQC, filtered sequences were aligned on the human reference genome (GRCh37) using TopHat. SNPs were called by VarScan on unique alignments and variants annotated by SeattleSeq. Validation by Sanger sequencing was performed.

Results: Preliminary Results showed 6999 variations that were exclusively shared by the two familial NB cases and not detected in the unlinked healthy relatives. We considered unknown or rare (MAF<0.01) missense mutations, which involved 30 genes. Sanger sequencing of all family members, including five NB patients, showed a proper segregation of four genes. Of the latter genes, only mutations harbored by GALNT14 and EIF2AK2 were predicted as damaging by PolyPhen2 and SIFT. Screening of these mutations in other 8 NB families and 167 sporadic cases showed only the GALNT14 mutation (chromosome 2p: g31167749 C>T) in the tumors of two heterozygotes NB twins and in both germ-line and somatic DNAs from one sporadic patient. This GALNT14 mutation was not detected in 132 unrelated healthy individuals.

Conclusion: GALNT14 is a member of the polypeptide N-acetylgalactosaminyltransferase protein family and it maps closely to ALK on 2p23.1, a region we previously discovered in linkage with NB in this family. In other tumors GALNT14 was reported as correlated with Apo2L/TRAIL sensitivity to proapoptotic signaling through O-glycosylation of Apo2L/TRAIL death receptors. Conclusively, we propose GALNT14 as a novel gene potentially involved in the predisposition of NB.

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POB016

Tumor Sphere Specific Transcription Factor CDX1 Regulates Stem Cell-Related Gene Expression and Aggressiveness in Neuroblastoma

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Background: CD133 (Prominin-1) was expressed in cancer stem cells of several malignancies and its functional roles in tumor cells have been studied recently. We previously reported that CD133 suppressed differentiation via RET suppression and p38MAPK and AKT phosphorylation in NB cells (Takenobu et al., Oncogene 2011). However, the role of CD133 in stemness of NB cells and its regulatory mechanism of transcription in NB cells remain to be elucidated.

Methods: Primary NB sphere and CDX1-expressed NB cells were analyzed by RNA seq using illumina® next generation sequencer. CDX1 knockdown and over-expression were performed by lentiviral system. Cells were subjected to proliferation, nude mice tumorigenic and sphere formation assays.

Results: To study the regulatory mechanism of CD133 transcription in NB tumor sphere, we analyzed CD133 P1-P5 promoter activities. CD133 wasmainly transcribed by P1 promoter upstream of exon 1A in NB tumor sphere. To identify the sphere specific transcription factors, we examined comprehensive expression analysis of transcription factors in NB spheres. The expression pattern of the CDX1 transcription factor was correlated with that of CD133 during NB tumor sphere formation and CDX1 directly bound to CD133 P1 promoter region and up-regulated CD133 expression. Intriguingly, RNA seq analysis indicated Wnt pathway activation in CDX1-expressing NB cells and several stem cell-related genes, e.g. OCT4, Klf5, Sall4, and Nanog were up-regulated in CDX1-expressing cells. We also found that CDX1 directly bound to OCT4 promoter region. Furthermore, CDX1 induced cell proliferation in vitro/in vivo and NB sphere forming efficiency and high expression of CDX1 was correlated to poor prognosis in MYCN amplified and non-amplified NB patients.

Conclusion: Tumor sphere specific transcription factor CDX1 induces stem cell-related genes, including CD133 and OCT4 expression, and contributes characteristics of NB tumor initiating cells and unfavorable prognosis of NB patients.

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POB017

Ataxia-Telangiectasia Mutated (ATM) Silencing Promotes Neuroblastoma Progression through a MYCN Independent Mechanism

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Background: Deletion of one copy of chromosome 11q occurs in approximately 40% of neuroblastoma (NB) with poor prognosis, where it behaves as an independent prognostic factor. The ataxia-telangiectasia mutated (ATM) tumor suppressor gene is located on 11q23. This suggests that ATM haploinsufficiency might contribute to NB progression.

Methods: Using three different shRNAs againts ATM, we stably silenced ATM expression in three different NB cell lines having no MYCN amplification and exhibiting a functional ATM/p53 response to DNA damage. We studied the phenotypic consequences of ATM silencing in vitro by means of the soft agar assay, and in vivo by subcutaneous injection into nude mice.

Results: In the three cell lines, ATM silencing resulted in increased proliferation in the soft agar assay. When injected into nude mice, NB cells with ATM silencing formed tumors up to 33.6-fold larger than the respective controls. These effects were dependent on the extent of ATM silencing, with partial silencing of ATM being sufficient to observe them. Following ATM silencing, we did not observe significant alterations of MYCN regulated genes. ATM silencing also strongly promotes NB progression in a NB cell line having undetectable MYCN expression.

Conclusion: Our Results show (i) that stable silencing of ATM by three different ATM shRNAs consistently confers a growth advantage in vitro and in vivo to three different NB cell lines; (ii) that this effect is dependent on the extent of ATM silencing; (iii) that partial silencing of ATM is sufficient to observe such growth advantage, and (iv) that this effect does not require the expression or the activity of MYCN. These Results provide the first experimental evidence that reduced expression of ATM, as it may result from 11q23 haploinsufficiency, directly contributes to NB progression. In addition, they are consistent with the notion that 11q23 deletion is an independent prognostic factor in NB.

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POB018

A Novel Long Noncoding RNA, lncNB, is Amplified in Human Neuroblastoma Tissues, and Promotes Neuroblastoma by Up-Regulating N-Myc Expression

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Background: MYCN oncogene amplification is often accompanied by the amplificationof genes at2q24and 17q in neuroblastoma patients. While the MYCN oncogene has been extensively studied, it is unknown whether long noncoding RNA genes at 2q24 and 17q play a role in neuroblastoma tumorigenesis.

Methods: Publically available bioinformatics data was employed to discover a novel long noncoding RNA, lncNB, and amplification of the lncNB gene was examined by SNP arrays in 341 human neuroblastoma samples. Modulation of N-Myc and N-Myc target gene expression by lncNB was investigated by RT-PCR, immunoblot and Affymetrix microarrays. RNA pull-down assays, mass spectrometry and RNA immunoprecipitation assays were employed to identify proteins which bound to lncNB RNA. Kaplan–Meier survival analysis and Cox regression were used to test for associations of lncNB expression with overall survival in three independent cohorts of patients with primary neuroblastomas. In addition, two groups of mice were xenografted with neuroblastoma cells and treated with antisense oligonucleotides targeting lncNB or mis-match sequence.

Results: Bioinformatics data generated by the HAVANA team at the Sanger Institute predicted lncNB gene and RNA, and RT-PCR analysis confirmed its three exons two introns. The lncNB gene was amplified in 88 out of 341 human neuroblastoma tissues. lncNB RNA bound to the RNA-binding protein NonO, leading to N-Myc RNA up-regulation, modulation of N-Myc target gene expression and neuroblastoma cell proliferation. High levels of lncNB and NonO expression in human neuroblastoma tissues correlated with high levels of N-Myc expression and predicted poor patient prognoses, independent of age and disease stage. Moreover, treatment with antisense oligonucleotides targeting lncNB in neuroblastoma-bearing mice significantly reduces N-Myc expression and hinders tumor progression.

Conclusion: Our data demonstrate the important roles of lncNB in regulating N-Myc expression and neuroblastoma oncogenesis, and provide the first evidence that amplification of the lncNB gene contributes to neuroblastoma.

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POB019

The ARID1-Containing Swi/Snf BAF Complex Is a Driver of Poor Outcome Neuroblastoma

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Background: ARID1 proteins are mutually exclusive members of the Swi/Snf chromatin remodeling BAF complex that has tumor suppressor activities. BAF complexes regulate differentiation through programmed subunit exchanges as neural cells progress from stem (esBAF) to neural progenitor (npBAF) to post-neuritogenic neuron (nBAF) states. We propose that disrupted BAF activities preserve an undifferentiated progenitor state and define an aggressive neuroblastoma phenotype.

Methods: Whole-genome sequencing identified ARID1A/B lesions; cell lines of diverse ARID1A/B status were characterized by immunoprecipitation and immunoblot of BAF complexes and BAF-activity biomarkers, including anaphase bridging, were assessed. Clinical correlates of BAF subcomplex expression were sought, and functional consequences confirmed in cell lines.

Results: Recurrent ARID1A and ARID1B mutations were identified in neuroblastomas with dismal outcome (median survival 386d). ARID1A mutations are biallelic (point mutation/deletion) nominating this as a bona fide 1p35 tumor suppressor; while ARID1B mutations are monoallelic (germline haploinsufficiency causes the neurodevelopmental Coffin-Siris syndrome). ARID1A/B mutant cells had altered BAF complex composition and increased anaphase bridge formation. In tumors, expression of all genes unique to the npBAF complex correlated directly with each other (r>0.3; p<0.001) and inversely with genes unique to nBAF (r>-0.3; p<0.001). Similarly, neuritogenesis gene expression correlated directly with nBAF and indirectly with npBAF members. High npBAF expression independently correlated with unfavorable outcome.

Conclusion: A hallmark of unfavorable neuroblastoma is defective neural differentiation and BAF complex chromatin remodeling activities govern this process. We identified BAF-scaffold genes ARID1A and ARID1B as recurrently mutated, more frequently in cell lines and poorest outcome tumors. We propose these changes disrupt BAF-mediated neural differentiation, and increase genomic instability by abrogating BAF-TOP2A chromatin binding. As with other principal oncogenic pathways, functional deregulation of BAF complex activities may not be restricted to tumors with bona fide BAF-complex mutations as evidenced by correlations between npBAF expression and poor outcome in unselected neuroblastoma populations.

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POB020

The Facilitates Chromatin Transcription (FACT) Protein Complex Acts in a Forward Feedback Loop with the Oncoprotein MYCN to Promote Neuroblastoma Tumorigenesis

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Background: Neuroblastoma tumor initiation is characterised by transient repression of cell deletion signals, causing embryonal neuroblasts to persist postnatally as cancer prone lesions. Mechanisms that escalate MYCN expression to very high levels are required to support this pre-malignant phenotype. Facilitates Chromatin Transcription (FACT) protein complex is a histone chaperone that regulates the dynamic structure of chromatin, driving transcriptional initiation and elongation. We have previously shown that FACT is potently upregulated in numerous cancers and enriched at the c-myc genomic region in HT1080 fibrosarcoma cells suggesting a FACT/MYC relationship. This study examined a potential role of FACT in MYCN driven neuroblastoma.

Methods: Fact´s role in regulation of tumour initiation was examined in neuroblastoma cell lines and primary ganglia cultures derived from TH-MYCN neuroblastoma mice. Prophylaxis experiments with FACT inhibitor Cbl137 were used in TH-MYCN mice to evaluate the role of FACT in neuroblastoma oncogenesis.

Results: High expression levels of the two FACT subunits, SSRP1 and SPT16, predicted poor patient prognosis in a dose-dependent manner, and strongly correlated with MYCN expression and amplification in primary human neuroblastoma tumour tissue (n = 476). FACT and MYCN participated in a positive feedback regulatory loop where MYCN directly bound the SPT16 promoter in ChIP assays. Chemical inhibition of FACT by Cbl137 treatment in vitro restored the death response of precancerous ganglia from TH-MYCN mice to trophic-withdrawal, and markedly reduced perinatal tumor initiation and subsequent tumourigenesis in vivo. SSRP1 was significantly upregulated in an immunohistochemical audit of TH-MYCN ganglia through tumourigenesis where SSRP1 and MYCN expression were strongly correlated and both had enriched expression in the neuroblast cell fraction of precancerous ganglia. Cbl137 was potently cytopathic against a panel of neuroblastoma cells.

Conclusion: Our data identified FACT as a novel effector of MYCN-driven neuroblastoma tumor initiation. FACT inhibitors may have potential in neuroblastoma prevention and/or treatment.

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POB021

MYCN Inhibition Causes Metabolic Changes in Human Neuroblastoma Leading to Accumulation of Lipid Droplets

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Background: Neuroblastoma (NB), which arises from the developing sympathetic nervous system is one of the most aggressive solid tumors of early childhood. Amplification of the MYCN oncogene can be foundin around 30% of NB patients and it is associated with rapid tumor progression and poor prognosis. Our recent findings show that a small chemical molecule, 10058-F4, previously identified as a c-MYC inhibitor also targets the MYCN/MAX complex resulting in apoptosis and neuronal differentiation in MYCN-amplified NB cells. Importantly, we demonstrated that inhibition of MYCN in NB cells results in metabolic changes including mitochondrial dysfunction leading to accumulation of lipid droplets. Similarly, treatment with the bromodamain inhibitor JQ1 leads to MYCN downregulation followed by lipid accumulation(Zirath et al, PNAS 100, 10258-10263, 2013).

Methods: We have used a high resolution quantitative proteomics approach which includes iTRAQ labeling and isoelectric focusing. Pathway analysis was performed by Ingenuity, PANTHER and GSEA. Proteins was analyzed by Western blots and immunofluoresence and metabolic changes using Seahorse.

Results: In order to explore possible effects of MYCN targeting therapy we have performed quantitative proteomics of MYCN-amplified NB cells treated with 10058F4 or with JQ1. For comparison, downregulation of MYCN expression using short hairpin RNA followed by proteomic analysis was performed. We identified around 7000 proteins of which 6500 have been used for identification of novel pathways involved in NB pathogenesis and for investigation of potential MYC related biomarkers. Our preliminary analysis shows that the largest numbers of affected proteins are involved in primary metabolic processes including protein, lipid and nucleic acid metabolic processes. Also, we demonstrate an impact of MYCN-driven lipid and fatty acid metabolism on NB pathogenesis.

Conclusion: Taken together, we have lighted important metabolic pathways in NB which may be the basis for future therapies.

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POB022

Rac/Rho GTPase Signaling in Neuroblastoma

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Background and Methods: The genomic landscape of neuroblastoma is starting to unravel through whole genome sequencing of large groups of tumors. We discovered neuritogenesis gene alterations to be one of the new molecular defects in high stage neuroblastoma. Several of these genes function as regulators in the Rac/Rho pathway, which mediates signaling guidance cues in neurotigenesis. By manipulating the pathway we study the importance of the balance of Rho and Rac activity in neuroblastoma and their effect on proliferation and differentiation in neuroblastoma cells.

Results and Conclusions: De overall expression of RhoA in neuroblastomas is high and high expression is associated with a poor prognosis. We found not only RhoA expression, but also RhoA activity (GTP bound) to be high in multiple neuroblastoma cell lines. Inducing shRNA-mediated knockdown of RhoA resulted in a strong reduction on the protein expression, although the active RhoA pool proves more difficult to inhibit. Nevertheless, we observed a strong inhibition of cell proliferation, the formation of neurite outgrowths and ultimately, the induction of apoptosis., We overexpressed RhoA and Rac1 variants (wildtype, dominant negative and constitutively active) in multiple cell lines and can demonstrate the influence on the activity of the pathways. Although activation of the Rac1 pathway does not seem to improve the induction of neuritogenesis, the inhibition of Rho-activation consistantly Results in the induction of differentiation and inhibition of proliferation., Rho signalling is mediated by the downstream effector Rho kinase (ROCK), which can be inhibited with small molecule drugs. Inhibition of ROCK in neuroblastoma cell lines resulted in an induction of neuronal outgrowths and inhibition of proliferation. In some cases treatment of neuroblastoma cells in combination with Retinoic Acid showed a synergistic differentiating effect, suggesting that this drugcombination may provide a therapeutic means.

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POB023

Physical Interaction Between RUNX3 and MYCN Facilitates Protein Degradation of MYCN in Neuroblastoma

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Background: Neuroblastoma is a common pediatric solid tumor of neural crest origin. Among the prognostic indicators of neuroblastoma, the deletion at chromosome 1p36 and MYCN amplification are strongly associated with advanced stages, rapid tumor progression, and poor outcome. RUNX3, mapped to chromosome 1p36.2, encodes a Runt-related transcription factor. Originally, RUNX3was identified as a candidate tumor suppressor gene in solid tumors of diverse origins, such as gastric, lung, and colon cancer. Previously, we reported that higher expression of RUNX3 is closely correlated with better prognosis in neuroblastoma patients in which MYCN oncogene is highly expressed.

Methods: We hypothesized that high expression of RUNX3 could overcome the oncogenic property of MYCN in neuroblastoma. To prove this, we employed cell biological and biochemical assays, such as colony formation in soft agar, immunoprecipitation, and immunofluorescence microscopy.

Results: RUNX3 inhibited cellular growth and migration of neuroblastoma cell lines, confirming the tumor suppressive capability of RUNX3. In MYCN-amplified neuroblastoma cells, the expression level of MYCN was significantly decreased at protein level by overexpression of RUNX3, while mRNA expression of MYCN was not affected. Overexpression of RUNX3 induced poly-ubiquitination of MYCN, indicating that RUNX3 destabilizes MYCN through the proteosome. RUNX3 and MYCN colocalized in the nucleus, and RUNX3 protein physically interacted with MYCN both in vitro and in vivothrough its runt domain.RUNX3-R122C, a transcriptionally incompetent mutant of RUNX3 could facilitate the degradation of MYCN, suggesting that the degradation is not a secondary effect of the transcriptional activation mediated by RUNX3.

Conclusion: Interaction between RUNX3 and MYCN facilitates protein degradation of MYCN via ubiquitin-proteosome pathway followed by their physical interaction, suggesting a novel molecular mechanism of RUNX3 to suppress MYCN-mediated tumorigenesis. We propose that clinical treatment targeting RUNX3 may provide with an important clue to develop a novel therapeutic strategy in neuroblastoma.

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POB024

Case Report of a Patient with Speech Delay and Behavioral Disorders Associated with Neuroblastoma. Possible role of CDKN2D and SMARCA4

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Background: Pediatric cancers can occur within a context of predisposition syndromes, the exploration of which can in turn contribute to the detection of genes implicated in oncogenesis. For neuroblastoma (NB), few associated malformative syndromes have been reported and these are only rarely characterized by genetic alterations identified to date.

Methods: Here, we report the case of a girl with a delay in language acquisition and behavioral disorders associated with an adrenal neuroblastic tumor discovered at the age of 4 years.

Results: At a constitutional level, a microdeletion (1.7 Mb) of the 19p13.2 region was detected. At a somatic level, no other genetic alteration was observed by array-CGH. The 1.7 Mb deletion encompassed, amongst others, the CDKN2D and SMARCA4 genes.

Conclusion: In NB, higher expression of CDKN2D has been reported in early stage disease, and more recently, a CDKN2D missense mutation has been identified in one case of high-risk NB by whole-exome sequencing. SMARCA4 mutations have been described to be involved in several pediatric cancers such as certain rhabdoid tumors and medulloblastoma, and have also been identified in NB cell lines but not in tumours. SMARCA4 belongs to the SWI/SNF complex and recently data from sequencing of large series of NB samples suggested that the ARID1A and ARID1B genes, which also belong to the SWI/SNF complex, can be involved in NB. Altogether, these observations suggest a possible involvement of genes of the INK4 family and of the SWI/SNF complex in NB oncogenesis. Further explorations including next generation sequencing will further elucidate the origin of this patient's tumor.

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POB025

GALNT2 Suppresses Malignant Phenotypes through Insulin-Like Growth Factor 1 Receptor and Predicts Favorable Prognosis in Neuroblastoma

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Background: Neuroblastoma (NB) is the most common extracranial solid tumor in childhood with a poor outcome in spite of aggressive treatment. N-acetylgalactosaminyltransferase 2 (GALNT2), one of the enzymes initiating mucin-type O-glycosylation, is differentially expressed in the neurite outgrowh of SH-SY5Y NB cells. However, the expression and role of GALNT2 in NB remain unknown.

Methods: GALNT2 expression was evaluated by immunohistochemistry in NB tumor tissues and correlated with clinicopathologic features of NB. GALNT2 overexpression and knockdown were performed to analyze effects of GALNT2 on NB cell lines. Xenograft tumor growth was analyzed in nude mice. Signaling was analyzed by western blotting.

Results: Our results showed that GALNT2 expression in NB tumor tissues correlated well with the histological grade of differentiation as well as younger age at diagnosis, early clinical stage, primary tumor originated from the extra-adrenal site, favorable Shimada histology, and MYCN non-amplification. Multivariate analysis showed that GALNT2 expression is an independent prognostic factor for better survival outcomes of NB patients. GALNT2 overexpression suppressed insulin-like growth factor 1 (IGF1)-induced cell growth, migration and invasion, as well as tumor growth of NB cells, whereas GALNT2 knockdown enhanced these phenotypes of NB cells. Mechanistic investigations demonstrated that GALNT2 overexpression modified O-glycans on IGF1 receptor (IGF1R) and, which in turn, suppressed IGF1-triggered dimerization of IGF1R and subsequent activation of downstream signaling. Conversely, these properties were reversed by GALNT2 knockdown in NB cells.

Conclusion: Our findings suggest that GALNT2 can regulate malignant phenotypes through IGF1R signaling in NB cells and suggest a critical role of GALNT2 in the pathogenesis of NB.

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POB026

The CDKN2/CDK4/CCND1 Genes are Altered by Various Mechanisms Including Copy Number Defects and Point Mutations in Neuroblastoma

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Background: Neuroblastoma is characterized by a great clinical and genetic heterogeneity. A number of previous reports documented genomic aberrations targeting cell cycle genes in neuroblastoma.

Methods: We performed a screening of a series of 32 neuroblastoma cell lines, 103 primary neuroblastoma tumors obtained at diagnosis and 24 pairs of samples available at diagnosis and relapse to evaluate the frequency of mutations in the CDKN2 family genes. Copy number alterations were investigated for these genes, as well as for the CDK4/CDK6 and CCND1 genes. Expression data were obtained for a subset of cases and methylation analysis was performed for CDKN2A and CDKN2B promoters. Finally, p16 protein expression was investigated by Western blot analysis in 29 cell lines.

Results: In the complete series including 159 cases, we identified 13 cases with at least one genomic aberration targeting one of the investigated genes. Four cases presented with a point mutation of the CDKN2A gene and a homozygous deletion was documented in two cases. Mutations in the CDKN2D gene were identified in two cases. Amplification of the CCND1 and CDK4 genes was observed in 2 and 3 cases respectively., Expression of the CDKN2A gene was highly variable both in tumors and cell lines. In cell lines, we documented a good correlation between mRNA and protein expression levels. Methylation analysis did not reveal hypermethylation of the CDKN2A promoter in the samples where expression could not be detected. Whereas the CDKN2B gene is expressed only in a subset of neuroblastic tumors, a high expression of the CDKN2C gene was observed in most of cell lines and primary tumors. Expression of the CDKN2D gene was higher in tumors compared to cell lines.

Conclusion: Altogether, our data demonstrate that a variety of mechanisms leads to alterations of G1-cell cycle genes in a subset of neuroblastoma.

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POB027

NCYM Promotes Production of Myc-nick of MYCN in Neuroblastoma

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Background: Neuroblastoma is one of the pediatric solid tumors originating from sympathetic nervous system. The patient over one year of age with MYCN amplification usually has poor prognosis. Recently, it has been shown that Myc family proteins such as c-Myc or MYCN are cleaved by calpain, resulting in Myc-nick production. Myc-nick increases acetylation of a-tubulin through GCN5 recruitment and the acetylated a-tubulin is involved in stabilization of a-tubulin, promoting mi-

gration and drug resistance. However, regulators of the calpain-mediated Myc-nick production have not been identified. Here, we show that NCYM, a MYCN cis-antisense gene product, promotes the production of Myc-nick.

Methods: CHP134 and SK-N-BE(2)c cells were cultured with lentiviral supernatant for transfection of the indicated shRNA. 48 hours after transfection, cells were harvested and processed for western blot, For the in vitro cleavage experiments, c-Myc, MYCN, NCYM and Calpeptin was incubated on ice and the samples were processed for western blot. , Double-thymidine exposure technique was employed to synchrinize cell in each phase. After double-thymidine exposure, cells were washed free of thymidine and harvested at indicated time. And then cells were subjected to cell-cycle distribution analysis and Western blot analysis.

Results: Knockdown of NCYM decreased Myc-nick in CHP134 human neuroblastoma cells. Purified NCYM promoted calpain-mediated cleavage of c-Myc and MYCN in vitro, whereas the increased Myc-nick production by NCYM was blocked in the presence of calpeptin, a calpain inhibitor. Next we synchronized CHP134 cells using double thymidine block system, and found that expressions of both NCYM and Myc-nick were increased in mitotic phase and were accompanied by the induction of acetylated-α-tubulin. The expression levels of calpain and α-tubulin remained unchanged throughout the cell cycle.

Conclusion: Our results indicate that NCYM regulates Myc-nick production via activation of calpain-dependent proteolysis in human neuroblastoma. NCYM may enhance cell division, migration, metastasis of neuroblastoma by activating Myc-nick formation.

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POB028

Loss of the Promyelocytic Leukemia Protein (PML) in Neuroblastoma Promotes Angiogenesis and is a Marker of Recurrence in Localised Disease

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Background: Patients with localised, resectable neuroblastoma have generally an excellent prognosis and can be treated by surgery alone. However, approximately 10% of these patients develop local recurrences or metastatic progression. Only a very small fraction of patients with localised disease (less than 1%) show genetic changes associated with high-risk disease, such as amplification of MYCN or ALK mutations. Thus, there is need to find additional biomarkers that would allow the identification of the patients at risk of recurrence benefiting from more aggressive therapeutic interventions., The Promyelocytic leukemia protein (PML) has been shown to act as tumour suppressor in a number of human cancers, and we have recently demonstrated that its expression is required for proper development of the neocortex via cell fate regulation in neural progenitor/stem cells. We hypothesise that PML could play a role in the pathogenesis of nervous system tumours, including neuroblastoma., The aim of this study is to investigate the role of PML in neuroblastoma pathogenesis.

Methods: We used a combination of in vitro and in vivo approaches, ranging from genetic modification of neuroblastoma cell lines, xenotransplantation and vasculogenesis assays.

Results: Here we show that PML is expressed in the sympathetic nervous system in mice, marking neural crest progenitor cells at different stages of pre-natal and post-natal development. PML expression is low or absent in most high-grade (stage 4) human neuroblastomas and in tumours arising in the TH-MYCN mouse model. Notably, loss of PML expression in stage 1/2 human neuroblastomas accurately predicts tumour recurrence. Mechanistically, we found that PML re-expression in neuroblastoma cell lines causes Schwann-like differentiation and promotes the secretion of extracellular factors that hamper tumour angiogenesis. Finally, PML expression inversely correlates with tumour angiogenesis in neuroblastoma patients.

Conclusion: Collectively, these results demonstrate that PML is a novel neuroblastoma suppressor gene acting on tumour angiogenesis and a biomarker for disease recurrence.

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POB029

TRIM16 Inhibits Cell Growth through Direct Interaction and Modulation of TDP43 Protein Stability in Cancer Cells

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Background: The tripartite motif (TRIM) protein family are involved in a diverse range of cellular processes ranging from innate immunity, oncogenesis and tumour suppression. We have shown a strong correlation between loss of TRIM16 expression and disease progression in three human cancers: neuroblastoma, squamous cell carcinoma and melanoma. TRIM16 acts as a tumour suppressor in these cancers through effects on cell cycle and migration. However, the mechanism by which this putative tumor suppressor influences cell proliferation was undetermined.

Methods: We used Yeast two-hybrid assays, co-immunoprecipitation, siRNA and plasmid co-transfections, cyclohexamide chase assay, Western immunoblot, RT-qPCR, BrdU proliferation assay and alamar blue viability assay in this study.

Results: In order to better understand the mechanism of TRIM16 in cancer cell growth inhibition, we have identified a novel protein binding partner of TRIM16 and investigated the functional outcome of the protein-protein interaction. Using the yeast two-hybrid screening assay, we identified transactive response DNA-binding protein 43 (TDP43) as a novel TRIM16 binding protein. TDP43 has been described as a RNA/DNA binding protein with a putative role in HIV transcription and a wide range of neurodegenerative diseases. Through co-immunoprecipitation studies, we demonstrated that TDP43 directly bound to TRIM16 protein in human BE(2)-C neuroblastoma and MCF7 breast cancer cells. Enforced over-expression of TRIM16 increased the protein half-life of TDP43. We also found that TDP43 expression was required for TRIM16 induced inhibition of neuroblastoma and breast cancer cell growth. Moreover, we have shown that the levels of two cell cycle regulatory proteins, E2F1 and pRb, were regulated by TRIM16 and TDP43.

Conclusion: Taken together, our studies suggest that the novel mechanism by which TRIM16 inhibits cancer cell growth is through the direct interaction and up-regulation of TDP43 protein expression and with consequent effects on E2F1 and phos-Rb.

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POB030

Modulation of Mxi1 and Mxi0 Expression impacts N-Myc-Mediated Neuroblastoma Tumor Pathogenesis and Chemosensitivity

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Background: Neuroblastoma is the most common extracranial malignancy of childhood. The Myc family regulates cell growth and proliferation is implicated in the etiology of many cancers. MYCN amplified neuroblastoma carries a poor overall survival. Investigating specific tumor pathways will further our understanding of neuroblastoma pathogenesis and lead to future therapeutic options. Mxi1 is a member of the MAD family which inhibits N-Myc function. Mxi0 is an alternatively-spliced variant of Mxi1 whose function has not been determined. We hypothesize that Mxi1 and Mxi0 impact N-Myc-dependent neuroblastoma cell growth.

Methods: We expressed Mxi1 and Mxi0 in SHEP neuroblastoma cells and SHEP cells stably transfected to express high levels of MYCN (SHEP/MYCN). We also utilized native neuroblastoma cell lines with inducible expression of Mxi1 and Mxi0. Cell proliferation and survival were quantified using BrdU and MTT assays. Apoptosis was measured by propidium iodide staining and caspase-3 immunohistochemistry. Cellular localization of Mxi1 and Mxi0 proteins was detected by immunofluorescence.

Results: Overexpression of Mxi1 inhibits N-Myc mediated cell proliferation. In the absence of N-Myc, Mxi1 overexpression independently inhibits cell proliferation and induces cell apoptosis. Conversely, overexpression of Mxi0 in neuroblastoma cell lines leads to enhanced proliferation, suggesting that Mxi0 has a counter-regulatory role to that of Mxi1. Expression of Mxi0 made the cells more chemoresistant. Finally, examination of Mxi1 and Mxi0 cellular location reveals that Mxi1 resides in the nucleus while Mxi0 is found primarily in the cytoplasm.

Conclusion: Overexpression of Mxi1 in neuroblastoma cell lines leads to inhibition of N-Myc-mediated cell proliferation while Mxi0 appears to promote cell growth. Mxi1 expression enhanced chemosensitivity of neuroblastoma cells, while Mxi0 had the converse effect. A better understanding of the interaction between Mxi1 and Mxi0 and how the balance of these proteins affect neuroblastoma physiology may aid in developing more effective targeted therapies to improve outcomes in children with neuroblastoma.

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POB031

NCYM, a Cis-Antisense Gene of MYCN, is a De Novo Evolved Gene

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Background: Gene evolution has long been thought to arise from pre-existing genes through duplication or rearrangement followed by rapid divergence. De novo gene birth from non-coding genomic regions has been generally believed to be exceptionally rare. The recent advances in whole genome sequencing technology have identified the presence of de novo proteins; however, their physiological significance have largely remained unclear. Recently, we found that NCYM, a natural antisense gene of MYCN encodes a protein stabilizing MYCN protein via GSK3β inhibition and promotes metastasis in human neuroblastomas (Suenaga et al., PLOS Genetics in press). MYCN, but not c-MYC, directly activates NCYM transcriptin to form positive feed back loop with NCYM in human MYCN-amplified neuroblastomas. Here we report that NCYM is a de novo gene which is posively selected during evolution.

Methods: We used Basic Local Alignment Search Tool (BLAST) to make an alignment between translated amino-acid sequences ending at the first terminal codon, and calculated K_a and K_s, which are the rates of non-synonymous and synonymous amino-acid changes, respectively. We measured a bias in the amino acid frequencies (or codon frequencies) through the deviation from the uniform usage of each amino acid.

Results: We searched for paralogs and orthologs of the human NCYM protein among other animals using the BLAST with an E-value threshold of 10^{-3}. We did not find any paralogs, but identified orthologs for a probable NCYM protein in olive baboons, chimpanzees and pigmy chimpanzees.The evolutionary rates between the indicated species suggest that the coding sequence of NCYM gene was exposed to positive selection in humans and chimpanzees, and the amino acid frequencies in these species were significantly different from a uniform usage of amino acids ($P < 0.001$).

Conclusion: These results suggest that NCYM is the first de novo protein whose precise function has been clarified in multicellular organisms, specifically in humans.

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POB032

Flotillin-1 Regulates Oncogenic Signaling in Neuroblastoma Cells through Receptor Endocytosis of Anaplastic Lymphoma Kinase

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Background: Recent studies revealed that amplification of anaplastic lymphoma kinase (ALK) and series of oncogenicmutation of ALK are potent oncogenic factors of neuroblastoma.
Methods: To elucidate the role of anaplastic lymphoma kinase (ALK) in oncogenesis of neuroblastoma, phosphotyrosine-containing proteins associated with ALK were investigated.

Results: Flotillin-1 (FLOT1), a plasma membrane protein involved in endocytosis, was identified as a binding partner of ALK that undergoes ALK-dependent tyrosine phosphorylation. Knockdown of FLOT1 in neuroblastoma cells caused dissociation of ALK from endosomes along with membrane accumulation of ALK, which resulted in activation of ALK and downstream signals. Suppression FLOT1 expression also enhanced oncogenic properties of neuroblastoma cells both in vitro and in vivo. On the other hand, oncogenic ALK mutants showed less binding affinity to FLOT1 than wild-type ALK. Lower expression levels of FLOT1 were observed in highly malignant subgroups of human neuroblastoma tissues.

Conclusion: Taken together, it was suggested that decreased levels of FLOT1 or defects in binding affinity between ALK mutants and FLOT1 may cause malignant phenotypes of neuroblastoma through the activation of ALK signaling.

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POB033

Analysis of the MYCN Amplicon in Neuroblastoma (NB) Cell Lines

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Background: MYCN amplification is observed frequently in neuroblastomas: ~25% in primary tumors and ~90% in cell lines. MYCN is the only gene that is consistently amplified in NB tumors and cell lines. However, little is known about the structure of MYCN amplicons or the mechanisms that give rise to MYCN amplification.

Methods: We performed SNP-Arrays on 27 NB cell lines and used this information to construct a map of amplicons. Real-time PCR was used to refine the boundaries of each amplified region. Once the the boundaries of each amplicon end were narrowed to <3 kb, PCR was used to define the junctions and determine the orientation (head-tail, head-head, tail-tail). DNA sequencing was used to precisely define the boundaries and to identify the exact nucleotides at the junction between amplicons or amplicon ends.

Results: We have sequenced the MYCN amplicons of three NB cell lines (Kelly and BE-2C with HSRs; LA-N-5 with DMs) so far, and our data show that the amplicon ends are composed of head-tail junctions of a contiguous region from 2p23 including the MYCN gene. The junctions are a perfect match with the genome sequence, i.e., no novel sequences was introduced or deleted during the amplicon ligation. Interestingly, short and imperfect hairpin structures were observed at or near these junctions.

Conclusion: Our data indicate that MYCN amplicons result from a precise end-joining mechanism that was similar for both HSR- and DM-containing NB lines. We found no evidence of repetitive or transposable elements, common sequence motifs, or recurrent sequence additions or deletions at these junctions. The precise mechanism that results in formation of the MYCN amplicon is still unknown. However, generation of a circularized DNA fragment containing both a replication-initiation and matrix-attachment region would be sufficient to allow for DM accumulation by random assortment and selection.

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POB034

Calreticulin Up-Regulates VEGF-A and VEGF-C Expression in Neuroblastoma Cell Lines

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Background: Neuroblastoma (NB) is a childhood cancer with low survival rate and great potential of metastasis. Even though there are several clinically relevant prognostic markers have been identified, the molecular mechanism underlying the tumorigenesis of NB is still unclear. Calreticulin (CRT), an endoplasmic reticulum chaperone protein, is one of the biomarker of NB. It was suggested to play a critical role

in the tumorigenesis and differentiation in NB. In gastric cancer cells, it was also found that CRT strongly enhances angiogenesis by promoting the expression of vascular endothelial growth factors (VEGFs). In this study, we aim to investigate the correlation between CRT and VEGFs in NB.

Methods: The CRT expression vector and siRNA were transiently transfected into SK-N-DZ and SH-SY5Y cells. The expression levels of VEGFs were analyzed by real-time PCR, western blot and ELISA analysis. CRT inducible expression stNB-V1 cell line was established and was employed in mice xenograft experiments.

Results: It was found that overexpression of CRT in SK-N-DZ and SH-SY5Y cells up-regulated VEGF-A and VEGF-C expression at both mRNA and protein level. CRT siRNA efficiently suppresses CRT expression resulting in downregulation of VEGF-A and VEGF-C. ELISA analysis showed that CRT also significantly enhanced VEGF-A protein secretion in the conditioned medium. By using CRT inducible stNB-V1 cell line in mice xenograft model, CRT expression induced by doxycycline in the drinking water significantly increased VEGF-A expression and suppressed the tumor growth. However, the underlying mechanism of how CRT affects NB tumor progression through modulating VEGFs expression still needs further clarification.

Conclusion: These results indicate that CRT plays a role in regulating VEGF-A expression, which may affect angiogenesis and tumor progression in NB.

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POB035

Neuroblastoma Contains Multidrug-Resistant Mesenchymal-Type Cells That Depend on NOTCH-PDGFRβ Signalling

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Background: Neuroblastoma is an embryonic tumour of the peripheral sympathetic nervous system. Despite intensive chemotherapy, high stage neuroblastoma often relapse as therapy resistant tumour. Hardly any insight in drug-resistance in neuroblastoma exists.

Methods: Here we characterize intra-tumoral heterogeneity of neuroblastoma. We describe that each neuroblastoma tumour includes two types of tumour cells with highly divergent characteristics. We cultured pairs of both cell types from a series of fresh primary neuroblastoma and analysed resistance to therapy.

Results: One type has neuro-epithelial characteristics (NE) and expresses all classical neuroblastoma and lineage differentiation markers, like PHOX2B, DBH and TH. The other cell type hardly expresses these markers, but instead has mesenchymal (MES) characteristics. MES-type cells are highly motile. When compared to NE cells, the MES cells were much more resistant to all currently used chemotherapeutics. In addition, they were resistant to retinoic acid, which is included in current standard therapies. In vitro, both cell types spontaneously interconvert at low frequency. Immunohistochemical analysis revealed that neuroblastomas of all stages include both cell types. These data suggest that MES-type cells might play an essential role in drug-resistant relapses and metastasis. Identification of drugs specific for MES cells thus seems important. MES-type cells showed active NOTCH signalling and inducible expression of NOTCH constructs efficiently converted NE-cells into MES-cells in vitro. NOTCH3 induced PDGFRβ expression, which essentially contributed to the mesenchymal phenotype. PDGFR signalling activated PI3K/AKT and MEK-ERK signaling cascades in response to PDGF-D.

Conclusion: The elucidation of key-pathways in MES-cell induction and maintenance allowed the development of targeted approaches for the specific eradication of MES-type cells.

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POB036

Mutant ALK Controls the RET-ETV5 Signaling Axis in Neuroblastoma: Implications for Normal Development, ALK Driven Tumor Formation and Novel Therapeutic Strategies

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Background: Neuroblastoma (NB), a pediatric cancer of the sympathetic nervous system, displays high metastatic potential and nearly 60% of the patients present with metastatic lesions at the time of diagnosis warranting the identification of therapeutically relevant driver genes. Targeting the RET signaling pathway has been of interest as a therapeutic option for NB due to its involvement in the control of proliferation, metastasis and angiogenesis. Importantly, an intrinsic component of the RET pathway during development is the oncogenic transcription factor ETV5 which is a driver of metastasis in multiple cancer types.

Results: With the aim of selecting novel nodes for therapy intervention in NB, we have previously generated a gene signature list reminiscent of mutant ALK activation in NB cells. Cross species genomics analysis of MYCN and ALKF1174 driven tumors and targeted ALK inhibition studies in vitro indicated that both RET and ETV5 function as downstream targets of aberrant ALK signaling in NB. Our data further show that knockdown of ETV5 in NB cell lines alters their clonogenic, adhesion and invasive potential and reduces growth of NB tumors in vivo. Additionally, treatment of NB cell lines with the RET inhibitor vandetanib ceases cell proliferation and results in downregulation of ETV5 expression, suggesting a preservation of the RET-ETV5 signaling axis. Zebrafish and mouse modeling of both ETV5 and RET is ongoing, offering a powerful tool for studying the role of these genes in NB formation in the context of ALK driven tumorigenesis.

Conclusion: In conclusion, we propose that ETV5 is an intrinsic component of aberrant ALK signaling in NB, providing a functional link between the RET and ALK oncogenic pathways and opening up future possibilities for the development of targeted therapy.

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POB037

Replicative Stress Induces Copy Number Alterations in Neuroblastoma Cell Lines and Might Contribute to Tumor Evolution

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Background: Inneuroblastoma (NB), copy number alterations (CNAs) correlate with tumor aggressiveness. Previous studies have demonstrated that replicative processes may account for genomic rearrangements in NB. We sought to further analyze the role of replicative stress in the generation of CNA in NB.

Methods: We submitted 4 cells lines (2 NB cell lines CLB-Ga and Gimen; 2 non NB cell lines A673 and G401) to replicative stress using low dose aphidicoline (0.3µM APH) and then isolated cellular clones. New Copy number alterations (CNAs) in the obtained clones were characterized using a cytoscan HD® array and compared to the genomic profile of the original cell line. The impact of replicative stress in tumor development was studied by xenografting a bulk of cells treated or not with APH in NOD/SCID mice.

Results: In the clones obtained after replicative stress, 100% of the NB clones (19/19 clones: CLB-GA 9/9 and GIMEN 10/10) contained new CNAs versus 60% of non NB clones (5/8 clones: A673 3/4 and G401 2/4). The mean number of new CNAs per clone was significantly higher in NB clones (2.36) compared to non NB clones (1.12) (Student test, p= 0.011). The majority of new CNAs were sized < 1Mb in both NB and non NB clones. Around 20% of the CNAs affected loci known to contain previously published fragile sites but no common region of overlap was targeted by the new CNAs. Pilot xenograft experiments indicate that tumor growth latency seems shorter in APH treated NB cells as compared to control NB cells. (34 days versus 44 days).

Conclusion: Our observations suggest that in NB due to a higher sensitivity to replicative stress accumulation of new CNAs might contribute to tumor aggressiveness.
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POB038

Role of MYCN in Neuroblastoma Rb-E2F1 Network

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Background: Neuroblastoma is a common extracranial tumor in children. It has been found that the MYCN is an important prognosis marker in 30% of the tumors. Additional studies have found that MYCN exerts a control in the Rb-E2F1 network but the exact mechanism remains still unclear. The work by Yao et. al, has shown that E2F1 expression exhibits a bistable switch response. This switch allows a controlled and irreversible commitment of cells into cell cycle upon certain threshold of serum stimulation.

Methods: To investigate the effects of MYCN on the Rb-E2F1 switch, Neuroblastoma cell lines with a Tet-inducible system for MYCN overexpression (SH-EP) and MYCN knockdown (IMR5/75) were used, and those cells lines were transduced with GFP-promoter constructs for E2F1 and Cyclin D1. Serum titration experiments and time series were performed to observe the response of E2F1 activation upon growth signals and to evaluate the integrity of the bistable switch.

Results: The initial results showed disparate behaviors. In the non-amplified cell line SH-EP, there is no bimodal behavior upon serum titration or either during serum-stimulated time course. Synchronizing cells with a thymidine block did not modify this result. In contrast, IMR5/75 cells, which harbor MYCN amplification, exhibited noisy bimodality upon serum stimulation, suggesting still the presence of a potential bistable switch.

Conclusion: These results suggest a strong perturbation of the bistable switch in the non-amplified case in contrast to a less severe damage in cells with MYCN amplification. A mathematical model was developed to integrate these results into a framework for a better understanding of the Rb-E2F1 network and potential prediction of new drug targets.

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POB039

MYCN Safeguards its Upregulated Expression through Negatively Controlling its Upstream miRNAs

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Background: MYCN plays a central role in neuroblastoma development and exerts a pleiotropic role through the regulation of many protein encoding genes and microRNAs (miRNAs). MYCN activity is strictly controlled at multiple levels, including transcription and protein stability. Previous studies already pinpointed several miRNAs controlling MYCN expression levels. To fully elucidate this regulation, we further explored the MYCN-miRNA interactome upstream of MYCN.

Methods: Therefore, we used the powerful combination of an unbiased high-throughput luciferase reporter screen for the 3\'UTR of MYCN, and a unique dynamic expression dataset, generated from the TH-MYCN mouse model.

Results: First, the unbiased MYCN 3\'UTR-miRNA library screen identified 29 miRNAs potentially targeting MYCN. Next, we determined which of these miRNAs were relevant in neuroblastoma context. Therefore we have evaluated the expression of these MYCN targeting miRNAs in a murine MYCN-driven neuroblastoma progression model. Interestingly, the majority of MYCN targeting miRNAs showed a decreasing expression pattern during tumor development, suggesting that MYCN induces their downregulation. Further, miRNAs downregulated during murine tumor development showed anti-correlation to MYCN expression in a large cohort of primary neuroblastoma tumors, supporting their role in regulation of MYCN expression.

Conclusion: In conclusion, our data strongly suggest that MYCN negatively controls the expression levels of miRNAs binding to it\'s 3\'UTR, thereby safeguarding its own upregulated expression levels.

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POB040

miR-129-3p Directly Binds and Regulates ALK Expression in Neuroblastoma

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Background: Anaplastic lymphoma kinase (ALK) has been identified as a major oncogene associated with familial and sporadic Neuroblastoma (NB). MicroRNAs (miRs) are non-coding, single-stranded 18-24 nucleotide RNA molecules that base-pair with target mRNAs and negatively regulate their stability and translation efficiency. We explored the mechanisms leading to overexpression of ALK in NB tumors.

Methods: We analyzed 61 NB primary tumors for the copy number changes (MLPA) of ALK, mutation status and expression levels of mRNA and protein. miR microarray analysis was performed on 47 primary tumors. Validation of miR expression was performed by quantitative RT-PCR. All the results were correlated to clinical parameters and outcome. Following transfection of miR-129-3p mimic or inhibitor into a MYCN amplified cell line, the levels of ALK protein and proliferation rates were evaluated. To validate if ALK is a direct target of miR-129-3p, we used the dual-luciferase assay.

Results: miR-129-3p was predicted to target ALK. Poor outcome was significantly associated with gain of the ALK gene, high ALK mRNA levels, high protein levels and low miR-129-3p levels (p=0.03; 0.004; 0.002 and 0.03, respectively). We identified a significant inverse correlation between miR-129-3p levels and cytoplasmic ALK protein levels defined by specific staining. This suggests that miR-129-3p may regulate ALK. By synthetically upregulating miR-129-3p we observed a significant decrease in ALK protein levels accompanied by reduction in the proliferation rate of the cells. Moreover, the dual-luciferase assay resulted in a reduction of 26% in the relative luciferase unit (RLU) of ALK following transfection with miR-129-3p-mimic.

Conclusion: We provided evidence that miR-129 directly binds and regulates ALK expression in NB, suggesting a new mechanism for the regulation of ALK. miR-129-3p can serve as a additional crucial biomarker by identifying patients with poor outcome. Over-expressing miR-129-3p could be an additional potential therapeutic approach in NB.

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POB041

Abrogation of ERK5 Activity Suppresses Neuroblastoma Growth and MYCN Expression Mediated by ALK (Anaplastic Lymphoma Kinase)

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Background: ALK, a receptor tyrosine kinase, has been identified as one partner in a wide variety of translocation events which mediate an oncogenic response in different cancer types. Genetic evidence reported in both familiar and sporadic forms of neuroblastoma support ALK as a significant driver of tumour formation.

Methods: A number of small tyrosine kinase inhibitors (TKIs) have been developed that act to inhibit ALK activity, the most studied of these being crizotinib, a small competitive ATP-binding inhibitor currently in clinical use in the treatment of ALK positive NSCLC patients.

Results: Initial treatment of ALK$^+$ neuroblastoma patients treated with crizotinib has not provided as clear cut responses as those observed in other cancer types. The data accumulated thus far suggests that monotherapy may not be the solution for all ALK$^+$ neuroblastoma patients, and that individualized combinations of specific drugs might be a future solution to address the disease. ERK5, a.k.a. BMK1, is suggested to play a vital role in proliferation, differentiation, and survival. We show that ERK5 activated by ALK through the pathway PI3K/Akt/PKB/MEKK3/MEK5, which contributes both to cell growth of neuroblastoma cell lines and initiation of MYCN transcription. Pharmacological inhibition or siRNA of ERK5 results in decrease in growth of neuroblastoma cell lines, whereas in combination with crizotinib, an ALK inhibitor seems to be more effective both in vitro and in vivo.

Conclusion: Taken together, our results indicate that ERK5 plays an important role in ALK mediated initiation of transcription of MYCN, suggesting that the targeting of ERK5 might be a potential therapeutic target worthy of future exploration for neuroblastoma patients.

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POB042

The Oncofetal Protein IGF2BP1 Cooperates with MYCN within a Positive Feedback Loop in High-Risk Neuroblastoma

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Background: Previous studies show chromosomal 17q21-ter gain in neuroblastoma is both a common and highly prognostic event. Insulin-like growth factor-2 mRNA-binding protein 1 (IGF2BP1), is located within this gained region. Strikingly, IGF2BP1 is essential for neurocrest migration during development, and influences the stability/translation of MYC, CD44, PTEN, IGF-2, MDR1, and other transcripts relevant to neuroblastoma. This study analyses for the first time the DNA copy number and expression of IGF2BP1 in neuroblastoma, and importantly dissects the previously unknown molecular interplay of the IGF2BP1 and MYCN oncogenes.

Methods: Clinical significance and expression of IGF2BP1 in untreated neuroblastoma tumours was examined in both Affymetrix gene arrays (R2 bioinformatic platform) and additionally in 30 frozen tumours received from the Cologne tumour bank (by qPCR and Western blot). In vitro studies were performed on tumour-derived cell lines. The binding of MYCN protein to IGF2BP1 gene promoter was tested by chromatin-immunoprecipitation assays.

Results: IGF2BP1 was found to be highly expressed in neuroblastoma and outperformed other described oncogenes, including MYCN, by Kaplan-Meier analyses. IGF2BP1 was also associated with MYCN amplification and MYCN mRNA expression. Additionally, in another patient cohort IGF2BP1 DNA copy number, mRNA and protein levels were all significantly higher in stage 4 disease, compared with localised disease. Significantly, high IGF2BP1 protein expression was associated with lower patient survival ($p=0.0249$). IGF2BP1 protein expression was significantly correlated with MYCN mRNA levels. In vitro studies confirmed IGF2BP1's promotion of MYCN, as IGF2BP1 knockdown decreased MYCN RNA, RNA half-life and protein expression as well as decreased cell viability. Moreover, we found MYCN promotes IGF2BP1 expression. MYCN protein binds the IGF2BP1 promoter and IGF2BP1 mRNA, and is greatly increased in pre-cancer cells overexpressing MYCN in the TH-MYCN mouse model.

Conclusion: These data demonstrate, for the first-time, IGF2BP1 as a potential oncogene and biomarker in neuroblastoma, which has an important role within MYCN-driven carcinogenesis.

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POB043

Putative Cross-Talk of the Two CXCL12 Receptors, CXCR4 and CXCR7, in Metastatic Dissemination of Human Neuroblastoma

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Background: CXCR4 and CXCR7 share chemokine CXCL12 as a common ligand. However, we recently suggested that the two receptors may display different expression pattern, and potentially opposite role(s) in neuroblastoma (NB). In contrast to CXCR4, commonly associated to poor prognosis, CXCR7 expression was detected in differentiated tumours. CXCR7 impaired CXCR4/CXCL12-mediated chemotaxis and growth in vitro, and delayed in vivo tumour take of CXCR4$^+$-NB cells in a CXCL12-producing orthotopic environment (mouse adrenal gland (AG)). Although pivotal interactions between CXCR4 and CXCR7 were recently described in cancer progression, contribution of CXCR4 and CXCR7 in NB metastatisation needs further elucidation.

Methods: CXCR7, CXCR4, or both receptors were ectopically expressed in different NB cell lines, and their subsequent in vitro cell adhesion properties on human endothelial cells (HUVEC) were explored. Intravenous (iv) injections of transduced NB cells into nude or NOD/SCID Gamma null (NSG) mice, and chick embryo chorioallantoic membrane (CAM) cell implantation model were developed to address impact of CXCR4/CXCR7 on NB cell selective invasion capacities.

Results: CXCR7 expression increased CXCR4+-NB cell adhesion capacities in vitro. Iv implantation in nude mice showed that NB cells disseminated (micro-metastases) to lungs, spleen, and AG, within 12 weeks, while much faster dissemination and growth (macroscopic metastases) were observed in liver, lungs, AG and bone marrow of NSG mice. Moreover, CAM assay revealed to be an alternative suitable model to rapidly evaluate invasive properties of CXCR4+/CXCR7+-NB cells, as tumour growth was observed already 4 days after implantation. The precise contribution of both receptors in NB dissemination is currently in progress.

Conclusion: Our data suggest CXCR4/CXCR7 cooperation in enhancing NB cell adhesion abilities on endothelial cells in vitro. Moreover, NB cell implantation models developed in NSG mice, and in chick embryo revealed promising tools to reconstitute NB-typical metastatisation pattern, and to further elucidate CXCR4/CXCR7 cross-talk in metastatic NB cell dissemination.

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POB044

MYCN, Proliferative Heterogeneity and Treatment Response in Neuroblastoma

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Background: MYCN amplification, occurring in 20% of neuroblastomas, is associated with drug resistant relapse and poor prognosis. Expression of MYCN has paradoxical effects in the cell: promoting cell cycle progression and sensitizing cells to apoptosis. Our aim is to understand how MYCN affects the cell cycle and influences cellular decisions after treatment with chemotherapeutic drugs.

Methods: We applied live-cell imaging to monitor how MYCN determines cell cycle progression in SHEP TET21N and IMR5/75 with tetracycline regulatable MYCN expression. To analyse how MYCN influences responses to chemotherapy, we collected quantitative, time-resolved RNA, protein and phenotypic data before and after treatment with doxorubicin. We used qPCR, flow cytometry, western blotting and reverse phase protein arrays.

Results: MYCNon cells progress rapidly through the cell cycle and have a greater proportion of cycling cells compared to MYCNoff cells. MYCN sensitized cells to apoptosis during treatment with doxorubicin. However, after drug-removal MYCNon cells showed clonogenic growth. In contrast, MYCNoff cells do not proliferate and undergo drug-induced senescence after treatment, which was shown by senescence-associated-beta-galactosidase activity and elevated p21 levels.

Conclusion: Here we show that MYCN expression levels control the switching between cycling and non-cycling states of the cell cycle. Thus, MYCN determines the proportion of apoptotic to senescent cell fates in response to chemotherapy. MYCN has a second function, driving clonal regrowth of surviving cells after therapy. The experimental model provides the basis for the quantitative understanding of the molecular mechanisms underlying MYCN-driven therapy resistance.

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POB045

CHD5 and MYCN Regulation Downstream of MAPK Pathways in Neuroblastoma

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Background: CHD5 is a tumor suppressor gene that is located on the deleted region of 1p. We analyzed functions of MAPK (ERK, p38, JNK) pathways and the downstream expression of MYCN and CHD5 in neuroblastoma cells.

Methods: Neuroblastoma cell lines NLF, SY5Y, and TrkA overexpressed SY-5Y (SY5Y-TrkA) were treated with NGF and/or each MAPK inhibitor. Phosphorylation of each ERK, p38 and JNK was analyzed by western blotting (WB), and expression of CHD5 and MYCN was analyzed by PCR and WB. The effects of inhibitors on cells were analyzed by SRB assay and flow cytometry.

Results: NGF induces the phosphorylation of ERK, p38 and JNK in SY5Y-TrkA. CHD5 expression was up-regulated in SY5Y-TrkA by NGF treatment for 4 hr, and it was suppressed by the JNK inhibitor SP600125 and also modestly by the p38 inhibitor SB20358. CHD5 expression in SY5Y-TrkA was increased by NGF treatment over 4 days, and it was gradually decreased with p38 inhibitor. On the other hand, MYCN expression was remarkably suppressed by the MEK inhibitor GSK1120212 (trametinib), which suppresses ERK phosphorylation. There was dramatic suppression of MYCN with trametinib also in the MYCN-amplified NLF cell line. In terms of pathway interaction, ERK and JNK showed higher activity with the p38 inhibitor SB203580, which suggested p38 activation suppressed ERK and JNK. We analyzed cell growth, apoptosis and cell cycle in NLF and SY5Y cell lines treated with each MAPK inhibitor. Both the MEK inhibitor trametinib and JNK inhibitor SP600125 significantly suppressed cell growth, trametinib by inducing cell cycle arrest and SP600125 by inducing cell death.

Conclusion: CHD5 expression is regulated initially by JNK and later by p38. MYCN expression is remarkably suppressed with MEK inhibitor trametinib. Our results suggest that p38 pathway plays a role for tumor suppression, and ERK and JNK are promising targets for neuroblastoma therapy.

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POB046

Aryl Hydrocarbon Receptor Suppresses Tumor Progression of Neuroblastoma

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Background: Neuroblastoma (NB) is the most common malignant disease of infancy. MYCN amplification is a prognostic factor for NB and is a sign of highly malignant disease and poor patient prognosis. However, how MYCN expression is regulated in NB remains unclear. In our previous study, aryl hydrocarbon receptor (AHR) was found to be inversely correlated to MYCN expression in NB. Positive AHR immunostaining correlated to the differentiated histology of NB and predicted better prognosis outcome of patients. By using NB cell lines as in nitro models, we found overexpression of AHR downregulated MYCN expression and promoted cell differentiation. The evidence suggested AHR indeed is an important factor that affects NB cell behavior. However, the role of AHR in regulating NB tumor progression still needs further investigation.

Methods: AHR inducible expression cell line (stNB-V1-AI) was established and employed in in vivo mice xenograft model. In addition, NB therapy experiments by using AHR endogenous ligand, Kynurenine (Kyn), were examined in vitro and in vivo.

Results: Doxycycline treatment in the daily drinking water significantly suppressed the tumor growth of stNB-V1-AI cells inoculated nude mice. Kyn promoted cell differentiation and downregulated MYCN expression in SK-N-SH cells. In vivo, Kyn significantly prolong the survival of TH-MYCN transgenic mice and suppress metastasis of NB in the xenograft models.

Conclusion: Our results suggested that AHR plays an important role in regulating NB tumor progression. The endogenous ligand of AHR, Kyn, has the great therapeutic potential for the NB treatment.

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POB047

No Evidence for Nucleotide Signatures of Tumor Viruses in Whole-Transcriptome and Whole-Genome Deep Sequencing Data of Neuroblastoma

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Background: 20% of all cancers are assumed to be associated with infections of oncogenic pathogens. The presence of oncogenic viruses in neuroblastoma is contested and recent results indicate the absence of viral cofactors in metastatic neuroblastoma [1].

Methods: In the first study of this scope, we analyzed 434 whole-transcriptome and 30 whole-genome deep-sequencing data (collectively comprising over 53 billion sequence reads) of neuroblastoma stages 1, 2, 3, 4, and 4S in order to identify nucleotide signatures of oncogenic viruses. Screening was conducted based on a recently published and well-validated in-silico approach that features unprecedented sensitivity [1] and assigns deep sequencing reads from tumor samples to the human genome and 3479 viral reference genomes while considering viral sequence divergence and human-viral homologues.

Results: Analysis of all sequencing data resulted in detection of five families of bacteriophages (Enterococcaceae, Inoviridae, Myoviridae, Podoviridae, Siphoviridae) and four families of viral commensals (Adenoviridae, Herpesviridae, Anelloviridae, Picornaviridae) that represent common lab contaminants and were present in all samples. In addition, two viral families were detected that are associated with non-malignant diseases of childhood (Parvoviridae, Astroviridae). The prevalence of viral families did not vary significantly between tumor stages. None of the known oncogenic viral species belonging to viral families Adenoviridae and Herpesviridae were identified in our data; rather, the identified species corresponded to non-oncogenic members of these families (Human adenovirus 1, HSV-1/2, present in 13.7% of samples). While the Torque-Teno virus (Anelloviridae) found in a single sample of our data has recently been suspected to be a viral cofactor of some forms of colon cancer, this association is not yet conclusive and the virus is also highly prevalent in the healthy human population.

Conclusion: Our results strongly indicate that human neuroblastoma does not have viral cofactors corresponding to any known oncogenic viruses., [1] Schelhorn,

S.-E. et al. (2013). PLoS Comput Biol, 9(10), e1003228.

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POB048

Neuropeptide Y Y5 Receptor in Neuroblastoma Chemoresistance

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Background: Neuropeptide Y (NPY) is a sympathetic neurotransmitter released from neuroblastoma cells. High systemic levels of NPY are associated with poor clinical outcome of the disease, which is in agreement with its Y2R-mediated proliferative effect in neuroblastoma cells and angiogenic properties. Some neuroblastoma cell lines express additionally NPY Y5R, which interacts with brain-derived neurotrophic factor receptor, TrkB, enhancing its pro-survival functions. Thus, the goal of our study was to test the role of Y5R in neuroblastoma chemoresistance.

Methods: Gene expression was assessed by real-time RT-PCR, Western Blot (WB) and immunohistochemistry (IHC), activation of signaling pathways by WB, cell survival by MTS assays and apoptosis by caspase activity and TUNEL. Experiments in vivo were performed using subcutaneous xenograft model.

Results: Expression of Y5R and NPY was significantly increased in NB cells treated in vitro with chemotherapy. This effect was more pronounced in cells derived from chemotherapy-treated, relapsing tumors. Additionally, these refractory NB cell lines had elevated basal levels of Y5R and NPY expression, as compared to corresponding cell lines derived from the same patients at diagnosis. In line with this observation, 100% of surviving NB cells in tissues derived from chemotherapy-treated NB tumors was highly positive for Y5R, while in non-treated tumors only single, isolated Y5R-positive cells were observed. Blocking Y5R in chemoresistant NB cells sensitized them to chemotherapy-induced apoptosis. This effect was associated with a decrease in activity of p44/42 MAPK. Consequently, Y5R antagonist significantly inhibited growth of NB xenografts derived from chemoresistant NB cells, which was associated with a 4-fold increase in cell death.

Conclusion: Y5R/NPY axis is an inducible pro-survival pathway activated in NB under cellular stress. This Y5R-mediated anti-apoptotic effect contributes to NB chemoresistance, implicating this receptor as a novel therapeutic target for patients with refractory NB, thus far lacking adequate treatment.

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POB049

MYCN-Dependent Expression of Sulfatase2 REgulates Neuroblastoma Cell Survival

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Background: Despite the well-demonstrated role of MYCN in the pathogenesis of neuroblastoma (NB), the mechanisms underlying its oncogenic function are not entirely understood and there is evidence that MYCN activity is regulated by the tumor microenvironment (TME). Heparan sulfate proteoglycans (HSPG) play a critical role in the interaction between tumor cells and the TME because of their ability to retain cytokines and chemokines and to control their bioavailability. This function is dependent on the sulfation pattern of HSPG that itself is controlled by sulfotransferases that add sulfate groups to the repeating disaccharide units and sulfatases (SULF) that selectively remove 6-O-sulfates.

Methods: The expression of sulfotransferase and SULF was examined in 8 NB cell lines and in 65 primary tumor samples. Loss of function (siRNA) and gain of function (pcDNA SULF2) experiments were performed on CHLA-255 (MYCN-NA), SK-N-BE(2) (MYCN-A) and SHEP-12N (inducible MYCN downregulation) cells.

Results: We observed by RT-PCR higher levels of expression of SULF2 in MYCN-amplified (MYCN-A) cell lines, an observation confirmed by Western blot. We then demonstrated that the knockdown (KD) of SULF2 in SK-N-BE(2) cells by siRNA resulted in a significant decrease in cell viability and in cell cycle entry associated with an increase in apoptosis. Overexpression of MYCN in CHLA-255 cells increased SULF2 expression whereas downregulation of MYCN expression in SHEP-21N cells downregulated SULF2. Underlying the importance of SULF2 in NB cell survival independently of MYCN, we demonstrated that overexpression of SULF2 in CHLA-255 cells increased cell viability without increasing MYCN expression. An analysis of SULF2 protein expression by immunohistochemistry in 65 primary human NB tumors revealed a high level of expression in MYCN-A tumors and almost complete absence of expression in MYCN- NA tumors.

Conclusion: The data thus identify SULF2 as a new and key contributor to MYCN function in human NB.

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POB050

Prostaglandin Signaling in Primary Neuroblastoma Reveals Novel Therapeutic Targets

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Background: Tumor promoting inflammation is important for neuroblastoma development and progression and pro-inflammatory prostaglandin E_2 (PGE_2) acts as a paracrine survival factor. Anti-inflammatory treatments show promising results in neuroblastoma models. We have previously shown that mPGES-1, essential for PGE2 synthesis, promotes growth and survival of several cancers., We now further characterized prostaglandins and their specific enzymes in primary neuroblastomas of different biological and clinical subsets in the aim of finding more specific therapeutic targets

Methods: The abundance of different prostaglandins was assayed in 40 primary human NB tumors from all different subsets by mass spectrometry (MS). The expression of COX-1, COX-2, mPGES-1, L-PGDS, H-PGDS and 15-PGDH was further analyzed in tumors by immunohistochemistry (IHC)., Double staining of mPGES-1 and the specific NB cell marker GD2 was performed using immunofluorescence. To consider the level of inflammation and PGE2 signaling in these tumors we analyzed cell markers of both the innate and adaptive immune system together with mPGES-1.

Results: Our results shows significantly elevated levels of PGE2 in 11q-deleted neuroblastomas compared to MYCN-amplified and low-risk tumors whereas elevated levels of prostaglandin D_2 (PGD_2) could be coupled to neuroblastoma with favorable clinical outcome. Expression of mPGES-1 and PGD_2-synthase, L-PGDS, was detected in all analyzed tumors. In low-risk tumors mPGES-1 is not expressed by the tumor cells whereas co-localization of GD2 and mPGES-1 indicate proper tumor cell origin in 11q-deleted high-risk tumors. Furthermore, mPGES-1 expressing cells are found in clusters closely together with T-cells, B-cells, dendritic cells and M1/M2 macrophages.

Conclusion: Our data further show the involvement of inflammatory prostaglandins in neuroblastoma development and tumor progression opening up new strategies of targeted therapies for specific neuroblastoma subsets.

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POB051

Heat-Shock Proteins as the Molecular Targets of Iron Chelators in Neuroblastoma Cells

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Background: Iron is an essential cellular nutrient being a critical component of many proteins and enzymes that are involved in cell growth and replication. Compared to normal cells, neoplastic cells require greater amount of iron because generally they proliferate at a greater rate than their normal counterparts. It has become clear that some iron chelators show promising anti-cancer activity by inducing cell cycle arrest and apoptosis. In this work we evaluated the effect of molecules with iron chelation activity on NB cells.

Results: Among 17 iron chelators tested, four molecules - ciclopirox olamine (CPX), piroctone, 8-hydroxyquinoline, deferasirox - were shown to efficiently chelate intracellular iron and at the same time decrease viability of two MNA NB cell lines. The more detailed investigation of CPX compound demonstrated that its effects on NB cells viability were dependent on iron and mediated by both deregulation of cell cycle and induction of apoptosis. Microarray analysis on the total and polysomal samples from CPX-treated vs untreated CHP134 cells demonstrated downregulation of the individual members of the human heat-shock protein (HSP) family. The down-regulation was validated by qPCR and subsequently confirmed on protein level for HSP90 protein. The CPX–mediated inhibition of HSP90 protein levels was shown to be iron dependent, since addition of iron reversed the effect. Moreover, the other iron chelators - piroctone, 8-hydroxyquinoline, deferasirox – resulted in similar HSP90 down-regulation.

Conclusion: HSPs contribute to tumorigenesis through their well-documented antiapoptotic activity. Our data point out the suppression of HSP90 as a potential mechanism of iron chelators-mediated cell-death.

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POB052

Inhibition of ALDH Activity Influences the Stem Cell Properties o Neuroblastoma Cells

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Background: The successful targeting of so-called cancer stem cells (CSC) or tumour-initiating cells (TICs), not yet identified in neuroblastoma (NB), is a real challenge to develop new therapeutical strategies and efficiently eradicate this disease. We have previously identified, by a microarray time course analysis of serial NB spheres passages, ALDH1A2, among other genes, as a potential TICs marker. ALDH1A2, with ALDH1A1 and ALDH1A3, belong to the subfamily of ALDH1 enzymes, which play a crucial role in the synthesis of retinoic acid,and have been already identified as functional CSC markers in different tumour types, including leukemia, breast, and melanoma cancers.

Methods: The ALDH activity and the expression levels of the three ALDH1A genes were measured in the serial NB cell spheres passages by FACS, and real-time qPCR, respectively. The consequence of ALDH activity inhibition was also explored on the defining functional characteristics of TICs.

Results: By using the Aldefluor assay, we demonstrated an increased ALDH activity in a restricted number of cells upon spheres passages of three NB cell lines. This suggests that an $ALDH^{high}$ cell subpopulation is pre-existing and is selected during the self-renewal process. ALDH1A1 and 1A2 transcripts were increased during spheres passages in every cell lines tested. In contrast, enhanced ALDH1A3 expression was cell-type dependent. Furthermore, specific inhibition of ALDH activity with DEAB resulted in the significant reduction of the 2D-proliferation capacity, and the 3D-clonogenic and self renewal potential of NB cells. Moreover, NB cells resistance to various chemotherapeutic drugs was partially abolished by inhibition of ALDH activity, however in a cell line- and drug-dependent fashion.

Conclusion: These findings reveal that ALDH isoenzymes could be potential NB biomarkers and therefore interesting therapeutic targets. The specific role of each ALDH1A isoforms is currently explored by using lentiviral specific shRNA-mediated silencing.

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POB053

Lack of Correlation Between Telomerase Activity and Individual Components of the Telomerase Complex (hTR, hTERT, Dyskerin) in Neuroblastoma Cell Line Panel

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Background: Telomere maintenance by the ribonucleoprotein telomerase allows continuous proliferation of cancer cells. Telomerase is associated with advanced disease and poor prognosis in neuroblastoma and is comprised of hTERT (catalytic reverse transcriptase domain), hTR (RNA template), and the nucleolar protein dyskerin.

Methods: In this study we first looked for telomerase gene amplification by qPCR and asked whether mRNA levels of each of the 3 components correlated with telomerase activity (measured by qTRAP) in 29 neuroblastoma cell lines. In addition, we induced differentiation in three cell lines (LAN-5, SH-SY5Y, KCNR) with 5 μM all-trans retinoic acid (ATRA) for 14 days and examined the effect of differentiation on mRNA levels of telomerase components and telomerase activity.

Results: While there was no evidence of TERC (encodes hTR) or DKC (encodes dyskerin) gene amplification, hTERT gene amplification was found in one cell line (CHLA- 171), which contained 9 copies of hTERT. Relative hTERT mRNA expression ranged from 0.15 to 8.15 (median 0.92) in the 29 cell lines; however, CHLA-171 did not have the highest hTERT despite gene amplification. hTR mRNA levels ranged from 0.28 to 5.47 (median 1.16), and dyskerin level ranged from 0.29 to 1.97 (median 0.96). Telomerase activity also varied across the panel of cell lines ranging from 0.75 to 73.8 (median 4.65). We found no correlation between the mRNA levels of each of the 3 telomerase components and telomerase activity ($P > 0.05$). While hTR and dyskerin levels remained unchanged in the differentiated cell lines, there was a 4 to 7 fold decrease ($P < 0.01$) in hTERT expression resulting in a significant reduction of telomerase activity in all three cell lines (7 - 22 folds; $P < 0.01$).

Conclusion: 1) hTERT amplification is rare in neuroblastoma, 2) mRNA expression does not correlate with telomerase activity in a large panel of cell lines, and 3) neuroblastoma differentiation is associated with a significant decrease in hTERT and downregulation of telomerase.

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POB153

Transcriptional Regulation of IGF-II Mediated HIF2A Expression in Neuroblastoma

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Background: During normal sympathetic nervous system (SNS) development, cells of the ganglionic lineage can malignantly transform and develop into the childhood tumor neuroblastoma. Hypoxia-inducible transcription factors (HIFs) mediate cellular responses during normal development and are central in the adaptation to oxygen shortage. Expression of the oxygen sensitive HIF subunits HIF-1a and HIF-2α is differentially regulated in many tumor forms including neuroblastoma. The mechanism underlying the differential HIFα regulation remain poorly understood but we have recently shown that hypoxia-induced insulin-like growth factor (IGF)-II activates hypoxia-driven transcription of HIF2A. High expression of HIF-2α in neuroblastoma correlates with stem cell-like features, poor clinical outcome and disseminated disease, illustrating the importance of understanding how HIF-2 activity is regulated.

Results: Here we demonstrate that the IGF-II-mediated HIF2A transcription is diminished when IGF-1- or insulin receptors are inhibited. IGF signaling is commonly mediated through the PI3K and mTOR pathways and inhibiting PI3K activity virtually abolished HIF2A expression. Inhibiting mTORC1 had no effect on HIF-2α mRNA or protein expression but diminished HIF-1α protein levels as expected. Instead, inhibition of both mTORC1 and mTORC2 using the dual mTORC inhibitor, PP242, completely blocked the expression of HIF2A and thus also HIF-2α protein levels in hypoxic cells.

Conclusion: We conclude that both PI3K and mTORC2 inhibition may represent conceivable strategies to target the HIF-2α-driven aggressive and immature phenotype in neuroblastoma.

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POB156

Inactivation of SMC2 Shows a Synergistic Lethal Response in MYCN-amplified Neuroblastoma Cells

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Background: The condensin complex is required for chromosome condensation during mitosis; however, the role of this complex during interphase is unclear. Neuroblastoma is the most common extracranial solid tumor of childhood, and it is often lethal. In human neuroblastoma, MYCN gene amplification is correlated with poor prognosis.

Results: This study demonstrates that the gene encoding the condensin complex subunit SMC2 is transcriptionally regulated by MYCN. SMC2 also transcriptionally regulates DNA damage response genes in cooperation with MYCN. Downregulation of SMC2induced DNA damage and showed a synergistic lethal response in MYCN-amplified/overexpression cells, leading to apoptosis in human neuroblastoma cells. Finally, this study found that patients bearing MYCN-amplified tumors showed improved survival when SMC2 expression was low.

Conclusion: These results identify novel functions of SMC2 in DNA damage response, and we propose that SMC2 (or the condensin complex) is a novel molecular target for the treatment of MYCN-amplified neuroblastoma.

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POB157

Epigenetic Role of the MYCN/LSD1 Complex in Neuroblastoma

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Background: Childhood neuroblastoma is the most common solid tumour of infancy, highly refractory to therapy. One of the most powerful prognostic indicators for this disease is the MYCN gene amplification, which occurs in approximately 25% of neuroblastomas. MYCN regulates transcription of hundreds genes by binding the E-box DNA sequence and through modulation of histone methylation and acetylation. A recent study has shown that the histone demethylase LSD1 (KDM1A) is highly expressed in undifferentiated neuroblastomas and that its high expression correlates with an adverse outcome. Moreover, we previously reported that c-Myc can recruit LSD1 on its target genes and that this interaction is relevant for early events of transcription. Here, we show that LSD1 can form a tight complex with MYCN and that this complex controls transcription of genes involved in MYCN-driven oncogenesis.

Methods: Several human neuroblastoma cell lines (SK-N-BE(2)C, SH-SY-5Y, IMR32, LAN1) including the TET21N carrying a conditional MYCN minigene, were employed to determine the interaction between MYCN and LSD1 (Co-IP), to define the occupancy of the MYCN/LSD1 complex on MYCN target regulatory regions (ChIP), and to estimate growth rate, cell cycle distribution, colony formation and wound healing as a function of LSD1 expression levels (LSD1 overexpression, shRNA mediated silencing) or inhibition of LSD1 activity.

Results: We provide lines of evidence that MYCN and LSD1 form a complex and that LSD1 is recruited at regulatory regions of MYCN responsive genes. LSD1 inhibition affects MYCN function, by either up-regulating or inhibiting canonical MYCN target genes. Moreover, inhibition of LSD1 activity reduces cell proliferation and blocks cells in the G1 phase.

Conclusion: Our results point to LSD1 as another critical regulator of MYCN activity, which can be targeted by specific drug inhibitors. These findings pose the bases to design novel therapeutic approaches to treat advanced neuroblastomas.

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POB161

Whole-genome Sequencing Analysis of Neuroblastoma's Clonal Evolution

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Background: Neuroblastoma, a clinically heterogeneous pediatric cancer, is characterized by distinct genomic profiles but few recurrent mutations. As neuroblastoma is expected to have high degree of genetic heterogeneity, study of neuroblastoma\'s clonal evolution with deep coverage whole-genome sequencing of diagnosis and relapse samples will lead to a better understanding of the molecular events associated with relapse.

Methods: Whole genome sequencing was performed on trios (constitutional, diagnose and relapse samples) from seven patients using Illumina Hi-seq2500, with 100x100 or 90x90 paired-end reads. Expected coverage was 100X and 50X for tumor and constitutional samples, respectively. Following alignment with BWA allowing up to 4% of mismatches, bam files were cleaned up according to GATK recommendation. Variant calling was performed using GATK and SAMTOOLS. In a first step, we focused on SNV (single nucleotide variants), within and around coding sequences, unknown as polymorphisms in the 1000 genomes and ExomeSequencingProject. Finally SNVs specific to diagnosis and/or relapse were filtered relative to constitutional variations.

Results: For all samples, at least 94% of CDSs and UTRs were covered at 20X. The mean number of exonic SNVs was 11 per sample (range: 3-27). A mean of 19+/-2% of SNVs were common to both paired tumor samples, 33+/-8% were specific to diagnosis and 46+/-5% were specific to relapse. Among the 77 predicted exonic SNVs, 4 were somatic ALK mutations in exon 23 or 25. In one case a mutation switch from 17.7% c.3522G>T:p.F1174L to 61.5% c.3522G>C:p.F1174L i.e. leading to the same amino acid change, was observed. Six other genes were found to be targeted in diagnosis or relapse samples of 2 different patients (CRIPAK, GOLGA6L10, NAB2, POM121, RBMX, TULP4).

Conclusion: These results confirm the high level of mutation heterogeneity between diagnosis and relapse tumors. Next steps will focus on validation of predictions and analysis of copy number alterations and structural variations to obtain an overall view of molecular events associated with relapse.

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POB165

Modelling MYCN-dependent Apoptosis in Neuroblastoma

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Background: Neuroblastoma is the most common extracranial solid tumour in childhood and the most common cancer in infancy. It is an extremely heterogeneous disease stratified in low-, intermediate- and high-risk tumours. Whereas low-risk tumours often undergo spontaneous regression, high-risk tumours can be very aggressive despite multi-modal treatments. The MYCN gene encodes for MYCN transcription factor and its amplification is commonly associated with high-risk tumours although it has been shown that MYCN gene overexpression also correlates with apoptosis sensitization. HMGA1 is one of the MYCN target genes and is involved in regulating apoptosis in response to DNA damage in a pathway involving ataxia-telangiectasia-mutated (ATM), HIPK2 and p53. On one hand, HMGA1 increases the DNA damage response by inducing ATM gene expression in a positive feedback loop. On the other hand HMGA1 prevents the activation of p53 by binding HIPK2 and trans-locating it to the cytosol. Here we propose a dynamic model in which MYCN protein regulates this DNA damage system with implications for the chemoresistance in MYCN-amplified neuroblastoma. In addition to the intricate regulation of the DNA damage response outlined above, the analysis of this model points towards an important role MYCN-independent HMGA1 expression and subsequent HIPK2 nuclear/cytoplasmic localization.

Methods: Dynamical modelling of biological networks using ordinary differential equations.

Results: Our simulations predict an optimum in apoptosis depending on MYCN concentration.

Conclusion: MYCN deregulates both anti- and pro-apoptotic components. While MYCN overexpression may sensitize cells to apoptosis, very high level of MYCN may reduce the ability of drugs to induce apoptosis in such cells.

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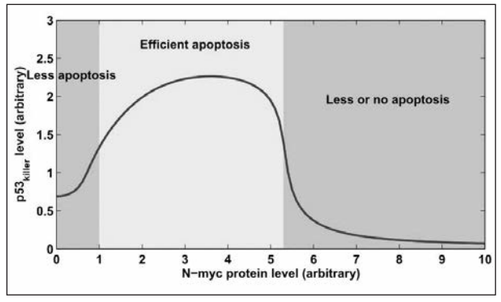

Basic Research: High Throughput Techniques

POB054

Identification of Potential Synthetic Lethal MYCN Target Genes or Interactors with High MYCN Using High-Throughput RNAi Screening

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Background: Identifying transcriptionally up-regulated MYCN target genes and functionally required genes that support oncogenic functions of MYCN or suppress apoptosis and/or senescence is likely to aid in developing novel therapeutic strategies for high-risk neuroblastoma patients with amplified MYCN.

Methods: To identify putative synthetic lethal interactions with high MYCN, we performed a druggable genome siRNA screen in an isogenic pair of a MYCN-non-amplified cell line (SH-SY5Y) harboring a tetracycline-regulatable MYCN transgene. Cell viability was measured 72 hours after transfection using CellTiterGlo reagent at MYCN high vs MYCN low condition. We defined two groups as hits: (i) genes that upon knockdown significantly reduce cell viability in both conditions MYCN high and MYCN low, and (ii) genes that show a higher cell viability-reducing effect with high MYCN as compared to MYCN low condition. Both hit lists were analyzed with Ingenuity Pathways Analysis program for enrichment of gene ontology (GO-) categories, pathways and functionally associated networks.

Results: (i) Knockdown of 49 genes resulted in significant reduction of cell viability in both conditions, MYCN high and MYCN low. (ii) 58 genes were identified that upon MYCN induction reduced cell viability to a significantly higher degree in the MYCN high condition as compared to the MYCN low condition. Genes from both hit lists were enriched in protein ubiquitination and EIF2-signaling pathways and in functionally associated networks related to cell death and survival, cell cycle and proliferation.

Conclusion: Large-scale RNAi screening performed in isogenic pairs of MYCN-regulatable cells is a promising tool to identify potential targets for future therapeutic approaches for high-risk neuroblastoma patients. A second druggable genome screen in an isogenic pair of a MYCN-amplified cell line that is stably transfected with a doxycycline-inducible shRNA directed against MYCN to allow conditional down-regulation of MYCN is in progress.

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POB055

Identification of Lead Organic Compounds Active against Stem Cell-Like Neuroblastoma Cells by High Throughput Screening

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Background: Cancer stem cells (CSCs) are thought to be responsible for distant metastases, drug resistance and recurrence. We previously reported the establishment of induced stable neuroblastoma (NB) stem cells (iCSC), which were stemness-enhanced NB sphere cultures. These cells recapitulated in vivo histological phenotypes of Large-Cell NB (LCN), the most aggressive and deadly subset of NB, including vesicular nuclei and prominent nucleoli (PNAS 110: 6097-102, 2013). In addition to the LCN phenotype, high-level expression MYC or MYCN is the consistent feature of the iCSC xenografts. The goal of this study is to identify lead organic compounds active against NB iCSCs by high throughput screening.

Methods: By using MTS assays, the SKNAS iCSC and SKNAS parental monolayer cells were used to screen the Prestwick Chemical Library®, containing 1200 FDA-approved small molecules. Growth suppressive compounds were defined as they exhibited cell survival reduction of greater than 80% compared to DMSO control.

Results: There were unexplored compounds with significant antineoplastic activity among existing antibacterial, antifungal, antiprotozoal, and anthelmintic compounds. Of 1200 compounds screened, we identified a dozen of the compounds that conferred the anti-growth effect preferentially to the SKNAS iCSC. Our data showed that Chlorhexidine (antiseptic), Benzethonium (antiseptic), and Digoxin (cardiac glycoside) were more effective in suppressing growth of the SKNAS iCSC than the monolayer cells. Benzethonium destabilized MYC only in SKNAS iCSC, and the other two compounds destabilized MYC in both SKNAS iCSC and monolayer cells. A similar MYCN destabilizing effect of the above three compounds was observed in the MYCN-amplified NB cells: SKNBE(2)C monolayer cells and SKNBE(2)C iCSCs. We are currently completing characterization of the small molecules identified, focusing on their anti-iCSC growth and anti-MYC/MYCN effects.

Conclusion: This study will reveal specific pathways regulating biological activities of CSC. It is also a promising approach that could lead to development of safe and effective anti-NB therapeutics.

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POB056

Gene Expression Analysis of Neuroblastoma Tumors According to MKI and Differentiation Status: An Interactive International Neuroblastoma Risk Database (iINRGdb) Study

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Background: Mitosis-karyorrhexis index (MKI) and tumor differentiation grade are established prognostic markers in neuroblastoma. To investigate the biological pathways underlying these histologic biomarkers, we mined the iINRGdb and the TARGET gene expression databases.

Methods: Patients in the iINRGdb with known MKI and differentiation grade were linked to patients in dbGaP TARGET data through Universal Serial Identifiers (USI). Background: correction, normalization, batch effect removal, and filtering were applied to identify differentially expressed genes. Machine learning techniques were applied to identify gene classifiers capable of separating the samples by MKI.

Results: 2128 Children's Oncology Group (COG) patients in the iINRGdb had known tumor grade (257 differentiating; 1871 undifferentiated) and 2103 had

known MKI (1770 low/intermediate; 333 high). Mapping to the TARGET database revealed 141 patients with expression data. Low numbers of samples with differentiating grade necessitated that we restrict analysis to MKI (low/intermediate: 78, high: 22). After normalization and filtering, 59 remained with low/intermediate MKI and 19 high MKI, with 212 genes differentially expressed between the groups (FC>=1.5, FDR<0.05). Functional enrichment analysis demonstrated significant differential expression of genes involved in mitotic checkpoint, cell division, and cell-cell adhesion. Classification algorithms including conditional random forest, standard random forest, and support vector machines, were used to generate candidate gene signatures. Mathew's correlation coefficient and AUC were used to evaluate signature performance. Three top performing signatures were cross validated with 100% MKI prediction accuracy. Signatures without MYCN contained cancer-associated genes such as DIRAS3 and SHC1, as well as CDCA7L (JPO2), which interacts with c-MYC to effect transformation in medulloblastoma cells.

Conclusion: The iINRGdb can be used to quickly perform a complex phenotype search of >12,000 patients, while linking to expression data. Understanding the gene expression changes correlating with tumor histology is likely to lead to more precise prognostication and may ultimately provide insights for novel therapeutics.

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POB057

The Neuroblastoma Translatome: Profiling Cancer-Related Translational Alterations in a Panel of Cell Lines

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Background: Cancer genetic instability is conventionally studied at the genome, epigenome and transcriptome levels, thus focusing essentially on the effects of genome alterations on transcription and splicing. Recent work is showing how translational control is a much more powerful determinant of what thought before of cell phenotypes: the occurrence of genomic lesion affecting RNA-binding proteins, noncoding RNAs and translation factors would thus result in perturbations of gene expression which are not detected at all by the conventional high throughput approaches.

Methods: To this end we report, for the first time, an integrative analysis of 13 MYCN-amplified and 3 non-MYCN-amplified neuroblastoma cell lines at the genome, transcriptome, miRome and translatome (mRNAs engaged in polysomes) levels; this dataset allows to investigate translational control networks normally acting in neural crest cells which may be altered in neuroblastoma and specific to its MYCN-amplified or non-MYCN amplified subtypes.

Results: We first investigated genome alterations, which were found to include RBP and translation factor loci. We then focused on transcriptome and translatome profiles: these are markedly different, with a number of translatome profiles of cell lines clustering with each other instead that with their corresponding transcriptome profile. Differentially expressed genes in the profiles, as computed by RankProd, are 12% of the total genes. Among them we highlighted a group of 64 translationally enhanced histone genes, potentially regulated by various RBPs and miRNAs such as SLBP and the mir-29 family.

Conclusion: Further investigations will be needed to elucidate this histone mRNA translational enhancing pattern, which could be selected in neuroblastoma cells to compensate for transcriptional alterations of the same mRNAs.
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POB058

An Epigenetic Focused siRNA Screen Identifies Novel Potentially Druggable Targets That Inhibit Growth and Induce Differentiation in Neuroblastoma

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Background: NexGen sequencing identified few novel druggable mutations in NB and these were mainly in chromatin and epigenetic regulators. Drug discovery targeting epigenetic regulators is a dynamic area of research, however which epigenetic enzymes drive NB tumorigenesis is unknown.

Methods: To identify epigenetic regulators of NB cell growth and differentiation, we used a high through-put format in which a focused Dharmacon Smartpool siRNA library of 400 known modulators of chromatin structure and function were reversed transfected into 2 NB cell lines (SY5Y-GFP & SK-N-BE) in 384-well plates. After 3 days at 37°C, plates were fixed and stained with Hoechst 33342. Cells were analyzed using an Opera High-Content Screening system in which 12 fields per well with five z-stacks 1um apart were imaged using a 20x objective lens.

Results: QQ-plot analyses identified 10 genes with oncogenic activity (defined as siRNAs causing a decrease in Nuclei number (NN) and an increase in Neurite length (NL)) that statistically deviated from the normally distributed bulk population of siRNA measurements. IPA analysis revealed the oncogenes are involved in embryonic and tissue development, cell cycle, DNA replication, recombination and repair (p= 10-5). Thirty percent of hits are associated with poor survival in stage 4 NB patients (R2 database). Two hits, CENPE and BRD4, have already been shown to be therapeutic targets in NB. EZH2, another hit, is dysregulated and represses tumor suppressor gene expression in NB. To verify EZH2, we transduced a TET-inducible EZH2shRNA into NGP cells and found that decreases in EZH2 expression were associated with decreased tumor xenograft growth and significantly prolonged murine survival (p=0.01). Additional hits, which include CHAF1A and members of the HMGN family, are under analysis.

Conclusion: This approach has identified epigenetic targets that are important in regulating NB growth and differentiation and may be druggable therapeutic candidates for high-risk NB patients.

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POB059

Exome Sequencing Suggests Candidate Genes Associated with Aggressiveness of Stage 4 Neuroblastoma Patients

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Background: Despite recent advances in the knowledge of neuroblastoma (NB) genetic determinants, stage 4 patients are still a clinical challenge. Particularly, we divided these patients into Short Survivors (SS; OS<60 months) and Long Survivors (LS; OS>60 months). We previously analyzed 91 NBs (45 SS vs 46 LS) by aCGH reporting a significant association between structural chromosomal alterations (1p loss, 11q and 17q gain, 19q loss) and the outcome of SS patients. In this study we searched for somatic variations within these genetic regions, in order to identify genes that might be related to SS tumor aggressiveness.

Methods: Exome Sequencing was performed on 7 SS and 7 LS MYCN single copy tumors and on their matched constitutive DNA in paired-end mode on the Illumina HiSeq 2000. The exome was enriched by 4000 SeqCap EZ v.2 kit (Nimblegen). Raw data from CASAVA (Illumina) were quality controlled by FastQC and SolexaQA. Filtered short-reads were aligned on the GRCh37/hg19 genome reference by Bowtie2, removing potential PCR duplicates with Picard tools. Finally, somatic variants were called by MuTect and filtered to retain only higher quality reads.

Results: After removing variants listed in NCBI dbSNP and those detected in the germ-line of patients, we sorted out 197 and 452 missense or nonsense variations exclusive to SS and LS cases, respectively. Next, we used PolyPhen2 to predict the functional effects of these variants, which involve 115 genes exclusive to SS and 61 shared by both SS and LS NBs. Eventually, we selected 81 genes with at least one variation predicted as damaging uniquely in SS NBs.

Conclusion: A list of candidate genes that are potentially involved in the aggressiveness of SS NBs has been achieved. Noteworthy, among the genes predicted to harbor damaging variations 11/81 were zinc finger transcription factors, including CASZ1 that is a known candidate NB suppressor gene.

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POB060

Identifying Mutation Enriched Protein Networks in Cancers with Heterogeneous Mutation Spectra

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Background: Recent analyses of the mutation spectra of cancer have revealed low mutation frequencies combined with low gene level recurrence in some malignancies, particularly in childhood tumors. In the absence of recurrence, identifying potential driver mutations can be challenging. We here present a method that aims to overcome these challenges by looking for recurrence at the level of gene interactions.

Methods: Based on the observation that cellular function tends to overlap with the modular structure of the protein interaction network (PIN), we hypothesize that potential driver mutations will be particularly enriched in such modular regions, whereas passenger mutations will be randomly distributed across the network. Here, we developed a method which clusters genes within the constraints of the PIN structure. Mutated genes are assigned to subnetworks within the PIN by combining the structure-based similarity metric with unsupervised clustering. The candidate subnetworks are then ranked using a score that reflects the modular nature of the subnetwork and the relevance of the mutated genes within the subnetwork, and compared to subnetworks found using sets of random genes to assess significance.

Results: We applied our method to three malignancies with low mutation rate and low recurrence: neuroblastoma, medulloblastoma and AML. Analysis of AML served as a positive control for the method, as its molecular pathogenesis is well-established already. In all tumour entities we found subnetworks with a statistically significant score that were enriched in mutations and contained known cancer-related genes. In line with our hypothesis, we found a subnetwork centered around the well-konwn genes FLT3 and KIT (p=0.0105) in AML, which validates the power of our method.

Conclusion: We show that meaningful genetic patterns can be found in mutation spectra in the absence of recurrence by interpreting them in the context of the PIN. Our method may thus provide entry points for elucidating the pathogenesis of neuroblastoma.

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POB061

Whole Genome Sequencing of Mitochondrial DNA in Low and High-Risk Neuroblastoma Patients

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Background: Mitochondrial DNA (mtDNA) mutations frequently occur in several human neoplasms and may contribute to tumor initiation and progression. mtDNA mutations have never been considered as causative or secondary events for neuroblastoma progression. To investigate the mutations of mtDNA in neuroblastoma we sequenced 36 full-length mitochondrial genomes belonging to 16 low-risk (LR) and 20 high-risk (HR) Italian neuroblastomas by Sanger method.

Methods: Mitochondrial FASTA sequences were aligned versus the newly mitochondrial reference sequence RSRS. Mutations were selected considering a variability <0.01 according to SiteVar algorithm with respect to 14,144 mitochondrial genomes from healthy individuals of HmtDB database. To determine whether the selected mutations were somatically acquired, we sequenced the matched germline DNAs.

Results: We found 3 somatic missense mutations in CO1, CO2, CYTB genesin HR patients and 1 in (mt)-tRNA gene in LR patients. Moreover, we identified 47 germline mutations: 5 were novel (2 missense and 2 tRNA mutations in HR and 1 insertion in LR patients); 10 (83.3%) rare missense mutations occurred in HR and 2 (16.7%) in LR patients. Genes with higher germ-line mutation frequency in HR patients than in low-risk ones included CYTB (20.0% vs 6.2%), ND1 (10.0% vs 0.0%),and (mt)-tRNA(25.0% vs 12.5%). All CYTB and ND1 mutations in HR patients were missense. The ND1 mutation G3391A caused amino-acid substitution in a highly conserved region (MutPred-Score=0.862). The highest rate of rare mutations was found in mt-tRNA gene (19.4% of cases). In HR patients, two mutations (one novel) in tRNA-thr altered (mt)-tRNA secondary structure and one novel mutation occurred in the anticodon loop of tRNA-gly.

Conclusion: In line with recent high-throughput screenings of nuclear DNAs, the rate of somatic mitochondrial mutations in neuroblastoma is low. This study suggests that rare and novel germ-line variants in CYTB, ND1 and(mt)-tRNA loci might be important contributors to HR neuroblastoma development.

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POB062

Next-Generation Exome Sequencing Reveals Actionable Genetic Alterations in Relapsed Neuroblastomas: The Memorial Sloan-Kettering Cancer Center (Mskcc) Experience

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Background: Prognosis in patients with neuroblastoma relapsing after intensive multi-modality therapy remains poor. Identifying genetic targets should optimize personalized therapies for relapse. Although recurrent exome alterations are rare in newly-diagnosed neuroblastomas, information on relapse samples is insufficient.

Methods: Soft-tissue samples from primary and recurrent neuroblastoma were tested by next-generation exome sequencing for >300 cancer-related genes using a commercially-available assay (Foundation Medicine, Cambridge, USA) . Samples comprised ≥40µm paraffin-embedded tissue sections with >20% neuroblastoma content. Data were analyzed with IRB approval.

Results: From June-December 2013, 21 samples (2 at diagnosis, 8 at definitive resection, 4 at first relapse [R1] and 7 at >first relapse) from 18 patients were tested. One sample did not yield adequate DNA. Known MYCN amplification (n=4) and ALK mutation (n=1) at diagnosis agreed with exome-sequencing performed at resection (n=4) and relapse (n=1). Besides MYCN amplification, 3/8 pre-relapse samples had alterations: one TP53 splice site mutation (at diagnosis and at resection) and one (from an adolescent) had a complex genotype: PTCH1 mutation; MCL1 and NFKBIA amplification. In another patient, 3 samples obtained at diagnosis, R1, and second relapse, respectively, all showed ATRX loss, ARID1A mutationand CDK4 amplification. However, R1 had an additional ALK1275Q mutation with a different ALK mutation at relapse. Overall 9/11 (88%) relapse samples had alterations. Two patients commenced therapy based on actionable alterations (follow-up too short for response evaluation).

Conclusion: Relapsed neuroblastoma samples carried a high rate of genetic alterations detected by commercially-available next-generation exome sequencing, offering promise for the implementation of personalized therapies.

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Pt No.	Age at diagnosis (years)/ Sex	*ALK* status	Site of relapse sample	Time of relapse sample after diagnosis (years)	Actionable alterations	Other alterations
First Relapse						
1	2.7/M	WT	Thoracic paraspinal	2.5	*JAK1* Y1034N missense mutation *CDKN2A* R58 mutation	*ATRX* loss
2	8.6/F	WT	Lung	1.8	*CDK4* amplification *MDM2* amplification	None
3	5.2/M	WT	Abdominal mass	1.3	None	None
>First Relapse						
4	4.8/M	WT	Axilla	3.9	*NRAS* Q61K mutation *CDKN2A/B* loss	None
5	19.8/M	WT	Epidural	5.5	*TP53* N235S	*EZH2* and
6	0.3/M	F1174	Abdominal	0.8	F1174 ALK	None
7	23.5/F	WT	Neck	8.1	None	*CSF1R* V32G
8	13/F	WT	Pelvic	6.5	None	None
9	5.3/M	WT	Neck	1	*RPTOR* amplification	*MAP3K14*

ABTRACTS

POB063

Single Cell Next-Generation RNA Sequencing Analysis in Neuroblastoma Tumor Initiating Cells for Identifying Novel Targets for Neuroblastoma

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Background: Neuroblastoma (NBL) is biologically and genetically heterogeneous and demonstrates both favorable and unfavorable outcomes. Cancer stem cell theory suggests that rare tumor initiating cells (TICs), resistant to conventional therapy, are responsible for relapse in unfavorable tumors. Here we apply single-cell RNA sequencing (RNA-Seq) analysis usign next generation sequencing (NGS) to individual neuroblastoma TICs.

Methods: TICs in NBL cell lines (SK-N-SH, NB6, etc.) were concentrated using morphological subtype （I-type） and their gene expression and surface marker profiles by GeneChip® Human Gene 1.0 ST Array and BD LyoplateTM Screening Panels. Then, a single cell sample (mainly CD133+, CD44+ CD144a+ cell) cell was isolated from using C1™ Single AutoPrep System. RNA-Seq analysis in each single isolated cell was performed using Illumina Nextera-seq system (n = 6 each) to identify highly expressing genes in TICs.Thereafter, we analyzed the expression levels of these candidate genes in 298 sporadic NBL samples.

Results: Comparison of RNA seq data between TIC and CD133-, CD44- CD144a+ cells showed 16,382 expressed genes detected in TICs including 2,434 long noncoding RNAs (lncRNAs) and differential splicing events including unknown splicing patterns.And Wnt and PDGF signaling pathways were suppressed but the STAT, TGF-β pathway genes were upregulated in individual TIC. However, some genes including MYC, CCND1, and SRC including these suppressed pathways were upregulated. The expression levels of these genes were significantly high in unfavorable NBL tumors.

Conclusion: A single cell RNA sequencing using NGS in neuroblastoma TICs revealed several candidate hallmark genes including MYC, SRC, and CCND1, which might be regulated by lnc RNAs. The expression profiling of TICs also indicated that heterogeneous subtypes exist in NBL cell lines. NGS can be used in research and diagnostic settings to screen for existing TIC in genetically heterogeneous neuroblastoma. Further NGS analysis provided important candidates of indicators for diagnostic and therapeutic targets for NBLs.

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POB064

Integrative Analysis Reveals Novel Subtypes of Neuroblastoma

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Background: Neuroblastoma is a paediatric malignancy considered to emanate from progenitors of the developing sympathetic nervous system and account for 15% of all childhood cancer-related mortality. Although the 5-year survival rate has increased over the last decades this gain is chiefly attributable to increased survival amongst low-risk patients. Despite considerable advances in risk stratification, current classification systems are based on surrogate markers for underlying biology such as specific cytogenetic aberrations, DNA ploidy, and clinical parameters. The current inability to define patient outcomes based on underlying biological subtypes illustrates a major impediment in advancing our understanding of neuroblastoma.

Methods: We use unsupervised consensus clustering and consensus NMF, build classifiers using a semi-supervised approach, infer pathway activity and cell fates using single-sample gene set enrichment analysis, infer subclonality and tumour purity using ABSOLUTE and ESTIMATE algorithms, generated an algorithm to infer MYC/FOXM1 pathway activity across platforms.

Results: We revaluated several previously published expression datasets encompassing 1749 patients using unsupervised Methods:and integrate copy-number and mutational data. Unsupervised analysis of transcriptomics uncovered three molecularly distinct subtypes termed 'mitotic', 'immunoreactive', and 'differentiated' with considerable clinical implications and with different subtype-specific chromosomal aberrations. Using a semi-supervised approach we generated a 522-gene classifier with reasonable accuracy (93.4 ± 0.34%, 10.000 times 10-fold CV) and validated it in 9 independent cohorts (1749 patients). Semi-supervised pathway analysis highlight distinct biological functions associated with the proposed subtypes. By applying recently published algorithms we infer the amount of infiltrating immune cells and tumour stroma and present correlations to survival and subtype-specific changes in the microenvironment. Lastly, we further characterize the commonalities of neuroblastoma subtypes in a pan-cancer analysis (~8,000 tumours) revealing inter-cancer similarities. To this end, we developed a platform-independent algorithm to quantify FOXM1/MYC pathway activation and compare across several tumour types. A similar analysis was performed for inflammation and stroma.

Conclusion: Taken together, we propose three robust mRNA-based neuroblastoma subtypes associated with distinct biological, structural, and clinical variations and highlight common nodes of therapeutic intervention across cancer types.

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POB065

Active PI3K/mTORC1 Signaling Predicts Poor Outcome in Neuroblastoma

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Background and Methods: We investigate the role of the PI3K/AKT/mTORC signaling pathway, a major pathway in regulating pro- and anti-survival responses. In vitro data using 24 neuroblastoma cell lines showed differential sensitivity to PI3K or mTORC inhibitors, suggesting that neuroblastoma cell lines can be classified in PI3K/mTORC dependent and independent lines. To analyze this for neuroblastoma tumors, we generated signatures of the PI3K/AKT/mTORC pathway by mRNA profiling of cell lines treated with PI3K/AKT or mTORC inhibitors.

Results and Conclusion: We analyzed the mRNA profiles of a panel of 122 neuroblastoma tumors for activity of different molecular pathways. Signatures specific for the activity of PI3K/AKT and of mTORC allowed us to identify a PI3K/AKT/mTORC-active and –inactive group in the series. The PI3K/AKT/mTORC-active group was associated with a very poor prognosis. Strikingly, the group with active PI3K/AKT/mTORC-signaling also had an active MYCN signaling, as evident from the analysis with our recently described MYCN-signature (MYCN-157, Valentijn et al; PNAS, 2012). To further dissect the relevance of mTORC in neuroblastoma, we generated mTORC1 and mTORC2 signatures using mRNA profiling of cell lines undergoing shRNA-mediated silencing of respectively RAPTOR or RICTOR. We found that the specific mTORC1 signature only partially overlaps with the specific PI3K/AKT signatures, suggesting that the mTORC1 axis can operate independently of upstream PI3K/AKT signaling. Additionally, our data suggest that side by side activity and cross regulation of PI3K/mTORC1 and MYCN pathways are of great importance in the more aggressive Stage 4 neuroblastoma tumors and that dual PI3K/mTORC inhibitors, which target both signaling pathways, can be more beneficial against all neuroblastoma.

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POB067

Neuroblastoma with Distinct Genomic Profiles Harbor Similar Mutational Patterns

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Background: In MYCN-non amplified neuroblastoma (NB) distinct genomic profiles can be distinguished: whereas neuroblastoma with numerical chromosomal alterations (NCA) only are associated with an excellent prognosis, neuroblastoma with segmental chromosomal alterations (SCA) have a higher risk of recurrence. The aim of this study was to analyze the mutational spectrum of MYCN-non amplified NB of different genomic profiles.

Methods: Whole-exome sequencing was performed on 16 neuroblastoma samples with distinct genomic profiles: NCA (n=5) and SCA (n=11). Whole exome sequencing was performed on Illumina Hiseq 2500 ® with 2x100 pair-end reads. Following alignment and standard GATK procedures including filtering on the constitutional sample, variant calling was done using Samtools and GATK. Annotations were performed using ANNOVAR.

Results: In each sample, at least 80% of exons was covered at 20X. Following appropriate filtering, a total of 192 somatic variations were identified in the 16 samples. The mean number of somatic variations was not significantly different between NCA and SCA tumors (NCA: mean number of variations 7.2, range 3-11; SCA mean number of variations 10.18, range 2-24; p=NS), with one sample in the SCA group showing a significantly higher number of somatic variations (n=55). Recurrent non-synonymous variants concerned previously described mutations in the ALK gene (2/16: 1 NCA, 1 SCA), as well as the CDC27 (2/16: 2 SCA) and FRG1 genes (2/16: 1 NCA, 1 SCA). Pathway analysis of variants revealed that genes involved in cellular assembly and organization were targeted in both NCA and SCA tumors, whereas genes involved in nervous system development were altered preferentially in the SCA group. Mutations were not observed more frequently in the vicinity of BPs, indicating that a phenomenon of kataegis is not observed recurrently in NB.

Conclusion: Our results suggest that there is no major difference in the mutational spectrum between NCA and SCA tumors, underlining the importance of the pattern of copy number alterations, rather than mutations, in NB oncogenesis.

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POB069

Reduced DICER1 Functionality in Neuroblastoma through Reduced Expression Levels or Somatic Mutations

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Background: Neuroblastoma has been faithfully modeled in mice. DICER1 is a known tumor suppressor gene in cancer whose function has not been extensively studied in neuroblastoma. As part of a broader study of dynamic microRNA/mRNA regulation during neuroblastoma development, we observed dynamic downregulation of Dicer1 in MYCN-driven hyperplastic lesions and in tumors in mice. Although it can be assumed that this effect is mediated by MYCN, we sought for alternative mechanisms of DICER1 inactivation.
Methods: This search was initiated by exome sequencing and CNV analysis of 9 TH-MYCN, 6 LSL-MYCN and 7 TH-MYCN;ALKF1174L driven mouse tumors. Additional profiling of these tumors using small RNA sequencing and miRNA expression profiling of primary neuroblastoma cases has provided additional evidence for a role for DICER1 in neuroblastoma biology.

Results: Compound heterozygous Dicer1 mutations where found in one TH-MYCN tumor (c.5669T>C, E1797G; c.5660T>C, D1794G), located within the RNase IIIb catalytic centers. This domain is critical for microRNA interaction and cleavage, and mutations are leading to selective reduced RNase IIIb activity. This bi-allelic Dicer1 mutation was accompanied by a reduced 5p/3p microRNA ratio compared to Dicer1 wild-type tumors. Although targeted Sanger sequencing of the RNase III domain in 300 human primary neuroblastoma did not reveal additional damaging mutations, additional genetic evidence for the importance of DICER1 is a heterozygous truncating mutation in the RNAse II domain in 1 whole genome sequenced primary neuroblastoma case and a focal partial exon deletion in one neuroblastoma cell line.
Conclusion: MicroRNA gene expression profiles indicate that a shift in the 5p/3p ratio of mature microRNAs, possibly due to reduced DICER1 levels or specifically observed splice variants, is correlated with survival in human neuroblastoma patients. These data warrant further investigation towards the exact role of DICER1 in neuroblastoma oncogenesis. Animal modeling is currently ongoing.

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POB070

Genomic Anatomy of Chemotherapy-Resistant Neuroblastoma

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Background: Relapse after multimodal treatment of neuroblastoma (NB) is associated with extremely poor outcomes. The goal of this study is to identify genetic/genomic changes that are enriched in NB tumor samples after induction therapy. We hypothesize that these changes are involved in the mechanism of chemotherapy resistance in high-risk NB patients.

Methods: Laser capture microdissection of neuroblasts was performed on tumor samples from high-risk NB patients at diagnosis (PRE) and after induction chemotherapy (POST). Somatic mutations and copy number changes were identified through whole exome sequencing and high density SNP array analysis of tumor and matched normal blood samples (NL).

Results: A total of 251 candidate somatic variants were identified in 11 trios (PRE, POST, NL) and one paired sample (PRE, NL), including 150 mutations in the pre-treatment specimens (mean of 13 variants/tumor) and 101 mutations in the post-treatment specimens (mean of 9 variants/tumor). The frequency of somatic mutations was similarly low in pre- and post-treatment specimens (0.34 and 0.28 muts/MB, respectively), as was the spectrum of mutation types. Preliminary analysis suggests mutational overlap between the two groups; however, mutations in several known cancer genes were identified exclusively in the pre-treatment (MCC, MSH3, CA9, POU4F2, AATK) or post-treatment specimens (CDH1, ERG, PDGFRB). The putative mutations identified are currently being validated using an orthogonal sequencing method. SNP array analysis revealed copy number alterations in previously described regions of the NB genome, including amplification of MYCN and amplification of 17q, as well as novel focal alterations.

Conclusion: Next-generation sequencing of NB at two different time points in treatment has provided insight into the genetic/genomic changes that occur during induction therapy. Analysis of the mutated genes may provide clues to mechanisms of chemotherapy resistance in high-risk NB patients and allow target discovery for novel therapies.

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POB071

A Submicroscopic Constitutional 3p Deletion in a Neuroblastoma Patient Leads to Identification of CHL1 Gene as a Novel Tumor Suppressor Gene Candidate

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Background: Constitutional chromosomal abnormalities lead to genes related to neuroblastoma tumorigenesis. We describe a submicroscopic constitutional 3p26.3 terminal deletion which occurred de novo in an adolescent neuroblastoma patient with otherwise normal phenotype. Deletion of 3p cause a variable phenotype ranging from null to severe neurological problems.
Methods: Constitutional and tumor DNA was tested by comparative genomic hy-

bridization (a-CGH) and SNP-array using the 4x180K Kit (Agilent). Bioinformatics analysis was performed on a merged dataset containing gene expression profiles and clinical information of 614 neuroblastoma patients of all stages.

Results: The patient, 17 years of age, carried a submicroscopic constitutional 3p26.3 deletion. The imbalance was less than 1.35 Mb in size and contained the genes CHL1 and CNTN6. The patient's tumor showed, as the only segmental alteration, a 3p26.3 overlapping deletion of 2.67 Mb in size that included CHL1, CNTN6, and CNTN4 genes. Low expression of CHL1 was observed in the patient's tumor by immunofluorescence. CHL1 is a neural cell adhesion molecule involved in other types of cancer. , The expression of the 3 genes was studied in the 614 patients cohort. CNTN6, and CNTN4 expressions did not stratify the patients. In contrast, high CHL1 expression was associated to good overall survival in 162 patients, whereas low CHL1 expression characterized 452 patients having worse overall survival. The Kaplan-Meier was highly significant ($P<0.0001$ even considering Bonferroni correction) indicating that haploinsufficient CHL1 gene was a potential risk factor for neuroblastoma patients.

Conclusion: This is the first report of a constitutional 3p26.3 deletion, containing only two genes, reported in a neuroblastoma patient. This small 3p deletion allowed the identification of CHL1 as a novel tumor suppressor gene candidate in neuroblastoma. Larger deletions of 3p occur frequently in neuroblastoma suggesting the presence of one or more tumor suppressor genes. We provide the first evidence that CHL1 gene may be one of them.
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POB073

Function-Based Genome-Wide Association Study Reveals cis- and trans- Modulators of BARD1 Expression are Associated with the Development of High-Risk Neuroblastoma

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Background: We previously showed that common genetic variation is associated with the development of high-risk neuroblastoma. Single nucleotide polymorphisms from genome-wide studies of human disease are enriched for expression quantitative trait loci (eQTLs). Using a novel method that annotates genetic variants with eQTL information, we undertook a gene-based test that leverages functional information increasingly available from whole genome data to improve power to detect association with high-risk neuroblastoma pathogenesis.

Methods: SNP genotypes for 3,404 patients with neuroblastoma enrolled on the Children's Oncology Group ANBL06B1 genome-wide association study were available for analysis. After sample-based quality control, genotypes from 2,709 patients formed the analytic cohort. We developed a gene-based method to test the aggregate effect of eQTLs for association with phenotype. Genome-wide analysis was performed using International Neuroblastoma Risk Classification risk group as a phenotype, comparing high-risk patients to non-high-risk patients. eQTL information in reference human lymphoblastoid cell lines derived from samples of European descent (n=90) was used.
Results: Ten variants within the gene XRCC5 were found to significantly influence the expression of nearby gene BARD1. In addition, both genic and non-genic variants on chromosomes distant from BARD1 influence the expression of the gene. These variants associated with the expression of BARD1, when taken in aggregate, reached genome-wide significance for the association with high-risk neuroblastoma (empirical $p < 10^{-6}$).

Conclusion: Our novel gene-based genome-wide study of high-risk neuroblastoma pathogenesis has implicated variants both cis- and trans- to BARD1. Common genetic variation within BARD1 has been previously implicated in susceptibility to high-risk neuroblastoma. This study demonstrates that additional variants throughout the genome are associated with BARD1 expression and suggests a novel transcriptional mechanism that contributes to high-risk neuroblastoma phenotype.

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POB074

Integrating Cell-Based and Clinical Genome-Wide Studies to Identify Genetic Variants Contributing to Treatment Failure in Neuroblastoma Patients

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Background: High-risk neuroblastoma is a particularly aggressive malignancy with high rates of treatment failure. Cyclophosphamide is an integral component of high-risk neuroblastoma treatment. We evaluated genetic variants associated with in vitro sensitivity to two derivatives of cyclophosphamide for association with clinical response in a cohort of patients (n=2,709) with neuroblastoma.

Methods: Cell lines of European, African and African-American ancestry were exposed to increasing concentrations of 4-hydroperoxycyclophosphamide [4HC (a 4-hydroxycyclophosphamide equivalent), n = 422] and phosphoramide mustard [PM (as its cyclohexylammonium salt), n = 428] to determine cellular sensitivity to the drugs. Genome-wide association studies (GWAS) were performed in each population to identify single nucleotide polymorphisms (SNPs) associated with 4HC and PM sensitivity. SNPs associated with sensitivity in European samples were then evaluated in African and African-American samples and those that replicated were analyzed for associations with event-free survival in patients with neuroblastoma.

Results: An enrichment of SNPs nominally associated with PM sensitivity ($P < 1x10^{-4}$) was observed among SNPs associated with high-risk neuroblastoma (empirical $P = 0.039$). Two linked SNPs within the IKZF3-ZPBP2 locus, rs9908694 and rs1453560, were found to be associated with PM sensitivity in Europeans ($P = 5.62x10^{-5}$), replicated in the cell lines of African origin ($P = 0.049$ in Africans and 0.05 in African-Americans) and were associated with both high-risk neuroblastoma phenotype ($P = 0.001$) and event-free survival ($P = 0.01$) in all patients, as well as in the high-risk subset ($P = 0.05$).

Conclusion: Our study highlights the value of cell-based models to identify candidate variants that may predict response to treatment in patients with cancer.

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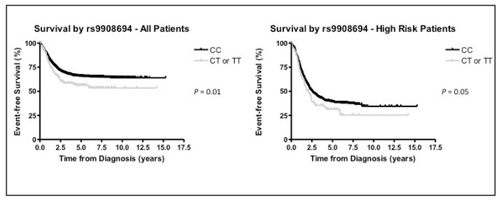

POB075

Anaplastic Lymphoma Kinase Signalling in Model Systems, from Flies to Man

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Background: Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) involved in the development of several human cancers. Of specific importance for ANR2014 are the described mutations of ALK in neuroblastoma.

Methods: We have posed a number of questions concerning ALK function using a combination of Drosophila, cell culture and mouse model systems.

Results: The first of these concerns ALK ligands. In vertebrates the ligand for ALK is currently unclear, with the small heparin-binding proteins Midkine and Pleiotrophin suggested as candidates. We have examined this genetically in Drosophila, where a well characterised ligand for dAlk – Jeb – exists, together with loci encoding homologues of Midkine and Pleiotrophin. The results of this analysis will be presented. Secondly, we have a long term interest in mapping the signaling pathways regulated by the dAlk/ALK receptor. For example, dAlk signaling regulates cell specification and subsequent fusion in the developing visceral muscle, and also regulates Dpp (TGF) signaling between the visceral muscle and the neighboring endodermal tissue. Drosophila dAlk also plays a key role in the regulation of the Gli family transcription factor lame duck (lmd) during development, regulating its nuclear localization and activity. Thirdly, recent results in the investigation of ALK signaling during development employing model systems will be discussed, including a role for ALK in puberty in mouse models. Finally, we will present results from our studies in mouse of activating ALK mutants described in human neuroblastoma.

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POB076

Subtype-Specific Whole Exome Sequencing of 101 Neuroblastomas Identified Genetic Mutations Involved in Cancer-Related Pathways Particularly in Aggressive Subgroups

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Background: We have previously established the risk classification system for neuroblastoma (NB) based on DNA copy number profiles of the tumor determined by array CGH analysis. According to our subgrouping results, the two most major subclasses with poor prognosis are P1a (with partial chromosomal gains/losses including MYCN amplification, 1p loss and 17q gain, but no 11q loss) and P3s (with partial chromosomal gains/losses including 11q loss and 17q gain, but no 1p loss and MYCN amplification), both of which exhibit aggressive behaviors with 8-year survival rates of 33% and 31%, respectively, in our database. On the other hand, Ss subgroup, characterized by almost no chromosomal aberrations, shows a favorable prognosis (8-year survival rate is 82%).

Methods: To determine and compare the spectrum of genetic alterations in each tumor subtype, we have so far conducted whole-exome sequencing of 101 NB cases (P1a:17, P3s:32, Ss: 22, others including metastasized or relapsed: 30).

Results: On average, P1a and P3s subgroups harbored approximately 14 and 19 non-silent somatic alterations, respectively, while Ss had very few alterations (average, 1.2 mutations per tumor). We identified 21 recurrent non-silent somatic mutations in P1a and Ss subgroups. Of those, SEMA6C, SLIT1 and NRAS were involved in axon guidance pathway. In addition, pathway enrichment analysis using DAVID as well as the KEGG database indicated that a part of these novel somatic mutations were concentrated in cancer-related signaling pathways, such as MAPK pathway (14 genes, adjusted $p<0.001$) and Wnt pathway (7 genes, $p=0.072$). These mutated genes mainly existed in P1a tumors. Ongoing efforts will validate mutation candidates in a larger scale sample set by independent analysis platforms as well as the correlations with prognosis of each tumor subset.

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POB077

Focused Exome Sequencing Frequently Identifies Actionable Genomic Alterations in Neuroblastoma Samples

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Background: Next generation sequencing (NSG) technology provides detailed molecular characterization of tumors, and identification of genomic alterations in cancer related genes. Our aim is to demonstrate the application of genomic profiling of neuroblastoma samples, and to identify specific genetic alterations that may provide insight into cancer pathogenesis and prognosis, and potentially guide choices for novel molecularly-targeted therapies.

Methods: Sequencing of DNA isolated from tissue sections from 21 non-sequential patients from Cincinnati Children's Hospital Medical Center who had primary, relapsed or refractory neuroblastomas or ganglioblastomas was performed for 4561 exons of 287 cancer-related genes and for 47 introns of 19 select rearrangements on indexed, adaptor-ligated, hybridization-captured libraries using the Illumina HiSeq 2000 (Illumina, Inc., San Diego, Calif). All sequencing assays were performed exclusively by Foundation Medicine Laboratories (Foundation Medicine Inc., Cambridge, MA).

Results: Genomic alterations in tumor related genes were identified in 16 of the 21 tumor samples analyzed, including 14 of 16 neuroblastomas and 2 of 5 ganglioneuroblastomas tested. In total, 32 genomic alterations were identified in 19 genes. Of these, there were 17 single nucleotide substitutions, 11 amplifications (including 4 NMYC amplifications), 2 gene losses (both in the ATRX gene), and 2 frameshift mutations. Of the 16 samples with mutations, 8 had ALK mutations. Specific ALK alterations included mutations at position F1174 in 4 samples, and at position R1275 in 3 samples. Also present was a mutation in position R1245 in a subclone of one sample, and one sample with amplified ALK.

Conclusion: Focused exome sequencing of cancer related genes in pediatric tumors has the potential to broaden our understanding of neuroblastoma biology and guide therapy.

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POB078

Next Generation Sequencing of Neuroblastomas Identified PPP3CB as a Novel Prognostic Marker of High-Risk Neuroblastoma

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Background: Neuroblastoma (NB) displays extremely heterogeneous biological and clinical behaviors. In this study, we sought to identify novel somatic mutations linking to tumorigenesis as well as different outcomes in NB.

Results: We have conducted whole-exome sequencing of 57 paired neuroblastoma samples using Illumina platform and identified a novel stop-gain mutation in PPP3CB, a catalytic subunit of calcineurin, which results in a constitutive active form of the PPP3CB protein. To investigate the functional role of PPP3CB in NB, we first detected its expression levels in 72 NB clinical samples by quantitative RT-PCR method. As a result, we found that high expression of PPP3CB was significantly associated with poor prognosisin our sample set. Furthermore, multivariate Cox regression analysisshowed that PPP3CB was an independent prognosis factor predicting poor outcome. Enforced expression of PPP3CB as well as its gain-of-function mutant promoted cell growth in NB cells, however knock-down of endogenous PPP3CB by shRNAs reduced cell growth in these cells. Moreover, treatment with two kinds of calcineurin inhibitors suppressed cell proliferation and induced apoptotic cell death in several NB cell lines.

Conclusion: These results indicate that PPP3CB is a novel indicator of prognosis predicting poor outcome in neuroblastoma and that inhibition of the activity of calcineurin might be a potentially effective therapeutic target against high-risk NB.

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POB079

Estimating of Copy Number Alteration in Neuroblastoma: Comparison of Exome Sequencing Data and SNP Microarrays

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Background: Neuroblastoma show high degree of clinical heterogeneity (from tumors with fatal outcome to cases of spontaneous regression). Analysis of recurrent chromosomal aberrations such as losses of 1p, 11q, gain of 1q, 17q and/or MYCN amplification are currently used for patient stratification and definition of therapeutic strategy. Different analysis techniques for detection of segmental abnormalities include FISH, CGH-microarrays or multiplex ligation-dependent probe amplification (MLPA). However, as next-generation sequencing (NGS) becomes available for clinical use, this could also be used for assessment of copy number alterations simultaneously with mutational analysis.

Methods: We compare genomic profiles generated through the bioinformatical tool Control-FREEC on Exome sequencing(ES) data with profiles generated from Affymetrix 250K or 50K SNP-microarrays on 20 NB tumors of different stages. ES was performed by paired-end sequencing on Illumina instrumentation after DNA enrichment with Agilent SureSelect All Exome. The sequencing were performed at three separate occasions with median raw coverage of 91X, 127X and 340X respectively. Neuroblastoma tumors were normalized with either corresponding constitutional DNA or normal control DNA for NGS data while microarrays were normalized against healthy control DNA. Gross genomic changes were extracted through visual and/or ratio inspection from the 20 neuroblastoma tumors.

Results: 128 larger segmental aberrations and 63 numerical aneuploidies were detected through SNP-microarrays. Discrepancies between the Methods:were detected in three cases; one segmental loss detected through microarray was not confirmed by NGS-profiling, one smaller deletion was only detected through NGS-profiling and in one instance both Methods:indicated 2p-gain but at different positions. Otherwise the results were concordant. Furthermore, through ES data we detected ALK-mutations in four patients, ATRX-deletions in two patients and chromothripsis of chromosome 5 in one patient.

Conclusion: Exome sequencing could be used for diagnosis of neuroblastoma tumors combining mutational screening with detection of common chromosomal/segmental aneuploidies such as 2p-gain, 17q-gain, 11q-deletion, MYCN-amplification and alterations of ALK or ATRX.

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POB080

Analysis for Neuroblastoma Tumors to Reveal Novel Target Using Next-Generation RNA Sequencing

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Background: A genetic association with the onset of Neuroblastoma (NB) has only been found for a small fraction of all cases. We have previously reported activating mutations in the coding gene for anaplastic lymphoma kinase (ALK) in about eight percent of all NB patients; unfortunately well-characterized targets are lacking in many NB patients. Many efforts have been undertaken to identify risk factors for aggressive NB, but amplification of the MYCN oncogene is still the most powerful single predictor of adverse outcome of NB.There is a need to more accurately divide NB types associated with an excellent or poor prognosis from those requiring more aggressive therapy. The hallmarks of many solid tumours are chromosomal translocations, which may lead to gene fusions. Recently, next-generation sequencing techniques at the transcriptome level (RNA-Seq) have been used to verify known and discover novel transcribed gene fusions. In this study, we used RNA-Seq to search for fusion transcripts in NB patient cells or cell lines and found fusion transcripts.

Methods: We screened 7 cases who developed Stage 3 or 4 of NB and 3 NB cell lines, ALK mutations in tyrosine kinase domain was not detected in all samples except for SK-N-SH which is one of NB cell lines.

Results: RNA-seq experiments confirmed expression of several predicted chimeric genes and genes with disrupted exon structure including neuritogenesis or cell cycle related genes.

Conclusion: Here, the authors review the applications and challenges of RNA-Seq in discovering novel potential therapeutic targets and candidate biomarkers in the premalignant progression of NB. This study contributes to our understanding of the genetic diversity of NB, an important step towards finding therapeutic targets for a disease that is refractory to current treatments.

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POB081

Kinome Expression Profiling of Human Neuroblastoma Tumours Identifies Potential Drug-Targets for Ultra-High Risk Patients

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Background: Although substantial improvement in outcome of well-defined subsets of neuroblastoma (NBL) patients has been observed during the past few decades, the outcome for children with a high-risk (HR)-NBL has improved only modestly, with estimated 5-year survival rates of 50%. Kinases, which constitute ~1.7% of human genes, are activated or overexpressed in cancers, and constitute current or future targets for successful therapies.In this study, we aimed to identify potential targets of an ultra-HR subset among HR-NBL patients by means of a specific kinome expression profiling.

Methods: We firstly identified HR-NBL patients based on COG risk assignment. Among these, we then distinguished two subsets: (i) ultra-HR patients, HR-NBs who died of disease within 18 months (ultra-HR); (ii) controls, HR alive without an event for at least 2 years (CTR-HR). Human genes codifying protein kinases (n=538) were selected from KinBase (kinase.com/kinbase/). DNA microarray-based gene expression and clinical data of 740 NBLs from two independent freely available datasets (GSE16476 and GSE45547) were collected. Gene classification was performed according to the DAVID 6.7 functional annotation system (david.abcc.ncifcrf.gov).

Results: We selected 49 and 19 ultra-HR, 63 and 5 CTR-HR patients per each dataset. We performed a meta-analysis of the two gene expression sets using the web tool INtegrative Meta-analysis of EXpression data (INMEX, www.inmex.ca). By means of combining standardized difference method, we obtained a set of 27 differentially expressed metagenes between ultra-HR and CTR-HR. Approximately 14% of the 27 genes belongs to the druggable MAPK signaling pathway. Of note, 5 metagenes are predicted as "clinically actionable" by Drug Gene Interaction database (DGIdb, dgidb.genome.wustl.edu).

Conclusion: Our approach could lead to the identification of target-pathways for novel therapies with already known clinically actionable drugs. In vitro functional studies are currently ongoing to evaluate the effect of diverse drugs on NB cell lines.

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POB082

R2: A Public User-Friendly Website for Integrated Analysis of Expression Data and Associated Clinical Parameters in Neuroblastoma

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Background: Microarray analyses have established gene expression profiles in neuroblastoma series and provided prognostic signatures. Such data also contain a blueprint of all pathways and genes relevant for neuroblastoma, but this information can be difficult to retrieve.

Methods: We designed a web-based program to facilitate functional analysis of mRNA expression data of neuroblastoma. Next to a series of 88 richly annotated neuroblastoma samples generated in our lab, the database also contains a range of other public neuroblastoma datasets and thousands of microarrays from other tumor types and normal tissues.

Results: The R2 program has a user friendly interface enabling a wide range of interconnected analyses. Any clinical or biological group can be analyzed for differentially expressed genes (e.g. MYCN amplified vs. single copy). Also significant correlations of any gene with all other genes can be calculated. The results are graphically displayed and the obtained gene lists can be analyzed for pathways and functional categories (Gene Ontology, KEGG mapping). Also microarray data from cell lines with ectopic expression of e.g. MYCN are included. MYCN-target genes can be identified and compared to the correlations found in the tumor series. In addition, such functional signatures can be used to classify patient cohorts. R2 can also calculate Kaplan Meier curves based on the expression of each gene, and scans for the strongest prognostic factors in any chosen subtype of neuroblastoma. Prognostically highly significant expression profiles are thus identified.

Conclusion: R2 provides a valuable resource for high throughput data of Neuroblastoma. The R2 program and database has been used in more than 125 publications and is publicly accessible via r2.amc.nl. R2 will help researchers in identifying important genes and biological processes in neuroblastoma.

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POB083

Integrative Genomic and Epigenomic Characterization of Stage 4S Neuroblastoma

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Background: Stage 4S neuroblastoma remains an enigma, with no unifying explanation for the unique clinical phenotype. A better understanding of the underlying biology driving this subset of patients may provide new insights into basic mechanisms of spontaneous regression and the development of novel treatment approaches.

Methods: Twenty-two matched tumor-normal pairs from stage 4S patients were whole-genome sequenced in the NCI-TARGET project by Complete Genomics. The same set of tumors were profiled for genome-wide DNA methylation using Illumina HumanMethylation450 arrays and will have mRNA expression assayed using Illumina-based RNA-sequencing.

Results: We observed an exceptionally low number of somatic non-silent coding mutations per sample (average 2.7, range 0-7); each occurred only once within the cohort. Somatic structural variants (SVs) were also exceedingly rare and non-recurrent. Nearly all somatic copy number alterations (CNAs) involved whole chromosomes, with very few segmental CNAs. Recurrent germline deletions of exons within the crystallin zeta gene (CRYZ) at chromosome 1p31 were detected in 15% of cases (3/22), a rate significantly higher than observed in the 1000 genomes project (0.6%, 7/1151; P=0.0006). Principal component analysis revealed global differences in the methylome of 4S vs. localized low-risk, high-risk and normal brain tissues, with 4S most resembling low-risk neuroblastoma. Differential promoter methylation was observed for 77 genes when comparing 4S vs. low-risk (FDR 0.05), with nearly all exhibiting reduced methylation in 4S (75/77). Differentially methylated promoters were enriched (FDR 0.05) for genes harboring binding sites for several transcriptions factors involved in cell differentiation (LEF1, TCF3, ETS2, and PITX2), suggesting these may play a role in spontaneous regression of 4S neuroblastoma. Significant enrichment was also observed for aberrant WNT signaling ($P=1.7x10^{-4}$; FDR 0.03) and metabolism of lipids and lipoproteins ($P=2.4x10^{-4}$; FDR:0.04).

Conclusion: A unifying mutation that explains 4S neuroblastoma behavior was not identified. Further examination of germline, transcriptomic and epigenetic alterations in 4S neuroblastoma may explain the initiation and evolution of explosive disease progression followed by spontaneous regression.

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POB084

Gene Expression Studies on Disseminated NB Cells: The Long Way to Reliability

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Background: Disseminated tumor cells (DTCs) in the bone marrow (BM) of neuroblastoma (NB) patients, presumably responsible for tumor relapse, are so far not characterized concerning their expression profile. Neither whether it differs to the tumor at diagnosis nor whether changes occur during disease progression and, importantly, whether altered pathways, which may help to apply drugs specifically acting against these deregulated genes, can be identified. In order to address these crucial questions, a series of unsolved technical issues, like the influence of material storage and DTC enrichment on the gene expression profile, had to be solved by testing different pre-treatment strategies.

Methods: LAN1 cells were spiked into peripheral blood/BM and enrichment of NB cells was facilitated by magnetic bead separation applying FITC labeled GD2 antibodies. Different conditions were simulated and expression signatures were compared between: i) non-enriched vs. enriched tumor cells; ii) freshly spiked tumor cells vs. spiked tumor cells stored up to 72 hours at room temperature and 4°C; iii) fresh vs. frozen tumor cells. Expression of 96 genes was quantified by dynamic array qPCR for every condition and by expression arrays.

Results: Tumor cell manipulations i.e. mononuclear cell (MNC) isolation and tumor cell enrichment had no significant impact on the expression of the tested RNA species. However, storage time at 4°C influenced the expression profile of the tested genes remarkably and differences were even stronger when spiked tumor cells were stored at room temperature. Importantly, freezing and thawing of the MNC/tumor cell fraction preceding manipulation procedures did not systematically alter their expression signatures.

Conclusion: DMSO frozen BM samples from 98 patients who meet the inclusion criteria i.e. transport within 24hours at 4°C can be used in our ongoing RNAseq analyses.

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POB085

A Kinome-Wide RNAi Screen Identifies ALK as a Synthetic Lethal Target in the Interaction with HDAC8-Inhibitors

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Background: Despite continuous intensification of chemotherapy, patients with advanced stage neuroblastoma remain to have very poor prognosis and the 5-year survival rate is less than 40%. Recently, mutations and dysregulation of anaplastic lymphoma kinase (ALK) was correlated to the tumorigenesis and malignancy of neuroblastoma. Mutations of ALK are main causes of hereditary neuroblastoma,

and these mutations were also found in about 8% of sporadic neuroblastoma patients. We previously reported that the expression of histone deacetylase (HDAC) 8 was correlated with poor outcome. Consistent with this, selective HDAC8 inhibitors slowed down cell proliferation and induced differentiation in neuroblastoma cell lines.

Methods: We performed a kinome-wide RNAi screening, combination index calculations for synergism as well as cell death and colony assays.

Results: A kinome-wide RNAi screening identified synthetic lethal interactions between the knockdown of ALK and HDAC8 inhibitors. The combination of ALK inhibitor crizotinib and HDAC8 inhibitor Cpd2 (1-Napthohydroxamic acid), efficiently killed a diversity of neuroblastoma cell lines, including those carrying the F1174L mutation in ALK (SH-SY5Y and Kelly cells). In order to characterize the effects of the combined treatment, we tested for synergism with the calculation of the combination index (CI). We observed synergistic actions of the inhibitors when concentrations at or above the IC50s were used. In colony forming assays, much less colonies were formed when cells received the combinational treatment. Finally, bioinformatics analysis revealed that the mRNA expression levels of HDAC8 were correlated with that of ALK in patient samples.

Conclusion: These findings identified the combinational targeting of ALK and HDAC8 as a novel strategy for the treatment of neuroblastoma and mechanistic studies are under way to unravel the link between both drug targets.

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POB086

A Search for Genes Differentially Expressed in Neuroblastoma Using Next-Generation Sequencing and Spheroid Culture

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Background: MYCN transgenic (Tg) is commonly used as a model of neuroblastoma. We employed this model to search for genes differentially expressed in neuroblastoma tumorigenesis, using the next-generation sequencer (NGS).

Methods: Superior mesenteric ganglion (SMG) of wild-type mice and primary tumors of MYCN Tg mice were subjected to NGS. Furthermore, we used allografted tumors derived from MYCN Tg primary tumors, which show more rapid tumor growth compared with MYCN Tg primary tumors. Furthermore, spheroid culture cells from MYCN Tg primary tumors and allografted tumors were also analyzed by NGS. Among them, data of spheroid culture cells from MYCN Tg primary tumors and allografted tumors were intensively analyzed.

Results: We found that three genes were up-regulated by more than 20 times in spheroid culture cells from allografted tumors than those from MYCN Tg primary tumors. These up-regulations were confirmed by RT-PCR. Among these, Aif1l showed selectively expressed in tumors of MYCN Tg hemizygotes and homozygotes, but not SMG of wild-type mice.

Conclusion: Our strategy of selection of genes through NGS analyses of spheroid culture cells with various potentials of tumorigenesis would be an alternative way to identify important genes for tumorigenesis or neuroblastoma therapy.

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POB087

Characterization of Anaplastic Lymphoma Kinase (ALK) Mutations in Neuroblastoma

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Background: ALK is a receptor tyrosine kinase belonging to the Insulin receptor (IR) superfamily. Since its discovery in 1994 in ALCL (Anaplastic large cell lymphoma), it has been described in wide range of cancer types including lymphomas, neuroblastomas and non-small cell lung cancer (NSCLC).

Methods: We are investigating the mechanisms of Anaplastic Lymphoma Kinase (ALK) activation in neuroblastoma. To do this we employ cell culture, and Drosophila model systems together with phosphotyrosine profiling and biochemical analysis.

Results: Neuroblastoma, a cancer type in which ALK mutations are found, is a neural crest derived embryonal tumour of the postganglionic sympathetic nervous system. A number of characteristic chromosomal aberrations are observed and activating mutations of ALK have been described. ALK mutations, which occur in 6.9 % of neuroblastoma, are most frequently observed in MYCN amplified tumours (8.9 %), correlating with a poor clinical outcome. Currently we are investigating whether all mutations observed in neuroblastoma patients are activating in character. In parallel, we have examined acquired resistance ALK mutations found in NSCLC patients treated with the ALK inhibitor crizotinib or with 2nd and 3rd generation inhibitors, addressing how these mutations change the ability of ALK to be activated.

Conclusion: We hope to provide experimental data which addresses the suitability of patients with ALK mutations for treatment with ALK TKIs, and to improve our understanding of the impact of ALK activation in neuroblastoma.

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POB154

MicroRNA-Mediated Feed-Forward Regulation Driven by MYCN in Neuroblastoma

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Background: Neuroblastoma (NB) is the most common extracranial solid tumor of childhood, and MYCN, a member of a family of oncogenic transcription factors, is a major driver of NB tumorgenesis. Recent studies revealed that a number of MicroRNAs (miRNAs), small non-coding RNA molecules, play critical roles in NB progress. To uncover the regulations between MYCN and miRNAs can assist us to understand the pathogenesis of NB. In this study, we focused on the common regulation format, feed-forward loop (FFL).

Methods: We performed ChIP-Seq and miRNA-Seq experiments on SK-N-BE (2), a MYCN-amplified NB cells, to determine MYCN binding sites and differentially expressed miRNAs by MYCN knockdown, respectively. We then integrated miRNA targets obtained from CoMeTa and MYCN-gene regulation relationships inferred from our ChIP-Seq studies and gene expression profiles of NB samples to find the common target genes of MYCN and MYCN-mediated miRNAs.

Results: To compile the results of ChIP-Seq and miRNA-Seq studies, 32 potential MYCN-mediated miRNAs were identified, with 17 and 15 miRNAs being activated and repressed by MYCN, respectively. A total of 1,392 FFLs with 26 miRNAs and 342 co-regulated genes were identified. The functional analyses of co-regulated genes revealed that these miRNAs and genes might play important roles in NB, including cell cycle and nervous system development. Some FFLs are supported by previous studies.

Conclusion: In this study, we provide a valuable resource for further understanding the complex regulatory mechanism in NB.

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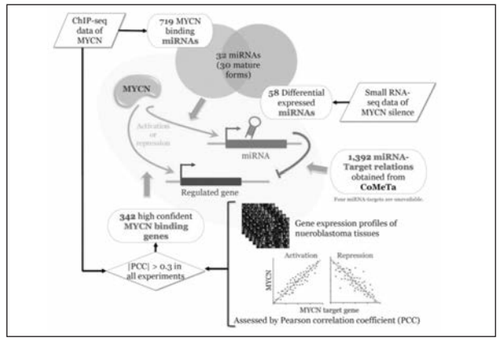

POB155

Single-cell Transcriptomics Reveals Mechanism of MYCN Promoting Cell Cycling

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Background: Elevated MYCN expression has paradoxical effects on neuroblastoma cells by promoting cell cycle progression and sensitizing cells to apoptosis. An accurate mechanistic understanding of how MYCN influences the cell cycle is of both fundamental and therapeutic interest. Our data suggest that MYCN controls the switching between cycling and non-cycling states of the cell cycle. We reasoned that capturing individual cells at different points along the cell cycle would reveal the molecular network that drives MYCN-dependent cell cycling.

Methods: To compare the behavior of cells with high and low MYCN expression, we used MYCN amplified IMR5/75 cells with a tetracycline-regulatable shRNA targeting MYCN. In addition, an E2F1-d2GFP reporter was introduced into the cells in order to measure E2F transcriptional activity. MYCN+ and MYCN– cells were grown in the presence or absence of serum. The transcriptomes in MYCN+ and MYCN– cells with low, intermediate and high E2F transcription were analyzed by RNA-seq in FACS-sorted subpopulations and single cells. ERCC spike-in controls were added to the samples according to cell number.

Results: Single-cell RNA-seq of MYCN amplified cells reveals extensive transcriptome heterogeneity. MYCN+ cells with low E2F transcription show bimodal gene expression, whereas E2F-GFP intermediate and high populations have unimodal transcriptomes and lose the peak of low-expressed genes. The bimodal distribution was concealed in RNA-seq samples of bulk sorted cells. Various quality controls showed that single-cell and bulk samples clustered according to MYCN level, serum concentration and E2F activity.

Conclusion: Single-cell RNA-seq provides a robust method to reveal unique gene expression patterns that are not evident in bulk sorted samples. ERCC spike-in standards that reflect cell number are essential for RNA-seq data normalization to accurately reflect the biological subtypes.

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POB158

Integrative Omics of MYCN Reveals Potential Novel Therapeutic Targets, including Modulation of Wnt Signalling

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Background: The MYCN oncogene was first discovered over 30 years ago in neuroblastoma. Since then it has been extensively studied and numerous functions of this enigmatic oncogene have been identified. Yet, despite over 10,000 MYCN publications (PubMed, as of March 2014), we still do not have a complete picture of the workings of MYCN or integration of its disparate roles. In addition, therapeutic approaches to tackle high-risk MYCN amplified neuroblastoma have been lacking, although some progress appears to be on the horizon. We have taken an unbiased omics approach to MYCN to identify the networks through which MYCN regulates neuroblastoma cell fate and outcome, in order to elucidate potential novel therapeutic targets.

Methods: We have integrated data from both MYCN overexpression and amplification at multiple molecular levels, protein-protein interactome, DNA binding, transcriptome and proteome, utilising a range of high-throughput methodologies.

Results: We identified the predominant repression of target genes, non-canonical MYCN binding patterns and interactions with numerous transcription factors and epigenetic regulators. We have applied the latest Systems Medicine techniques to these data to identify the key changes induced by MYCN and to reveal potential vulnerabilities of neuroblastoma cells. The validity of our findings was confirmed using patient tumour micro-array data. We functionally validated the role of one of the recurrently identified signalling pathways, the Wnt signalling pathway. We demonstrated how drug-induced modulation of this pathway (small molecule activation or inhibition) leads to the death of MYCN amplified neuroblastoma cells.

Conclusion: Integrative Omics and Systems Medicine approaches elucidated MYCN regulated signalling networks revealing novel therapeutic avenues.

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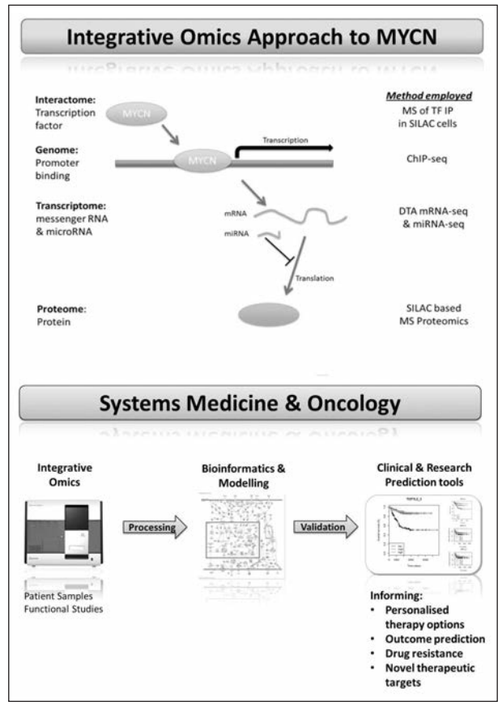

POB159

Identification of key exploitable vulnerabilities in Neuroblastoma by integrative functional genomics screening

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Background: Despite advances in multimodal treatment, neuroblastoma (NB) is often fatal for children with high-risk disease. Additionally, survivors often suffer from long-term side effects due to high-dose chemotherapy and radiation. Identification of novel mutation-based targeted therapies is hampered by a general scarcity of actionable mutations in this cancer and currently limited to a small group of ALK-mutated cases. Recently, high-throughput loss-of-function siRNA screens have been used to identify genetic dependencies in cancer cell lines including ki-

nome and apoptosis-specific screens in neuroblastoma. This study is aimed at identifying actionable genetic vulnerabilities and their targeting compounds by integrating siRNA and small molecule screening data.

Methods: We used the QIAgen human druggable genome kit containing 13910 siRNAs to target 6878 genes for the discovery of genetic dependencies in four NB cell lines, two MYCN-amplified (IMR5 and IMR32) and two non-amplified (SKNAS and NBEB) lines. Cell titer Glo assays were used to measure cell viability. Identified target genes from the primary screen were verified in a secondary screen with three independent Ambion siRNAs per gene. Functional annotation was performed by using Ingenuity pathway analysis. Additionally, we tested 462 small molecules comprised in the MIPE3 compound library for activity in the same cell lines. By integrating both datasets, we were able to narrow down our list of genetic vulnerabilities to candidates targeted by currently available inhibitors.

Results: Of the 213 genes identified in the primary screen, 61 were verified. Pathway analysis revealed predominance for genes associated with cell cycle progression and regulation of cell death and survival. The drug screening data offered 9 classes of inhibitors with considerable activity targeting the identified dependencies directly.

Conclusion: Data integration of different functional screens is a powerful approach to identify targets for therapeutic intervention in Neuroblastoma. Our candidates converged on playing important roles during cell cycle progression.

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POB163

Interaction Proteome of N-Myc in Neuroblastoma

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Background: Neuroblastoma is the most common childhood cancer in infancy with heterogeneous outcomes. Spontaneous regression may occur due to neuronal differentiation or apoptosis; however, the majority of neuroblastomas show malignant progression. One of the key prognostic factors is the status of the MYCN oncogene, since amplification of MYCN confers a highly malignant phenotype. Therefore, our aim is to elucidate the N-Myc interaction proteome in neuroblastomas with different MYCN status.

Methods: We identified the N-Myc interactome in MYCN-amplified (SMS-KCN, SMS-KCNR, IMR-32) and non-amplified (SH-SY5Y) neuroblastoma cell lines using a label-free quantitative interaction-proteomic approach. N-Myc interacting partners were refined using t-test followed by false discovery rate correction and fold change analysis; then consecutively clustered using k means and Gap statistic algorithm.

Results: We identified eight hundred molecules that associate with N-Myc. Approximately two hundred N-Myc bound proteins were common in all tested neuroblastoma cell lines. Sixty N-Myc interacting proteins, that are primarily responsible for regulation of gene expression, were present only in the MYCN-amplified neuroblastoma cells. Forty proteins, mainly participating in differentiation, cell cycle regulation and gene expression, were unique to the non-amplified cells.

Conclusion: Our datahighlights the heterogeneity of the N-Myc interactome across cell lines with different MYCN status.We hypothesize that N-Myc might function not only as a transcriptional activator, but also as a transcriptional repressor. Additional work is required to elucidate the roles of N-Myc interaction partners in the pathogenesis of neuroblastoma.

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POB166

Unraveling TrkA Signaling in Neuroblastoma Using a Quantitative MS-based Approach

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Background: Neuroblastoma (NB), a cancer of the sympathetic nervous system, comprises the most common extracranial tumor of early childhood. High expression of the receptor tyrosine kinase, TrkA is associated with a favourable NB prognosis and TrkA signaling is believed to be the key mediator of spontaneous tumor differentiation and regression, an intriguing feature seen for a high proportion of NBs.

Methods: We used a quantitative mass spectrometry (MS)-based proteomics approach to unravel TrkA signaling associated with NGF-induced neurite outgrowth in a NB cell line, SH-SY5Y-TR-TrkA with tetracycline-inducible expression of TrkA. Using stable isotope labeling by amino acids in cell culture (SILAC) samples were generated to study signaling dynamics in terms of interactome, phosphoproteome and proteome changes. All samples were analyzed by nanoflow LC-MS/MS on a Q-Exactive mass spectrometer and processed with MaxQuant software.

Results: Phosphoproteomics identified and quantified more than 10,000 phosphorylation sites and classification according to their temporal dynamic profiles led to 7 distinct clusters. With a focus on sustained signaling clusters and inhibitor-based targeting of active protein kinases we identified several kinases with importance to NGF-induced neurite outgrowth. In the dynamic interactome we identified several known and also novel dynamic interactors of TrkA. Among them, the signaling adaptor, GAREM2 was validated by western blotting. Furthermore, we identified 447K and 775K as TrkA ubiquitination sites. Interestingly, we also identified an E3 ligase not previously linked to TrkA signaling. Ongoing work focuses on the potential of this E3 ligase to influence TrkA ubiquitination and stability as well as neurite outgrowth.

Conclusion: Quantitative proteomics is a powerful tool to unravel signaling dynamics in NB. The combined analysis of the NGF-TrkA interactome, phosphoproteome and proteome changes associated with neurite outgrowth has a strong potential as valuable resource to better understand TrkA signaling and ultimately to expand the biomarker repertoire for NB patients.

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Basic Research: Biological Models

POB088

Hypoxia Promotes Transdifferention of Neuroblastoma Cells into Endothelial Cells through Epithelial-Mesenchymal Transition

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Background: Tenascin-C (TNC)+ neuroblastoma (NB) cells trans-differentiate into genetically unstable tumor-derived endothelial cells (TDEC), that may be targeted by antibody-mediated immunotherapy.

Methods: We treated immunodeficient mice carrying orthotopic NB formed by the HTLA-230 human cell line with TDEC-targeting cytotoxic human (h)CD31 or isotype-matched mAbs.

Results: hCD31 mAb treatment did not affect survival of NB-bearing mice, but increased significantly hypoxia in tumor microenvironment where coexistence of apoptotic and proliferating TDEC was detected, suggesting the occurrence of vascular remodeling. Tumor cells from hCD31 mAb treated mice showed i) up-regulation of epithelial-mesenchymal transition (EMT)-related and vascular mimicry (VM)-related gene expression, and ii) expression of endothelial (CD31, VE-cadherin) and EMT-associated immunophenotypic markers. The latter included i) nuclear Twist-1, ii) reduction of E-cadherin expression and iii) up-regulation of N-cadherin and TNC expression. HTLA-230 and IMR-32 NB cells cultured under hypoxic conditions underwent EMT and endothelial transdifferentiation, thus demonstrating that hypoxia was the common driver of both phenomena. Furthermore, tumors from hCD31 treated mice displayed up-regulation of high mobility group box-1 (HMGB-1) expression, that was also detected in the above NB cell lines cultured under hypoxic conditions. Finally, human recombinant HMGB-1 induced expression of EMT-related and endothelial markers in the same cell lines cultured under normoxic conditions, thus mimicking the effects of hypoxia.
Conclusion: TDEC targeting with hCD31 mAb increases tumor hypoxia which upregulates in NB cells expression of VM-related and EMT-related genes, setting the stage for new waves of TDEC transdifferentiation. Hypoxia-induced HMGB-1 contributes to amplify these phenomena. These events in combination are responsible for refractoriness of NB tumors to TDEC targeting with hCD31 mAb.

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POB089

The Epigenetic Modifier CHAF1A Opposes Neuroblastoma Differentiation via Metabolic Reprogramming

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Background: Neuroblastoma (NB) arises from embryonal neural crest secondary to a block in differentiation and long-term survival inversely correlates with the degree of neuronal differentiation. Treatment with differentiation agents such as retinoic acid (RA) has modestly improved survival. We have recently demonstrated that the histone chaperone CHAF1A plays a critical role inhibiting neuronal differentiation in high-risk NB. CHAF1A is a subunit of the Chromatin Assembly Factor-1 (CAF-1) which regulates H3K9-trimethylation and DNA methylation.

Methods: Loss-of-function studies in neuroblastoma RA-resistant cell lines with inducible CHAF1A knockdown were performed. Gene expression profiling of CHAF1A knockdown and control cell lines was performed on Affymetrix U133 + 2.0 arrays. GSEA (Gene Set Enrichment Analysis) defined the transcriptional response to CHAF1A silencing. Quantitative-PCR assays were used to validate the most significant enriched metabolic gene sets.

Results: We show that CHAF1A loss-of-function effectively drives neuronal differentiation in multiple neuroblastoma RA-resistant lines. GSEA reveals that genes regulated by CHAF1A were associated with major metabolism and oncogenic pathways. CHAF1A silencing significantly (nominal p-value <0.05 and FDR q-value <0.25) enriches for cell metabolism pathways (valine, leucine, and isoleucine degradation, glutamate metabolism, and insulin pathways) and suppresses pathways with known oncogenic function in neuroblastoma (KRAS, ALK, AKT, and BMI1). Quantitative-PCR confirmed CHAF1A modulation of critical genes involved in glucose metabolism and insulin pathway.

Conclusion: Our findings support the hypothesis that CHAF1A aberrant expression contributes to the resistance of NB tumors to retinoic acid. They also suggest that CHAF1A expression restricts neuronal differentiation by regulating glucose metabolism. Better understanding of the metabolic changes induced by CHAF1A will identify vulnerable points impairing NB growth and guide the development of novel differentiating therapies.

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POB090

Modeling Human Neuroblastoma in Mice

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Background: There is a lack of relevant well characterized orthotopic xenograft models of human neuroblastoma.. Our aims were to characterize and compare neuroblastoma cell line-derived orthotopic xenografts and patient-derived xenografts (PDXs) in mice.

Methods: The human neuroblastoma cell line SK-N-BE(2)C was transduced with lentivirus expressing eGFP and injected orthotopically into the adrenal gland, and patient-derived neuroblastoma explants were implanted orthotopically into immunodeficient mice. Immunohistochemistry was performed on tissue sections of xenografts and mouse organs. Single Nucleotide Polymorphism (SNP) array was carried out on the SK-N-BE(2)C cell line and its' xenograft tumors, and on PDXs and the corresponding patient tumors.
Results: SK-N-BE(2)C orthotopic xenografts express neuroblastoma-associated markers NCAM, TH and Chromogranin A and PDX tumors retained the expression of patient-specific markers NCAM, TH, Chromogranin A and Synaptophysin. Both xenograft types are highly proliferative as shown by Ki67 staining. SK-N-BE(2)C and PDX orthotopic tumors display infiltrative growth into surrounding tissues and metastasize spontaneously, with the PDX showing a more infiltrative and metastatic character. SNP array showed that SK-N-BE(2)C tumors retain neuroblastoma-associated chromosomal aberrations (1p deletion, MYCN amplification, 3p deletion, 11q LOH) and that PDXs retain patient-specific aberrations (1p deletion, MYCN amplification, 9p deletion, 17q gain).

Conclusion: We conclude that both SK-N-BE(2)C orthotopic xenografts and neuroblastoma PDXs 1) express neuroblastoma-associated markers; 2) display infiltrative growth and spontaneous metastasis; and 3) maintain neuroblastoma-typical chromosomal aberrations. However, neuroblastoma PDXs better resemble the histopathological and genomic features of the patient disease. The models will be valuable for investigating neuroblastoma growth and metastasis and for preclinical drug testing.

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POB091

FOXP1 Inhibits Cell Growth and Attenuates Tumorigenicity of Neuroblastoma

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Background: Segmental genomic copy number alterations, such as loss of 11q or 3p and gain of 17q, are well established markers of poor outcome in neuroblastoma, and have been suggested to comprise tumor suppressor genes or oncogenes, respectively. The gene forkhead box P1 (FOXP1) maps to chromosome 3p14.1, a tumor suppressor locus deleted in many human cancers including neuroblastoma. FoxP1 belongs to a family of winged-helix transcription factors that are involved in processes of cellular proliferation, differentiation and neoplastic transformation.

Methods: Microarray expression profiles of 476 neuroblastoma specimens were generated and genes differentially expressed between favorable and unfavorable neuroblastoma were identified. FOXP1 expression was correlated to clinical markers and patient outcome. To determine whether hypermethylation is involved in silencing of FOXP1, methylation analysis of the 5′ region of FOXP1 in 47 neuroblastomas was performed. Furthermore, FOXP1 was re-expressed in three neuroblastoma cell lines to study the effect of FOXP1 on growth characteristics of neuroblastoma cells.

Results: Low expression of FOXP1 is associated with markers of unfavorable prognosis like stage 4, age >18 months and MYCN amplification and unfavorable gene expression-based classification (p<0.001 each). Moreover, FOXP1 expression predicts patient outcome accurately and independently from well-established prognostic markers. Array-based CGH analysis of 159 neuroblastomas revealed that heterozygous loss of the FOXP1 locus was a rare event (n=4), but if present, was associated with low FOXP1 expression. By contrast, DNA methylation analysis in 47 neuroblastomas indicated that hypermethylation is not regularly involved in FOXP1 gene silencing. Re-expression of FoxP1 significantly impaired cell proliferation, viability, migration and colony formation in soft agar. Moreover, induction of FoxP1 expression led to cell cycle arrest and apoptotic cell death of neuroblastoma cells.
Conclusion: Our results suggest that down-regulation of FOXP1 expression is a common event in high-risk neuroblastoma pathogenesis and may contribute to tumor progression and unfavorable patient outcome.

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POB092

Profound Therapy Resistance in Neuroblastoma is Characterized by a Mitochondrial Phenotype

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Background: Profound therapy resistance is a hallmark of lethal neuroblastoma progression yet its mechanisms remain poorly understood. Effective therapies activate a death signal in cancer cells sufficient to engage mitochondrial apoptosis. We describe a novel model of therapy resistance defined by altered mitochondria-endoplasmic reticulum (mito:ER) association.

Methods: Using 7 isogenic neuroblastoma cell line pairs from diagnosis ("pre"-treatment) and after relapse ("post"-treatment), the latter demonstrating chemotherapy resistance, we studied stress responses of isolated mitochondria (BH3 response profiles), inferred survival dependencies and confirmed these in vitro and in vivo. We directly compared cell line mitochondria using 2D-proteomics, mtDNA sequence and quantitation, mitochondrial biomass, and ultrastructure (EM) of whole cells and mitochondrial (heavy membrane) fractions.

Results: Neither mtDNA nor mitochondrial biomass differed between isogenic pairs, yet for all cases post-relapse cells showed markedly attenuated mitochondrial signaling to all stress signals (like Bid, Bim) consistent with profound apoptosis resistance. Cell-based and xenograft models confirm that targeted agents with potent activity against pre-Rx tumors fail to retain activity against post-relapse tumors despite still engaging their drug target due to this terminal apoptotic blockade. Comparative proteomics identified ER stress proteins as most differentially expressed, and EM imaging identified marked reductions in ER:mitochondria tethering in resistant cells. Add-back of ER membrane microsomes to mitochondria restored apoptotic signal transduction.

Conclusion: We propose a unifying phenotype of therapy resistance that consists of attenuated mitochondrial apoptosis signaling associated with disruption of the mito:ER functional unit. Altered mitochondrial lipids and/or calcium signaling (both derived from ER) may be responsible. The model explains the infrequent activity seen in Phase 2 trials as even drugs that activate stress signals will have absent clinical activity against such tumors, and this must be considered as agents like ALK inhibitors move to the clinic. The model enables testing approaches to restore disrupted stress signaling in cancer cells.

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POB093

BMCC1, a Multifunctional Scaffold Protein, Promotes Mitochondrial Apoptosis and Attenuates AKT Survival Signal after E2F1-Dependent Induction in Neuroblastoma

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Background: As we reported, BMCC1 is expressed at significantly high levels in favorable neuroblastoma (Oncogene, 2006). BMCC1 has a conserved BNIP2 and Cdc42GAP homology (BCH) scaffold domain that may modulate signaling networks and multiple cellular functions. Our previous study demonstrated that BMCC1 promotes neuronal apoptosis induced by depletion of nerve growth factor (NGF). However, the molecular mechanism remains unknown.

Methods: To understand the precise role of BMCC1 in inducing apoptosis, we analyzed the function of BCH domain and transcriptional regulation of BMCC1.

Results: By immunoprecipitation, we demonstrated that the BH3-containing BCH domain of BMCC1 is associated with anti-apoptotic BCL2 in human cells. Moreover, full-length BMCC1 overexpression inhibited AKT phosphorylation of residue T308 and its upstream kinase PDK1, both of which are required to promote apoptosis in neuroblastoma cells, whereas deletion of the BCH domain did not inhibit them. BMCC1 overexpression induced mitochondrial apoptosis through these mechanisms in association with cleavage of caspase-9 but not caspase-8. In addition to NGF-depletion-induced BMCC1 accumulation, we demonstrated BMCC1 upregulation following cisplatin- and adriamycin-mediated DNA damage, an intrinsic apoptosis stimulus. Therefore, DNA damage-induced apoptosis was enhanced by BMCC1 overexpression and diminished by inhibiting BMCC1 expression. Furthermore, we demonstrated E2F1-dependent regulation of BMCC1. A ChIP assay revealed E2F1 binding to the BMCC1 promoter, and a luciferase assay demonstrated E2F1-dependent BMCC1 promoter activation. Consistent with these results, we found BMCC1 upregulation during S-phase of the cell cycle, after DNA damage, and during NGF-depletion-induced apoptosis accompanied by E2F1 accumulation. AKT abrogated the pro-apoptotic function of E2F1; thus, inhibition of the PI3K–AKT pathway by LY294002 accelerated the BMCC1 expression after DNA damage.

Conclusion: E2F1-accumulated BMCC1 triggers mitochondrial apoptosis and facilitates drug sensitivity by BCL2 association and AKT inhibition. Since high level of phosphorylated form of AKT is associated with unfavorable neuroblastoma, the AKT-dependent downregulation of BMCC1 may be a cause of aggressive neuroblastoma.

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POB094

MYCN Promotes Neuroblastoma Cell Migration by Regulating TRPM7 Channel and Kinase Activities

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Background: Neuroblastoma (NB) is the most common extra-cranial solid cancer in children. MYCN is a transcription factor that regulates the expression of genes that are involved in cell proliferation, growth, cell migration, etc. MYCN gene amplification is a prognostic indicator of poor outcome in NB. Recent studies have shown that the multiple steps involved in cell migration (e.g. lamellapodia formation, focal adhesion turnover, and cytoskeletal dynamics) are dependent on the availability of intracellular calcium. Although significant advances have been made in understanding the role of calcium during migration, little has been achieved towards understanding its impact on the progression of diseases such as cancer where cell motility is critical for the spread of tumor cells. Interestingly, previous studies showed thatTRPM7 regulate cell migration by mediating calcium influx, and by modulating the phosphorylation and activity of myosin-HIIA. The objective of the current study was to elucidate the mechanism by which TRPM7 regulates NB cell migration.

Methods: Whole cell lysates from NB cells with different MYCN status were analyzed via western blot in order to determine the relationship between MYCN and TRPM7 expression. In addition, the cleavage of the TRPM7 kinase domain was examined by western blot analysis of these lysates. Electrophysiological measurements and live cell calcium imaging were used to determine the regulation of TRPM7 channel activity in NB cells with different MYCN status.

Results: The results showed that MYCN increased TRPM7 expression, enhanced calcium signaling, induced constitutive TRPM7 channel activity, and promoted cell migration in NB cells. The results also showed that MYCN expression induced cleavage of the TRPM7 kinase domain. In addition, cleavage of the TRPM7 kinase further potentiated TRPM7 channel activity, increasing intracellular free calcium.
Conclusion: Overall, the results from this study suggest that MYCN promotes NB cell migration and invasion through a process that may involve TRPM7 channel and kinase activities.

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POB095

miR-18a Inhibits Differentiation of MYCN-Amplified Neuroblastoma by Deregulation of Estrogen and NGF Signaling

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Background: The aim of this study is to explore the regulatory pathways controlling differentiation of neuroblastoma and what consequences deregulation of these pathways has on tumorigenesis. Neuroblastoma exhibits heterogeneous clinical behavior, from spontaneous regression to rapid progression and fatality. One of the few prediction markers for poor prognosis is amplification of MYCN. Expression of the nerve growth factor (NGF) receptors TrkA and p75NTR negatively correlates with MYCN amplification and are associated with differentiated tumors with good prognosis.

Methods: We have generated MYCN-amplified neuroblastoma cells expressing either anti-miR-18a, ERα or the corresponding control plasmids. These cells have been treated with 17-β-estradiol (E2), NGF and inhibitors of ERα signaling and mRNA expression has been analyzed with qPCR and protein levels with western blot and immunofluorescence.

Results: In an effort to delineate the molecular consequences in MYCN-driven neuroblastoma we have identified a role for the MYCN-regulated miRNA miR-18a in maintaining an undifferentiated phenotype. We have demonstrated that MYCN overexpression as well as miR-18a overexpression downregulates estrogen receptor alpha (ERα) a transcription factor involved in neuronal differentiation and development of the sympathetic nervous system (Lovén et al., PNAS 107, 1553-8, 2010).We have now found that activation of estrogen signaling either through ectopic ERα expression or by inhibiting miR-18a expression leads to induction of NGF receptors. In addition, co-activation of estrogen and NGF signaling in neuroblastoma cells resulted in prominent neuronal differentiation and expression of neuronal markers. Preliminary data indicate that p75NTR may play a role in cytoskeletal modulation which is currently investigated.

Conclusion: Our results propose a novel pathway of how MYCN can maintain an undifferentiated phenotype in neuroblastoma through the activation of miR-18a, which targets ERα leading to disruption of NGF signaling and inhibition of differentiation.

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POB096

The Neurodevelopmental Gene, COUP-TF1, Mediates Differentiation and Apoptosis to Affect Therapy Response and Survival in High-Risk Neuroblastoma (HR NB)

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Background: The COG trial, A3973, showed that HR NB patients with MIBG-avid (+) disease at diagnosis had worse overall survival compared to non-MIBG-avid (-) disease (Yanik, 2013). We hypothesized that MIBG (+) and (-) NBs arise during different stages of neuroblast development to harbor different genes affecting therapy response. We interrogated published microarrays of HR NBs based on norepinephrine transporter (NET) expression (MIBG uptake receptor) and found the COUP-TF1 gene to be significantly decreased in NET-high, inferring MIBG (+), NBs. Prognostic data showed that low COUP-TF1 expression in primary HR NBs is associated with inferior (<15%) survival (ANR 2012). COUP-TF1 is an orphan steroid transcription factor involved in neural development as well as 13-cis-retinoic acid (RA) response.

Methods: To expand on our previous results, we genetically manipulated COUP-TF1 expression in HR NB cell lines and assessed for changes in gene expression, differentiation and apoptosis in response to therapy.

Results: Stable inhibition of COUP-TF1 by short hairpin-(sh)RNA in RA sensitive CHLA15 inhibited RA-induced differentiation. COUP-TF1 knockdown in CHLA15 abolished the basal gene expression of RA-effectors, RARα, RARβ, CRAPBP1, CRAPBP2, and prevented their induced expression following RA treatment. Inhibition of COUP-TF1 in CHLA15 also prevented doxorubicin-induced apoptosis. Furthermore, retroviral transfection of COUP-TF1 into COUP-TF1-low, RA-resistant NB1691 restored RA-induced apoptosis, as evident by Parp-1 cleavage and increased expression of apoptosis genes, Nur77 and FOXO3A. COUP-TF1-expressing NB1691 was exquisitely more sensitive to doxorubicin as well.

Conclusion: We now show that COUP-TF1 is necessary for RA-induced differentiation and apoptosis in HR NBs. We are the first to show that COUP-TF1 regulates cytotoxic response in cancer as well. Mouse xenograft studies of NB1691 with and without COUP-TF1 and COG primary tumor IHC are ongoing to validate these findings hold true in vivo. Lastly, low COUP-TF1 tumor expression may be a biomarker for an ultra high-risk population who warrant alternative maintenance therapies than RA to improve survival.

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POB097

Hypoxic Preconditioning Promotes Invasion of Neuroblastoma Cells In Vivo and Enables the Metastasis of Adjacent Non-Hypoxic Cells

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Background: More than 60% of neuroblastoma cases are invasive and despite treatment advances in the last decade, metastasis is still one of the major obstacles to overcome. It has been suggested that hypoxia promotes metastatic dissemination and tumour aggressiveness however, the molecular mechanisms occurring in vivo remain elusive.
Methods: To visualise the invasive processes we used the chick embryo as a model. Fluorescently labelled NB cells preconditioned in 1% or 21% O_2 were either implanted on the chorioallantoic membrane of the chick embryo at embryonic day 7 (E7) or co-injected intravenously at E3 prior to live real-time imaging in or ex ovo.

Results: We showed that hypoxic preconditioning promoted the entire metastatic cascade including intravasation, margination, extravasation and proliferation of the secondary tumour. In addition, it was observed that cells subjected to hypoxia could trigger the invasion of adjacent normoxic preconditioned and non–metastatic cells.

Conclusion: At a molecular level, exposure to hypoxia initiated the expression of several genes involved in adhesion to the vascular endothelium as well as extravasation and matrix degradation. Some of the genes activated by hypoxic pre-conditioning had long-term effects on NB and are hence contributing to the emergence of an invasive aggressive phenotype rendering them a potential new treatment target for hypoxic invasive tumours. We thank the Neuroblastoma Society and the Alder Hey Oncology Fund for funding.

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POB099

γ-Secretase Mediated Intramembrane Proteolysis of NLRR3 Leads to the Liberation of its Intracellular Domain: Its Role in Inducing Neuroblastoma Differentiation

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Background: NLRR3 gene is a member of NLRR family and encodes a type I transmembrane protein with unknown function. We have previously reported that high-level of NLRR3 expression was significantly associated with favorable outcome in neuroblastomas (NBs), and its expression was negatively regulated by MYCN in association with Miz-1 (Clin. Cancer Res., 2011). The expression of NLRR3 is closely associated with the differentiation of NB cells. We thus aimed to characterize the functional role of NLRR3 in the molecular mechanism governing the neuronal differentiation of NB.

Methods: NLRR3 function in cell proliferation was analyzed by WST-8 assay and neuronal differentiation was confirmed by measuring the neurite outgrowth. Expression and cellular localization of NLRR3 in NB cells and primary tumors were examined by confocal microscopy and western blotting.

Results: Overexpression of NLRR3 promoted neuronal differentiation in NB cells, whereas NLRR3 knockdown by siRNA significantly reduced the differentiation upon retinoic acid treatment. Furthermore, ectopic expression of NLRR3 repressed proliferation and reduced the colony numbers. Immunostaining using anti-NLRR3 C-terminal antibody showed that NLRR3 was strongly expressed in favorable NB tumor cells, especially in the cell nuclei and also in the NB cells nucleus. Additionally, western-blot analysis showed multiple short fragments of overexpressed NLRR3.

Our data showed that NLRR3 are substrates for γ-secretase cleavage and the cleavage is inhibited by γ-secretase inhibitors. Like most γ-secretase substrates, the ectodomain cleavage was inhibited by a metalloprotease inhibitor, suggesting that α-secretase cleavage is followed by γ-secretase cleavage. The intracellular portion of NLRR3 which contains putative nuclear localization signal was detected in the nucleus by immunocytochemistry.

Conclusion: Present evidences have suggested that the NLRR3-ICD is cleaved by γ-secretase and translocates to the nucleus, which may implicate signalling for neuronal differentiation mediated by NLRR3.

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POB100

Accumulation of Cytosolic Calcium Induces Necroptotic Cell Death in Human Neuroblastoma

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Background: Necrosis has been studied extensively since the early days of medicine, with some patterns of necrosis found to be programmed like apoptotic cell death. However, the mechanisms of programmed necrosis (necroptosis) has yet to be fully elucidated. In this study, we investigated how the hemagglutinating virus of Japan-envelope (HVJ-E) induces necrosis in human neuroblastoma cells.

Methods: Human neuroblastoma cell line SK-N-SH which defects the expression of caspase-8 was used in these in vitro experiments. Cell viability was assessed by MTS assay. Morphological changes in cells were observed by transmission electron microscopy. The phosphorylation of the death receptor kinase RIP1 was assessed by labeling cells with ^{32}P-orthophosphate and immunoprecipitation. The reactive oxygen species (ROS) generation was detected using a fluorescence probe MitoSOX. Cytosolic calcium was estimated with the Fura2 and the phosphorylation of calcium-calmodulin kinase (CaMK) II was detected by Western blot.The small molecule necrostatin-1 and CK59 were used as inhibitors for RIP1 and CaMK II, respectively.

Results: HVJ-E induced cell death in SK-N-SH cells in a dose-dependent manner, with necrotic morphological changes such as swollen nuclei. HVJ-E-induced cell death was found to depend on the phosphorylation of RIP1 and on the production of ROS. This process was interpreted as necroptosis, based on its suppression by necrostatin-1. We also demonstrated that increased concentrations of cytoplasmic calcium triggered necroptosis by activating CaMK II. Finally, we determined that RIP1 phosphorylation was mediated by CaMK II activation.

Conclusion: We are the first to report that an increase in cytosolic calcium followed by CaMK II activation is one of the upstream pathways of necroptosis in neuroblastoma cells, with potential therapeutic implications, because most aggressive neuroblastoma cells reportedly do not express caspase-8 which suppresses RIP1.

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POB101

BMCC1, a pro-apoptotic gene upregulated by E2F1 after DNA damage, facilitates the drug sensitivity in neuroblastoma

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Background: The BCH motif-containing molecule at the carboxyl terminal region 1(BMCC1) was identified from our cDNA libraries of primary neuroblastoma. Our previous study revealed that BMCC1 was highly expressed in favorable neuroblastoma and had a pro-apoptotic function (Oncogene, 2006). In addition to its role in NGF depletion-induced neuronal apoptosis, BMCC1 was shown to be upregulated during DNA damage-induced apoptosis. However, the underlying molecular mechanisms of transcriptional regulation during apoptosis remain elusive.

Methods: RT-PCR and immunoblot analysis were performed to investigate mRNA and protein expression. BMCC1 promoter activity was measured using the luciferase reporter assay. Recruitment of E2F1 onto BMCC1 promoter was analyzed using chromatin immunoprecipitation (ChIP) assay.

Results: Computational analysis revealed several possible E2F1 binding sites in the BMCC1 promoter region. E2F1, a dual-functional transcription factor, plays a critical role both in cell proliferation and apoptosis, including DNA damage-induced apoptosis. Luciferase reporter assay showed E2F1-dependent BMCC1 promoter activation. The ChIP assay revealed the recruitment of E2F1 onto the BMCC1 promoter, suggesting that BMCC1 is the direct transcriptional target of E2F1.siRNA-mediated knockdown of E2F1 reduced BMCC1 expression, whereas E2F1 overexpression induced BMCC1 expression at both mRNA and protein levels. We observed that accumulation of BMCC1 after cisplatin treatment of neuroblastoma cells is accompanied with ATM dependent phosphorylation of E2F1. ATM inhibitor attenuated both DNA damage-induced E2F1 phosphorylation and BMCC1 induction. We observed that shRNA knockdown of E2F1 reduced the BMCC1 accumulation and subsequently showed drug resistance by cisplatin treatment in neuroblatoma cells. In addition, increased expression of BMCC1 restored E2F1 knockdown-attenuated cisplatin sensitivity in neuroblastoma cells.

Conclusion: We conclude that BMCC1 is an important transcriptional target of E2F1 in DNA damage-induced apoptosis. Inhibition of the pro-apoptotic role of E2F1 in cancer resulted in BMCC1 downregulation that may provide greater insight into mechanisms leading to drug resistance of unfavorable neuroblastomas.

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POB102

A Schwann Cell Expression-Profile Study - Normal, Benign and Neuroblastoma-Associated Schwann Cells Differ from F-Cells

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Background: In contrast to aggressive Neuroblastomas (NBs), maturing/mature NBs harbour a special characteristic, the Schwann cell (SC) stroma which closely correlates with NB growth inhibition and favourable prognosis. Our previous results revealed a normal genomic status of the NB-associated SCs. Together with our in-vitro data on co-cultivation of primary SCs and NB cell lines (showing a proliferative response of SCs alongside with a NB growth impairing effect) we conclude a non-neoplastic origin of the SC stroma in NB. However, due to the occurrence of two morphologically distinct cell types, i.e. neuronal-like (N-cells) and flat (F-cells) in NB cell line cultures, the derivation of SCs in NB is still discussed controversially. Our current investigations on the genomic status and the yet unknown expression profile of SCs in all physiological & pathological conditions in comparison to F-cells shall help to solve this issue.

Methods: DNA and RNA is extracted from SCs obtained from Ganglioneuromas (GNs), Schwannomas (SNs) and human peripheral nerves (hPN) and analysed alongside with purified N- & F-cell DNA/RNA from several NB cell lines by RNA-sequencing and HD-SNP-arrays. In addition, S100, S100A6, p75NTR, Vimentin and CD44 expression was tested on grown hPN-SCs, F-cells and cryosections of GNs and SNs by immunofluorescence.

Results: Method optimization resulted in the successful development of an hPN-SC expansion protocol with primary cultures up to 96% purity. Furthermore, hPN-SCs, SN-SCs and GN-SCs share the same expression profile whereas F- & N-cells lack the expression of S100 and p75NTR. Notably, the presumed SC-marker S100A6 is expressed but not specific for SCs as also found in fibroblasts and endothelial cells.

Conclusion: Our findings clearly indicate that F-cells differ from SCs in physiological and pathological conditions supporting the assumption that F-cells are no SCs, thus, depriving the basis for the assumption of the neoplastic origin of SCs in GN/GNBs.

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POB103

Elucidating Molecular Mechanisms of Oncogene and Non-oncogene Addiction in Pediatric Neuroblastoma

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Background: Amplification of MYCN, loss of chromosomes 1p and 11q and gain of 17q are associated with high risk Neuroblastoma (NB). ALK mutations are frequent, despite which NB exhibits paucity of recurrent somatic mutations. Neither genomic nor gene expression separately are sufficient to explain NB heterogeneity. We apply integrative Systems biology approaches to identify subtype-specific Master Regulator (MRs) of tumor progression and propose novel therapeutic targets.

Methods: Molecular subtypes identification by unsupervised clustering of expression data; ARACNE reconstruction of NB specific regulatory networks and MARINA to identify MRs associated to high risk NB. Genome wide CNV and DNA methylation data to identify up-stream causal modulators de-regulating MRs. Patient versus cell lines similarities assessed to select the most appropriate validation system. We tested oncogenic MRs by cell viability assays in cell lines following lentiviral-shRNA silencing.

Results: NB samples co-segregate into more subtypes than previously reported. We identified three high risk subtypes: a mesenchymal/immunogenic subtype and two highly proliferative: a MYCN addicted and another characterized by 11q loss and 17q gain. Stage1 subtype hereafter used as control group for the identification of tumor progression MRs., Cell viability assays on top 25 MRs from the MYCN subtype in IMR5 and SKNAS: MYCN plus another 4 MR silencing reported strong response; TRIM28 and PRDM8 specific of MYCN subtype; RCOR2 and PTTG1 also MRs of the 11qLOH subtype, indicate that confluent signals could drive both subtypes.

Conclusion: Our pipeline successfully identifies regulatory bottlenecks main-taining aggressive phenotypes. Newly identified subtypes have impact in understanding NB heterogeneity and potentiate future personalized medicine strategies.

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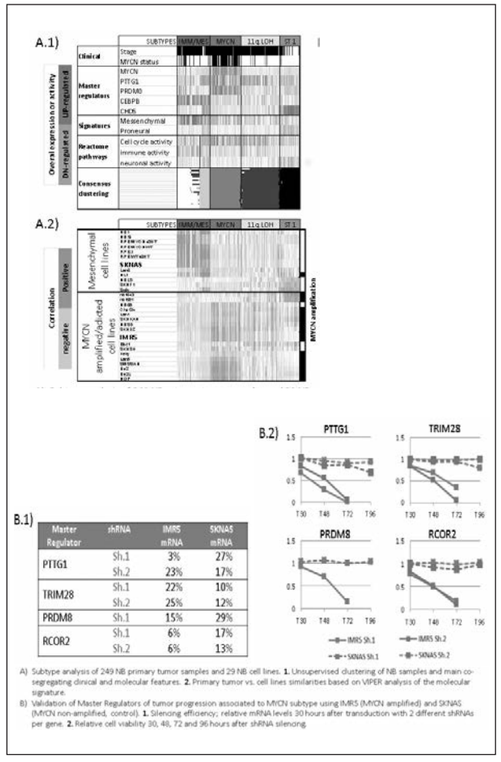

A) Subtype analysis of 249 NB primary tumor samples and 29 NB cell lines. **1.** Unsupervised clustering of NB samples and main co-segregating clinical and molecular features. **2.** Primary tumor vs. cell lines similarities based on VIPER analysis of the molecular signature.
B) Validation of Master Regulators of tumor progression associated to MYCN subtype using IMR5 (MYCN amplified) and SKNAS (MYCN non-amplified, control). **1.** Silencing efficiency; relative mRNA levels 30 hours after transduction with 2 different shRNAs per gene. **2.** Relative cell viability 30, 48, 72 and 96 hours after shRNA silencing.

POB104

The Children's Oncology Group Cell Culture and Xenograft Repository Neuroblastoma Cell Lines and Patient-Derived Xenografts

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Background: Cell lines and patient-derived tumor xenografts (PDX) enable biological and pre-clinical therapeutic laboratory studies. The Children's Oncology Group (COG) Cell Culture and Xenograft Repository (www.COGcell.org) establishes, banks, and distributes cell lines and PDXs from childhood cancers.

Methods: Tumor, bone marrow aspirates, and blood are obtained with informed consent from neuroblastoma patients at COG institutions. Cell lines are established (including in hypoxic and/or serum-free conditions) and xenografts established in NSG immunocompromised mice and are only banked if negative for Epstein-Barr virus and identity validated by short tandem repeat assay. Cell lines are verified to be mycoplasma free and PDXs infectious virus negative. Lines are cryopreserved, and expression of tyrosine hydroxylase and telomerase, amplification of MYCN, and mutations of ALK, are determined.

Results: The COG repository has 127 neuroblastoma cell lines and 11 PDXs that are continuous and characterized from patients with stage 1 (2), 2 (2), 3 (7), 4 (102), 4S (2) and unknown (12); 59 cell lines were established pre-therapy, 38 after chemotherapy, 7 after myeloablative therapy, 18 at post-mortem, and 5 unknown therapy. PDXs are pre-therapy (1), post-mortem (9), unknown (1); 8 PDXs have a matching cell line. MYCN gene amplification was tested in 102 lines: 76 cell lines and 5 PDXs were MYCN amplified; 26 cell lines and 3 PDXs were MYCN non-amplified. Of 95 sequenced lines, 67 cell lines and 5 PDXs were ALK wild-type and 28 cell lines and 4 PDXs had ALK mutations. There are matched pairs from 10 patients of pre-therapy/post-therapy lines. Whole genome sequencing comparing cell lines and PDXs to original tumors and germline for selected lines is in progress.
Conclusion: The COG repository neuroblastoma cell lines encompass all stages and a wide variety of therapeutic exposures in patients. The number of PDX models is expanding. These lines are a valuable and freely available resource for the research community.

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POB105

The Inhibition of Hypoxia Inducible Factors Combined with Retinoic Acid Treatment Enhances Glial Differentiation of Neuroblastoma Cells

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Background: Neuroblastoma (NB) tumor shows notable biological heterogeneity being characterized by cells arrested at various stages of differentiation, where highly malignant tumors express low levels of neuronal differentiation markers and present an immature sympathetic neuroblast phenotype. Hypoxia promotes the undifferentiated phenotype of NB cells either by dedifferentiation or inhibition of differentiation and may contribute to tumor cell aggressiveness through these mechanisms. Targeting hypoxia through different approaches might be a promising strategy to reduce tumor intrinsic and differentiating treatment-resistance.

Methods: To dissect the role of Hypoxia inducible factors HIF1-α and HIF2-α (HIFs) in NB tumor progression and responsiveness to the pro-differentiating agent retinoic acid (RA) we combined the RA treatment with the lentiviral silencing of HIFs expression. We assessed how this combination affects tumorigenic and differentiation ability in several NB cell lines.

Results: RA single agent treatment enhances neuronal differentiation but the treatment combined with HIFs expression decrement shows an enhanced expression of glial differentiation markers and glial phenotype. Moreover, HIFs inhibition can combine with RA to generate a novel trigger for senescence in NB cells.

Conclusion: Our data provide evidence that HIFs regulate the equilibrium between neuronal and glial differentiation in NB cells, and the absence of HIFs let the tumor versus a more benign phenotype not prone to trans-differentiate back. These findings suggest a role of HIFs in high-risk NB relapse after high-dose chemotherapy and differentiating agents treatment. Thus this proposed combination might offer a potential therapeutic advantage to overcome NB resistance to conventional treatment.

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POB106

In Vivo Imaging of Human Orthotopic Neuroblastoma in Mice

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Background: Modeling human orthotopic neuroblastoma in mice requires advanced in vivo imaging techniques. The aims were to establish cell-line derived orthotopic tumors and neuroblastoma patient-derived xenografts (PDXs) and detect tumors by advanced in vivo imaging. We also aimed to develop a model for surgical resection of primary orthotopic neuroblastoma in mice and monitor tumor growth and relapse by in vivo imaging.

Methods: SK-N-BE(2)C, SH-SY5Y neuroblastoma cells or neuroblastoma patient-derived explants were implanted orthotopically into immunodeficient mice. Tumor-bearing mice underwent partial surgical resection of the primary tumor. Magnetic resonance imaging (MRI), fluorodeoxyglucose-positron emission tomography (FDG-PET) and in vivo bioluminescene imaging were used to detect tumor growth.

Results: Orthotopic tumors were established in all cases after injection of SK-N-BE(2)C and SH-SY5Y into the adrenal gland of immunodeficient nude or NSG mice. Neuroblastoma patient-derived xenografts (PDXs) were established in NSG mice. Orthotopic tumors were detected by FDG-PET and MRI. MRI was also used to detect neuroblastoma bone marrow growth following intrafemoral injection of neuroblastoma cells. Partial surgical resection of orthotopic tumors prolonged the lives of tumor-bearing mice and pre- and postoperative tumor growth was monitored by bioluminescence imaging in vivo.

Conclusion: We have established various models of orthotopic neuroblastoma in mice by implantation of cell lines or patient-derived explants. Tumors were detected by MRI, FDG-PET and/or bioluminescene imaging in vivo. We also developed a model for partial surgical resection of primary orthotopic tumors. The in vivo models and the imaging techniques will be important tools for studying mechanisms of orthotopic neuroblastoma growth and for preclinical drug testing.

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POB107

Investigation of ADCC Enhancement by NKT Cells Toward Neuroblastoma

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Background: Natural killer T (NKT) cells play an important role in tumor immunity. NKT cells are activated by a specific glycolipid ligand, α-Galactosylceramide (aGalCer) presented on CD1d molecules, and activated NKT cells enhance both innate (NK cells) and type I acquired (CTLs) immunity. Several clinical studies of NKT-cell based immunotherapy have been conducted to date in Chiba University Hospital, Japan recruiting lung cancer and head-and-neck cancer patients., Anti-GD2 antibodies work through NK cell-mediated ADCC (Antibody dependent cellular cytotoxicity) and have demonstrated clinical benefit for children with neuroblastoma. We hypothesized that NKT cells may enhance NK cell activity in ADCC, and thus planned to investigate the feasibility of the combination therapy using NKT cells and anti-GD2 antibody in neuroblastoma patients.

Methods: GD2 expression in several neuroblastoma cell lines and NK markers including FcgR on NKT cells were investigated. Human NKT cell- or NK cell- mediated ADCC toward GD2-expressing neuroblastoma cell lines was examined using anti-GD2 antibody (14G2a). ADCC of NK cells in combination with activated NKT cells was also examined.

Results: The surface expression of FcgR (CD16) was very low on freshly isolated NKT cells. After in vitro expansion with aGalCer, NKT cells came to express FcgR and other NK markers. NK cell mediated ADCC toward GD2-expressing neuroblastoma cell was dependent on intensity of GD2 expression (NMB>IMR-32>NLF). NK cell mediated ADCC was enhanced by activated NKT cells, though NKT cell itself didn't mediate ADCC.

Conclusion: Although NKTcells are not directly associated with ADCC, activated NKT cells may enhance ADCC through NK cell activation.

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POB108

Ultrasound Evaluation of Tumor Latency and Disease Progression in the Hemizygous TH-MYCN Transgenic Mice

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Background: The TH-MYCN transgenic mouse is a preclinical model that recapitulates human ultra-high-risk neuroblastoma with MYCN amplification. Most studies define palpation of an abdominal mass as tumor onset. This study aims to establish a standard protocol on its screening and follow-up with ultrasound.

Methods: After weaning and genotyping, female hemizygous TH-MYCN transgenic mice of 129/SvJ Background: were evaluated twice a week with Vevo 2100 High-Frequency Ultrasound using a 40-MHz transducer (VisualSonics). The chest and abdomen were scanned ventrally with transverse and sagittal views.

Results: Screened with ultrasound since age 34.7±4.3 (mean±SD) days, 46 (87%) of 53 mice were revealed with a tumor at a median age of 56 days. The tumors showed intermediate echogenicity (kidney > tumor > liver) and arose from one of three sites: 1) Pre-aortic (n=37; 70%); 2) juxta-adrenal (n=6; 11%); and 3) thoracic (n=3; 6%). The thoracic tumors were visualized deep in the posterior mediastinum, taking advantage of the heart or liver as an "acoustic window".

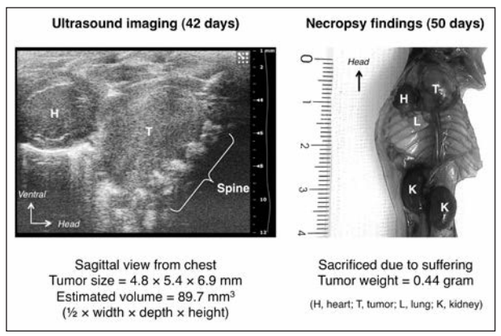

Figure. Ultrasound detection of thoracic tumor in a hemizygous TH-*MYCN* mouse

The thoracic tumors were difficult to be detected until reaching a substantial volume, in contrast to pre-aortic tumors (volume at detection, 98.9±53.5 mm^3 vs. 10.9±1.9 mm3, $P < 0.0001$). During the initial 14 days after tumor detection, the pre-aortic tumors had enlarged 35 folds (from 10.9±11.0 mm3 to 382.6±282.6 mm^3), reflecting rapid progression. After tumor detection, the median time-to-death was 29 days, at a median age of 83 days. Histopathology showed morphological features resembling poorly-differentiated neuroblastoma in human.

Conclusion: Ultrasound enables early detection and longitudinal evaluation of both abdominal and thoracic tumors in hemizygous TH-MYCN transgenic mice. Our results complement previous studies by Teitz et al., 2011 and Rasmuson et al., 2012 on this model.

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POB109

The Resistant Cancer Cell Line (RCCL) collection

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Background: The heterogeneity and individuality of cancer diseases is tremendously high. Recent genomic investigations revealed a tremendous genetic complexity in the cells from solid cancer diseases. Cancer cell (sub)populations may differ substantially between primary tumours and metastases as well as within primary tumours. This heterogeneity is a consequence of cancer clonal evolution processes. Among other models, comprehensive cancer cell line collections will be required to address this wide complexity. Resistance acquisition to anti-cancer therapies represents a major obstacle to the development of effective anti-cancer therapies. Major cancer cell drug resistance mechanisms have been discovered in drug-adapted cancer cell lines including the ABC transporters ABCB1 (also known as P-glycoprotein or MDR1) and ABCC1 (also known as MRP1) and clinically relevant resistance mechanisms to so-called "targeted therapeutics" (e.g. EGFR tyrosine kinase inhibitors, oncogenic BRAF inhibitors).

Methods: Cancer cell lines are adapted to growth in the presence of clinical concentrations of anti-cancer drugs in order to establish readily growing resistance models.

Results: Currently, the Resistant Cancer Cell Line (RCCL) collection consists of about 1000 cell lines from 15 different cancer entities with acquired resistance to a broad range of cytotoxic and targeted anti-cancer drugs including > 200 drug-resistant neuroblastoma cell lines.

Conclusion: The RCCL collection is a tool for the studying of acquired cancer cell resistance mechanisms, the investigation of anti-cancer agents, and the examination of drug-induced clonal evolution processes.

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POB110

A Developmental Approach to Analyze Neuroectodermal Childcancer in Matching Human Microenvironment in Vivo

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Background: Congruent with biological differences between childhood and adult cancers there is a need for new models better employed for studying tumors developing early in life. Most tumors originate from cells that have dysregulated their innate instructions to mature, and are strongly influenced by their microenvironment. Aiming for the principle of a better match between tumor and microenvironment, we have applied an in vivo model representing embryonic human tissues supporting the growth of childhood neuroectodermal tumors (1,2).

Methods: Experimental teratoma induced from human pluripotent stem cells with normal karyotype can be described as a failed embryonic process (3,4) and includes besides advanced organoid development also large elements with a prolonged occurrence of immature neural components (3). Such immature components, although benign, exhibit strong morphological resemblance with childhood tumors of embryonic neuroectodermal origin. Lit.Refs: 1) Int J Cancer. 2013 Oct 1 [Epub ahead of print]; 2) Int J Oncol. 2013;43:831-8; 3) Stem Cells Dev 2004;13:421–35; 4) PLoS ONE 2011;6:e27741.

Results: A novel finding emanated from a histologic demonstration of non-random integration of childhood neuroblastoma (cell lines and fresh tumors) into morphologically identifiable human embryonic tissues with matching histology, recapitulating also the overall tumor histology (1,2). The specific tropism in homologous environment implies an advantage over xenografts for clinical relevance and usefulness of the model for sorting out information significant for the treatment of patients.

Conclusion: The findings provide a proof of principle and open new possibilities for studies on growth promoting factors and guiding of treatment strategies targeting the tumor microenvironment of childhood neuroectodermal tumors. Current work focuses on using the model for pre-clinical predictions of patient therapy response. Specifically, we assess in vivo effects of therapy taking into account the influence of the most proximal microenvironment of matching human tissue. We aim to to answer the important question: Which preclinical biological parameters are most relevant for the patient therapy response and resistance mechanisms?

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POB111

Isolation and Characterisation of Neuroblastoma Cells from Bone Marrow Aspirates: A NCRI CCL CSG Neuroblastoma Group Study

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Background: Metastatic disease in the bone marrow (BM) of children with neuroblastoma (NB) is an indicator of poor outcome, and is associated with relapse and resistance to treatment. In this study we have isolated and characterised NB cells from BM aspirates of children with high-risk disease.

Methods: NB cells were isolated from diagnostic BM aspirates (n=33) using immune-magnetic bead selection for the cell surface disialoganglioside GD2. The percentage of viable GD2 positive cells isolated from BM aspirates was calculated using the trypan blue exclusion assay. Putative markers of NB were examined by reverse transcriptase quantitative polymerase chain reaction and immunocytology. Self-renewal and migratory capacity were analysed by hanging drop/colony formation in soft agar and imaging of isolated NB cell spheroids on gelatin respectively.

Results: The mean frequency of GD2 positive cells detected in BM aspirates was 18% (0.2-57%). GD2-positive cells expressed the NB-mRNAs tyrosine hydroxylase, paired-like homeobox2B, doublecortin, GD2-synthase and nestin, consistent with the NB phenotype. Expression of NB84 was heterogeneous. A mean self-renewing capacity of 12% (range 2-43%) was revealed using a single-cell hanging drop assay. Mean colony formation efficiency in soft agar was 4% (range 0.6-10%). GD2-positive cells isolated from BM aspirates migrated in gelatin; mean migration index 6 (range 1-13).

Conclusion: NB cells with self-renewing capacity and a migratory phenotype were successfully isolated and maintained in culture from BM aspirates taken at diagnosis from children with high-risk neuroblastoma. These results form the basis for the characterisation of NB cells in the BM compartment that may be responsible for the development of metastasis. Future investigations could identify novel ways to identify and eradicate these cells to improve outcome for children with NB.

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POB112

A Comparison of 2D vs. 3D Culture Methods:in Supporting Freshly Isolated Pediatric Neuroblastoma Cells

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Background: Neuroblastoma (NB) is the most common extracranial solid cancer in childhood arising from neural crest cells. Despite advances in treatment, children presenting with advanced disease still have a poor prognosis. The ability to reliably grow patient tumor cells in culture would enable direct testing. Current studies using cell lines do not necessarily mimic the cells found in pediatric patients. We hypothesize that patient specific cells would more reliably mimic a patient's tumor biology. Herein we compare the use of 2 dimensional Transwell (2D) and 3 dimensional Methylcellulose (3D) culture systems +/- mouse adrenal glands to determine optimal conditions for growth of freshly harvested NB cells.

Methods: Feasibility was first tested in immortalized cell lines SK-N-BE(2) and CHLA-20 were cultured for 5 days: 3D +/- Mouse Adrenal Glands and 2D +/- Mouse Adrenal Glands. Following these results, a pediatric tumor was freshly isolated (HH IRB# 13-035), cultured in the most optimal condition and analyzed by FACS.

Results: Cell line viability was highest in the 2D (72-76%) cultures compared to 3D (19-26%) cultures. In addition CD166+, CD114+, TUJ1+, CD166+/CD24+, and CD166+/CD44+ populations increased by 70-80% compared to routine culture. Freshly isolated tumor cells were cultured in 2D +/- Adrenal glands with the highest viability in those cultured without adrenal glands (60%). 2D without adrenal glands increased CD24+, CD44+, CD166+, TUJ1+, and CD133+/Nestin+ populations by more than 100% compared to cells at isolation.

Conclusion: The use of NB cell lines provides an unlimited number of cells for study, but does not mimic each patient's tumor biology. Freshly isolated cells from patients clearly require defined or specific growth conditions in order to be used for therapeutic screening. Therefore optimizing conditions to enable patient derived tumor growth may improve our ability to identify patient specific treatments.

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POB113

Live Cell Imaging Reveals Heterogeneous Proliferative Behaviour in Neuroblastoma

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Background: Genetic and epigenetic heterogeneities within tumour entities contribute to varying degrees to poor prognosis, such as the association of MYCN amplification and drug resistant relapse. However, several studies in a variety of cancer types demonstrated that even clonally identical cells exhibit heterogeneity in their expression levels, proliferation rate and their response to drug treatment. This study aims at quantifying the heterogeneity within clonally identical populations of neuroblastoma, to ultimately be able to understand cell fate decisions and the role of MYCN in these events.

Methods: Time-lapse microscopy was used to record cell attributes such as the proliferation rate, morphology and velocity of individual cells and their progeny generating family lineage information.

Results: Live cell imaging revealed a high variability in interdivision times and apoptotic events. Both MYCN knock-down and growth factor depletion in cells with high MYCN expression decreased the proportion of cycling cells and proliferation rate. Under the combination of low serum and low MYCN levels proliferation almost ceased., Cell lineage analyses showed a high similarity in cellular fates between sibling cells. However, this correlation deteriorated quickly in inter-generation comparisons.

Conclusion: The single cell proliferation dynamics suggest that MYCN expression levels highly influence the switch between a cycling and non-cycling state. The similarity in sibling cell behaviour indicates that this decision is partly predetermined by the state of the mother cell. The timing of cellular decision-making is being clarified further using fluorescent cell cycle and gene expression markers.

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POB114

Hypoxia Regulated Proteins: Promising Targets for Neuroblastoma Treatment

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Background: Neuroblastoma (NB) is a pediatric solid tumor arising from the cells of neural crest. Hypoxic microenvironment affects NB differentiation and contributes to therapy resistance. HIF1/2α (HIFs) are attractive therapeutic targets, but because of HIFs O_2 mediated degradation it is required to focus on HIFs-regulated proteins to open the way to new drug targets in resistant NB.

Methods: To get insights into the hypoxia-driven NB dedifferentiation we generated NB stable clones over-expressing HIFs proteins, and we depleted cells for HIFs expressions with lentiviral plasmids sh- HIFs. We applied a proteomic approach and identified HIFs-regulated proteins in NB cell clones which were also validated in several NB cell lines.

Results: We observed that HIFs have a crucial role for in vitro tumorigenesis and differentiation. Furthermore, the most interesting identified HIFs-regulated proteins belonged to Complement Inhibitor Protein class whose function is to suppress T cell immunity.

Conclusion: Taken together our data suggest HIFs expression contributes to NB tumorigenesis and pushes NB cells versus an undifferentiated stem-like phenotype. The identification of HIFs-regulated proteins provides new potential therapeutic targets to fight tumor immune-escape and therapy resistance in NB hypoxic areas.

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POB115

Expression of the Monocyte Chemotactic Protein-1-Induced Protein 1 (MCPIP1) Decreases Human Neuroblastoma Cell Survival

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Background: The recently discovered MCPIP1 (monocyte chemotactic protein-1-induced protein 1) multidomain protein encoded by the MCPIP1 (ZC3H12A) gene, has so far been described as a new transcription and differentiation factor, a ribonuclease, and a deubiquitinase. Recent analysis of microarrays data showed a lack of expression of the MCPIP1 transcript in primary neuroblastoma tumours. Enforced MCPIP1 gene expression in BE(2)-C cells caused a significant decrease in neuroblastoma proliferation and viability. Aim of the present study was to investigate the role of MCPIP1 in neuroblastoma.

Methods: Enforced MCPIP1 gene expression in BE(2)-C cells was obtained through transfection of plasmid constructs bearing MCPIP1wt or a mutant form lacking the RNase domain (MCPIP1ΔPIN). MCPIP1 protein expression was assessed by immunoblotting. Transcript levels were determined by RT-PCR. Changes in the transcriptome and microRNA expression were analyzed using expression DNA microarrays and microRNA microarrays, respectively.

Results: We detected significant increase of MCPIP1 either wild type, or mutant transcript and protein expression at 4th day after transfection. Detailed microscopic examination of the MCPIP1wt or MCPIPΔPIN pool of cells revealed clearly visible changes in their growth morphology and growth rate compared to the control cells. Expression microarray analysis showed few interesting genes which encode among others neuronal proteins, cytokine receptor and a C3H type zinc finger protein and their content was verified using the real time RT-PCR. The microRNA profiling identified a subset of 8 microRNAs which were differentially expressed in MCPIP1wt-transfected and MCPIP1ΔPIN-transfected cells. Candidate microRNAs were further studied using the real time RT-PCR system.

Conclusion: These results may help to gain more insight into the MCPIP1 gene expression levels importance in cancer development and indicate the possible signalling pathways involved in neuroblastoma cell survival.

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POB116

The Modulation of Attachment, Survival and Differentiation of Neuroblastoma Cells by Nanofilms with Tunable Stiffness

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Background: The mechanical microenvironment cues influence cellular fate and behavior. Thus, tuning the microenvironment stiffness impacts the attachment, motility, survival and differentiation of different cell lines., Polyelectrolytes multilayers (PEM) are versatile scaffolds with constantly growing application field, including biomaterials functionalization, regenerative medicine research and cell culture. Their stiffness can be modulated by crosslinking. In our work, we used a biocompatible PEM composed of Chondroitine Sulfate A (CSA)/Poly-L-Lysine (PLL) to elaborate a biomimetic neuroblastoma culture system with tunable stiffness.

Methods: PEM films composed of $(CSA/PLL)_6$ were builtup and post-crosslinked with a biocompatible crosslinking agent (Genipin : 0%, 0.25%, 0.5%, 0.75%, and 1%). Topography and elastic moduli were determined using Atomic Force Microscopy. The MycN negative SHSY5Y and the MycN positive IGR-N-91 cell lines were used to study cell cycle and apoptosis with the propidium iodide FACS assay. Cytoskeleton and focal adhesion points were visualized by immunofluorescence. Cell proliferation was assessed by using the WST-1 assay and cell differentiation was determined by measuring the average neurites length and assaying synaptophysin.

Results: Atop uncrosslinked PEM, cells were rounded and adopted a spheroid-like organization. They displayed a high apoptosis rate and a poor differentiation. Stiffening the PEM dramatically improved cell attachment, survival and both spontaneous and retinoic acid -induced differentiation.

Conclusion: The genipin-crosslinked CSA/PLL system is a valuable biomimetic system for neuroblastoma cell culture. Tuning its stiffness modulates cell survival and differentiation. This scaffold could also be loaded with numerous bioactive molecules (cytokines, growth factors, drugs…). Because of its ability to enhance neuronal differentiation, it should also be developed as a neuroregenerative tool.

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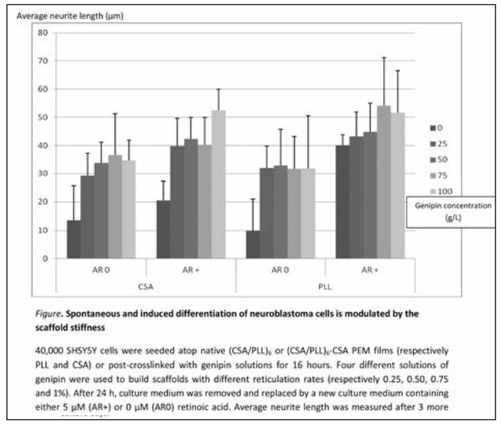

Figure. **Spontaneous and induced differentiation of neuroblastoma cells is modulated by the scaffold stiffness**

40,000 SHSY5Y cells were seeded atop native $(CSA/PLL)_6$ or $(CSA/PLL)_6$-CSA PEM films (respectively PLL and CSA) or post-crosslinked with genipin solutions for 16 hours. Four different solutions of genipin were used to build scaffolds with different reticulation rates (respectively 0.25, 0.50, 0.75 and 1%). After 24 h, culture medium was removed and replaced by a new culture medium containing either 5 µM (AR+) or 0 µM (AR0) retinoic acid. Average neurite length was measured after 3 more

POB160

Screening of Plant-derived Extracts for an Anti-proliferative Effect on Neuroblastoma Cells and Associated Changes in Intracellular Calcium Levels

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Background: Despite the advances in modern medicine, the options of effective neuroblastoma (NB) treatment are still limited and we are in need for new strategies. NB with amplification of the MYCN gene poses a special challenge. The pool of potent chemotherapeutic drugs often diminishes during the course of treatment due to the development of chemoresistance mechanisms. As the major part of compounds used in chemotherapy is plant-derived or based on naturally occurring scaffolds, a screening of plant extracts for an anti-proliferative effect in neuroblastoma cell lines can help identify new active substances for chemotherapy. Apart from cytotoxicity, we are interested in induced alterations of intracellular calcium levels. Calcium, as second messenger, is involved in various cellular signaling pathways that control many cell functions, including tumor progression or apoptosis.

Methods: We used Fluo4-AM staining and fluorescence microscopy followed by Sulforhodamine B (SRB) staining on MYCN2 and SKNBe(2c) cells for the screening process of aqueous and organic extracts from plants that are indigenous to the USA.

Results: After screening a total of 500 extracts, we were able to identify extracts from Juniperus oblonga and Scrophularia orientalis as highly potent agents that significantly reduce cell survival and induce an increase of intracellular calcium. The mitochondrial transition pore assay and western blot analysis revealed that cell death is associated with a mitochondrial calcium overload and Caspase-3- and PARP-cleavage.

Conclusion: In conclusion, extracts derived from Juniperus oblonga and Scrophularia orientalis effectively kill neuroblastoma cells with and without MYCN amplification by inducing apoptosis via the mitochondrial pathway and, thus, have the potential to lead to the development of new chemotherapeutic drugs. Still, further purification is necessary to isolate and identify the active ingredient so it can be tested in vitro and in animal models for its suitability for clinical use.

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POB162

ERK5 Positively Regulates HIF-1&α; Activity in Neuroblastoma SY5Y Cells

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Background: Extracellular signal regulated kinase 5 (ERK5), the most recently discovered and least-studied mammalian mitogen-activated protein kinase (MAPK), is reported to be a key mediator of endothelial cell function, and also involved in neuronal differentiation and cancer progression. Hypoxia-inducible factor-1α (HIF-1α) is a transcriptional activator which plays an important role in mammalian development and tumor progression. A negative regulation of HIF-1α by ERK5 has been reported in epithelial cells, but no studies have elucidated their association in cancer cells or neuronal cells.
Methods: In the present study, we investigated the regulation of HIF-1α by ERK5 in neuroblastoma SY5Y cells which have the neuronal characteristics using the dual luciferase reporter assay and genetic manipulation of ERK5 activity.

Results: We found that the protein expression and activity of HIF-1α, as well as the phosphorylated-ERK5 (P-ERK5) increased in response to hypoxia. ERK5 activation by constitutively active MEK5 (CA-MEK5) enhanced the HIF-1α activity under hypoxic condition, or in cells transfected with exogenous HIF-1α (wild type HIF-1α (WT-HIF-1α), or double mutated HIF-1α (DM-HIF-1α)). This CA-MEK5-induced increase of HIF-1α activity was blocked by dominant negative ERK5 (DN-ERK5). Furthermore, either CA-MEK5, or DN-ERK5 had no effect on the HIF-1α protein expression.

Conclusion: These data indicated that ERK5 is a positive regulator of HIF-1α activity in neuroblastoma SY5Y cells.

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Basic Research: Developmental Neuroblastoma Biology

POB117

Neuroblastoma in Dialog with its Stroma: NTRK1 is a Regulator of Cross-Talk with Schwann Cells

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Background: Tumor-stroma interactions can be regarded as a molecular battle to reprogram the other side towards a malignant or normal phenotype, respectively.

In neuroblastoma (NB) the degree of infiltration by stromal Schwann cells (SC) is correlated with disease outcome. Low-stage NB are characterized by extensive Schwann cell content. High-level NTRK1/TrkA expression, which is known to mediate tumor cell differentiation, but low expression of NTRK2/TrkB is characteristic of this NB subgroup. We hypothesized that Schwannian stromal development and neuroblastic differentiation is based on bidirectional interaction of NB and SC.

Methods: Microarray data from human SY5Y NB cells stably transfected with either NTRK1 or NTRK2 were reanalyzed for identification of SC stimulating factors. SC were isolated from the sciatic nerve of rats. We used western blotting, real-time cell proliferation analyses, Boyden chamber assays and phalloidin staining to characterize NB cell and SC interaction and validate involved regulators. The NB cell and SC interrelation was analyzed in vivo in a xenograft model of SY5Y cells with tetracycline inducible expression of NTRK1.

Results: The SC growth factor NRG1 was found to be upregulated in NTRK1-positive cells. Supernatants of NTRK1-expressing NB cells induced SC proliferation and migration, while neutralization of NRG1 could decrease these effects. Vice versa, NRG1-stimulated SC secreted the NTRK1-specific ligand, nerve growth factor (NGF). SC-conditioned medium could activate the NTRK1 receptor in a neuroblastoma cell culture model with conditional expression of the NTRK1 receptor and caused induction of differentiation markers in NTRK1-expressing cells. Finally, NTRK1 induction in NB xenografts mixed with primary SC significantly reduced tumor growth in vivo.

Conclusion: We propose a model for NTRK1-mediated and NRG1-dependent attraction of adjacent SC, which in turn induce neuroblastic differentiation by secretion of the NTRK1-specific ligand, NGF. These findings have implications for understanding the less malignant NB phenotype associated with NTRK1 expression.

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POB118

MYC Proteins Promote Neuronal Differentiation by Controlling the Mode of Progenitor Cell Division

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Background: MYC family members are expressed in multiple organs and, in particular, have been demonstrated to be critical during development of the nervous system. However, the functional role of MYC proteins in neural cells during development is poorly understood. We have taken advantage of a chick in vivo model to examine their role in progenitor cells of the developing neural tube.

Methods: In ovo electroporation, immunohistochemistry and in situ hybridizations on chick embryos. For time-lapse imaging spinal cord transversal slices (400 µm) were dissected from E3 chick embryos using a Leica vibratome.Video imaging of the in situ dividing cells was performed with a Zeiss LSM510 META NLO (Carl Zeiss, Germany) 2-photon laser scanning microscope. Stacks of ~50 µm were acquired every 3-5 min for 4-6 hours.

Results: Our results show that downregulation of endogenous MYC in radial glial precursors (RGPs) prevents differentiation and conversely, that overexpression of MYC induces neurogenesis independently of premature or upregulated expression of proneural gene-programs. Unexpectedly, the neurogenic function of MYC is dependent on the integrity of the polarized neural tissue, in contrast to the situation in dissociated RGPs where MYC is mitogenic. Within the polarized RGPs of the neural tube, MYC drives differentiation by eroding Notch signaling and by increasing neurogenic cell division, eventually resulting in a depletion of progenitor cells.

Conclusion: These results reveal an unexpected role of MYC in the control of stemness versus differentiation of neural stem cells in vivo. Our results also suggest that loss of cell polarity may fuel tumor development by unleashing MYC driven proliferation.

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POB119

From Human Embryonic Stem Cells to Sympathetic Neurons: A Model for Understanding Neuroblastoma Pathogenesis

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Background: Neuroblastoma is an embryonal tumour originating from neural crest (NC) cells which give rise to the sympathetic nervous system (SNS). Human embryonic stem cells (hESC) are a powerful tool to model developmental aspects of human disease. By directing stem cells down a pathway of sympathetic neuronal differentiation we can understand more about the biological drivers of neuroblastoma development and pathogenesis. Our aim is to differentiate hESC along NC and autonomic lineages to sympathetic neurons, providing a model to increase our understanding of the pathogenesis of neural crest derived malignancies, including neuroblastoma.

Methods: Using the stromal-derived inducing activity (SDIA) of murine PA6 cells in combination with B27 supplement, BMP4 and NGF, H9 and Ncl(R)14 hESC were induced to differentiate to neural crest stem cells and sympathetic neurons.

Results: Following 7 days of PA6 differentiation, mRNA expression of Snail and Sox-9 neural crest specifiers increased as well as Peripherin. Expression of the pluripotency marker Oct 4 decreased post differentiation, whereas p53 and LIN28B expression remained high at levels similar to SHSY5Y and IMR32. A marked increase in expression of the catecholaminergic marker Tyrosine Hydroxylase(TH) and the noradrenergic marker Dopamine Beta Hydyroxylase (DBH) was observed by day 7 of differentiation. The use of Neuronal MACS media® led to a significant increase in the yield of p75+ cells at day 8 (44.7%+/-6) compared with neural BHK media (32.5+/-1.43) (n=3 - p=0.0399). Fluorescence activated cell sorting for the neural crest marker p75, enriched for cells expressing p75, DBH, TH and Peripherin. SN were identified by immunofluorescence by co-expression of TH & Peripherin or DBH & PHOX2B. p75+ sorted cells showed increased migration compared with p75- cells, consistent with a migratory neural crest phenotype in-vitro.

Conclusion: We have established a human in-vitro model of noradrenergic SNS development using two hESC lines to improve our understanding of normal human SNS development and the mechanisms underlying neuroblastoma development and pathogenesis.

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POB121

Association of the MTHFR C677T Gene Polymorphism with Childhood Neuroblastoma Susceptibility and Progression

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Background: Neuroblastoma (NB) is the most common extracranial solid tumour in childhood, arising from developing neural crest cells of the sympathetic nervous system. The cause of NB remains largely unknown, despite numerous genetic factors identified to be associated with the clinical phenotype of disease. The aim of our study was to reveal the association of the MTHFR C677T polymorphism with childhood NB susceptibility and progression.

Methods: The case group comprised 146 children with NB (age: 1 month–17 years; stages I-II: 15, stages III-IV: 131; MYCN+: 40) and 151 healthy donors with no present or previous history of cancer. The polymorphic variants of the MTHFR gene (c.677 C>T) were analyzed by Allelic Discrimination Real-Time PCR using specific primers and TaqMan MGB probes.

Results: The frequency of CC, CT and TT genotypes in donors were 50.3% (76/151), 43.1% (65/151), 6.6% (10/151) and in NB patients – 42.5% (62/146), 39.5% (58/146), 17.8% (26/151), respectively. The distribution of MTHFR genotypes was consistent with Hardy-Weinberg equilibrium in donors ($\chi2=0.33$, p=0.56) as well as in patients ($\chi2=1,69$, p=0.19). Observed mutant T-allele frequency in donors was 0.28, similar to previous reports for healthy Caucasians. In NB patients T-allele frequency was 0.37. We observed a threefold significant increase of NB risk in patients homozygous for the mutant T-allele of the MTHFR gene (OR=3.06; 95%CI=1.42-6.59; $\chi2=8.85$, p=0.01) compared to the patients with one or two wild alleles. We did not notice association between patient's demographic characteristics (age and sex) or disease stage with MTHFR genotype. Noteworthy the frequency of MYCN gene amplification was significantly higher in patients with TT genotype compared to patients with CT or CC genotypes (46% (12/26) versus 17% (10/58), 29% (18/62); $\chi2=7.69$, p=0.02).

Conclusion: The obtained results suggest that polymorphism of the MTHFR gene is associated with NB susceptibility and MYCN gene amplification. Investigation of the role of folate-associated gene factors in NB may reveal potential risk factors involved in the carcinogenesis of disease.

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POB122

Neuroblastoma Oncogene Ortholog Lmo3 Cooperates with Hen2 on Induction of Aberrant Neurogenesis in Mice

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Background: We have previously reported that LMO3 and HEN2 act as oncogenes in development of neuroblastoma through up-regulating MASH1 transcription by interfering with HES1. To confirm these results in vivo, we generated transgenic mice (Tg) of these genes.
Methods: Lmo3 or Hen2 was expressed under the control of Wnt1 promoter that is expressed in central nervous system and neural crest from the sympathoadrenal lineage of that neuroblastoma develop.

Results: Heterozygous Lmo3 Tg (Lmo3/+) and Hen2/+ developed hydrocephalus with higher frequency as compared with the genetic Background: C57Black6. All homozygous Hen2 Tg (Hen2/Hen2) developed hydrocephalus, though not all Lmo3/Lmo3 did (67%). Furthermore, all double-Tg (Lmo3/Hen2) developed hydrocephalus. The thickness of the cortex was reduced in hydrocephalic mice as compared with wild-type mice at 3 weeks after birth. Although the thickness of the neocortex and the cortical germinal epithelium was reduced in Lmo3/Hen2 at E18.5, it didn't decrease in Lmo3/Hen2 at E13.5 when neurogenesis proceeds. Since no difference was observed in the postmitotic neuronal layer in Lmo3/Hen2 at E13.5, Lmo3 might cooperate with Hen2 in regulating the production of progenitors in the developing brain and not in causing a shift in the type of division of progenitors from symmetric to asymmetric. Furthermore, Lmo3/Hen2 showed increase in the number of intermediate progenitor cells in the developing neocortex as compared with the wild type mice at E12.5 and E13.5. These results uggest that Lmo3 and Hen2 interfere with neocortical development.

Conclusion: The similar phenotype was reported in Robo1/2$^{-/-}$ mice in which Hes1 expression level was decreased in ventricular zone progenitors. Thus, it is suggested that expression levels of Lmo3 and/or Hen2 could determine the fate of stem cells by inhibiting Hes1 function during nervous system development and might be a trigger of aberrant neurogenesis in vivo.

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POB123

Elucidation of Mechanisms Underlying Fate Determination of Neuroblastoma in MYCN-Tg Mice

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Background: Although frequent genome-wide alterations and few driver mutations are known to be common in neuroblastomas (NBs), the initial events and subsequent processes of tumorigenesis accompanied by such features remain elusive. We and other researchers previously found that MYCN hemizygous mice show clusters of neuroblasts in the superior mesenteric ganglion (SMG) around 3 weeks of age. In addition, we have recently found that neuroblasts can be observed at SMG in both wild-type and MYCN hemizygous mice during mid- to late-generation period. If we can characterize these neuroblasts, we will be able to address mechanisms underlying early tumorigenesis of NB.

Methods: We investigated clusters of neuroblasts and subsequent tumor development in MYCN hemizygous mice. Furthermore, we established a novel spheroid culture method for neuroblasts.

Results: Our study has provided three important findings. First, our new culture method revealed that neuroblasts at 3 weeks of MYCN hemizygous mice provided spheres which could be passaged and gave rise to tumors. These data suggest that neuroblast clusters represent early stage of tumor. Second, while neuroblast clusters could be detected around 3 weeks of age at 100% incidence in MYCN hemizygous mice, only 80% of them developed tumors. This suggests that these mice could be a model of spontaneous regression. Third, neuroblasts at E13.5 of MYCN hemizygous mice generated spheres that were passageable and gave rise to tumors, but spheres from wild type mice at the same period could not be passaged. Therefore, although neuroblasts at this embryonic day from wild type and MYCN hemizygous mice are not distinguishable by histology, important molecular events may have occurred in such an early stage of embryogenesis.

Conclusion: Our new spheroid culture method opened an avenue which allowed us to address the fate determination of neuroblasts in NB tumorigenesis (i.e., tumor initiation and spontaneous regression).

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POB124

PBX1 is a Strong Favorable Prognostic Biomarker in Low-Risk and High-Risk Neuroblastoma and is Critical to Retinoid-mediated Differentiation

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Background: There are no clinically validated biomarkers in neuroblastoma that identify low-risk patients who will require adjuvant chemotherapy nor to identify high-risk patients who will not benefit from current therapies, including the differentiating agent 13-cis retinoic acid (13-cisRA). Neuroblastoma is an embryonic cancer with defined errors in normal differentiation pathways. The Three-Amino-acid Loop Extension (TALE) family of genes are critical regulators of differentiation but have not been comprehensively evaluated in neuroblastoma.

Methods: mRNA and protein expression of nine TALE family genes were measured in 15 neuroblastoma cell lines, with and without 13-cis RA. PBX1 expression was modulated in both RA-sensitive and RA-resistant neuroblastoma cell lines,and proliferation and differentiation evaluated. PBX1 expression was measured in low-risk and high-risk primary tumor samples, and for survival analysis in three independent clinical cohorts.

Results: Increased PBX1 expression correlated with sensitivity to 13-cisRA in neuroblastoma cell lines. RA-resistant cells showed no significant increase in PBX1 expression. No other TALE family genes correlated with RA sensitivity. Five MYCN-amplified and nonamplified cell lines were derived with modulated PBX1 expression. Increased expression caused decreased proliferation ($p<0.01$) and decreased anchorage-independent growth ($p<0.001$). Reduced PBX1 protein levels correlated with increased proliferation and anchorage-independent growth ($p<0.05$). PBX1 expression correlated directly with differentiation markers, including increased β-3 tubulin and TrkA/B protein levels and neurite extension ($p<0.01$). High PBX1 expression was observed in surgically-cured low-risk neuroblastoma and low expression in recurrent low-risk and high-risk disease ($p<0.001$). High PBX1 expression was a favorable prognostic marker in 3 independent clinical cohorts ($p<0.0001$), including in low-risk and MYCN-nonamplified metastatic disease subsets.

Conclusion: PBX1 expression correlates with RA sensitivity and potentiates differentiation in neuroblastoma. PBX1 is strongly significantly prognostic in both low-risk and high-risk disease, particularly MYCN-nonamplified high-risk disease. Clarification of the mechanisms which suppress PBX1 expression in RA-resistant, high-risk disease may identify new therapeutic targets for improved outcome.

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POB125

O-GlcNAcylation in Neuroblastoma

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Background: Despite advances and intensification in therapy, high-risk neuroblastoma (NBL) is fatal for half of affected children. Neuroblastoma is strikingly heterogeneous, and the markers that predict outcome, such as age, stage, and MYCN oncogene status are surrogates for underlying biological pathways responsible for tumor aggressiveness. Here, we investigate two separate but interrelated protein modifications, phosphorylation and O-GlcNAcylation, within MYCN-amplified and non-amplified NBL. Differential O-GlcNAc modifications, along with phosphorylation-associated "cross-talk," wherein both modifications compete for the same Ser/Thr site, have been implicated in many other cancers, however this is the first systematic study of these modifications in NBL.

Methods: LC-MS/MS analysis using HCD and CID fragmentation techniques, in conjunction with sWGA lectin enrichment (specific for GlcNAc), was performed on MYCN non-amplified SY5Y cells and MYCN amplified NLF cells. The detection of oxonium ions in HCD spectra was used to confirm the presence of O-GlcNAc peptides. The associated CID spectra were searched using standard procedures with GlcNAc as a variable modification both cell lines.

Results: Western blot analysis using O-GlcNAc antibodies confirms that O-GlcNAc modifications are increased in MYCN non-amplified SY5Y cells compared to MYCN amplified NLF cells. Analysis has identified 92 O-GlcNAc modified peptides from 84 proteins in NLF cells and 68 O-GlcNAc modified peptides from 66 proteins in SY5Y cells. Only thirteen O-GlcNAc modified peptides were found to be common between SY5Y and NLF samples, suggesting differential O-GlcNAcylation-based signaling between the two cell lines. Work is ongoing to validate these findings and to identify phosphorylation target sites in SY5Y and NLF cells.

Conclusion: Combining glycoproteomics and phosphoproteomic results using ANOVA-based approaches is a powerful method for revealing the presence of phosphorylation–O-GlcNAcylation interplay within proteins in both MYCN-amplified and non-amplified NBL. These studies will provide insight into the underlying signaling mechanisms in neuroblastoma resulting in better diagnostic and therapeutic strategies.

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POB126

Mechanisms Involved in As2O3 and ATRA Induced Differentiation

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Background: Neuroblastoma malignant cell growth depends on their differentiated status. All trans retinoic acid (ATRA) induces in vitro neuroblastoma cells differentiation and restores promyelocytic leukemia nuclear bodies (PML-NB). Arsenic trioxide (As_2O_3) may also trigger neuroblastoma neuritogenesis, but mechanisms still remains unknown. We explored the differentiation process induced by As_2O_3 treatment.

Methods: We used three human neuroblastoma cell lines (SH-SY5Y, IGR-N-91, LAN-1) that differ from their MYCN and p53 status to explore the activation of ERK pathway by As_2O_3 and its effects on differentiation markers such as neurite outgrowth and neurofilaments isoforms expression. In parallel, we observed PML-NB assembly by immunolabeling.

Results: As_2O_3 (2 µM) induced neurite outgrowth in all three cell lines in an ERK-dependent manner, despite different MYCN and p53 status. Similar results were obtained using either a sustained (3 days) or a transient (2 hours) treatment, suggesting that very early events trigger the differentiation process. In parallel, As_2O_3 induced a rapid assembly of PML-NB in an ERK dependent manner for SH-SY5Y and IGR-N-91, and in an ERK-independent manner for LAN-1 (Petit et al., 2013). These results are similar to those observed with 5 µM of ATRA suggesting potential similar mechanisms. The expression level of neurofilaments M, a neuronal differentiation marker, increased in SH-SY5Y following As_2O_3 or ATRA treatment. A combination of 0.5 µM of As_2O_3 and 1.25 µM of ATRA induced similar differentiation process and PML-NB formation than those obtained with 2 µM of As_2O_3 or 5 µM of ATRA alone.

Conclusion: This results suggest a synergistic effect between arsenic derivates and ATRA on neuroblastoma differentiation, which has to be confirmed in vivo. A combination of As_2O_3 or ATRA treatment, used at lower doses, could help to avoid resistance to treatment and to reduce side effects observed in patients.

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POB127

The Phosphorylation Status of Ascl1 Regulates Neuroblastoma Self-Renewal and Differentiation

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Background: Neuroblastoma (NB) bares striking similarity to undifferentiated neuroblasts of the sympathetic nervous system with an overdriven cell cycle due to overexpression of cyclin D1 and amplification of MYCN. We sought to understand how cell cycle and differentiation are linked within NB by focusing our efforts on Ascl1, a proneural transcription factor that is both necessary and sufficient for neural differentiation of noradrenergic neurons and has been shown to be regulated by the cell cycle. We hypothesized that Ascl1 phosphorylation by cell cycle dependent kinases (CDKs) critically regulates its ability to induce differentiation.

Methods: Ascl1 expression and phosphostatus was determined by western blot. NB cell lines with WT Ascl1 and a phosphomutant form, where CDK phosphorylation sites are mutated, were induced with doxycycline and analyzed for downstream target analysis by qPCR. NB cell lines were treated with palbociclib (Pfizer) for analysis of proliferation and differentiation.

Results: Paradoxically, Ascl1 is both highly expressed and associated with poor prognosis in primary NB tumor samples. Ascl1 is also expressed and phosphorylated across multiple NB cell lines. siRNA knockdown of Ascl1 results in decreased proliferation and overexpression of WT Ascl1 actively promotes the G1-S transition by upregulating E2f, Skp2, and Cdk2. However the phosphomutant form arrests cells in the G1 phase by preferentially upregulating Ebf3 and Btg2 which induce p27.

Conclusion: We hypothesized that pharmacologic inhibition of CDK activity would induce differentiation via Ascl1. In a panel of NB cell lines, we observed neuronal differentiation across multiple cell lines, but greatest differentiation in cell lines that express high levels of Ascl1. Treatment with CDK inhibitor may provide benefit to NB patients, particularly in those patients with high Ascl1, but may likely be more broadly applicable as primary NB tumors express Ascl1.

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Basic Research: Epigenetics

POB129

Silencing of CHD5 Expression by H3K27me3 in Human Neuroblastoma (NB)

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Background: CHD5 is a tumor suppressor gene located on 1p36. CHD5 is preferentially expressed in the nervous system and testis, and expression is very low or absent in high-risk NBs, especially those with 1p deletion and/or MYCN amplification. EZH2, a Polycomb group protein subunit of the Polycomb repressive complex 2 (PRC2), binds to promoter regions and causes histone-3 lysine-27 trimethylation (H3K27me3), leading to transcriptional suppression. This is an important regulation mechanism of gene expressions that are necessary for normal neural differentiation. Recent evidence suggests that MYCN contributes to the regulation of PRC2. We analyzed the H3K27me3 status, as well as the binding of EZH2 and MYCN, around the CHD5 transcription start site.

Methods: ChIP assay: Nuclear proteins were prepared from NB cell lines NLF (1p deletion), NGP (1p translocation, no deletion), NBLS (no 1p deletion) by crosslinking and sonication. Immunoprecipitation was performed with antibodies binding to EZH2, H3K27me3, MYCN and control IgG. After purification of bound DNA, PCR and qPCR were performed with primers designed around the CHD5 transcription start site, as well as negative control primers.

Results: H3K27 trimethylation was found in CHD5 promoter -250 bp and inside intron 1 in NLF and NGP cell lines. Both cell lines showed very low CHD5 expression. EZH2 binding was also found, consistent with the H3K27me3 in both NLF and NGP. We also found MYCN binding to the E-boxes around -250bp and -800 bp of the CHD5 promoter in NLF and NGP. These 3 factors (H3K27me3, EZH2, and MYCN) were not found around the CHD5 promoter of the NBLS cell line, which shows high CHD5 expression.

Conclusion: Our data strongly suggest that H3K27 trimethylation by EZH2 contributes to the epigenetic suppression of CHD5 expression, and that MYCN binding may also contribute to the suppression of CHD5 expression.

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POB130

Role of MicroRNAs in the Epigenetic Silencing of CHD5, a Tumor Suppressor in Neuroblastoma (NB)

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Background: MicroRNAs (miRNAs) are small RNAs that bind to the 3' UTR to mediate downregulation of target gene expression by translational repression or mRNA instability. In NB with 1p deletion, there is strong evidence that mutation of the remaining allele of CHD5, a tumor suppressor gene, is rare. Therefore, it is likely that epigenetic mechanisms play a role in CHD5 downregulation. Dysregulation of CHD5 via miR-211 has been implicated in colon cancer. In order to understand the role of miRNA regulation of CHD5 in NB cell lines we tested additional miRNAs that are predicted to target CHD5.

Methods: We used TargetScan, miRanda, MirTarget2 and others to identify miRNAs that were predicted to bind to the CHD5-3'UTR in 2 or more programs. We identified 18: miR-204-5p, -211, -216b, -17, -19ab, -20ab, -93, -106ab, -130ab, -301ab, -454, -519d, -3666. We used a renilla-luciferase reporter plasmid that contains 103 bp of the 3'UTR of CHD5 targeted by all miRNAs except miR-204 -211- 216b, and miR-3666. We also created plasmids with no CHD5 3'UTR insert and a mutated CHD5 3'UTR insert as controls. Allstar siRNA served as an additional negative control. We performed transient transfections in NLF cells, an NB cell line, with the reporter plasmid and miRNA mimic.

Results: Our results indicate that at least four (miR-17, -93, -20b, and miR-106b) of the 12 miRNAs tested were potent suppressors of the CHD5 3'UTR construct. Interestingly, MYCN upregulates three of these four miRNAs: miR-17, -93, and -20b. We then confirmed consistent downregulation of CHD5 by transfection with all four miRNAs.

Conclusion: Our results suggest that CHD5 expression may be downregulated in MYCN-amplified NBs at least in part by upregulation of miR-17, -93, and -20b. This may explain the inverse relationship between MYCN and CHD5 expression in NBs.

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POB131

DNA Hypomethylation Affects Cancer-Related Biological Functions and Genes Relevant in Neuroblastoma Pathogenesis

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Background: Neuroblastoma (NB) pathogenesis has been reported to be closely associated with numerous genetic alterations. However, underlying DNA methylation patterns have not been extensively studied in this developmental malignancy. Here, we generated microarray-based DNA methylation profiles of primary neuroblastic tumors.

Methods: A total of 25 primary neuroblastic tumors (22 NBs, 2 ganglioneuromas (GN) and 1 ganglioneuroblastoma (GNB)) were used for DNA methylation profiling using Infinium HumanMethylation27 BeadChip array (Illumina, USA). An independent cohort of 13 NBs and 2 GN was used for bisulfite pyrosequencing, gene expression and DNA copy number variation analyses. Normal human fetal brain (FB) and adrenal gland (AG) tissues were used as reference samples. Data were analyzed using the BeadStudio software (version 3, Illumina Inc, USA). CpG beta-value (β value) methylation levels ranged from 0 for completely unmethylated to 1 for completely methylated cytosines. Gender-specific and low quality CpGs were excluded from the study. Methylation microarray data have been deposited at Gene Expression Omnibus data repository (GSE39626).

Results: Stringent supervised differential methylation analyses allowed us to identify epigenetic changes characteristic for NB tumors as well as for clinical and biological subtypes of NB. We observed that gene-specific loss of DNA methylation is more prevalent than promoter hypermethylation. Remarkably, such hypomethylation affected cancer-related biological functions and genes relevant to NB pathogenesis such as CCND1, SPRR3, BTC, EGF and FGF6. In particular, differential methylation in CCND1 affected mostly an evolutionary conserved functionally relevant 3' untranslated region, suggesting that hypomethylation outside promoter regions may play a role in NB pathogenesis. Hypermethylation targeted genes involved in cell development and proliferation such as RASSF1A, POU2F2 or HOXD3, among others.

Conclusion: The results derived from this study provide new candidate epigenetic biomarkers associated with NB as well as insights into the molecular pathogenesis of this tumor, which involves a marked gene-specific hypomethylation.

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POB132

CASZ1b Interacts with Chromatin to Suppress Tumor Cell Proliferation

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Background: The Zinc finger transcription factor CASZ1b is a chr1p36 neuroblastoma tumor suppressor. How CASZ1b regulates gene transcription to exert its biological functions is poorly characterized.

Methods: To identify CASZ1b interactors that contribute its transcription activity, co-immunoprecipitation (co-IP) and mass spectrometry was performed using 293T cells. Western blot analyses were confirmed in both 293T cells and neuroblastoma cells.

Results: We found that CASZ1b binds to the nucleosome remodeling and histone deacetylase (NuRD) complex. Unlike other transcription factors, CASZ1b also binds to chromatin proteins including histones, H3 and H4. Serial mutagenesis of the CASZ1b protein in combination with co-IP demonstrated that the N-terminus of CASZ1b is required for NuRD binding, and a putative Poly(ADP-ribose) (PAR) binding motif in the zinc finger region of the CASZ1b protein is required for histone binding. PARP inhibitor treatment doesn't affect CASZ1 binding to H3 indicating this interaction isn't PAR-dependent. Realtime PCR results showed that CASZ1b mutations that abrogate NuRD or histone binding cause a >3-fold decrease in CASZ1b's ability to regulate NGFR and TH transcription ($p<0.01$). Unlike wild type CASZ1b, CASZ1b mutations that abrogate NuRD or histone binding have a 30% decrease in their ability to suppress neuroblastoma cell proliferation in vitro ($p<0.01$). Aside from neuroblastoma, low CASZ1 expression is significantly associated with high-grade breast cancer (AMC R2 database). To determine whether these functions are restricted to NB cells, we over-expressed CASZ1b and its mutants in breast cancer cells. Similar to neuroblastoma, wild-type CASZ1b overexpression suppressed breast cancer cell proliferation and mutations in CASZ1b significantly decreased CASZ1's ability to suppress breast tumor cell proliferation in vitro ($p<0.01$).

Conclusion: Our study indicates that CASZ1b binds to chromatin and recruits NuRD complexes to orchestrate epigenetic-mediated transcriptional programs that are

critical for cancer cell proliferation.

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POB133

Histone H3 Methyltransferase G9A Epigenetically Activates the Serine Synthesis Pathway to Sustain Cancer Cell Survival and Proliferation

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Background: Increased activation of the serine biosynthetic pathway is an integral part of cancer metabolism that drives macromolecule synthesis needed for cell proliferation. Whether this pathway is under epigenetic control is unknown. We addressed this question by examining the role of G9A in the control of neuroblastoma cell metabolism. G9A has a primary role in catalyzing monomethylation and dimethylation of H3K9 (H3K9me1 and H3K9me2) in euchromatin, with H3K9me1 being associated with transcriptional activation and H3K9me2 with transcriptional repression. Increased G9A expression has been observed in many types of human cancers.

Methods: G9A activity in neuroblastoma cells was modulated through loss- and gain-of-function approaches, and G9A target genes were identified by microarray and ChIP-seq assays. Metabolite profiling and [U-13C] glucose flux analysis were conducted using Gas chromatography-mass spectrometry and liquid chromatography tandem mass spectrometry, respectively.

Results: We found that G9A is essential for the survival of all the neuroblastoma cell lines examined. G9A inhibition or silencing induced cell death with autophagy as a result of depletion of serine and its downstream metabolites. Gene expression profiling and ChIP-seq revealed that G9A is required for maintaining the serine synthesis pathway enzyme genes in a transcriptionally active state marked by H3K9me1. Moreover, we obtained evidence that G9A is a component of the molecular pathway that couples serine sensing to the transcriptional control of serine production, ribosome biogenesis and cell proliferation. Finally, we found that higher G9A expression is prognostic for reduced overall survival in neuroblastoma patients, and G9A overexpression promoted cancer cell survival and proliferation by increasing the production of serine and its downstream metabolites.

Conclusion: Our findings identify a G9A-dependent epigenetic program in the control of cancer metabolism, providing a rationale for G9A inhibition as a therapeutic strategy for neuroblastoma.

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POB134

GRHL1 Acts as Tumor Suppressor in Neuroblastoma and is Negatively Regulated by MYCN and HDAC3

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Background: Neuroblastoma is an embryonic solid tumor of neural crest origin and accounts for 11% of all cancer-related deaths in children. Novel therapeutic strategies are therefore urgently required. MYCN oncogene amplification, which occurs in 20% of neuroblastomas, is a hallmark of high risk. Here we aimed to exploit molecular mechanisms that can be pharmacologically addressed with epigenetically modifying drugs, such as HDAC inhibitors.

Methods: Transcriptional changes were analyzed via gene expression profiling in time-course. Clinical relevance of candidate gene expression was evaluated in primary tumors. Functional studies were performed in preclinical neuroblastoma models.

Results: GRHL1, a gene critical for Drosophila neural development, belonged to the genes most strongly responding to HDAC inhibitor treatment of neuroblastoma cells in a genome-wide screen. An increase in the histone H4 pan-acetylation associated with its promoter preceded transcriptional activation. Physically adjacent, HDAC3 and MYCN co-localized to the GRHL1 promoter and repressed its transcription. High-level GRHL1 expression in primary neuroblastomas correlated on transcriptional and translational levels with favorable patient survival and established clinical and molecular markers for favorable tumor biology, including lack of MYCN amplification. Enforced GRHL1 expression in MYCN-amplified neuroblastoma cells with low endogenous GRHL1 levels abrogated anchorage-independent colony formation, inhibited proliferation and retarded xenograft growth in mice. GRHL1 knock-down in MYCN single-copy cells with high endogenous GRHL1 levels promoted colony formation. GRHL1 regulated 170 genes genome-wide and most were involved in pathways regulated during neuroblastomagenesis, including nervous system development, proliferation, cell-cell adhesion, cell spreading and cellular differentiation.

Conclusion: The data presented here indicate a significant role of HDAC3 in the MYCN-mediated repression of GRHL1 and suggest drugs that block HDAC3 activity and suppress MYCN expression as promising candidates for novel treatment strategies of high-risk neuroblastoma.

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POB135

Epigenetic Modulation of MYCN-Driven Transactivation in Neuroblastoma Cell Lines and Tumors

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Background: A unique feature of neuroblastoma (NB) is the high frequency of spontaneous regression occurring mostly in stage 4S tumors. On the other hand, amplified MYCN oncogene marks an unfavorable subgroup and is associated with poor patient prognosis. Paradoxically, high MYCN levels are also encountered in stage 4S tumors. The present study aims at elucidating whether differentiation pathways, which show elevated activity in stage 4S tumors, (epigenetically) repress MYCN-driven transactivation in NBs.

Methods: Customized oligonucleotide microarrays were used to determine expression of MYCN targets in 251 primary NBs. Reverse phase protein arrays (RPPA) were applied to assess MYCN expression in 93 NB tumors. Chromatin imunoprecipitation on chip (ChIP-chip), ChIP-sequencing (ChIP-seq), and ChIP-qRT-PCR were performed to assess MYCN binding and histone mark-defined epigenetic activity state dynamics in neuroblastoma cell lines, xenografts and tumors.

Results: Expression analysis reveals that, despite high MYCN protein levels, activation of MYCN targets in stage 4S tumors remains low. Intriguingly, experimentally-induced differentiation of NB cells causes relocation of MYCN from the core promoter of crucial target genes (e.g. ALK) to distal regions. This is accompanied by decreased activating histone marks (H3K4me3) and increased repressive histone marks (H3K4me27) on the genes/promoters and by reduced expression of the respective genes. To assess the in vivo situation we currently extend the ChIP protocol to allow analysis of tumor material. At present, reproducible results can be obtained for MYCN- and histone mark-tumor ChIP with subsequent qRT-PCR of specific loci in NB xenografts and in primary NBs. Now we approach tumor ChIP-sequencing for analyses on a genome-wide scale.

Conclusion: MYCN-driven transactivation is repressed in favorable neuroblastomas with high MYCN protein levels. Evidence is provided that epigenetic processes including MYCN relocation and chromatin remodeling on target promoters participate in this process.

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POB136

Histone Deacetylase 10 Promotes Autophagy-Mediated Cell Survival

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Background: Despite intense multimodal therapy and many improvements through basic scientific and clinical research, the successful response of advanced stage neuroblastoma patients to chemotherapy remains poor. Autophagy is a cytoprotective mechanism that helps advanced cancer cells to survive stressful conditions such as chemotherapy. Interference with this survival mechanism is a potential novel strategy to sensitize for chemotherapy.

Methods: We examined HDAC1-11 expression levels and their correlation with clinical outcome of advanced neuroblastoma treated with multimodal chemotherapy (INSS stage 4). Functional assays were used to decipher the function of the class IIb histone deacetylase family member HDAC10.

Results: We unraveled a novel mechanism of the so far poorly studied histone deacetylase 10 (HDAC10) in pediatric cancer biology. We show that both knockdown and inhibition of HDAC10 effectively disrupted autophagy associated with sensitization to cytotoxic drug treatment in different neuroblastoma cell lines, in contrast to non-transformed cells. HDAC10-depletion in neuroblastoma cells interrupted autophagic flux and induced accumulation of autophagosomes, lysosomes and a prominent substrate of the autophagic degradation pathway, p62/SQSTM1. Enforced HDAC10 expression protected neuroblastoma cells against doxorubicin treatment through interaction with HSP70 family proteins, causing their deacetylation. HDAC10 expression levels correlated with autophagy in gene set analysis and predicted treatment success in patients with advanced stage 4 neuroblastomas.

Conclusion: Our results demonstrate that HDAC10 protects neuroblastoma cells from cytotoxic agents by mediating autophagy, and identify this HDAC isozyme as a druggable regulator of advanced-stage tumor cell survival. Moreover, these results propose a new and promising way to considerably improve treatment response in the neuroblastoma patient subgroup with the poorest outcome.

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POB137

CHD5 Forms a Novel Chromatin Remodeling Complex and Protein Interactions in Neuroblastoma (NB) Cell Lines

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Background: CHD5 is a tumor suppressor gene that maps to 1p36.31 in NBs and other tumors. The CHD5 gene encodes a protein with chromatin remodeling, helicase and DNA-binding motifs that is preferentially expressed in nervous system and testis. CHD5 is highly homologous to CHD3 and CHD4, which are the core subunits of nucleosome remodelling and deacetylation (NuRD) complexes. We performed studies to determine if CHD5 forms a similar chromatin-remodeling complex.

Methods: The NB line NLF was stably transfected with CHD5 cDNA in the sense or antisense orientation, and stable clones were isolated. The NB lines NBLS and SY5Y express endogenous CHD5. Immunofluorescence microscopy was used to determine subcellular localization of CHD5 protein. CHD5 NuRD components were detected either by immunoprecipitation or after immunodepletion of CHD4 followed by using GST-FOG1 as an affinity reagent to purify the NuRD complex. Proteins were detected by SimplyBlue staining and by Western blot. LC-MS was used to confirm the presence of CHD5 and other associated proteins in the complex.

Results: Immunofluorescence demonstrated nuclear localization of CHD5 protein. We examined nuclear extracts from CHD5-transfected NLF, NBLS and SY5Y cells, to determine if CHD5 forms a NuRD complex similar to CHD4. V5/His-tagged CHD5 was immunoprecipitated from nuclear extracts with either V5 or CHD5 antibody, or pulled down with GST-FOG1 after CHD4 depletion. CHD5 associated with all canonical NuRD components, including MTA1/2, GATAD2A/B, HDAC1/2, RBBP4/7, and MBD2/3, as determined by western blotting and tandem MS. In addition, CHD5 was uniquely associated with the dynein heavy chain (DYNC1H1), as well as other novel proteins including TOP2A, AHNAK, ARID4A, by Western blotting and LC/MS.

Conclusion: Our data suggest that CHD5 forms a NuRD-type chromatin remodeling complex, similar to CHD4, but CHD5-NuRD associated uniquely with other proteins, which may contribute to normal development and to tumor suppression.

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POB138

Genetic Alterations Associated with Neuroblastoma Cause EZH2 Mediated Epigenetic Dysregulation Which Can Be Reversed by Pharmacologic Targeting of EZH2

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Background: High expression of the epigenetic regulator EZH2 in undifferentiated, poor prognosis neuroblastoma (NB) tumors leads to epigenetic repression of tumor suppressor genes. What drives EZH2 expression in NB is unknown. We hypothesize that genetic alterations associated with NB lead to dysregulation of EZH2 causing disruption of normal developmental programs.

Methods: Analyses performed on 6 NB cell lines and in ALK mutant or WT TET-MYCN SHEP cells.

Results: Increases in MYCN alone or with ALKWT or ALKF1174L increase expression of the EZH2 methyltransferase and its target H3K27me3. Silencing of MYCN caused decreases in EZH2 and H3K27me3. Targeting EZH2 using EZH2 shRNA or the pharmacologic inhibitor DZNep, decreases levels of EZH2 and H3K27me3 but not MYCN

mRNA. This indicates EZH2 functions downstream of MYCN. Six NB cell lines were treated with DZNep, which selectively depletes EZH2 and methylation of its target H3K27. DZNep inhibits proliferation in 6/6 lines tested (mean IC50=1.5uM, range 0.5-5uM). All showed increases in subG1, but caspase dependent apoptosis was detected in only 2/6 lines. In these 2 cell lines, DZNep induces a 2-fold increase in caspase3/7 activity and pre-treatment with the caspase inhibitor Z-VAD-FMK blocks DZNep induced cell death. In 4/6 NB cell lines, inhibition of EZH2 induces differentiation. Microarray analyses revealed that a commonly induced gene is NTRK1, a known marker of good prognosis in NB patients. Treatment with DZNep renders NB cells sensitive to NGF and induces increased differentiation. In animal studies, pharmacologic inhibition of EZH2 using DZNep inhibits tumor xenograft growth.

Conclusion: This study indicates that the genetic alterations in MYCN and ALK contribute to the dysregulation of EZH2. The finding that EZH2 suppressess NTRK1 suggests these genetic alterations in NB render cells less sensitive to important developmental factors and thus contribute to tumorigenesis. Targeting EZH2 may have therapeutic benefit in NB.

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POB140

Genome-Wide Methylation Analysis Unravels Differential DNA Methylation Patterns Between (Prognostic) Neuroblastoma Patient Subgroups

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Background: Neuroblastoma (NB) has been considered as a disease driven by genetic aberrations. However, recent studies have shown that the NB biology is also strongly determined by the epigenetic profile of the tumor, an important finding underscored by the fact that whole-genome sequencing of primary NB tumors revealed no highly recurrent mutations, apart from activating ALK mutations. By profiling the methylome of more than 100 primary NB tumors using methyl-CpG-binding domain (MBD) sequencing we reveal new insights into the NB epigenome and highlight DNA methylation changes as alternative targets of prognostic biomarker research.

Methods: For MBD sequencing, primary tumor DNA was sheared and methylated fragments were captured using the high affinity of the MBD of human MeCP2 towards methylated cytosines. This methylated fraction was then paired-end sequenced on the Illumina GAIIx or HiSeq2000 system. After demultiplexing, performing QC and mapping of the raw sequencing reads, peak calling and absolute methylation quantification analyses were conducted in order to identify methylation enriched regions and differentially methylated regions (DMRs) between (prognostic) patient subgroups.

Results: We show remarkable different methylation patterns between tumors with and without MYCN amplification, stage 4S and other tumors, and different prognostic patient subgroups (low-risk survivors, high-risk survivors and high-risk non-survivors). Top ranking differentially methylated regions were subsequently selected for further validation on an independent cohort of more than 200 primary tumors using our previously established methylation-specific PCR (MSP) technology.

Conclusion: In conclusion, this unique data set sheds new light on molecular alterations of the NB methylome and demonstrates the clinical utility of DNA methylation biomarkers in NB risk stratification.

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POB141

Epigenetic Regulation of Transcription Factor Binding Limits Differentiation Potential

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Background: Neuroblastomas are comprised of sympathetic neuronal precursor cells that are unable to differentiate appropriately, and the molecular mechanisms preventing their differentiation are unknown. We have developed a model system to study barriers to differentiation through the expression of 'master regulator' transcription factors. NeuroD2 and MyoD were among the first factors shown to induce transdifferentiation, or the direct conversion from one cell type into either neuronal or muscle cells, respectively. The ability to transdifferentiate is dependent on cell type specific regulatory factors, and we have determined that epigenetic regulation of transcription factor binding sites is a critical mechanism for limiting the differentiation capacity of different cell types.

Methods: We expressed the transcription factors NeuroD2 and MyoD as well as a chimeric factor in which the MyoD DNA binding region was replaced with that of NeuroD2 in two different cell types: 1) fibroblasts, which can convert to muscle upon expression of MyoD, but not neurons, and 2) P19 cells, which can convert easily to neurons upon expression of NeuroD2, but not muscle. We performed RNA-seq and ChIP-seq to determine the gene expression profiles and genome-wide DNA binding, respectively, associated with these factors in each cell type.

Results: The MD(ND2) chimeric bHLH factor induces the expression of both neuronal and muscle genes in P19 cells, and this is due to binding to both NeuroD2 and MyoD sites. Interestingly, by mutating the PBX/MEIS interaction domain, an important MyoD co-factor, activation of the myogenic program by the chimeric factor is abolished and neurogenesis is enhanced.

Conclusion: Re-targeting of a transcription factor to an alternative binding site is sufficient to induce expression of an alternative differentiation program, indicating that epigenetic regulation of transcription factor binding is a critical mechanism for determining differentiation potential. Additionally, transcription factor activity can be rationally manipulated through alteration of co-factor interactions.
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POB142

Genome-Wide Methylation Analysis in Neuroblastoma

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Background: Neuroblastoma (NB) is a childhood tumor originating from sympathetic nervous system cells. Much effort has been made to identify genes involved in the initiation/progression of neuroblastoma, but is still poorly understood. The best-characterized genetic alterations include amplification of proto-oncogene MYCN, amplification/mutation of ALK gene, and losses of 1p and 11q. On the other hand, aberrant DNA hypermethylation driven gene silencing of tumor suppressor genes is today recognized as a key event in pathogenesis of human cancers. Although DNA-methylation of CASP8 and RASSF1A are known to be associated with pathogenesis of neuroblastoma, comprehensive studies on methylation status in neuroblastoma have still limited.

Methods: To explore methylation phenotype of neuroblastoma, we performed DNA methylation microarray analysis of 50 neuroblastoma samples using illumina Infinium HumanMethylation450 BeadChip. Unsupervised hierarchical clustering has been applied to classify subgroups.

Results: Fifty specimens were clustered into 2 groups. These groups were clustered independently of the clinicopathological findings, such as age, stage, or outcome. MYCN or ALK status, 1p deletion, or 11q deletion was not related with these 2 groups. Four thousands selected genes were clustered into 6 groups. GSEA was performed with following clinicopathological findings; stage, age, 1p del, 11q del, MYCN amplification, ALK mutation. In MYCN amplification positive specimens, a gene set about ectoderm development was enriched. Several genes were enriched as hypomethylated genes. TFAP2A was included in these genes, which is known as a transcription factor that required for early neural crest development. This finding might be involved in the pathogenesis of NB.

Conclusion: Our results indicated that not only genetic heterogeneity but also epigenetic heterogeneity exists in neuroblastoma genomes. Comparing expression patterns and methylation subgroups would be necessary to disclose the roles of epigenetic regulation in neuroblastoma.

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POB143

Extracellular miRNA Expression Profiling of Conditioned Media Derived from In Vitro Models of Multidrug Resistant Neuroblastoma

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Background: The acquisition of multidrug resistance is the principle obstacle to the successful treatment of neuroblastoma. Thus, the elucidation of mechanisms involved in multidrug resistance is vital for the discovery of novel biomarkers and therapeutics. The aim of our work is to ascertain the contribution of miRNAs in the development of multi-drug resistance in neuroblastoma.

Methods: To this end we have developed three cell lines, KellyCis83, CHP212Cis100 and SKNASCis24 that are significantly resistant to cisplatin and other agents. MiRNA expression profiling of the lines has led to the identification of a panel of miRNA common across all drug resistant variants. Each miRNA is predicted to target genes involved with drug resistance, providing a firm basis for testing our hypothesis that these miRNAs modulate drug resistance. Based on the recent discovery that extracellular miRNAs are present in the bloodstream and that such circulating miRNAs are remarkably stable, we also demonstrate here that both drug resistant and parental neuroblastoma cell lines rapidly export a significant amount of miRNAs into their culture medium.

Results: These extracellular miRNAs have been recovered from filtered conditioned media and include all that are expelled into the media i.e., microvesicles, exosomes and free secretory miRNAs. Using miRNA Taqman low density arrays we identified a number of miRNAs differentially expressed in KellyCis83, SKNASCis24 and CHP-212Cis100 respectively, when compared to their parental counterparts. This list includes miRNAs which have previously been implicated in neuroblastoma pathogenesis as well as those with prior associations with an acquired multidrug resistance phenotype in cancer. Of particular note is the observation that miR-34a, a known tumour suppressor, although undetectable in the drug resistant cell lines is abundant in the media compared to the parental cells.

Conclusion: Our findings lend further support to the idea of a novel miRNA trafficking system concurrent with the cell–cell communication hypothesis.
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POB144

Histone Deacetylase 2 and N-Myc Reduce p53 Protein Phosphorylation at Serine 46 and p53 Activity by Repressing Gene Transcription of Tumor Protein 53-induced Nuclear Protein 1

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Background: Myc oncoproteins and histone deacetylases (HDACs) exert oncogenic effects by modulating gene transcription. Paradoxically, N-Myc induces p53 gene expression. Tumor protein 53-induced nuclear protein 1 (TP53INP1) phosphorylates p53 protein at serine 46, leading to enhanced p53 activity, transcriptional activation of p53 target genes and programmed cell death. We aimed to identify the mechanism through which N-Myc overexpressing p53 wild-type neuroblastoma cells acquired resistance to apoptosis.

Methods: Gene and protein expression was analysed by real-time RT-PCR and immunoblot. Cell survival/death was examined by flow cytometry study of Annexin V-staining. The prognostic value of TP53INP1 expression in tumor tissues was investigated in three independent cohorts of neuroblastoma patients.

Results: TP53INP1 was one of the genes most significantly repressed by HDAC2 and N-Myc according to Affymetrix microarray gene expression datasets. HDAC2 and N-Myc reduced TP53INP1 gene expression by direct binding to the TP53INP1 gene promoter, leading to transcriptional repression of TP53INP1, p53 protein dephosphorylation at serine 46, neuroblastoma cell proliferation and survival. Moreover, low levels of TP53INP1 expression in human neuroblastoma tissues correlated with high levels of N-Myc expression and poor patient outcome, and the BET bromodomain inhibitors JQ1 and I-BET151 reduced N-Myc expression and reactivated TP53INP1 expression in neuroblastoma cells.

Conclusion: These findings identify TP53INP1 repression as an important co-factor for N-Myc oncogenesis, and provide further evidence for the potential application of BET bromodomain inhibitors in the therapy of N-Myc-induced neuroblastoma.

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POB145

The mir-15 Family Members Impair Neuroblastoma Growth by Reducing Cell Proliferation and Angiogenesis

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Background: High-risk NBL are very aggressive and often become refractory to all current forms of treatment. Intrinsic or de novo acquired therapy resistance is characterized by the multiple drug resistance (MDR) phenotype, which is composed of diverse cellular factors and signal transduction pathways. Owing to the multiple mechanisms that lead to chemoresistance, targeting single components of a pathway may not suffice to render NBL vulnerable to therapy. Thus, it is desirable to find molecules that can regulate multiple cellular processes or different components of the same pathway, thereby overcoming MDR and improving the therapeutic response. We propose the use of microRNAs (miRNAs) to target the oncogenic properties of aggressive NBL and render them vulnerable to treatment.

Methods: The expression of genes related to MDR was analyzed in mRNA expression NBL data sets. The 3'UTR of the genes differentially expressed in the highest-risk neuroblastoma group (Stage 4, MYCN amplified) was scanned with miRNA-binding sites prediction algorithms. Twenty-eight miRNAs predicted to regulate the expression of 3 or more overexpressed MDR genes, were transfected in two different chemoresistant NBL cell lines and cell proliferation was monitored. The function of the miRNA with higher therapeutic potential was evaluated in a xenograft NBL model.

Results: MicroRNA-binding prediction algorithms revealed that several MDR genes overexpressed in high-risk NBL could potentially be regulated by microRNAs. Functional analysis in chemoresistant NBL cell lines identified several microRNAs that regulate proliferation, the best of which were the miR-15 family members. The overexpression of miR-497 reduced de proliferation of multiple chemoresistant NBL cell lines and induced apoptotic cell death in MYCN-amplified cell lines. Moreover, the conditional expression of miR-497 in NBL xenografts reduced cell proliferation and inhibited angiogenesis.

Conclusion: Several MDR-related genes are deregulated in high-risk NBL and can be simultaneously targeted with miRNAs, providing a new therapeutic approach for chemoresistant neuroblastomas.

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POB146

Activation of the Calcium-Sensing Receptor in Neuroblastoma Cells Induces Apoptosis Dependent on Activation of Phospholipase C and ERK1/2

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Background: We have previously reported that the calcium-sensing receptor (CaSR) is expressed in benign, differentiated neuroblastic tumors, but it is downregulated in unfavorable neuroblastomas.

Methods: We have analyzed epigenetic mechanisms of transcriptional silencing and allelic loses by interphase fluorescence in situ hybridization (i-FISH). Also, the effects of CaSR ectopic overexpression and reactivation have been examined.

Results: Hypermethylation of a specific region within the CpG island encompassing the CaSR gene promoter 2 was detected in 25% primary neuroblastomas, in association with reduced CaSR mRNA expression and several predictors of poor outcome in neuroblastomas, including MYCN amplification. Hypermethylation of this region was also detected in MYCN-amplified neuroblastoma cell lines in which the CaSR expression was reduced or absent. Treatment with 5'aza-2-deoxycitidine (Aza) and/or trichostatin A restored the expression of the CaSR in these cell lines and, following Aza exposure, concurrent decreased percentages of methylated CpG sites were observed at the abovementioned region. Monosomy of chromosome 3, where the human CaSR gene resides, was seen in >90% of primary neuroblastic tumors of all clinical, histological and biological subgroups. Furthermore, overexpression of the CaSR in MYCN-amplified cell lines in which this gene is silenced by promoter hypermethylation significantly reduced their in vitro proliferation rates and almost abolished their capacity to generate xenografts in immunocompromised mice. Finally, upon acute activation of this receptor with several ligands, neuroblastoma cells with natural or ectopic expresion of the CaSR underwent apoptosis dependent on activation of phospholipase C and ERK1/2.

Conclusion: These data would support the hypothesis that epigenetic silencing of the CaSR gene is a mechanism relevant for survival of neuroblastoma cells and provide proof-of-principle indicating that activation of the CaSR might be a new therapeutic approach for malignant neuroblastomas.

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POB147

miR-324-5p Triggers Mitochondria-mediated Cell Death in Neuroblastoma

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Background: MiRNAs are post-transcriptional regulators of gene expression and their deregulation can contribute to pathogenesis of many different cancer types, including neuroblastoma. The expression levels of specific miRNAs can be significantly associated with clinical outcome.

Results: MiRNA-324-5p is significantly down regulated in MYCN amplified tumours (Bray et al, PLoS One. 2009 4:e7850) and analysis of 328 tumours in the Neuroblastoma Research Consortium dataset (Amsterdam, Dublin, Essen, Genoa, Ghent) revealed that down-regulation of this miRNA is significantly associated with poor overall patient survival (see Figure; $p = 4.3e^{-4}$). Ectopic over-expression of miR-324-5p mimics transfection into Kelly and CHP212 cells significantly reduced cell viability ($p=0.016$), indicating a potential tumour suppressive function. The TargetScan miRNA target site prediction algorithm indicated that the voltage-dependent anion channel 1 (VDAC1), a primary constituent of the outer mitochondrial membrane, was a potential target. Over-expression of miR-324-5p in Kelly, NB1691, SKNAS cells led to a significant decrease in both VDAC1 mRNA and protein, while the use of luciferase reporter plasmids confirmed that miR-324-5p directly targets the VDAC1 3' UTR ($p=0.008$). siRNA mediated inhibition of VDAC1 resulted in a reduction in mitochondria genes MT-CYB ($p=0.0002$), MT-ND4L ($p=0.0003$) in Kelly cells, suggesting a mitochondria-mediated cell death, though the precise mechanism of action has yet to be understood.

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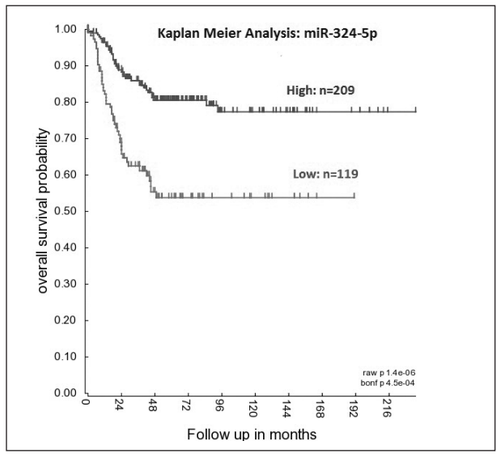

POB148

MYCN-Amplified Neuroblastoma Cells Depend on HDAC11 for Mitotic Cell Cycle Progression and Survival

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Background: Expression of HDAC11, the most recently identified histone deacetylase, is restricted to the cell nuclei in poorly differentiated neuroblastomas. We here aimed to decipher the functional relevance of distinct alterations in gene expression caused by HDAC11 depletion.

Methods: Whole-genome gene expression was evaluated in MYCN-amplified neuroblastoma cell lines following HDAC11 depletion. The biological function of genes consistently regulated over time across each cell system was assessed by analyzing gene ontology term over-representation. Cell cycle and cell death assays were conducted after HDAC11 depletion combined with candidate gene enforced expression. In primary neuroblastomas, differential expression of candidate genes was assessed in three independent datasets.

Results: HDAC11 depletion strongly induced neuroblastoma cell death, mostly mediated by apoptotic programs. The induction of cell death was highest in the presence of high-level MYCN expression. On a molecular level, genes necessary for mitotic cell cycle progression and cell division were most prominently enriched. All genes, among them CENPA, KIF14, KIF23 and RACGAP1, were found strongly repressed. Enforced expression of selected candidate genes partially rescued the induction of apoptosis caused by HDAC11 depletion. Candidate gene expression was significantly higher in MYCN-amplified primary neuroblastomas, suggesting a role in mediating the more aggressive clinical phenotype of MYCN-amplified tumors.

Conclusion: This study identified a group of cell cycle-promoting genes regulated by HDAC11, being both predictors of unfavorable patient outcome and essential for tumor cell viability. Thereby, the data indicates a significant role of HDAC11 for mitotic cell cycle progression and survival of MYCN-amplified neuroblastoma cells.

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POB149

Promoter DNA-Methylation in the TH-MYCN Neuroblastoma Mouse Model

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Background: High-resolution genome-sequencing efforts have discovered a wealth of mutations in genes encoding epigenetic regulators, providing evidence for their predominant role in cancer development. In a previous study, we identified prognostic methylation biomarkers in neuroblastoma using genome-wide promoter methylation analysis. However, therapeutic targeting of the neuroblastoma epigenome remains poorly explored.

Methods: Therefore, as a prelude to testing of novel epigenetic drugs, we performed MBD-based genome-wide methylation sequencing to establish the methylation pattern in a series of seven tumors from a MYCN-driven neuroblastoma mouse model.
Results: Seven hundred nineteen promoters, representing 516 unique genes, are commonly methylated in all seven mouse tumors. This list of methylated genes mainly consists of protein coding genes and pseudogenes, but also contains long noncoding RNAs, microRNAs and small nucleolar RNAs, of which long noncoding RNAs and microRNAs rank amongst the most extensively methylated genes (on the basis of peak height). Interestingly, the protocadherin alpha and beta clusters, which are methylated in virtually every human neuroblastoma tumor, are fully methylated in all seven mouse tumors, supporting the notion that this model can be applied to study DNA methylation in neuroblastoma. Finally, initial cross-species methylation analysis, suggest several cancer-associated genes as interesting hyper-methylated candidate genes for further study.

Conclusion: Together, these data show that the TH-MYCN mouse model is valuable in the study of neuroblastoma epigenetics, and provide the rationale for testing of novel epigenetic drugs.

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POB150

Epigenetic Mechanisms in Retinoic Acid Sensitive and Resistant Neuroblastoma Cells

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Background: Amplified MYCN is associated with poor patient outcome in neuroblastoma. Retinoic acid (RA) is an established element of high-risk neuroblastoma therapy. RA induces neuronal differentiation in some but not all high-risk neuroblastomas. The mechanism of RA neuronal differentiation as well as resistance remains still unclear. In this study, the landscape of chromatin states and epigenetic changes upon neuronal differentiation using all-trans retinoic acid (ATRA) treatment were elucidated.

Methods: This was obtained by performing chromatin-immunprecipitation sequencing (ChIP-seq) and 450k DNA methylation arrays. These two epigenetic mechanisms were compared to gene expression using RNA-seq and gene expression arrays. The neuroblastoma cell line SK-N-BE-2C served as a well-established in vitro model for neuronal differentiation.

Results: In some neuroblastoma cell lines RA treatment causes cell growth arrest and morphological differentiation. Additionally, MYCN and MYCN-bound genes are down regulated upon RA treatment. 144 h after ATRA application distinct changes in MYCN binding and epigenetic marks were observed at many different genes. Most noticeable was a shift of MYCN binding to promoter distal regions, e.g. in case of the ALK gene. This was associated with transcriptional down regulation of ALK. Moreover, there was an enrichment of H3K4me3 at the promoter region of many active genes (e.g. NTRK1). H3K4me3 enrichment implies transcriptional activity, which correlates with gene expression results. Besides the changes of histone modification there was no difference in DNA methylation after neuronal differentiation in the mentioned time frame.

Conclusion: Accordingly, we suggest a mechanism for gene expression regulation that is initially affected by a rapid chromatin modification in our in vitro model. A subsequent long term gene regulation by DNA methylation might occur later in time and has to be confirmed in further studies. Additionally, an in vivo model of differentiation with RA sensitive cells in an orthotopic mouse model is in planning stage.

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POB151

CHD5, a Chromatin Remodeling Protein, is Required for Spermiogenesis and Chromatin Condensation

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Background: Haploid spermatids undergo extensive cellular, molecular and morphological changes to form spermatozoa during spermiogenesis. Abnormalities in these steps can lead to serious male fertility problems, from oligospermia to complete azoospermia. CHD5 is a chromatin-remodeling nuclear protein expressed almost exclusively in the brain and testis. It is of great importance to learn the role that CHD5 protein plays in the extensive chromatin compaction in late spermatogenesis.

Methods: We generated Chd5 knockout (KO) by gene targeting. Sperm were harvested for protein analysis, and testis slides were stained with PAS or H&E, and immunohistochemistry. Electron microscopy was used to identify ultra-microscopic structures in seminiferous tubules.

Results: Male Chd5 knockout (KO) mice have deregulated spermatogenesis, characterized by immature sloughing of spermatids, spermiation failure, disorganization of the spermatogenic cycle and abnormal head morphology in elongating spermatids. This results in the inappropriate placement and juxtaposition of germ cell types within the epithelium. Sperm that did enter the epididymis displayed irregular shaped sperm heads, and retained cytoplasmic components inappropriately. These sperm also stained positively for acidic aniline, indicating improper removal of histones and lack of proper chromatin condensation. Electron microscopy showed that spermatids in the seminiferous tubules of Chd5 KO mice had extensive nuclear deformation, with irregular shaped heads of elongated spermatids, and lack the progression of chromatin condensation in an anterior-to-posterior direction. However, the mRNA expression levels of other important genes controlling spermatogenesis were not affected. Chd5 KO mice also showed decreased H4 hyperacetylation beginning at stage IX, step 9, which is vital for the histone-to-transition protein replacement in spermiogenesis.

Conclusion: In Chd5 KO mice, later spermatids showed defects in chromatin condensation related changes in cellular phenotypes. Our data indicate that CHD5 is required for normal spermiogenesis, especially for spermatid chromatin condensation.

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POB152

microRNAs and Chemoresistance in Neuroblastoma

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Background: Neuroblastoma is a neoplasm of the sympathetic nervous system representing the most frequently diagnosed solid tumor in infants. Despite continued improvements in cancer treatment, the overall survival of patients with high risk neuroblastoma is still only 40-50%. Irrespective of risk factors, neuroblastomas generally respond well to initial therapy. However, the majority of high risk patients relapse with tumors refractory to standard chemotherapeutic agents. Therefore, the understanding of biological and molecular aspects of drug resistance in neuroblastoma may provide new opportunities for therapy of aggressive neuroblastoma., Recent evidence has revealed a substantial role of microRNAs (miRNAs) in multidrug resistance in various cancer types. MicroRNAs are small (18-24 nucleotides) non-

coding RNA molecules that regulate the expression of genes at the post-transcriptional level by either direct cleavage of target mRNAs or repression of translation. Several studies indicate that deviant expression of certain miRNAs correlate with poor clinical outcome in neuroblastoma. However, the role of miRNAs in neuroblastoma cell resistance to chemotherapeutic drugs is poorly understood.

Methods: To explore the role of miRNAs in the resistance of neuroblastoma cells to anticancer drugs, we generated miRNA cDNA libraries from six isogenic human neuroblastoma cell line pairs established from the same patients at the time of initial diagnosis and relapse following therapy. To analyze expression patterns of miRNAs, a deep sequencing analysis (SOLiD sequencing) was performed using the miRNA cDNA libraries.

Results: Deep sequencing analysis (SOLiD sequencing) revealed differential expression patterns of miRNAs before and after treatment. Systematic analysis of these miRNA expression patterns identified potential alterations in pathways associated with drug resistance suggesting that dysregulation of miRNAs might influence sensitivity to therapy.

Conclusion: We anticipate that our findings will provide new insights into the molecular mechanisms of drug resistance in neuroblastoma.

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POB164

MYCN in Neuroblastoma: A Transcriptional and Epigenetic Regulator

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Background: Neuroblastoma (NB) is a type of embryonal tumours that arises from precursor cells of the sympathetic nervous system. The MYC gene family members are crucial for understanding the biology of NB, since they affect almost every aspect of their behaviour, driving poor outcome.

Methods: Our initial experiments were aimed at the identification of the genomic regions bound by MYCN, with particular emphasis on its target genes. We addressed this by a ChIP-seq (Chromatin Immuno Precipitation sequencing) approach in the human neuroblastoma SH-SY5Y cell line with inducible MYCN expression. In silico analysis of the ChIP-seq prompted us to further investigate the interplay of MYCN and epigenetic regulation, with a specific focus on CpG methylation and interaction with epigenetic writers and readers.

Results: Bioinformatics analysis revealed that MYCN binds numerous targets across the genome creating a dynamic pattern of transcriptional regulation. Interestingly, we observed just a small proportion of binding sites within promoters. However, the majority of binding occurred in intergenic, enhancer/silencer regions or introns, thus suggesting a strong interplay between epigenetic mechanisms and transcriptional regulation mediated by MYCN. In silico pathway analyses showed that a number of MYCN targets are part of the neuronal regulatory networks involved in signalling, survival, drug resistance and differentiation. Testing a panel of six NB cell lines, both MYCN amplified and non-amplified, with a demethylating agent revealed strong response in most of the cell lines. Also, treatment with a panel of forty five epigenetic leads showed strongly reduced viability for at least four compounds.

Conclusion: Specific epigenetic profile of neuroblastoma cells affects the MYCN transcriptional network and probably the progression of NB. Further experimental work will be aimed at the assessment of global epigenetic patterns in NB, integration of experimental results with other -omics datasets, mathematical modeling and identification of therapeutically targetable nodes.

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POSTER EXHIBITION

TRANSLATIONAL RESEARCH

Translational Research: Immune Response

POT001

Galectin-1 Secreted by Neuroblastoma NXS-2 Cells Induces IL-10 Production by B Cells

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Background: B cells with regulatory activity emerge as novel cellular mediators of immune balance. CD19+ B cells were found to suppress T effector cell function in cancer, mainly by IL-10 secretion. This suggests that IL-10 producing B cells, so-called B10 cells, modulate the immune response against neuroblastoma (NB) and are an interesting cellular target to evaluate when designing therapeutic strategies to boost the host´s immune system against one challenging childhood tumor.

Results: We were able to show that NB elicits a Gal-1-dependent program of immune suppression that hinders the immune system eradicating the tumor. In a syngenic NB mouse model (NXS2 cells provoke NB), we showed that knockdown transfectants expressing low amounts of Gal-1 (NXS2/L) showed reduction of primary tumor growth and prevented spontaneous liver metastases in contrast to NXS2 cell variants expressing high amounts of Gal-1. Here, we investigated whether B10 cells are involved in Gal-1-induced immunosuppression. We performed in vitro experiments on CD19+ B cells purified by fluorescence-associated cell sorting (FACS). Harvested B cells from A/J PBMC population were cultured for 24 hour in serum-free condition medium (SFCM) derived from NXS2 cultures or in DMEM. We were able to detect an increase in the IL-10 production in tumor-touched $CD19^+$ B cells compared to CD19+ B cells cultured in DMEM. The increase in the IL-10 production was comparable to that after LPS stimulation. Interestingly, the incubation of $CD19^+$ B cell in DMEM containing recombinant Gal-1 also enhanced IL-10 production in the $CD19^+$ B cell population.

Conclusion: We introduce Gal-1 as a novel modulator of Breg in NB. Our data highlights the therapeutic importance of B10 as their suppression may help to combat this severe childhood cancer.

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POT002

Crosstalk of Neuroblastoma Cells, Monocytes/Macrophages, and Mesenchymal Stromal Cells Promotes Growth and Impairs Anti-Tumor Efficacy of Activated Natural Killer (NK) Cell

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Background: Continuous crosstalk of neuroblastoma and normal cells in primary and metastatic sites likely creates a complex milieu that promotes tumor growth and suppresses immune responses. Models of tumor microenvironments were developed with neuroblastoma cells, monocytes, and mesenchymal stromal cells (MSC) to investigate effects on tumor cell growth, cytokine production, and NK cell anti-tumor activity.

Methods: Neuroblastoma cell lines (CHLA-255, CHLA-136, SMS-KCNR, and SK-N-BE2), bone marrow-derived MSC, blood monocytes, and K562mbIL21 grown/activated blood NK cells (aNK) were used. Conditioned medium (CM) was from 72-hour co-cultures of neuroblastoma cells, monocytes, and MSC (NMM-CM) and various combinations. Cytokines in CMs were quantified with Luminex® assays. Neuroblastoma cell growth in vitro (CellTiter-Glo assay), aNK cytotoxicity/ADCC in vitro (bioluminescence of luciferase [Fluc] labeled neuroblastoma cells), and neuroblastoma growth subcutaneously in NOD/SCID mice (bioluminescence of neuroblastoma-Fluc cells and tumor volume) were determined.

Results: CHLA-255 NMM-CM (50% v/v) increased growth of CHLA-255 4-fold and CHLA-136 cells 2-fold after 72 hours, which was greater than CHLA-255 N-CM. Co-injection of monocytes and MSCs with CHLA-136 or CHLA-255 increased tumor growth in mice compared to neuroblastoma alone or neuroblastoma with monocytes or MSC. NMM-CM generated with all neuroblastoma cell lines contained >500pg/ml CCL2/MCP1, CXCL1/GRO, CXCL8/IL8, IL-6, and VEGF, whereas N-CM from all had only VEGF >500pg/ml. TGFβ1 was >200pg/ml in NMM-CM and N-CM. Culturing aNK cells with NMM-CM (50% v/v) or TGFβ1 for 48 hours suppressed aNK cytotoxicity and anti-GD2 mAb ch14.18 ADCC against neuroblastoma cells, and suppression was blocked by TGFBR1 inhibitor LY2157299. Co-injected aNK cells (5%) + intravenous ch14.18 were significantly less effective against NMM tumors than N tumors. Culturing aNK for 48 hours with NMM-CM or TGFβ1 markedly suppressed their anti-tumor efficacy in the same NMM tumor model.

Conclusion: Neuroblastoma, monocyte, and MSC interactions promote tumor cell growth, generate multiple cytokines, and suppress cytotoxic NK cells.

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POT003

NKp30 Isoforms Dictate Clinical Outcome in High Risk Neuroblastoma

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Background: Despite intensive multimodal treatment, prognosis of childhood with high-risk neuroblastoma (HR-NB) remains poor with 5-year survival rates of 40%. Neuroblastoma (NB) is known to be sensitive to Natural Killer lymphocytes (NK) lysis. In a recent study we showed that the alternative splicing of the NCR3/NKp30 gene can affect NK cell function and gastrointestinal stromal tumors (GIST) patient's outcome. We proposed to investigate the NK cell receptor NKp30/NCR3 role in NB.

Methods: Exhaustive immunomonitoring has been performed on fresh peripheral (PBMC) and bone marrow NB blood samples. B7-H6-NKp30 ligand expression and its soluble form, sB7-H6, was explored on bone marrow NB cells and on NB cell lines. The NKp30 transcriptional status was investigated and correlated with clinical data in two independent HR-NB cohorts.

Results: In contrast to localized NB and chemotherapy-responsive HR-NB, chemotherapy-resistant HR-NB patients were characterized by a selective downregulation of NKp30 expression on $CD56^{dim}$ bone marrow NK cells. NB cells were found to express B7-H6 and stimulated NKp30 positive effector cells in a B7-H6-dependent manner. The soluble NKp30 ligand B7-H6 accumulated in the serum of HR-NB patients, correlating with NKp30 downregulation on peripheral blood NK cells, disease dissemination and resistance to chemotherapy. The transcriptional status of the NKp30/NCR3 gene dictates the event-free survival of HR-NB with minimal residual disease: dominant expression of the stimulatory NKp30B versus the inhibitory NKp30C isoform (DBC^{high}) on circulating NK cells predicts 10-year survival in two independent cohorts of HR-NB in complete remission after induction chemotherapy. Moreover we generated anti-NKp30C directed silencing RNA (siRNA) molecules able to abrogate the immuno-suppressive NKp30C expression.

Conclusion: NB appears to be controlled by NK cells, through a functional interaction between NKp30 and B7-H6, and the expression pattern of stimulatory versus inhibitory NKp30 isoforms dictates the clinical fate of HR-NB patients.

NKp30C-siRNA nanoparticles are promising for new modality of NB treatment.

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POT004

Monitoring Immune Modulation Induced by Anti-GD2 Monoclonal Antibody Therapy in High-Risk Neuroblastoma

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Background: Neuroblastoma, a childhood tumour of neuroectodermal origin, accounts for 15% of paediatric cancer deaths; often metastatic at diagnosis, and despite aggressive therapies has poor long-term prognosis with high-risk of recurrence. Monoclonal antibody (mAb) therapy targeting GD2, a disialoganglioside expressed on neuroblastoma, has shown promise in recent trials with natural-killer cell (NK) mediated antibody-dependent-cellular-cytotoxicity (ADCC) thought to be central to efficacy, although other immune effectors may be important. To further enhance therapy, immunomonitoring of patients is essential to further elucidate the in-vivo mechanisms of action. Our aim was to establish a 'real-time' ex-vivo whole blood (WB) immunomonitoring strategy to perform within the logistical constraints such as limited sample volumes; anti-coagulant effects; sample stability and shipping time.

Methods: A fluorescent-dye release assay, using calcein-acetoxymethyl ester (calcein-AM) to measure target-cell lysis was coupled with flow cytometry to monitor specific effector response. Heparinised and EDTA anti-coagulated WB was used as a source of effectors as an alternative to peripheral blood mononuclear cells (PBMC), with serum from clotted blood used to monitor the level of complement-mediated cytotoxicity.

Results: Significant target-cell lysis with anti-GD2-antibody ($p>0.05$) was abrogated following NK depletion from PBMC, confirming their role in ADCC. NK up-regulation of the activation markers CD107a and CD69, positively correlated with target-cell lysis ($r>0.6$). The ADCC activity of heparinised WB correlated with peripheral blood mononuclear cells (PBMC) ($r>0.95$), although WB showed overall greater target-cell lysis that was attributed to the combination of NK-mediated ADCC and CD16+ granulocyte degranulation and complement-dependent-cytotoxicity. Response was maintained in heparinised samples stored for 24hours at room temperature, but not 4°C. Critically, the assay showed good reproducibility (mean %CV<6.4) and was successfully applied to primary neuroblastoma samples.

Conclusion: WB provides a more holistic analysis of the multiple immune response mechanisms for efficient endpoint monitoring with small volume samples, to correlate immune-modulation with clinical outcome

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POT005

The T Cell Immune Landscape in Neuroblastoma and its Impact on Clinical Outcome

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Background: Neuroblastomas grow within an intricate network of different cell types including epithelial cells, endothelial venule cells, circulating factors and infiltrating immune cells. Despite the fact that the T cell immune infiltrates are one of the most important predictive criteria for patient survival, the complex interactions between neuroblastoma cells and their microenvironment remain to be elucidated. Herein, we examined composition, density, architecture and functional organization of specific intratumoral immune cell populations within neuroblastoma lesions representing all genetic subsets.

Methods: Eight-five neuroblastoma cases were selected for immunoistochemical detection of CD3+, CD4+, CD8+, CD25+ and Foxp3+ cell infiltration. Intratumoral proliferating CD3+ T cells were visualized by ki67 co-staining and quantitative imaging analysis was performed.

Results: Most of the T cell markers, including CD3+, CD4+ and CD8+, were longranked significant and associated with a good prognosis (disease-free survival), while the CD25 marker of regulatory T cells exerted only a moderate positive effect. Interestingly, CD3+, CD4+ and CD8+ markers were variably distributed between tumour nests and stromal septa and highly correlated each other. Furthermore, neuroblastoma of stage 4S and stage 4 displayed different T cell immune landscapes: the first were characterized by proliferating T lymphocytes that come into close contact with the tumour cells, while the latter displayed only non-proliferating tumor cells far away from tumour cells. Consistent with their role in suppressing tumor progression, a high density of intra-tumoral proliferating T cells in stage 4S neuroblastoma may explain their prognostic impact on tumor development or progression.

Conclusion: This study reveals that the T cell immune landscape in neuroblastoma is a hallmark of the tumour microenviroment associated with tumor progression and prognosis.

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POT006

A Role of Surface Sialylation in Immune Evasion of Neuroblastoma

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Background: Immune evasion is one of the hallmarks of cancer. The aim of this study was to analyze if aberrant glycosylation pattern of neuroblastoma cells, a typical alteration to be found on malignant transformed cells, contributes to the immune escape mechanism of this tumor. Abnormal surface sialylation leading to increased α2,8-linked sialic acids is characteristic for neuroblastoma cells. These ligands are bound by sialic acid-binding immunglobulin-like lectins (siglecs), which are expressed on immune effector cells including NK cells. It was hypothesized that neuroblastoma cells escape from NK cell-mediated lysis by engagement of this inhibitory receptor.

Methods: The siglec expression on immune cells was analyzed by flow cytometry. The extracellular binding domains of human siglec-5 and siglec-7 were cloned and expressed with a biotinylation sequence. Biotinylated siglecs were then tetramerized with streptavidin-phycoerythrin (SA-PE) to analyze siglec-ligand expression on neuroblastoma cells. Functional relevance of neuroblastoma cell sialylation for NK cell mediated lysis was investigated in cytotoxicity assays.

Results: Flow cytometry showed the expression of siglec-5 and siglec-7 on immune cells, especially siglec-7 on NK cells. Using siglec-tetramers in flow cytometry experiments, it was shown that neuroblastoma cell lines express ligands for both siglec-5 and siglec-7. Binding of siglecs was abrogated by neuraminidase treatment of the cells, which demonstrated that the sialylation of the ligands was crucial for binding. In addition, the functional relevance of siglec-ligands for immune escape was analyzed. It was shown that desialylation of neuroblastoma cells by neuraminidase treatment lead to an increased NK cell mediated lysis indicating the involvement of sialylation in immune escape mechanisms of neuroblastoma cells. Specifically, addition of monomeric siglec-7 to the cytotoxicity assay increased NK cell-mediated lysis.

Conclusion: These findings demonstrate that neuroblastoma cell sialylation plays a role in immune escape. Specifically, engagement of siglec-7 on NK cells by its ligand on neuroblastoma cells reduced their effector function.

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POT007

Myeloid-Derived Suppressor Cells, MDSCs, Contribute to Immune-Suppression in MYCN-Driven Neuroblastoma Providing Understanding of Tumor Development and Novel Targets of Therapy

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Background: New developments in tumor-immunotherapy show promising results for several cancers and might provide new opportunities for neuroblastoma treatment. To better define suitable immunological targets, it is crucial to understand the role of the immune system and define mechanisms underlying neuroblastoma-induced immune suppression. We have previously demonstrated accumulation of pro-inflammatory immune cells within tumor tissues and effective anti-inflammatory treatment with low-dose aspirin in homozygous animals of the TH-MYCN mouse model. Thus, this model can be further used to dissect detailed mechanisms aiming at defining new targets for neuroblastoma immunotherapy.

Methods: Flow-cytometric analysis and in vitro coculture assays with splenocytes of neuroblastoma-bearing TH-MYCN mice were performed. Immune-cell status and functions were compared to wildtype controls. Moreover, reagents targeting different immune suppressive mechanisms were tested.

Results: Neuroblastoma -bearing animals develop splenomegaly, indicating the active role of the peripheral immune system during MYCN-driven neuroblastoma development. Flow-cytometric analysis of spleens showed accumulation of $CD11b^+Ly6c^+Ly6g^+$ myeloid-derived suppressor cells (MDSC) in tumor-bearing mice compared to wildtype controls. Total numbers of $CD4^+$ and $CD8^+$ T cells were reduced, while the CD4/CD8 ratio and activation markers of T cells remained unchanged. To our surprise, even though accumulation of regulatoryT cells could be found in tumor tissues, their frequency was not significantly enhanced in spleens of cancer-bearing animals, indicating that MDSCs play a major role in peripheral immune suppression. Sorted from spleens of tumor-bearing mice, these MDSCs were capable of suppressing T cell proliferation in vitro through the productionof indolamine 2,3-dioxygenase (IDO) and cyclooxygenase-2 (COX-2).

Conclusion: Tumor-promoting inflammation contributes to neuroblastoma development with active involvement of the peripheral immune system but further studies are needed to define factors of the tumor microenvironment inducing direct or indirect immune-suppression. Finally, immunological interventions by targeting various suppressive mechanisms will be evaluated in this model aiming at future clinical application.

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POT120

Natural Killer Cell Functions are Impaired in Patients with Neuroblastoma

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Background: While prognosis for Neuroblastoma (NB) patients presenting at <18 months is usually favourable, approximately 45% of patients present with high risk tumours where prognosis for survival is poor. Therefore, identifying factors involved in NB development and progression is important for understanding the molecular basis of the disease and the potential for the development of new therapeutic approaches. Natural killer (NK) cells play a key role in the immuno-surveillance of tumours and have been shown to kill both NB cell lines and primary tumour cells. Some of the receptor interactions involved in adult NK cell killing of NB cells have been identified. However, the expression of these receptors as well as the efficacy of NK cell function in NB patients is unknown.

Methods: To investigate modulation of NK cell function in NB, we analysed surface expression of CD107a, a marker of NK cell degranulation, as well as intracellular IFN cytokine expression in NB patients. Surface expression of a panel of activatory NK cell receptors was also examined to determine if expression of key triggering receptors correlated with NK cell activity in NB.

Results: While expression of the activatory receptors investigated was unaltered, NK cell-mediated induction of IFN and cytolytic activity was substantially impaired in NB. Comparison of important NK cell regulatory cytokines showed that IL12/IL15-mediated activation of NK cell function was more dramatically reduced than IL2-induced NK cell functions.
Conclusion: Impaired NK cell functions in NB may represent an evasion mechanism to facilitate disease progression. Our data showing more severe impairment of IL12/IL15-mediated NK cell activation may have implications for immunotherapy regimes using IL12 that are currently being investigated in murine models for the treatment of NB.

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POT126

Galectin-1 Modulates the Immune Response and Angiogenic Properties in a Transgenic Model of Neuroblastoma

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Background: Galectin-1 (Gal-1) is a multifunctional protein that enhances tumor aggressiveness by inducing angiogenesis and contributing to the tumor immune escape. In neuroblastoma (NB), Gal-1 expression is correlated to invasive properties of NB cells in vitro. Here, we aimed to assess the effect of Gal-1 on the immune system and also on tumorigenesis using modulation of Gal-1 expression in immune effector cells and a transgenic NB model, respectively.
Methods: We first assessed the uptake of Gal-1 by immune cells using fluorescence-tagged recombinant Gal-1. To further examine the role of Gal-1 in immune cells, we ectopically expressed Gal-1 in CD4+ T-cells isolated from wild type mice., We next investigated the immune phenotype of neuroblastic tumors and control tissue in the established TH-MYCN NB mouse model as a function of the Gal-1 gene dosage using Gal-1 knockout mice. Furthermore, we analysed immune cell infiltration and angiogenesis by immunohistochemistry.

Results: Gal-1 was localized to the cell membrane of lymphocytes whereas antigen presenting cells ingested the protein. Ectopic expression of Gal-1 resulted in autocrine- as well as paracrine-mediated inhibition of T-cell proliferation in line with an immunosuppressive role of Gal-1., Tumor tissue from TH-MYCN mice differed in lymphocytic infiltration compared to double transgenic TH-MYCN, Gal-1-/- mice. While the frequency of CD4 T cells tended to decrease in Gal-1 deficient NB mice, the fraction of CD11c-positive dendritic cells was significantly elevated. Reduced Gal-1 gene dose correlated with reduced tumor angiogenesis. Interestingly, the tumor incidence was higher in the double transgenic TH-MYCN, Gal-1-/- mice compared to TH-MYCN mice, although this did not reach statistical significance.

Conclusion: These results confirm a role for Gal-1 in modulating the immune phenotype and angiogenesis in a transgenic NB model. The implications of targeting Galectin-1 in therapeutic settings, as it is currently being developed for other tumor entities, remain to be explored for NB.

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Translational Research: Preclinical Experimental Therapies

POT008

Exome Sequencing Reveals a Genomic Drift in Chemoresistant versus Chemosensitive Neuroblastoma Cell Lines

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Background: Resistance to chemotherapy limits the effects of treating relapsed neuroblastoma. To understand the molecular mechanisms of chemoresistance, a precise understanding of the genomic alterations in cells exposed to cytotoxic drugs is required.

Methods: We obtained exome sequences, DNA methylation patterns and mRNA expression profiles for the parental Kelly NB cell line as well as Kelly cells continuously exposed to either doxorubicin or cisplatin. Exome sequence data were analysed using the in-house developed platform "Exomate" and compared to epigenetic and transcriptome changes.

Results: Few additional mutations were acquired in Kelly cells resistant to doxorubicin, which were instead found to harbor an amplification of the MDR1 locus. In contrast, more than 4.000 single nucleotide variants were accumulated in cisplatin-resistant Kelly cells compared to the parental cell line. Interestingly, these mutations were not randomly distributed, but showed an enrichment of CT over TT di-

nucleotides in line with a kataegis-like event. Correlation of mutational status to mRNA expression and DNA methylation patterns was gene-specific. Moreover, cisplatin-resistant cells presented with multiple single nucleotide variations within individual genes including resistance modifiers such as BCL11A and PTEN.

Conclusion: When designing personalized approaches for treating relapsed NB, not only acquisition of MDR but also the mutational profile of patients pretreated with DNA-binding drugs have to be taken into account.

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POT009

The Histone Deacetylase Inhibitor, Panobinostat Induces Apoptosis and Prolongs Survival in the TH-MYCN Murine Model of High-Risk Neuroblastoma

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Background: Deregulated acetylation of histones plays a key role in the pathogenesis of haematological as well as solid tumours by changing the chromatin structure and consequently altering transcription of genes involved in cell cycle control, differentiation and apoptosis. Inhibitors of histone deacetylases (HDACs) can therefore result in multiple cellular responses, and are considered to be potent inducers of cell death. Thus, there is considerable interest in HDAC inhibition as a potential therapeutic modality in haematological and solid tumour malignancies.

Methods: Homozygous TH-MYCN transgenic mice underwent serial abdominal ultrasounds (US) from five weeks of age until neuroblastomas of between 50mm^3 and 200 mm3 were detected. Mice were treated continuously for 9 weeks with low dose (5mg/kg) panobinostat, a pan-HDAC inhibitor. Repeat US were performed twice weekly. Tumours were harvested 48hr post-dose for western blot and immunohistochemical analysis of key proteins of the apoptosis pathways as well as biomarkers of HDAC inhibition. In-vitro analyses of sensitivity and apoptotic response to panobinostat were also performed in the neuroblastoma cell lines, IMR32, SK-N-SH and NH02A.

Results: Treatment with panobinostat significantly improved survival with 100% of TH-MYCN mice alive at day 63 compared with vehicle (0.0%, mean survival 7 days; $p<0.0001$). One hundred days after the withdrawal of drug 88.9% of panobinostat treated mice remained alive ($p<0.0001$). Panobinostat induced rapid tumour regression as determined by US, and resulted in significant apoptosis mediated through the caspase-dependent pathway via up-regulation of the BH3 only pro-apoptotic proteins, BMF and BIM. These findings were confirmed in the in vitro studies utilising the neuroblastoma cell lines.

Conclusion: Treatment of TH-NMYC mice with panobinostat significantly improved survival and reduced tumour burden, primarily via induction of apoptosis. These preclinical findings indicate that HDAC inhibition is a promising therapeutic strategy, and supports the further evaluation of panobinostat as a treatment option for high-risk neuroblastoma patients.

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POT010

AT7519 Potently Inhibits Tumour Growth of MYCN-Amplified Neuroblastoma Tumours

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Background: Neuroblastoma patients with MYCN amplification (i.e. 20% of all patients) have a poor clinical outcome. We previously showed that CDK2 inhibition is synthetic lethal to MYCN-overexpressing neuroblastoma cells. This made CDK2 a promising target for the future treatment of high risk neuroblastoma patients with MYCN-amplification. We tested the efficacy of the novel available CDK2 inhibitor AT7519 in vitro and in vivo and additionally studied the in vivo pharmacokinetics and –dynamics of the drug.

Results: Evaluation of the in vitro efficacy of AT7519 in a large panel of neuroblastoma cell lines showed that MYCN-amplified neuroblastoma cells were more sensitive to AT7519 than non-MYCN-amplified cells. Treatment of MYCN-amplified neuroblastoma cells with AT7519 resulted in apoptosis, as shown by increased PARP cleavage and increased subG1 fractions. No or moderate apoptosis was observed for the non-MYCN-amplified cells., Treatment of mice bearing xenografts of the MYCN-amplified neuroblastoma cell line AMC711T with 5-, 10- or 15 mg/kg/day AT7519 resulted in dose-dependent tumour growth inhibition. Drug responses correlated with the total tumour exposure to AT7519, as shown by the estimated AUC values of the intratumoural drug concentrations at multiple time points. AT7519 reduced the phosphorylated levels of retinoblastoma protein (Rb) and nucleophosmin (NPM) in the tumour. CDK2 targets p-Rb and p-NPM can therefore be used as efficacy biomarker. The efficacy of AT7519 was additionally tested in Th-MYCN transgenic mice with spontaneously developed neuroblastoma tumours. A stronger antitumour response was observed as compared with the AMC711T neuroblastoma xenograft model. Treatment with 15 mg/kg/day AT7519 resulted in strong tumour regression and improved long-term survival. The improved efficacy observed in Th-MYCN transgenic mice could be explained by the higher tumour exposure to AT7519.

Conclusion: Current study showed that the CDK2 inhibitor AT7519 is a promising drug candidate for the future treatment of high risk neuroblastoma patients with MYCN amplification.

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POT011

Continous Long-Term Low-Dose Topotecan Treatment Induces Tumor Cell Senescence, Regression and Tumor-Inhibiting Functions in a Neuroblastoma Mouse Model

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Background: There is an unmet need for novel treatment strategies for high-risk neuroblastoma (NB) patients, frequently encountering recurrent disease. In contrast to high-dose induction regimens, mainly triggering apoptosis, continuous low-dose treatment with DNA-damage-inducing drugs in vitro leads to tumor cell senescence, a state of proliferative arrest. However, little information on the role of senescent tumor cells is available and none in the context of NB. We have explored senescence-induction as treatment strategy for high-risk NB by establishing an in vivo model for low-dose-therapy-induced senescence and studied the role of senescent tumor cells in vitro and in vivo.

Results: Screening of candidate drugs, targeting cell cycle regulation and/or inducing DNA-damage, in MYCN-amplified cell lines for anti-proliferative activity and senescence, identified camptothecin (CPT), a topoisomerase I-inhibitor, to trigger apoptosis and senescence within 3 weeks at low concentration (1-5nM). Importantly, secretome analysis revealed that CPT-treatment did not result in the secretion of a panel of angiogenesis- and metastasis-associated proteins, while treatment with BrdU does. In vivo experiments in xeno-transplanted mice confirmed that topotecan (TPT), a CPT derivative, applied daily at low dose (0.1mg/kg/d) over 2 weeks, enhances the frequency of senescent tumor cells and reduces tumor size and vascularization. A tumor cell inhibiting/anti-proliferative function of senescent on non-senescent NB-cells could be verified in co-culture experiments. In line with a less aggressive phenotype, a reduction of the MYCN-copy number and expression was observed in low-dose TPT treated NB-cells in vitro and in vivo. Furthermore, expression profiles of TPT-treated tumors, revealed known and new senescence markers/pathways.

Conclusion: Thus, we demonstrated that low-dose continuous drug-treatment induces senescence and – depending on the drug - this is associated with tumor-inhibiting properties in vitro and in vivo. Moreover, we have successfully established an in vivo model to study drug-response in NB, including both, apoptosis and senescence, allowing further evaluation of novel drugs.

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POT012

Preclinical Development of Therapeutic Combinations Targeting ALK to Overcome Primary Resistance

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Background: Our cataloguing of ALK mutations from diagnostic tissues, correlating sequence variations with oncogenicity, and the phase 1 Children's Oncology Group (COG) trial of the ALK inhibitor crizotinib has revealed that some of the observed mutations have properties suggestive of intrinsic resistance to direct ALK inhibition. We hypothesize that, even if not effective as a single agent, crizotinib in combination with cytotoxic agents is necessary for cooperative and durable inhibition of ALK-mutant NB.

Methods: To assess the effects of ALK inhibition on DNA damage-mediated cell death, we combined continuous dosing of crizotinib with topotecan and cyclophosphamide (topo/cyclo) at clinically achievable doses in patient-derived (PDX) and conventional xenograft models with varying ALK status and crizotinib sensitivity.

Results: In SHSY5Y (ALK-F1174L, resistant mutation) xenografts, combination therapy achieved complete tumor regressions and significantly prolonged survival ($P<0.0001$) compared to crizotinib or topo/cyclo alone. In COG-N-426x (ALK-F1245V, resistant mutation) PDXs, similar anti-tumor efficacy was observed in the combination arm, with only mild growth delay in the crizotinib-alone group and moderate growth delay in the topo/cyclo group. In NB1643 xenografts (ALK-R1275Q, sensitive mutation), continuous single agent crizotinib achieved complete but only transient regressions with regrowth of tumors at five weeks; topo/cyclo achieved transient regression with regrowth at eight weeks, but the combination arm achieved complete and sustained responses ($P<0.0001$). Combination treatment also considerably delayed tumor growth in a wild type-ALK xenograft expressing phosphorylated ALK (NB-EBC1, $p<0.0001$). In xenografts harboring p53 aberrations (NB1691, NBSD, SKNAS), even with co-occurrence of an ALK mutation, crizotinib enhanced activity of topo/cyclo but all tumors regrew rapidly on therapy. Systematic assessment of these drug combinations in vitro is ongoing to elucidate molecular mechanisms of the interactions.

Conclusion: These data have provided the rationale for the currently accruing COG phase 1 trial combining crizotinib with chemotherapeutic agents, and provisionally support integration of crizotinib upfront in patients with ALK-aberrant neuroblastoma.

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POT013

Treatment with a Glutamine Antagonist Inhibits Growth of Neuroblastoma Tumors, Rachelle Olsen, St. Jude Children's Research Hospital, Oncology, Memphis, TN, United States

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Background: Oncogenic Myc can cause metabolic dependence on exogenous glutamine, and MYCN-amplified neuroblastoma is associated with very poor prognosis. Identifying therapeutic agents effective against neuroblastoma (NB), particularly MYCN-amplified tumors, could significantly increase patient survival. We used the glutamine antagonist DON (6-diazo-5-oxo-norleucine) as a tool to inhibit glutamine metabolism in neuroblastoma. DON is a competitive inhibitor which irreversibly alkylates glutamine-utilizing enzymes. DON was first explored as a cancer chemotherapeutic in the 1950s, although it was associated with severe nausea which limited its use in patients.

Methods: DON sensitivity of six human neuroblastoma cell lines (IMR-32, Kelly, SK-N-AS, SK-N-BE2, SK-N-FI, and SK-N-SY5Y) and BJ fibroblast control cells was determined by CyQuant assay. For tumor experiments, cells were injected subcutaneously into nude mice. Established tumors (200mm^3) were randomized into DON (100mg/kg) or water control groups and treated i.p. twice/week. To examine the mechanism of DON-induced cell death, NB cells were treated with DON combined with 20μM pan-caspase inhibitor QVD and cell viability was quantified. Lastly, we performed an in vitro screen to identify compounds that increase the effects of DON.

Results: DON inhibited growth of NB cells and DON susceptibility correlated with glutamine addiction ($R^2=0.73$). In preclinical models, DON treatment was cytostatic and significantly inhibited both tumor growth and BrdU incorporation. Furthermore, DON-treated SK-N-BE2 tumors had increased levels of cleaved caspase-3, an apoptotic marker. In vitro studies showed that DON induces cell death partially by activating apoptosis, and we identified three pro-apoptotic compounds which sensitized cells to DON. Navitoclax, a Bcl-2 family inhibitor, caused the greatest increase in DON activity across the entire panel of cell lines tested.

Conclusion: DON is a broadly effective inhibitor of neuroblastoma cell lines and tumors. In addition, our results suggest that targeting glutamine metabolism while reducing the threshold for tumor cell apoptosis may be beneficial for treatment of neuroblastoma.

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POT014

NCL-1 Inhibits KDM1A and Has Antitumoral Activity by Inducing a Less Aggressive Phenotype in Neuroblastoma Cells In Vitro and In Vivo

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Background: Epigenetic changes in DNA and histone methylation are hallmarks of most cancers. Several histone demethylases have been identified, most of which catalyse the removal of methyl groups from histone H3 lysine residues to influence gene expression. The KDM1A histone demethylase regulates demethylation of dimethyl marks on H3K4 and H3K9. A recent report from our group indicates that targeting KDM1A could be a therapeutic option for neuroblastoma. Here we tested the potential usefulness of the new KDM1A-specific small molecule inhibitor, NCL-1, against neuroblastoma cell lines in culture and grown as xenograft tumours in mice.

Methods: We analyzed the effect of NCL-1 on 4 neuroblastoma (NB) cell lines. KDM1A activity was accessed using an ELISA-based assay. We performed cell viability analysis (MTT) and analyzed markers for apoptosis and proliferation. Differentiation markers were analyzed by qPCR. In vivo efficacy of NCL-1 was assessed in SKNAS xenografts.

Results: NCL-1 effectively inhibited demethylation of H3K4me2 and H3K9me2 by KDM1A in the neuroblastoma cell lines, SHEP, SK-N-BE, SK-N-AS and IMR5. IC50s for NCL-1 ranged between 38-48μM for the cell lines tested. NCL-1-mediated inhibition was more effective for KDM1A than the monoamine oxidase inhibitor, tranylcypromine. Inhibition mimicked the effects of siRNA-mediated KDM1A knockdown in vitro and suppressed cell viability and proliferation, while inducing expression of the neuronal differentiation marker, neurotensin. Interventional NCL-1 treatment of neuroblastoma xenografts in mice significantly delayed tumour growth, as compared to vehicle treated mice.

Conclusion: High levels of KDM1A expression in neuroblastoma cells are likely to contribute to the maintenance of an undifferentiated state. Inhibiting KDM1A shifts the balance from proliferation towards differentiation, as demonstrated by the decrease in neuroblastoma cell viability and proliferation accompanied by induction of neurotensin expression. These results indicate that targeting KDM1A specifically with NCL-1 could be a beneficial addition to treatment regimens for patients with aggressive neuroblastomas.

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POT015

Novel Therapeutic Strategy for Neuroblastoma with the Combination of 13-cis-retinoic Acid and Oncolytic Virus

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Background: Recently, antitumor activities of inactivated Sendai virus particle (Hemagglutinating virus of Japan-envelope, HVJ-E) were found and clinical trials of HVJ-E for adult cancers are ongoing. We investigated the antitumor effects of HVJ-E alone and the combination with 13-cis-retinoic acid(13cRA) against neuroblastoma.

Methods: 1) The cell viability of seven human neuroblastoma cell lines after HVJ-E treatment was assessed by MTS assay, and the expression of GD1a and SPG, receptors for HVJ, was assessed by HPLC analysis. The xenograft tumor was created in SCID mice using SK-N-SH cells, the most sensitive to HVJ-E, and intratumoral injections of either PBS or HVJ-E were performed, then the tumor volume was compared between the two groups. 2) NB1 cells, the most resistant to HVJ-E, were pretreated with 13cRA, then the same experiments as SK-N-SH were performed. The antitumor effects were compared between PBS, HVJ-E, 13cRA, and 13cRA+HVJ-E groups. All animals were handled according to the approved protocols and guidelines of the animal committee of our institute.

Results: 1) The sensitivity of each cell line to HVJ-E significantly correlated with the expression level of GD1a and SPG, with Pearson's correlation coefficient of 0.80. Complete eradication of SK-N-SH-derived xenograft was achieved by HVJ-E treatment without tumor recurrence or signs of toxicity. 2) HVJ-E significantly decreased the survival rate of NB1 cells from 86.8±3.4% to 40.9±3.6% by the pretreatment with 13cRA which enhanced the expression rate of GD1a from 0.6% to 5.0%. Partial eradication of NB1-derived xenograft was achieved by the combination of 13cRA and HVJ-E, although only growth inhibition of the tumor was achieved by HVJ-E alone.

Conclusion: We are the first to demonstrate the antitumor activity of HVJ-E against neuroblastoma and the potentiality of 13cRA as an adjuvant to HVJ-E both in vitro and in vivo. These findings will open new therapeutic strategies for neuroblastoma.

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POT017

Galectin-1 Minigene DNA Vaccine is Effective against Neuroblastoma

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Background: We recently identified Galectin-1 (Gal-1), a multifunctional glycan-binding protein, as an immunoregulator in neuroblastoma (NB). Gal-1 negatively regulates T cell and dendritic cell function resulting in a blockage of host immune responsiveness against NB. Due to its increased expression and secretion by NB cells Gal-1 emerges as an excellent antigen target for novel active vaccination strategies. Here, we generated minigene DNA vaccines encoding exclusively for Gal 1-derived peptides with superior MHC class I binding affinities and tested its efficacy in a syngeneic NB mouse model.

Methods: Vaccination was performed in a prophylactic setting by oral gavage of attenuated SL7207 (AroA-, 108 per mouse) carrying minigene or pU control plasmids. Thereafter, A/J mice were challenged with lethal dosages of NXS-2 NB cells (2 x 106 s.c.).

Results: Epitope screening was performed with online syfpeithi database (www.syfpeithi.de) and computer docking experiments. "FDQADLTI" (FDQ), "GDFKIKCV" (GDF) and "AHGDANTI" (AHG) were predicted with superior H2-Kk and "KFPNRLNM" (KFP), "DGDFKIKCV" (DGD) and "LGKDSNNL" (LGK) with superior H2-Dd binding affinities. DNA sequences encoding for i) FDQ-GDF-AHG (G1-KK), ii) KFP-DGD-LGK (G1-DD) as well as iii) a triplet of highest affinity G1-epitopes FDQ-GDF-KFP (G1-H) were generated by overlapping PCR and resulting minigenes were cloned into a ubiquitin containing plasmid (pU). Mice receiving the pUG1-KK or pUG-1H presented up to 80% reduction in s.c. tumor volume and tumor weight in contrast to mice treated with empty vectors and untreated controls. Vaccination with pUG1-DD plasmid showed less suppressive capabilities on primary tumor progression. Isolated splenocytes from succesfull vaccinated mice were more cytotoxic as indicated by an increased NXS2 target cell lysis.

Conclusion: Vaccination with DNA plasmids encoding for rational designed Gal-1 epitopes is effective against NB.

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POT018

Targeting Mcl-1 Dependence with EGFR Inhibition in High-Risk and Relapsed Neuroblastoma (NB)

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Background: High-risk (HR) NBs resist apoptosis through Bcl-2 or Mcl-1 sequestration of pro-apoptotic Bim. ABT-737 potently kills Bcl-2 dependent NBs, yet no therapies target Mcl-1 directly. Receptor tyrosine kinases (RTK) regulate Mcl-1 in many cancers and play a role in NB proliferation, yet how they regulate Bcl-2 family interactions in NB was unknown.

Methods: We performed an RTK phosphoprotein microarray on Bcl-2 dependent CHLA15 and an ABT-737 resistant, Mcl-1 dependent clone, CHLA15-ABTR. RTK's identified were evaluated for functional effects on Mcl-1 dependent SKNBE(2), NLF, and CHLA15-ABTR.

Results: Phospho-EGFR is increased in CHLA15-ABTR compared to CHLA15. Immunoblots of 10 NB cell lines confirm increased pEGFR, pERK, pAKT in NBs with de novo Mcl-1 dependence and in Mcl-1 dependent NB cell lines derived from the same tumor at diagnosis and following relapse (equally high EGFR) compared to Bcl-2 dependent pairs (equally low EGFR). Following shRNA inhibition of EGFR or Erlotinib treatment of Mcl-1 dependent NBs, Bim and Noxa protein increases and Bim moves from Mcl-1 to the binding pocket of Bcl-2, despite redundant Mcl-1/Bcl-2. The same occurs with ERK (U0126) but not PI3K (LY294002) inhibition. Erlotinib and U0126 treatment of NLF cause Bim to run lower/faster on immunoblot, suggesting loss of a post-translational modification. Lambda phosphatase treatment of NLF decreases Bim in an Mcl-1 immunoprecipitate, confirming Bim de-phosphorylation displaces Bim from Mcl-1. Consequently, Erlotinib synergizes with ABT-737 against Mcl-1 dependent NBs in vitro.

Conclusion: EGFR regulates acquired and de novo Mcl-1 dependence in HR and relapsed NBs via ERK-mediated phosphorylation of Bim and downregulation of Noxa. This may explain why Erlotinib was ineffective in Phase I trials in NB, because Bim is sequestered by Bcl-2 following EGFR inhibition. Our results show EGFR inhibitors are most effective when combined with Bcl-2 antagonists and support that therapies that increase Noxa (Bortezimib, HDACi) will also enhance ABT-737 in HR NB.

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POT019

Targeting the Retinoic Acid Receptor α and thymosin-β4 with a Combination of Fenretinide and Vorinostat Impairs Cell Survival and Migration of Neuroblastoma Cells

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Background: Retinoids are an important component of advanced neuroblastoma therapy at the stage of minimal residual disease, yet 40-50% of all patients treated with 13-cis-retinoic acid (13-cis-RA) still relapse and die, indicating the urgent need for more effective retinoid therapy.

Methods: The effects of fenretinide (4-HPR), a novel synthetic retinoid combined with Vorinostat (SAHA), a histone deacetylase inhibitor, were examined in human neuroblastoma BE(2)-C and SH-SY5Y cells, and compared with 13-cis-RA+ SAHA. We used flow cytometry, cell migration assay, gene-expression analyses, siRNA knockdown, chromatin immunoprecipitation assay and a xenograft tumour mice model in this study.

Results: At clinically relevant concentrations, the 4-HPR (1.33-3μM) + SAHA (0.22-0.5μM) combination exerted synergistic cytopathic effects in two neuroblastoma cell lines (combination index < 1). 4-HPR+SAHA increased Caspase 3 activity and was a more potent inducer of apoptosis than 13-cis-RA+SAHA. The combination also blocked colony formation and significantly decreased cell migration. In vivo xenograft experiments of BE(2)-C cells in nude mice treated with 4-HPR (1.45mg/kg, i.v.) + SAHA (35mg/kg, i.p.) significantly decreased tumour growth. The combination was associated with down-regulation of retinoic acid receptor α (RARα) and up-regulation of thymosin-beta-4-X (Tβ4) mRNA expression. Importantly, low RARα ($p \leq 0.05$) and high Tβ4 ($p \leq 0.001$) expression was associated with good patient prognosis among 102 primary neuroblastoma tumour tissues. Down-regulation of RARα and up-regulation of Tβ4 was necessary for the 4-HPR+SAHA cytotoxic effect shown by siRNA knockdown and plasmid overexpression studies. RARα directly bound the Tβ4 gene promoter and acted as a trans-repressor for Tβ4 transcription. Tβ4 siRNA knockdown blocked the effect of 4-HPR+SAHA on cell migration and focal adhesion formation, indicating Tβ4 was necessary for the 4-HPR + SAHA therapeutic effects.

Conclusion: 4-HPR + SAHA was a substantially more effective therapeutic than 13-cis-RA alone or with SAHA, and, should be considered in future neuroblastoma clinical trials.

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POT020

Hu3F8 Bispecific Antibody to Engage T cells against Neuroblastoma

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Background: The ability to utilize T cells intelligently can lead to a fundamental shift in cancer immuno-therapeutics. Bispecific antibodies (BsAb) permit the targeted engagement of polyclonal T cells and exploitation of their effector functions through HLA-non-restricted CD3-mediated activation, hence overcoming previous hurdles, such as low T cell clonal frequency and loss of tumor HLA. Humanized anti-GD2 antibody hu3F8 has proven to be less immunogenic and safe in patients. It targets neuroblastoma, as well as many GD2(+) solid tumors and cancer stem cells.

Methods: We have successfully engineered an IgG-scFv BsAb by attaching the huOKT3 (anti-CD3) single chain Fv fragment (ScFv) to the carboxyl end of the hu3F8 IgG1 light chain. The resulting hu3F8-BsAb was expressed in mammalian CHO-S cells, purified on protein A affinity chromatography, size and purity verified by HPLC and SDS-PAGE.

Results: In vitro assays showed strong binding of this hu3F8-BsAb to GD2 on tumor cells and to CD3 on T cells. In the presence of cultured T cells, it showed potent cytotoxicity against neuroblastoma with EC_{50} at femto-molar concentrations. It activated T cells in the presence of GD2(+) tumor targets only with hu3F8-BsAb but not with control BsAb (Calcium flux assay), and induced the formation of clear immunologic synapses. TH1 cytokines (TNF-α, IFN-γ and IL-2) were induced only when GD2(+) tumors and fresh PBMC were co-cultured together. This BsAb targeted well to GD2(+) tumors in vivo, and eradicated neuroblastomas in three different humanized mouse xenograft models (sc tumor plus sc effector cells, iv tumor plus iv effector cells, sc tumor plus iv effector cells).

Conclusion: Given the safety profile of OKT3 in humans for decades, and the high expression of GD2 on neuroblastoma and other human cancers, BsAbs built with hu3F8 and huOKT3 offer an Fc-independent polyclonal T cell based approach with considerable clinical potential.

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POT021

Effect of Concomitant Inhibition of VEGFR and MET Signaling on Angiogenesis, Migration and Cell Proliferation in Neuroblastoma Models

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Background: Neuroblastomas are highly vascularized tumors with strong potential for metastatic spread. Increased microvessel density, expression of vascular endothelial factor (VEGF) and its receptors, and downregulation of physiological angiogenic growth inhibitors correlate with advanced stage. MET is expressed in most neuroblastoma cells, indicating that hepatocyte growth factor may interact in a paracrine manner to stimulate tumor invasion. MET activation triggers tumor growth, neoangiogenesis and metastasis, and has been correlated with poor prognosis. MET upregulation has also been proposed as an escape mechanism to various anticancer agents.

Methods: Combined inhibition of VEGFR and MET signaling was evaluated in neuroblastoma models using cabozantinib, an inhibitor of tyrosine kinases including VEGFR2, MET and RET. Cell proliferation and migration in vitro were followed by phase-contrast with Incucyte system. Cabozantinib was explored in vivo using the orthotopic (adrenal) IGR-N91-Luc and the systemic IMR-32-Luc neuroblastoma xenograft models. Mice were treated by oral gavage with vehicle or cabozantinib at 30 or 60 mg/kg/day. Tumor growth was followed by ultrasonography and bioluminescence. Pharmacodynamic read-outs were performed using immunohistochemistry and Western Blotting analyses.

Results: In vitro, cabozantinib inhibited proliferation of IMR-32-Luc and IGR-N91-Luc neuroblastoma cells in a dose dependent manner with IC50s of 2.8 μM and 1.8 μM respectively. It also inhibited cell migration in both cell lines. In orthotopic IGR-N91-Luc xenograft tumors in nude mice, cabozantinib resulted in a dose-dependent tumor growth inhibition at Day 29 of 60% and 87% at 30 and 60 mg/kg/day, respectively. Growth inhibition was associated with decreased vascularization as determined by CD34 staining. The evaluation of systemic IMR-32-Luc model, which develops metastatic spread to the bone and lung, is currently ongoing.

Conclusion: Concomitant inhibition of VEGFR and MET signaling using cabozantinib exhibited antitumor activity in the orthotopic IGR-N91 neuroblastoma model associated with inhibition of neovascularization and inhibition of cell migration.

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POT022

Joining Forces: Exploring the Combined Role of HDAC8 and HDAC10 in Neuroblastoma Outcome and Treatment

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Background: Targeting histone deacetylases (HDACs) has yielded promising treat-

ment alternatives for neuroblastoma. Since trials with broad-spectrum histone deacetylase inhibitors in several malignancies have shown only moderate efficacy associated with class-specific undesirable toxicities, the importance of investigating therapies targeting specific HDAC isozymes with oncogenic function in distinct tumor types, thereby maximizing efficacy and limiting the spectrum of toxicity, is highlighted. Based on our previous work showing that i) HDAC8 is expressed at higher levels in stage 4 neuroblastoma, ii) its specific inhibition slows tumor growth and favors differentiation, iii) inhibition of HDAC10 interferes with autophagy, thereby sensitizing neuroblastoma cells to cytotoxic chemotherapy, and, iv) elevated HDAC10 expression in stage 4 patients is associated with poor outcomes, we are now investigating the combined role of these two HDACs in small round blue cell tumors.

Methods: Expression and survival data from independent neuroblastoma cohorts were analyzed for survival and outcome differences. Additionally, colony assays were performed with HDAC8 inhibitor- and HDAC10/HDAC6 inhibitor-treated neuroblastoma cells.

Results: The analysis of microarray data from several independent cohorts revealed that those patients expressing both HDAC8 and HDAC10 mRNA at a high level have poor overall and event-free survival probability compared with the rest of the patients. In line with these results, the combined and selective inhibition of HDAC8 and class IIb (HDAC10 and 6) in a diversity of neuroblastoma cell lines (e.g. BE(2)-C, IMR-32, SH-SY5Y) induced complete growth inhibition, while the same treatment did not affect the growth of normal human fibroblasts. Additional studies are under way to unravel the mechanism behind the synergism observed when both HDACs are inhibited.

Conclusion: Though their individual roles in neuroblastoma are distinct and important, the combination of HDAC8- and HDAC10-inhibition may yield an enhanced impact on both outcome and minimally-toxic treatment of neuroblastoma patients.

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POT023

GD2-Specific Genetically Engineered NK Cell Therapy is Effective in a Drug-Resistant Neuroblastoma Xenograft Mouse Model

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Background: Drug-resistant neuroblastoma remains a major challenge in pediatric oncology. A human NK cell line NK-92-scFv(ch14.18)-z engineered to express a GD2-specific chimeric antigen receptor (CAR) may help to address this problem. We investigated the cytotoxicity of NK-92-scFv(ch14.18)-z in a panel of GD2+ drug-resistant neuroblastoma cell lines and analyzed the anti-tumor efficacy of NK-92-scFv(ch14.18)-z in a drug-resistant neuroblastoma xenograft mouse model.

Methods: Cytotoxic activity of GD2-specific NK-92-scFv(ch14.18)-z towards a panel of GD2+ cell lines (CHLA-20, SK-N-BE(2), CHLA-136, CHLA-79, LA-N-1, LA-N-5), some of which exhibit partial or multidrug resistance, was analyzed in a ^{51}Cr release assay. We investigated the impact of GD2 recognition on NK-92-scFv(ch14.18)-z-mediated lysis by blocking the CAR through the addition of an anti-idiotype antibody (anti-IdAb) and also by downregulating GD2 on target cells induced by the glucosylceramide synthase (GCS) inhibitor PPPP. We then employed ELISA to determine the production of effector molecules granzyme B and perforin in response to activation with immobilized GD2. Anti-tumor efficacy of NK-92-scFv(ch14.18)-z and IL-2 was analyzed in a drug-resistant GD2+ xenograft mouse model by peritumoral injections of the GD2-specific NK cell line.

Results: NK-92-scFv(ch14.18)-z effectively lysed $GD2^+$ drug-resistant NB cell lines. This effect was almost completely abrogated by blocking the CAR with an anti-IdAb. Decreased GD2 expression on target cells also resulted in diminished lysis mediated by NK-92-scFv(ch14.18)-z. Quantification of granzyme B and perforin production with ELISA revealed that the plate-bound antigen GD2 alone was sufficient to induce activation of NK-92-scFv(ch14.18)-z. Importantly, repeated peritumoral subcutaneous injections of a combination of NK-92-scFv(ch14.18)-zeta and IL-2 significantly prolonged survival time of mice challenged with aggressively growing subcutaneous CHLA-20 tumors in a xenograft mouse model.
Conclusion: These encouraging results indicate that GD2-directed immunotherapy with genetically engineered NK cells is an appropriate treatment strategy especially in relapsed NB that exhibit drug resistance.

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POT024

Non-Invasive Functional MRI Biomarkers of Response to Targeted Therapy in the TH-MYCN Genetically-Engineered Mouse Model of Neuroblastoma

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Background: Advanced magnetic resonance imaging (MRI) techniques can define non-invasive and quantitative functional biomarkers that inform on the tumour microenvironment, its heterogeneity and response to treatments. Neuroblastoma is highly sensitive to single-agent inhibition of the DNA damage response protein checkpoint kinase 1 (CHK1). Here, we investigated functional MRI biomarkers of tumour response to the highly selective, orally available, CHK1 kinase inhibitor CCT244747 in the TH-MYCN model of neuroblastoma.

Methods: Homozygous TH-MYCN mice were treated with either a single 150mg/kg dose of CCT244747 p.o. (n=4) or vehicle alone (n=4). MRI was performed prior to and 24h following treatment, and included quantitative assessments of tumour volume and microenvironment, including haemodynamic vascular function (transverse relaxation rate R_2*), oedema, cellular density (apparent water diffusion coefficient, ADC), molecular crowding (spin lattice relaxation time T1) and iron-containing molecules (T1 and R_2*).

Results: Treatment with CCT244747 resulted in a 30% decrease in tumour burden at 24h, compared to a 36% increase in volume in the control cohort (p=0.003). This anti-tumour activity was associated with both a significant 49% increase in R_2* (92±12s-1 to 136±17^{s-1}, p=0.008) and 11% decrease in T_1 (1946±19ms to 1722±53ms, p=0.02); there was no significant change in either parameter in the control cohort. No significant changes in ADC were detected in either cohort.

Conclusion: CCT244747 is active in the TH-MYCN model of neuroblastoma. Changes in R_2* and T_1 may reflect an increase in iron content associated with macrophage infiltration. In addition to conventional and vascular-targeted chemotherapies, these data support R_2* and T_1 as generic functional biomarkers of response to molecularly-targeted treatment in neuroblastoma, and which are currently being evaluated in MRI-embedded paediatric clinical trials.

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POT025

Combined Inhibition of CDK4/6 and MEK1/2 in Preclinical Models of Neuroblastoma

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Background: MAPK-Ras/Raf and CyclinD-CDK4/6 signaling is hyperactive in subsets of high-risk neuroblastoma. We sought to define biomarkers of anti-tumor activity and the molecular mechanisms conferring sensitivity to the inhibition of both pathways.
Methods: Orally available MEK1/2 (MEK162) and CDK4/6 (LEE011) inhibitors are currently in clinical trials. We established single agent IC_{50} values across a panel of 23 neuroblastoma cell lines. The IC_{50} rankings for both MEK162 and LEE011 were cross-referenced with expression microarrays and exome sequencing to identify the genetic underpinnings predictive of response to inhibition of each pathway. To identify differential expressed genes univariate analysis was performed using the Limma package comparing the most sensitive (IC_{50} < 250 nM) and resistant (IC_{50} > 3 mM) lines for each compound.

Results: Neuroblastoma cell lines showed a wide range of sensitivity to single agent MEK162 (median IC_{50} = 771 nM, range 5 – >10,000 nM) and LEE011 (median IC_{50} = 328 nM, range 126 – >10,000 nM). Univariate analysis demonstrated an inverse relationship between the response to MEKi and CDK4/6i. MYCN amplification was associated with MEK162 resistance (N=14, p=0.002) and LEE011 sensitivity (N=8, p= 0.0227). Furthermore, CDK4/6i resistant lines were enriched for genes within the MAPK-Ras/Raf pathway (r= 0.83, p= $7.81x10^{-05}$), a signature associated with sensitivity to MEK162. Combination studies revealed synergistic inhibition of growth compared with either agent alone (CI range 0.114–0.747).

Conclusion: Dual inhibition of CDK4/6 and MEK kinases is potently synergistic in high-risk neuroblastoma models. Due to emerging evidence for enrichment of these pathways in chemotherapy resistant neuroblastomas, clinical development of this combination should be considered. Robust biomarkers predicting sensitivity to these compounds individually or in combination are being developed.

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POT026

A Dual Specific Anti-IGF-I/IGF-II Human Monoclonal Antibody Alone and in Combination with Chemotherapy or Temsirolimus for Therapy of Neuroblastoma

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Background: The insulin-like growth factors (IGF), IGF-I and IGF-II, have been implicated in the growth, survival, and metastasis of a broad range of neoplasm including pediatric tumors. As autocrines or paracrines, they bind to the IGF-1R receptor and insulin receptor-A isoform (IR-A). IGF-1R blockade does not inhibit IGF-II mediated activation of IR-A. If both IGFs are neutralized, such escape mechanism can be prevented.

Methods: We identified a human monoclonal antibody (m708.5) with dual specificity for both human IGF-I and IGF-II, affinity matured by light chain shuffling, mutagenesis, yeast display, and reshaping as IgG1 to be expressed in CHO cells with high affinity to IGF-I (K_D = 26 pmol/L) and to IGF-II (K_D = 13 pmol/L).

Results: IgG1 m708.5 inhibited both IGF-I- and IGF-II-induced phosphorylation of IGF-1R as well as IGF-II-induced phosphorylation of the IR. m708.5 exhibited strong anti-neuroblastoma activity as a single agent, and showed very strong synergy with temsirolimus and with standard chemotherapeutic agents in vitro. In a xenograft mouse model, it significantly inhibited neuroblastoma growth (Fig.1) and prolonged survival.

Conclusion: The dual specific anti-IGF-I and anti-IGF-II human monoclonal antibody m708.5 showed promising preclinical activity against a broad spectrum of human neuroblastoma. The clinical development of this agent is planned.

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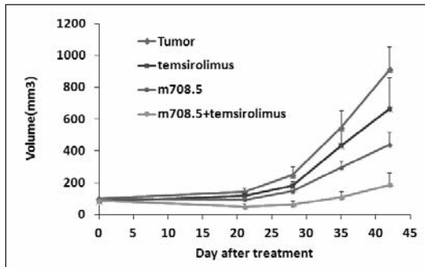

Figure 1. m708.5 inhibits the growth of neuroblastoma i n xenograft models as a single agent and in combination with temsirolimus. Tumor-bearing mice were treated with either 0.1 mg m708.5 (i.v., twice weekly for 4 weeks), 0.025 mg temsirolimus (i.p., 5 times weekly for 4 weeks), or both m708.5 and temsirolimus. Graphs are presented with the mean tumor lead for each treatment group (n-5); bars are standard error of means.

POT027

Novel Small Molecules That Sensitize Neuroblastoma to Cisplatin and 6-MP

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Background: The development of agents that sensitize tumours to existing therapeutics is an attractive goal in cancer therapy. We previously used cells over-expressing the multidrug resistance protein MRP4 to screen for compounds that sensitize to the purine analogue 6-mercaptopurine (6-MP), an MRP4 substrate. In addition to MRP4 inhibitors, we identified a compound (Hit 52) that inhibits nucleotide phosphatases, enzymes that oppose the incorporation of thiopurine compounds into DNA and RNA. Notably, this compound also inhibits the growth of cultured neuroblastoma cells as a single agent.

Methods: We conducted focused library screening around Hit 52 to identify additional related compounds. These compounds were tested for inhibition of recombinant nucleotide phosphatases in enzyme assays and for inhibition of cell growth and sensitization to 6-MP using cultured neuroblastoma cell lines. In vivo activity was assessed using the TH-MYCN transgenic mouse model of neuroblastoma both in a prophylaxis setting as a single agent and in a combination chemotherapy setting in established tumours.

Results: We identified a series of compounds with >95% homology to the original molecule. The majority of these "Hit 52 series" compounds also inhibit nucleotide phosphatases in enzyme assays, sensitize to 6-MP and inhibit neuroblastoma cell growth in culture. Inactive and partially active analogues were also identified, enabling future structure-activity studies. The active compounds delayed tumour growth as single agents immediately post-weaning and prior to overt tumour formation, and sensitized established tumours to 6-MP, substantially prolonging survival. Surprisingly, these compounds also strongly synergized with the DNA cross-linking agent cisplatin in established tumours, but did not sensitize to the topoisomerase I inhibitor irinotecan and the microtubule inhibitor vincristine.

Conclusion: We have identified new agents that sensitize established tumours to both cisplatin and 6-MP in the TH-MYCN mouse neuroblastoma model. We are currently investigating the basis of cisplatin sensitization and determining their efficacy in xenograft models.

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POT028

Additional Receptor Tyrosine Kinases Compensate Survival Signals in ALK Inhibitor-Treated Neuroblastoma Cells

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Background: Blockade of aberrant anaplastic lymphoma kinase (ALK) signaling caused by gene amplification, missense point mutations and mutation-independent high expression, offers a therapeutic benefit in high-risk group of neuroblastoma (NB). Indeed, the first clinically available ALK tyrosine kinase inhibitors (ALKi), crizotinib, showed tumor regression in a recent clinical study, while it was implied that the de novo mechanisms might influence NB sensitivity to the inhibitor. Therefore, we have pursued the alternative pathway which modulates ALKi susceptibility.

Methods: To investigate acute response of NB cells to ALKi, ALK-mutated NB cell lines treated with ALKi (crizotinib and alectinib) were subjected to microarray analysis. Transcriptional alteration and signal pathways were examined by RT-PCR and western blot. Cell survival was evaluated by living cell imaging system and WST-8 assay. Plasma levels of growth factors in NB patients were measured by ELISA, and apoptosis was assessed by a flow cytometer.

Results: We identified three growth factors (NGF, EGF or bFGF) that significantly rescued NB cells from death induced by ALKi. In ALKi-treated NB cells, the expressions of NGF receptors, TrkA and $p75^{NTR}$, were upregulated among growth factor/neurotrophin receptors. In the survived cells, the receptors and downstream signal molecules were stimulated by the growth factors even in the presence of ALKi. The lowest concentration of EGF sufficient for NB cell survival was comparable to the plasma level in NB patients. On the other hand, without NGF treatment, TrkA-expressing cells underwent apoptosis after ALKi treatment, suggesting that the upregulated NGF receptors function to induce apoptosis.

Conclusion: We have identified a novel cellular response to ALKi in NB cells with aberrant ALK status. The present study suggests that co-targeting the bypassed survival signals contributes to efficacy of ALKi therapy in NB patients.

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POT029

NK Cell Clones Exert Alloreactivity According to the KIR Receptor/ligand Model against Neuroblastoma Cell Lines In Vitro

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Background: Haploidentical stem cell transplantation provides a therapeutical platform for the transfer of expanded potentially alloreactive NK cells. The combination with GD2 monoclonal antibody (CH14.18/CHO) treatment is likely to exceed antitumor activity. To investigate the alloreactivity of NK cell clones with and without KIR receptor-ligand (R/L) mismatch, we sorted and expanded defined NK subsets.

Methods: NK cell clones expressing only one KIR receptor (CD158a or CD158b) were high efficiently expanded (6.0-6.6 logs) using K562mb15 4-1BBL feeder cells and IL-2. Cellular cytotoxicity as well as ADCC-mediating GD2mAB was measured in a our BATDA release assay against neuroblastoma cell lines LAN-1 (expressing only HLA-C ligands for CD 158b inhibitory NK receptor) and LS (expressing HLA-C ligands for both CD158a and b NK receptors). NK clones were matched by K562 lysis.

Results: CD158a+ (R/L-mismatched, non-inhibited) NK clones of all investigated donors (n=3) lysed LAN-1 significantly higher without ($p=0.0047$) and with GD2mAB ($p=0.0001$) than CD158b+ (no R/L-mismatched) clones, which were inhibited by the HLA ligands of the targets. Moreover, there was no significant difference in the lysis of LS by CD158a+ clones vs CD158b+ clones without and with GD2mAB, since these targets inhibited both types of clones and therefore prevent alloreactivity. GD2 antibody enhanced specific lysis significantly in LAN-1 and LS (LAN-1 $p<0.0001$; LS $p<0.0001$) independent from phenotype of NK cell clones. Increase of GD2mAB-mediated ADCC was significantly higher in LAN-1 than LS ($p<0.0001$).

Conclusion: NK alloreactivity in neuroblastoma may be significantly influenced by KIR R/L-mismatch and should therefore be taken into account for donor selection strategies in HLA mismatched haploidentical stem cell transplantation. Alloreactivity may occur not only against leukemias but also against neuroblastomas. Ex vivo expanded, highly activated alloreactive haploidentical NK cells could be used for NK cell transfer posttransplant in combination with GD2mAB to augment antitumor activity.

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POT030

Targeted Drug Delivery for the Treatment of Neuroblastoma

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Background: The long term survival rates for High risk Neuroblastoma (HRNB) in the United States are still only 30–50%. Current chemotherapy regimens also have a serious limitation due to adverse effects at higher doses and the off-target toxicity. In the present work, we evaluated the potential application of reconstituted high density lipoprotein (rHDL) nanoparticles as a drug carrier and compared this novel approach to current NB therapeutics.

Methods: The rHDL particles were prepared using the cholate dialysis method and various drugs such as Fenretinide, all-trans Retinoic Acid, Imatinib and sequinavir were encapsulated and tested. The particles were then characterized for their physical properties and chemical composition. Size of the particles was determined using dynamic light scattering and electron microscopy. Cytotoxicity, cell viability and uptake inhibition studies were also conducted. Pilot studies done on mice models also demonstrated selecive targeting ability of these synthetic nanoparticles.

Results: The characterization and stability studies of rHDL particles showed small size (<100 nm) and a high encapsulation efficiency while the cytotoxicity studies of free drugs vs. rHDL toward the NB cell lines showed up to 2-fold enhancement with rHDL.

Conclusion: Our studies show that this novel approach of encpsulating the anticancer agents into rHDL nanoparticles greatly increases their efficacy against NB cell lines, thus indicating the potential of this system in improving the long-term prognosis of NB patients.

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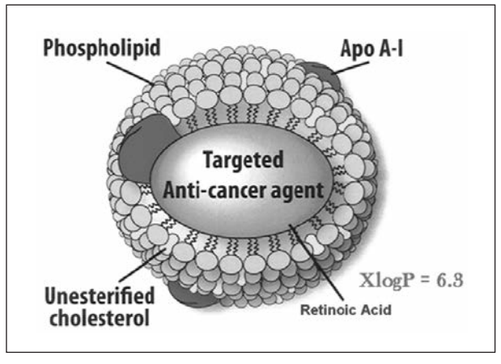

POT031

Notch is a Therapeutic Target in Neuroblastoma

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Background: Dysfunction of developmental signaling pathways in peripheral sympathetic progenitors is implicated in the maintenance of neuroblastoma (NB). Little is known about Notch signalling in this regard. We therefore investigated Notch as a potential therapeutic target in NB.

Methods: Patient NB, primary NB cultures and NB cell lines were investigated. In silico analysis of Notch signalling was performed in clinically annotated NB expression arrays of more than 500 patients. Notch expression was analysed by qRT-PCR in NB cell lines and primary cultures, and by immunohistochemistry in more than 100 NB tumors. 5 g-secretase inhibitors (GSI) inhibiting Notch were screened in NB cells. In vitro proliferation, cell cycle, apoptosis, clonogenicity and tumor sphere integrity were determined after GSI treatment. Orthotopic NB xenografts were GSI-treated, monitored by in vivo bioimaging and analysed by histology and immunohistochemistry.

Results: Primary cultures and NB cell lines expressed at least one of the Notch receptors and one of the ligands leading to para- or autocrine activation of Notch target genes., High mRNA expression of Notch pathway genes in silico is associated with worse prognosis. In situ expression of pivotal Notch signalling genes including Notch2, JAG1 and JAG2 was confirmed by immunohistochemistry and analysed for prognostic significance., GSI-I was the most potent GSI. GSI-I decreased NB cell viability, reduced clonogenicity and killed putative NB stem cells. Notch target genes decreased following GSI-I treatment. GSI-I-treated NB cells arrested at G2/M associated with increased expression of p21, p27, cyclin B and CDC25C. Arrested NB cells proceeded to mitotic catastrophe and apoptosis. Importantly, MYCN activation enhanced sensitivity to GSI-I. Finally, systemic GSI-I treatment reduced growth of orthotopic xenografts, associated with signs of mitotic catastrophe, decreased proliferation and attenuated vascularisation.

Conclusion: Notch is a promising therapeutic target in NB.

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POT032

The ALK Inhibitor NMS-202 Shows In Vivo Efficacy in an Orthotopical Model of ALK-F1174L Mutant Neuroblastoma

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Background: Constitutive activation of ALK results from either missense mutations or genomic amplification, that occur in about 8-10% of Neuroblastoma (NB) patients and 4% of high-risk NBs, respectively. Therefore, survival of this subset of patients might be improved by ALK tyrosine-kinase inhibitors. However, the efficiency of inhibition varies depending on specific ALK alteration. Particularly, F1174L mutation proved to be highly oncogenic and showed relative resistance to inhibition. We tested NMS-202 ALK inhibitor in NB cell lines with different ALK alterations (mutations, amplification, wild-type) and in an orthotopic mouse model.

Methods: The in vitro efficacy of NMS-202 was tested by MTT assay and phospho-ALK status analyzed by immuno-blotting, after treating NB cells with increasing doses of compound in time-course experiments. For in vivo studies, a luciferase reporter vector was stably integrated into SK-N-SH cells. Twenty CD1 nude mice (Charles River) were orthotopically inoculated with 5x105 SK-N-SH luciferized cells and tumors were allowed to grow for 24 days before treatment. Mice were treated with NMS-202 (60 mg/kg; route: os), twice a day for 10 days and tumor growth monitored by Xenogen IVIS Imaging System (Caliper).

Results: After 24 hours of NMS-202 treatment, IC_{50} values of NB cells were as follows: NB1 (ALK-amplified), 22nM; UKF-NB3 (ALK-R1275Q), 307nM; SH-SY5Y, KELLY, SK-N-SH (ALK-F1174), 1387nM, 2533nM, 348nM, respectively; SK-N-BE2(C), SK-N-AS (ALK-wt), 4036nM, >5000nM, respectively. The compound therefore showed highly potent activity in ALK amplified NB1 cells. Surprisingly, in SK-N-SH cells, which carry the F1174L mutation, we additionally observed an efficacy comparable to that obtained with UKF-NB3 cells, which instead harbor ALK R1275Q. In vivo, a 60% decrease of estimated tumor mass was observed for SK-N-SH inoculated mice following 10 days of orally administered treatment with NMS-202, as compared with a 650% increase in vehicle treated controls.

Conclusion: ALK inhibitor NMS-202 showed efficacy in inhibiting tumor growth in mice orthotopically implanted with ALK-F1174L NB tumors.

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POT033

Inhibition of Exportin 1 (XPO1) Potently Suppresses Growth of Human Neuroblastoma Cell Lines

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Background: Exportin 1 (XPO1; Chromosome Region Maintenance 1 Protein Homolog, CRM1) is the sole nuclear exporter of TP53, FOXO1, RB1, CDKN1A, and other critical tumor suppressor proteins (TSPs). KPT-330 is a selective inhibitor of nuclear export (SINE) that irreversibly binds XPO1 and inhibits XPO1-mediated nuclear export across multiple tumor types leading to activation of TSPs and cancer cell death.

Methods: A panel of 14 genomically-characterized cell lines was treated with KPT-330 across a 5-log range. Growth inhibition was measured using viability assays and a real-time growth monitoring system. NOD/SCID mice with neuroblastoma xenografts (both flank and tail-vein injected) were treated orally with KPT-330 (15 mg/kg thrice weekly or 20-25 mg/kg twice weekly); tumor size was monitored by direct measurement and luciferase luminescence.

Results: KPT-330 showed cytotoxicity against 14 neuroblastoma cell lines with a median IC_{50} of 236 nM (range 51-568). Sensitivity to KPT-330 was independent of MYCN amplification status. Combination of KPT-330 with commonly used cytotoxic agents (irinotecan, topotecan, cisplatin, and doxorubicin) showed additive effects. Flank xenografts using SH-SY5Y showed significantly decreased tumor growth after administration of KPT-330. After allowing tumors to grow following 28 days of drug withdrawal, re-exposure to the drug again showed growth suppression. In a model of metastatic neuroblastoma after tail-vein injection of luciferase-transfected cell lines (SH-SY5Y, IMR-5, and Be2c), KPT-330 showed significant tumor growth inhibition ($p<0.001$). KPT-330 treatment resulted in decreased Myc family protein levels and activation of apoptotic pathways. Major toxicity was weight loss which was overcome with nutritional supplementation.

Conclusion: Neuroblastomas show sensitivity to XPO1 inhibition both in vitro and in vivo. Ongoing work is focused on discovering the cellular and genomic factors responsible for increased sensitivity to nuclear export inhibition and potential synergistic combinations. With the completion of the first-in-human phase I trials, treatment with KPT-330 has the potential to be rapidly translated into a clinical trial for children with neuroblastoma.

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POT034

Optimizing Neuroblastoma-Specific CD171-Targiting CARs

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Background: Adoptive immunotherapy using chimeric antigen receptor (CAR) expressing T cells is a promising therapeutic modality for cancer, including neuroblastoma (NB). In this study we designed a CAR using a tumor-restricted epitope of the antigen CD171 (L1CAM), expressed in 100% of high risk NB. Our aim was to optimize the extracellular spacer (short, medium, long) and intracellular co-stimulatory domains (41BB-zeta +/- CD28) for therapeutic CD171-targeted CAR development.

Methods: We analyzed CAR-transduced CD8 central memory T cells (Tcm) for effector function using chromium and cytokine release assays, for phenotypic analysis of activation and exhaustion markers and for tumor eradication in vivo using an NSG mouse NB xenograft model.

Results: The long spacer CAR-expressing cells displayed the best in vitro lytic capacity (44% lysis; E:T 10:1) and cytokine release (IFN-γ, TNF-α) of all 41BB-zeta (2nd generation) CARs, yet failed to inhibit tumor growth in vivo. In contrast, the short spacer 41BB-zeta CAR-expressing cells were less effective in vitro (25% lysis; E:T 10:1) but able to completely eradicate tumor in vivo. Further, short spacer CAR cells exhibited diminished activation induced cell death and lower exhaustion marker expression levels relative to long spacer CAR-expressing cells following serial tumor cell co-culture challenge (viability 33% versus 14.8%; Tim3+PD1+ 23% versus 41%). A 3rd generation (CD28-41BB-zeta) short spacer CAR enhanced CD8 Tcm lytic capacity and cytokine release in vitro (1.5-fold higher lysis, 3-fold higher TNF-α release) but exhibited inferior anti-tumor activity in vivo relative to the 2nd generation CAR.

Conclusion: Short spacer CD171-targeted CARs with 41BB-zeta as intracellular domain had superior in vivo efficacy. Co-stimulation with CD28-41BB-zeta may lead to T cell exhaustion. A clinical trial examining toxicity and efficacy of short spacer CD171- 2nd and 3rd generation CARs is underway.

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POT035

Evaluation of Targeted Drugs in Combination with the MDM2 Antagonist RG7388 in Neuroblastoma

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Background: Neuroblastoma is the second most common solid malignant tumor in children and accounts for approximately 15% of all pediatric cancer-related deaths. As in most pediatric cancers, TP53 mutations are relatively rare in neuroblastoma. The TP53 tumor suppressor protein is mainly kept inactive by aberrations in the $P14^{ARF}$/MDM2/TP53 axis resulting in increased activity of MDM2, a central TP53-inhibitory protein. These biological conditions make neuroblastoma specifically suitable for treatment with targeted inhibitors of the TP53–MDM2 interaction. Well-matched combination therapy is considered decisive for targeted therapeutics by increasing therapeutic efficacy and preventing the development of treatment resistance. This study aims to identify appropriate drug combination partners for the small-molecule MDM2 antagonist RG7388.

Methods: Using an in vitro cell viability screen, 15 targeted drugs were evaluated in combination with RG7388 in a panel of four neuroblastoma cell lines, three of which have wild-type TP53 (NGP, IMR-32 and SH-SY5Y) and one with a TP53 mutation (SK-N-BE(2c)).

Results: Several compounds displayed synergism in combination with RG7388 in at least one of the cell lines studied. The highest degree of synergism was observed when RG7388 was combined with the BCL2 inhibitor ABT-199. Potent induction of pro-apoptotic TP53 pathway genes as a result of TP53 activation complemented with the inhibition of anti-apoptotic proteins by ABT-199 resulted in enhanced induction of apoptosis and could account for the observed synergism.

Conclusion: Dual targeting of MDM2 and BCL2 is highly synergistic in neuroblastoma cells.

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POT036

Sensitization for chemotherapy-induced apoptosis by Smac mimetic LCL161 in neuroblastoma is independent of ripoptosome-formation, NF-κB and TNF-α

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Background: In neuroblastoma (NB) an upregulation of XIAP the most potent IAP was detected. Targeting IAPs using Smac mimetics (SM) sensitized NB for chemotherapy-induced apoptosis. cIAP-1/-2 degradation, blockade of canonical but activation of non-canonical NF-κB and TNF-α secretion are thought to be the most important mechanisms of SM-mediated cell death. Canonical NF-κB activates survival signals but if blocked can lead to caspase 8-mediated apoptosis. TNF-α independent ripoptosome assembly consisting of RIP1, FADD and caspase-8 is another possible way how SM can induce apoptosis. Therefore, a more detailed analysis of NF-κB involvement in SM-mediated cell death is warranted. This could help to better understand and overcome the frequent chemotherapy-resistance of NB and lead to the development of more effective treatment options for this malicious disease.

Methods: Cell lines served as an experimental model for in vitro treatment of NB. The vinca alkaloid vincristine was used in combination with SM. Influence of canonical (pro-survival) and non-canonical NF-κB signaling on apoptosis induction was evaluated. Possible activation of NF-κB pathways was detected and effects of disrupted NF κB signaling using chemical inhibitors or siRNA knock-down on apoptosis induction were analyzed. Inhibition of RIP-1 to prohibit ripoptosome formation was conducted to evaluate its involvement in SM-mediated cell death

Results: Inhibition of canonical (pro-survival) NF-κB signaling led to mild induction of apoptosis. No significant effects were detected if non-canonical NF-κB was blocked. Strong apoptosis induction by combined vincristine and SM treatment was not abrogated if NF-κB signaling pathways were inhibited. RIP-1 depletion and prevention of ripoptosome assembly again had no effect on vincristine and SM effected apoptosis.

Conclusion: NF-κB signaling is not involved in SM-mediated cell death in NB. Assembly of the ripoptosome can't be ruled out completely but likely it has no impact on SM-mediated and vincristine-induced apoptosis in NB.

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POT038

Differential Expression of Uridine-Cytidine Kinases in Neuroblastoma. Implications for Development of a Targeted Therapeutic Approach

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Background: Uridine-cytidine kinase (UCK) is a pyrimidine ribonucleoside kinase that catalyzes the phosphorylation of uridine and cytidine to UMP and CMP, respectively. UCK also catalyzes the phosphorylation, and thereby the pharmacological activation, of several cytotoxic pyrimidine ribonucleoside analogues. Two human UCKs have been reported with UCK1 being ubiquitously expressed in several tissues whereas the expression of UCK2 seems to be confined to human placenta and various tumor cells. In this study, we investigated the functional role of UCKs in neuroblastoma.

Methods: Expression of UCKs in neuroblastoma cell lines was investigated with quantitative PCR and Westernblotting using antibodies generated against purified UCK1 and UCK2 (Abcam). Subcellular localization of UCKs was investigated using UCKs expressed in fusion with green fluorescent protein. Cytotoxicity towards 3-deazauridine was assessed using a dsDNA Quantitation Kit in neuroblastoma cells, transfected with the pcDNA3.1Zeo(+) vector containing UCK1 or UCK2, or after transfection with siRNA targeting UCK2.

Results: Analysis of mRNA and protein levels coding for UCK1 and UCK2 showed that UCK2 is by far the most abundantly expressed UCK in neuroblastoma cell lines. Subcellular localization studies showed that the UCK1-GFP protein was localized in the cell nucleus whereas UCK2-GFP was located in the cytosol. To investigate the role of UCKs in metabolizing pyrimidine analogues we tested the cytotoxicity of 3-deazauridine which is activated primarily by UCK2. Transient overexpression of UCK2 in neuroblastoma cells resulted in an increased cytotoxicity of 3-deazauridine whereas knockdown of endogenous UCK2 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. However, overexpression of UCK1 protected the neuroblastoma cells from 3-deazauridine-induced toxicity. Subcellular localization studies showed that co-expression of UCK1 with UCK2 resulted in a nuclear localization of UCK2 instead of its normal cytosolic localization.

Conclusion: The discovery that UCK2 is highly expressed in neuroblastoma opens the possibility for selectively targeting neuroblastoma cells using UCK2-dependent pyrimidine analogues, while sparing normal tissues.

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POT039

Pre-Clinical Studies of the MDM2-p53 Antagonist RG7388 Alone and in Combination with Chemotherapy in Neuroblastoma

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Background: p53 is the most commonly mutated gene in human cancer occurring in >50% of cases. In neuroblastoma, p53 mutations are rare, however p53 pathway inactivation through MDM2 amplification and inactivation of $p14^{ARF}$ has been reported. Therefore, reactivating wild-type (wt) p53 using MDM2-p53 antagonists offers a novel therapeutic strategy for the treatment of neuroblastoma. RG7388 is currently undergoing early phase clinical evaluation in adults.

Methods: Aim: To investigate the efficacy of RG7388 as a single-agent and in combination with existing chemotherapies in pre-clinical models of neuroblastoma.

Results: GI50 values were determined in a panel of 21 p53 wt and mutant neuroblastoma cell lines of varying MYCN, MDM2 and $p14^{ARF}$ status, together with MYCN-regulatable Tet21N cells. Consistent with the mechanism of action of MDM2-p53 antagonists, the primary determinant of response is the presence of wt p53, and overall there was a ~200 fold difference in GI_{50} values of p53 wt compared with p53 mutant cell lines ($P < 0.005$). Sensitivity to RG7388 was not significantly associated with MYCN or MDM2 amplification or p14ARF abnormalities, however Tet21N MYCN+ cells had a significantly lower GI_{50} value compared to Tet21N MYCN- cells ($P < 0.005$). Using Calcusyn median-effect analysis, selected combinations of RG7388 with cisplatin, doxorubicin, topotecan and temozolomide were shown to be synergistic (CI values <1). Consistent with this, combination treatments led to increased levels of apoptosis as evident by the higher levels of caspase 3/7 activity, compared to either RG7388 or chemotherapy agent alone. A preclinical in vivo study with human NGP cells as xenografts in mice treated with RG7388 and temozolomide is currently underway.

Conclusion: Our data shows that RG7388 is highly potent against neuroblastoma cells with wt p53, and should be further evaluated alone or in combination with existing chemotherapies to improve the survival and potentially reduce toxicity of patients with high-risk neuroblastoma.

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POT040

Biodegradable Nanoparticles Loaded with a Rapidly Activatable SN38 Prodrug Effectively Inhibit Growth of Neuroblastoma (NB) Xenografts

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Background: The majority of NB patients have advanced stage disease and a poor prognosis, so more effective, less toxic therapy is needed. We wanted to determine if more targeted delivery of conventional agents would improve efficacy and limit systemic toxicity. We developed a novel nanocarrier-based strategy for tumor-targeted delivery of a prodrug, SN38, the active metabolite of Irinotecan.

Methods: We formulated ultrasmall-sized (<100 nm) biodegradable poly(lactide)-poly(ethylene glycol) based nanoparticles (NPs) containing SN38-tocopherol succinate (SN38-TS). We conducted studies assessing alternative dosing schedules of SN38-TS NPs compared to Irinotecan (10 mg/kg, 10 mice per arm), on nu/nu mice xenografted with the Trk-null SY5Y cell line transfected with TrkB. Irinotecan was given 5x/wk x 4 weeks (20 doses), whereas the SN38-TS NPs were given as either 1x/2 weeks x 4 weeks, 1x/week x 4 weeks, or 2x/week x 4 weeks (2, 4 and 8 doses, respectively). We then compared Irinotecan 5x/wk x 8 weeks (40 doses) to SN38-TS NPs given 2x/wk x 4 weeks, 1x/wk x 8 weeks, and 2x/wk x 8 weeks (8, 8, and 16 doses, respectively).

Results: In vitro, SN38-TS NPs showed greater inhibition of cell growth than Irinotecan using SRB assays. In vivo, SN38-TS NPs given 1x/2 weeks x 4 weeks had similar efficacy to Irinotecan given 5x/wk x 4 weeks (20 doses), but none were cured. All SN38-TS NP regimens were far superior to Irinotecan, and cures were obtained in all NP arms (no cures with Irinotecan). SN38-TS NP delivery resulted in 200x the amount of SN38 in NB tumors at 4 hr post-treatment, and 25% of the 4-hr SN38 level remained at 72 hr post-treatment, compared to no SN38 detected at 24 hr post-treatment for the Irinotecan arm.

Conclusion: We conclude that this SN38-TS NP formulation dramatically improved delivery, retention, and efficacy, without causing additional systemic toxicity.

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POT041

Generation and Characterization of Ganglidiximab for Active Immunotherapy of Neuroblastoma

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Background: Passive immunotherapy targeting disialoganglioside GD_2 emerges as an important treatment option for high-risk neuroblastoma (NB) patients. In order to maintain anti-GD_2 activity, vaccination with GD_2 mimotopes may further consolidate remission in the minimal residual disease setting. For this purpose, we generated and characterized ganglidiomab, a murine anti-idiotype antibody (anti-Id Ab) of anti-GD_2 Ab ch14.18 and demonstrated induction of an active GD_2-specific humoral immune response.To further tailor this response to murine variable regions in humans, we engineered a new chimeric anti-Id Ab ganglidiximab by replacing murine constant fragments with corresponding human IgG1 regions and demonstrate its GD_2 surrogate function.

Results: DNA fragments encoding for murine variable heavy (VH) and light chain (VL) of ganglidiomab were amplified, purified and cloned into the pdHL7 vector in frame with coding sequences for constant regions of human IgG1. Dhfr-deficient CHO cells were stably transfected with the generated plasmid and ganglidiximab-producing clones were selected and cultured using Dhfr/methotrexate gen amplification system. Binding of purified ganglidiximab to anti-GD_2 Abs of the 14.18 family (ch14.18, ch14.18-dCH2, ch14.18-IL-2, hu14.18 and hu14.18-IL-2) as well as to NK-92 cells expressing scFv(ch14.18)-zeta receptor was demonstrated using standard ELISA and flow cytometry analysis, respectively. Finally, concentration-depended inhibition of ch14.18-mediated GD2-specific lysis of NB target cells was shown after pre-incubation with ganglidiximab in a functional cytotoxicity assay confirming anti-idiotypic characteristics.

Conclusion: We engineered and characterized ganglidiximab, a new human/mouse chimeric anti-Id Abfor active immunotherapy against NB. This agent may be useful to tailor immune response to the paratope regions mimicking GD_2 in NB patients.

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POT042

Generation and Characterization of Two Expression Plasmids Encoding for Single Chain Variable Fragments of Ganglidiomab for Active Immunotherapy in Neuroblastoma

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Background: Disialoganglioside GD_2 is a highly expressed but poorly immunogenic tumor associated antigen in neuroblastoma (NB). Protein vaccination with ganglidiomab, a new anti-idiotype antibody (Ab) mimicking GD_2, resulted in induction of an active immune response against GD_2 in vivo. To further increase this response, we generated and characterized two expression plasmids encoding for single chain variable fragment (scFv) of ganglidiomab (variable light (VL) and variable heavy chain (VH) genetically linked via a short peptide linker (ln; VL-ln-VH and VH-ln-VL)).

Methods: DNA sequences encoding for VL and VH of ganglidiomab were amplified, linked and cloned into the expression vector phCMV3 using standard molecular biology techniques. After sequence analysis of the correct scFv insertion followed by plasmid transfection into CHO cells, sufficient amounts of scFv protein were isolated from cell supernatants and characterized using competitive GD_2-ELISA, flow cytometry and Calcein-AM-based cytotoxicity assay.

Results: DNA sequences encoding for scFv of ganglidiomab cloned into the expression vector phCMV3 were identified using Kabat Database. Both VL-ln-VH and VH-ln-VL scFv proteins isolated from CHO cell supernatants competitively inhibited binding of anti-GD_2 Ab ch14.18 to the nominal antigen GD_2. Furthermore, scFvs showed similar binding to NK-92 cells expressing scFv(ch14.18)-zeta receptor (NK-92tr) compared to ganglidiomab. Moreover, GD_2-specific lysis of NB cells by NK-92tr was inhibited after pre-incubation with CHO cell supernatants clearly showing anti-idiotype characteristics of scFvs. Finally, two scFv-based DNA-vaccines were generated using attenuated Salmonella typhimurium as a plasmid transport vehicle.
Conclusion: We generated and characterized two expression plasmids based on scFv of ganglidiomab which mimics GD_2 providing an important baseline for the

development of DNA- and protein-vaccines against NB.

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POT043

Sensitivity to CCNB1/cdk1 Complex Inhibition is Modulated by TP53 Mutational Status in Neuroblastoma Cell Lines

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Background: Cell-cycle inhibitors are an emerging class of anti-cancer drugs, which are currently under clinical evaluation in many solid tumors, including neuroblastoma (NB). Specifically, the complex comprising cyclin B1 (CCNB1)/ cyclin dependent kinase 1 (cdk1), which is involved in regulating G2-M transition, has been recently identified as a promising cancer target.

Methods: Exon-level mRNA profiling was performed in 101 patients with neuroblastoma and analysed for expression of cell cycle regulating genes. In order to evaluate the CCNB1/cdk1 complex as a potential therapeutic target in NB, we used either small molecule inhibitors of the CCNB1/cdk1 signalling pathway or siRNA mediated knock-down of CCNB1 or cdk1.The effects of interfering with CCNB1/cdk1 were analysed in vitro with respect to regulation of proliferation, cell cycle distribution and induction of apoptosis.

Results: Expression of both CCNB1 and cdk1 mRNAs were found significantly upregulated in aggressive tumor stages. CCNB1/cdk1 inhibition by small molecules or siRNA-mediated down-regulation of either CCNB1 or cdk1 caused decreased cell viability in NB cell lines. Interestingly, the anti-proliferative effect was more pronounced in p53 wild-type cells as compared to p53 mutant cell lines. This co-occurred with differential activation of p53 downstream targets, including p21 and Bax. We could also demonstrate that effects of CCNB1/cdk1 inhibition on cell cycle distribution were dependent on p53 status.

Conclusion: As relapsing neuroblastoma often present with TP53 mutations, these findings have implications for therapy approaches targeting cdks.

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POT044

Testing of SNS-032 in a Panel of Human Neuroblastoma Cell Lines with Acquired Resistance to a Broad Range of Drugs

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Background: Many neuroblastomas respond initially well to therapy but eventually acquire resistance. Thus, novel treatment options are needed for the successful therapy of high-risk neuroblastoma patients, particularly in an acquired resistance setting. Here, the effects of the cyclin-dependent kinase (CDK) inhibitor SNS-032 were studied in a panel of 109 neuroblastoma cell lines consisting of 19 parental cell lines and 90 sub-lines with acquired resistance to 14 different anti-cancer drugs.

Methods: IC_{50} and IC_{90} values were determined by MTT assay. Moreover, the roles of p53, ABCB1 and ABCG2 expression, the CDKs 7 and 9, RNA synthesis, and anti-apoptotic proteins were studied in neuroblastoma cell response to SNS-032.

Results: 73% of the investigated neuroblastoma cell lines and all four investigated primary tumour samples displayed IC50 values in the range of the therapeutic plasma levels reported for SNS-032 (< 754nM). 62% of the cell lines and two of the primary samples displayed IC_{90} values in this concentration range. SNS-032 also impaired the growth of the multi-drug resistant cisplatin-adapted UKF-NB-3 subline UKF-NB-3^{r}CDDP^{1000} in mice. ABCB1 expression (but not ABCG2 expression) conferred resistance to SNS-032. The anti-neuroblastoma effects of SNS-032 did not depend on functional p53. The anti-neuroblastoma mechanism of SNS-032 included CDK7 and 9 inhibition-mediated suppression of RNA synthesis and subsequent depletion of anti-apoptotic proteins with a fast turnover rate including XIAP, Mcl-1, cIAP1, and survivin.

Conclusion: CDK7 and CDK9 represent promising drug targets and SNS-032 represents a potential treatment option for neuroblastoma including therapy-refractory cases.

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POT045

Role for Sirtuins in Chemoresistance in Neuroblastoma

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Background: Chemoresistance is a major obstacle in the successful treatment of high-risk neuroblastoma (NB). Mechanisms of chemoresistance include the activation of survival pathways such as the unfolded protein response (UPR), an adaptive mechanism in response to endoplasmic reticulum (ER) stress, and the PI3K/AKT/MTOR pathway. AKT pathway promotes UPR and contributes to cell survival in response to chemotherapy. At the intersection of these pathways are sirtuins (SIRTs), which are NAD+ dependent deacetylases, which are activated during periods of stress leading to cellular protection., Objective. Determine the therapeutic potential of SIRT inhibition as a novel method to increase NB chemosensitivity to ER stressors and AKT pathway inhibition.

Methods: Cell viability was determined by MTS assay and cell signaling pathways were evaluated by western blot analysis. NB cells were treated with celecoxib and velcade to induce ER stress, and perifosine and everolimus to inhibit the PI3K/AKT/MTOR pathway. Sirtinol was used as sirtuin inhibitor.

Results: Treatment of NB cells with the SIRT1 and 2 inhibitor, sirtinol, induced NB cell death (IC_{50}: 67mM, and 44mM for NB1691 and SK-N-BE2C, respectively). Sirtinol blocked expression of GRP78, an UPR survival protein, increased expression of the pro-death protein CHOP and significantly increased NB1691 cell death (viability; vehicle=100±2.3%, sirtinol=63±1.6%, celecoxib=67±1.1%, celecoxib+ sirtinol=10±0.6%, velcade=68±2.0%, velcade+sirtinol=28±1.3%). Combined SIRT and AKT pathway inhibition induced PARP cleavage and NB1691 cell death (viability; vehicle=100±1.9%, sirtinol=70±1.9%, perifosine=76±1.9%, sirtinol+ perifosine=28±0.9%, everolimus=87±3.2%, sirtinol+everolimus=23±2.2%).

Conclusion: Our data indicates that SIRTs regulate UPR and cell survival following chemotherapeutic insult. Most SIRT inhibitors are in pre-clinical trials, however NAMPT, needed to generate NAD+, inhibitors are in clinical trials and could potentially be used to inhibit SIRTs thereby enhancing the therapeutic effect of AKT and UPR targeting agents. Here we provide novel insights in role of SIRTs in NB and suggest a new therapeutic regimen for a cancer with minimal survival.

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POT046

Interrelation Between PI3K, Akt, mTOR and P53 Signaling Routes in GD2 Ganglioside - Targeted Neuroblastoma Cell Lines

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Background: Better understanding of tumor-relevant signaling pathways led to cancer treatment being shifted from conventional cytotoxic drugs to target-based agents. We aimed to study PI3K/Akt/mTOR and P53 signaling pathways, being aberrantly regulated in neuroblastoma (NB) and altered upon GD2 ganglioside (GD2)-targeted treatment with the 14G2a monoclonal antibody (mAb).
Methods: Immunobloting analysis, cell viability and caspase activity tests, flow cytometry analysis.

Results: In this study we performed immunobloting analysis of the expression and phosphorylation status of PI3K and Akt kinases in IMR-32, LA-N-1 and CHP-134 neuroblastoma cell lines for which cytotoxic effects of the mAb were observed. We found that phosphorylation of Akt was the most significantly decreased in IMR-32 cell line, especially 24 and 48 h after the mAb treatment, possibly as a consequence of simultaneous upregulation of phosphorylated form of PTEN phosphatase. Inhibitory effects of the mAb were also noted for phosphorylated and unphosphorylated forms of mTOR and its downstream effectors: p70S6K and 4EBP1. We showed that in the mAb-mediated cytotoxicity toward IMR-32 and CHP-134 cells (both expressing wt P53) the statistically significant inhibitory effect on Akt/mTOR cascade was correlated with upregulation of P53 protein. Additionally, we evaluated combinatorial treatment of mAb and PI3K/Akt/mTOR pathway inhibitors e.g. LY294002, perifosine, SAR245409 on IMR-32, LAN-1, LAN-5, CHP-134 and HTLA-230 neuroblastoma cell lines. We found that combination of the mAb with PI3K kinase inhibitor (LY294002) significantly decreased the viability of IMR-32, LA-N-1, CHP-134 cells, as compared to both agents used alone.

Conclusion: Our findings suggest that the dominance of signals such as upregulation of proapoptotic P53 protein and inhibition of pro-survival PI3K/Akt/mTOR pathway in GD2-targeted IMR-32 and CHP-134 cells may promote the commitment to cell death. These findings have important implications for the development of anti-GD2 mAb and PI3K/Akt/mTOR inhibitors-based combination therapies., The study was supported by grant 2012/07/B/NZ1/02808 from the Polish National Science Center.

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POT047

The Combination of 13-cis Retinoic Acid and Isorhamnetin Exerts Synergistic Anticancer Activity against Neuroblastoma Cell Lines

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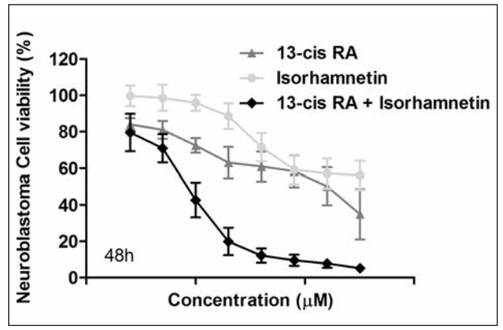
Background: 13-cis retinoic acid (13-cis RA) is a synthetic retinoid that causes differentiation and decreased proliferation of neuroblastoma cells. When administered after myeloablative therapy with autologous stem cell transplantation, 13-cis RA increases 3-year event-free survival in children with high-risk neuroblastoma. In the last 10 years, research for new drugs to be used in oncology has refocused on natural molecule, especially, on flavonoids. The purpose of this study was, thus, to identify flavonoids potentiating the effect of 13-cis RA against neuroblastoma cells.

Methods: CHP134 cell line was challenged with a library of flavonoids in the presence of a sublethal dose of 13-cis RA for 48h. Drug combination experiments of 13-cis RA and isorhamnetin were carried out in different cell lines and their synergism was determined by combination index analysis. Changes in cell cycle and cell death were investigated by flow cytometry, fluorescence microscopy. Microarray gene expression analysis was performed on polysomal RNA isolated from control cells and treated cells (24h).

Results: 13-cis RA and isorhamnetin, hit of the primary screening, exerted synergistic inhibition of the growth of different neuroblastoma cell lines



accompanied by cell cycle arrest in G0/G1 phase and enhanced apoptosis. At gene expression level the drug combination caused negative regulation of cell proliferation and specific neural cell differentiation gene pathway and up-regulation of specific tumor suppressors, G protein-coupled receptor downstream signaling, cell cycle arrest, apoptosis genes.

Conclusion: On the basis of these findings we advanced hypotheses on the molecular mechanism of this pharmacological synergism with potential clinically therapeutic application.

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POT048

Targeted Therapy by Smac Mimetic LCL161 Sensitizes Neuroblastoma for Chemotherapy-induced Apoptosis in a Drug Class-dependent Manner

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Background: In neuroblastoma (NB) an overexpression of XIAP, most potent member of the IAPs (inhibitor of apoptosis proteins), recently was shown. Inhibition of IAPs using Smac mimetics (SM) significantly sensitizes NB-cells for chemotherapy, however strongly dependent on the used cytotoxic drug. Therefore a systematic analysis of the impact of SM in combination with different classes of drugs is of great interest. This should lead to a better understanding why NB-cells are often resistant against chemotherapy and to the development of novel effective therapy regimens in the treatment of neuroblastoma.

Methods: Cell lines served as an experimental model for in vitro treatment of NB. Cytotoxic drugs from the substance classes of anthracyclines, platinum derivatives, topoisomerase inhibitors and vinca alkaloids used for standard neuroblastoma therapy were used in combination with SM. Effects on proliferation and apoptosis induction were evaluated.

Results: Vinca alkaloids in general revealed the strongest synergistic effect in combination with SM. With this drug class the most significant enhancement of proliferation inhibition and induction of apoptosis in the combination treatment were detectable. Application of topoisomerase inhibitors resulted in only weak synergistic effects together with SM.

Conclusion: The mechanisms of action of the used cytostatic drugs obviously have a big impact on the occurrence of synergistic effects if used in combination with SM. More detailed analyses on the molecular regulation mechanisms, the activated signaling pathways and the thereby induced cell death are warranted to evaluate a possible targeted use of SM in neuroblastoma therapy in the future.

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POT049

Synergistic Induction of Apoptosis by Dual PI3K/mTOR Inhibitor NVP-BEZ235 and Chloroquine in Neuroblastoma Cells via Mitochondrial-Lysosomal Amplification Loop

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Background: Based on our previous identification of aberrant phosphoinositol-3-kinase(PI3K)/AKT signaling as a poor prognostic factor in neuroblastoma, we evaluated the dual PI3K/mTOR inhibitor NVP-BEZ235 (BEZ). Further we investigated the ability of BEZ to induce autophagy as a survival mechanism and whether inhibition of autophagy by chloroquine (CQ) can promote cell death induction.

Methods: BEZ alone or in combination with CQ was analyzed for its effect on autophagy induction, the lysosomal compartment, reactive oxygen species (ROS), and the activation of apoptosis signaling pathways in neuroblastoma cell lines. Functional relevance was determined by knockdown or specific pharmacological inhibition.

Results: BEZ as a single agent is insufficient to induce cell death but strongly induces autophagy. CQ cooperates with BEZ to induce mitochondrial apoptosis in a highly synergistic manner. Against the initial hypothesis, inhibition autophagy is unlikely the primary mechanism of this synergism, since neither inhibition of autophagosome formation nor disruption of the autophagic flux enhances BEZ-induced apoptosis. BEZ causes hyperpolarization of the mitochondrial membrane potential prior to ROS generation as well as an enhancement of the lysosomal compartment. CQ on the other hand accumulates in lysosomes and promotes lysosomal membrane permeabilization (LMP). Finally, BEZ and CQ cooperate to trigger LMP in a ROS-dependent manner. This is a critical step in the synergism, as inhibition of lysosomal enzymes, LMP or scavenging of ROS significantly rescues cells from apoptosis.

Conclusion: Since BEZ is currently being evaluated in phase-II trials and CQ is a clinically well-established drug, the combination appears to be a promising and feasible therapeutic approach against neuroblastoma.

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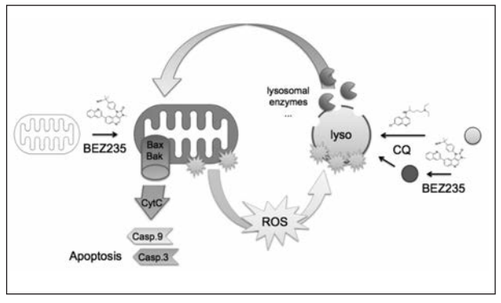

POT050

Targeted Nuclear Imaging and Radiotherapy with 68Ga-DOTA-TATE and 177Lu-DOTA-TATE on Neuroblastoma Preclinical Models

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Background: Somatostatin receptors (SSTRs), including SSTR2, are expressed in most neuroblastomas (NB), making molecular imaging and radiotherapy with radiolabeled somatostatin analogues, ^{68}Ga-DOTA-TATE and ^{177}Lu-DOTA-TATE, an attractive option for selected patient populations. Here, we evaluated the role of SSTR2 on predicting ^{68}Ga-DOTA-TATE tumor imaging and ^{177}Lu-DOTA-TATEtherapy in NB preclinical models.

Methods: SSTR2 expression was determined by RT-PCR and Western blot using eight NB cell lines representing different biological and genetic backgrounds. To assess the relationship between ^{68}Ga-DOTA-TATE uptake and SSTR2 expression, a high SSTR2-expressing cell line, CHLA-15, and a low SSTR2-expressing cell line, SK-N-BE(2), were selected to generate tumor xenografts and imaged using positron-emission tomography (PET). Standardized uptake values (SUV) of ^{68}Ga-DOTA-TATE were calculated and compared between CHLA-15 and SK-N-BE(2) tumors. Colocalization of SSTR2 expression and ^{68}Ga-DOTA-TATE uptake was analyzed by combined autoradiography-immunohistochemistry. Anti-tumor effect of ^{177}Lu-DOTA-TATE was further evaluated in the CHLA-15 NB model.

Results: NB cell lines expressed SSTR2 at variable levels, and SSTR2 mRNA levels did not directly correlate with protein levels. CHLA-15 had the highest SSTR2-expression and SK-N-BE(2) the lowest. There was no correlation between SSRT2 expression and MYCN status. We demonstrated a significant increased SUV in the higher SSTR2-expressing CHLA-15 xenografts compared to SSTR2 low expressing SK-N-BE(2) tumors ($p<0.05$). Histologically, we observed co-localization of SSTR2 expression and ^{68}Ga-DOTA-TATE uptake in CHLA-15 tumors, while autoradiography-immunohistochemistry signals in SK-N-BE(2) tumors were too low for autoradiography and co-localization analysis. For radiotherapy, a single low dose of ^{177}Lu-DOTA-TATE (20MBq/animal) provided significant tumor growth inhibition compared to the vehicle control in the CHLA-15 xenograft model ($p<0.05$).

Conclusion: SSTR2 expression levels are associated with ^{68}Ga-DOTA-TATE uptake and correlate with antitumor efficacy of ^{177}Lu-DOTA-TATE. Radioactive ^{68}Ga-DOTA-TATE can be used for NB tumor imaging to predict candidates for ^{177}Lu-DOTA-TATE targeted therapy leading the road to personalized targeted radiation therapy.

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POT051

Structurally Diverse MDM2-p53 Antagonists Act as Modulators of MDR-1 Function in Neuroblastoma

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Background: A frequent mechanism of acquired multidrug resistance in human cancers is overexpression of ATP binding cassette transporters such as the Multidrug Resistance Protein 1 (MDR-1). Nutlin-3, an MDM2-p53 antagonist, has previously been reported to be a competitive MDR-1 inhibitor, reversing MDR-1 mediated multidrug resistance in MDR-1-overexpressing p53 mutant neuroblastoma cells.

Methods: Aims: To assess whether structurally diverse MDM2-p53 binding antagonists, MI-63, NDD0005, and RG7388 are also able to modulate MDR-1 function, particularly in p53 mutant neuroblastoma cells.

Results: XTT based cell viability assays demonstrated that verapamil, Nutlin-3, MI-63 and NDD0005, but not RG7388, potentiated vincristine mediated growth inhibition in a dose-dependent manner when used in combination in high MDR-1 expressing p53 mutant neuroblastoma cell lines at concentrations which did not affect the viability of cells when given alone. Liquid chromatography-mass spectrometry analyses showed that verapamil, Nutlin-3, MI-63 and NDD0005, but not RG7388, led to increased intracellular levels of vincristine in high MDR-1-expressing cell lines. These results show that in addition to Nutlin-3, other structurally unrelated MDM2-p53 antagonists can also act as MDR-1 inhibitors, and reverse MDR-1 mediated multidrug resistance in neuroblastoma cell lines in a p53-independent manner. ,

Conclusion: These findings are important for future clinical trial design of several classes of MDM2-p53 antagonists when used in combination with other drugs which may be MDR-1 substrates, and support a role for their development in combination with p53 mutant tumours which express high levels of MDR-1.

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POT052

HDAC8 Selective Inhibitors Exhibit Anti-Neuroblastoma Activity In Vitro and In Vivo and Enhance Retinoid Acid Mediated Differentiation

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Background: Histone deacetylase (HDAC) inhibitors are currently under investigation for the treatment of a broad spectrum of cancer diseases. However, one clinical drawback is class-specific toxicity of unselective inhibitors, limiting their full anticancer potential. Selective targeting of individual HDAC isozymes in defined tumor entities may therefore be an attractive alternative treatment approach.

Methods: In vivo and in vitro studies with isozyme-selective HDAC inhibitors alone and in combination with retinoid acid.

Results: We have previously identified HDAC family member 8 (HDAC8) as a novel drug target in childhood neuroblastoma. Using small molecule inhibitors, we here demonstrate that selective inhibition of HDAC8 exhibits anti-tumoral effects without toxicity in a xenograft mouse model of MYCN oncogene amplified, relatively chemotherapy-resistant neuroblastoma. In contrast, unselective inhibitor SAHA was more toxic and less efficacious in the same model. The anti-neuroblastoma activity of selective HDAC8 inhibition is associated with induction of cell cycle arrest and differentiation in vitro and in vivo. Upon combination with retinoic acid, differentiation is significantly enhanced and tumor cell growth markedly reduced.

Conclusion: In conclusion, selective HDAC targeting can be effective in cancers exhibiting HDAC isozyme dependent tumor growth in vivo.

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POT053

GNF-4256 Enhances Chemotherapeutic Antitumor Efficacy in a Neuroblastoma (NB) Murine Xenograft Model

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Background: The TrkB/BDNF autocrine pathway plays an important role in the aggressive behavior of high-risk NBs. GNF-4256 (GNF4) is a novel and potent inhibitor of the Trk family of receptor tyrosine kinases. We wanted to determine the efficacy of GNF4 as a single agent, and whether co-treatment with GNF4 could enhance the efficacy of chemotherapeutic agents, Irinotecan and Temozolomide (Irino-TMZ), in vitro and in vivo.

Methods: We tested the effect of GNF4, alone or in combination with Irino-TMZ, on the human NB cell line (SY5Y), and a TrkB-expressing subclone (SY5Y-TrkB), in vitro. We verified that GNF4 enhanced inhibition of TrkB expression and cell growth of SY5Y-TrkB cells (and not SY5Y) using Western blots and SRB cell growth assays. Then, SY5Y-TrkB cells were grown as xenografts in nu/nu mice for a 4-arm study (vehicle, GNF4, Irino-TMZ and combination). GNF4 was administered twice daily (40 mg/kg BID). Irino (0.63 mg/kg QD) and TMZ (7.5 mg/kg QD) were administered together either alone, or with GNF4. Studies were continued for three weeks, or until tumor size exceeded 3 cm^3.

Results: GNF4 had little effect on SY5Y cell growth, but it dramatically inhibited TrkB phosphorylation and in vitro cell growth of SY5Y-TrkB cells in a dose-dependent manner. GNF4 in combination with Irino-TMZ had the greatest effect on TrkB-expressing NB cell growth. Similarly, in our NB xenograft mouse model, the combination of GNF4 and Irino-TMZ significantly slowed NB tumor growth compared to mice treated with either GNF4 or Irino-TMZ alone.

Conclusion: Combining the Trk inhibitor, GNF4, with conventional chemotherapeutic agents (e.g., Irino-TMZ) produced a significantly enhanced therapeutic effect, with no additional side effects, suggesting that inhibition of TrkB with GNF4 could be added to the initial treatment of recurrent/refractory high-risk NBs.

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POT054

Preclinical development of meta-[^{211}At]astatobenzylguanidine ([^{211}At]MABG) Targeted Radiotherapy for Neuroblastoma

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Background: Neuroblastoma (NB) is a radiosensitive malignancy and NB cells express the norepinephrine transporter (NET) enabling uptake of NET ligands. Meta-[^{131}I]iodobenzylguanidine ([^{131}I]MIBG) is a NET ligand radiotherapeutic showing response rates in NB of 40-50%. However, the longer path-length and lower relative biological effectiveness of ^{131}I β particles compared to α particles may contribute to failure with [^{131}I]MIBG. Here we report our progress with preclinical studies designed to optimize NET-targeted radiotherapy with the α-emitting radiopharmaceutical, [^{211}At]MABG.

Methods: We first determined NET (SLC6A2) mRNA and protein expression in 35 human NB cell lines. We then used lentiviral constructs for dual forced overexpression of human NET and luciferase cDNAs in NB cells. [^{125}I]MIBG was used for uptake assays and biodistribution experiments, and [^{131}I]MIBG was used for multi-log cytotoxicity assays in NB cell lines and therapeutic trials (25 mCi/kg) in mouse models. In parallel, [^{211}At]MABG was synthesized and uptake studies performed.

Results: Unlike primary human NBs, NET expression was low in the majority of 35 cell lines studied but all transduced lines showed significant NET overexpression. Transduced lines showed 4-10 fold higher uptake of [^{125}I]MIBG than non-transduced isogenic lines and demonstrated tumor-specific uptake in vivo with tumor-muscle ratios ranging from 13.80 to 29.48 at 24 hours post injection. In vitro cytotoxicity experiments using [^{131}I]MIBG showed NET-expressing cell lines to be more susceptible to treatment compared to low-NET expressing cells (IC_{50} of 2.94 nCi vs. 15.99 nCi). [^{131}I]MIBG treatment of mice bearing NB1691-NET xenografts showed no significant impact on growth, while SKNSH-NET xenografts showed complete maintained remissions. We subsequently showed that NB1691 was highly radioresistant. Lastly, we synthesized [^{211}At]MABG (average radiochemical yield ~20%, radiochemical purity > 99%) and showed NET-specific uptake of [^{211}At]MABG in NB1691-NET cells.

Conclusion: Robust models of NET-overexpressing NB will allow for the rapid preclinical development of [^{211}At]MABG for high-risk NB therapy.

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POT055

Targeting the Warburg Effect and Metabolic Plasticity in Neuroblastoma with FDA Approved Ritonavir and Metformin

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Background: Cancer cells are highly dependent on glycolyis to sustain growth; Warburg effect

Methods: We used six different neuroblastoma cell lines including both NMYC amplified and non amplified ones.

Results: The number of live cells decreased considerably when deprived of glucose. We did not see a similar dependence on glutamine or fatty acid oxiation using the fatty acid oxdation inhibitor Etomoxir. Western Blot and PCR for various glucose transporters showed that GLUT 4 was highly expressed across all cell lines while the expression of GLUT 1 was variable. In the patient specimens examined >50% were

positive for GLUT 1 and GLUT 4. On knocking down GLUT 4 using lentivirus sh-RNA in the IMR 5 cell line we observed a large difference between the morphology of the 2 groups (control sh-RNA and the ones with GLUT 4 knocked down). The cells where GLUT 4 had been knocked down were found to be more vacuolated and failed to form any neural processes which is a characteristic of this cell line at the end of 72 hours. We also observed that the cell line IMR5 (NMYC amplified) showed marked cell death in the presence of metformin and ritonavir compared to SHY5Y (single copy of NMYC) where the cell death was not so remarkable.

Conclusion: Inhibition of glucose transport by the use Ritonavir, that has an off-target inhibitory effect on GLUT4, in Neuroblastoma, results in cell death. In addition, the cells, which are resistant to ritonavir, are sensitized upon treatment with a complex 1 inhibitor (metformin) that targets compensatory mitochondrial metabolism.

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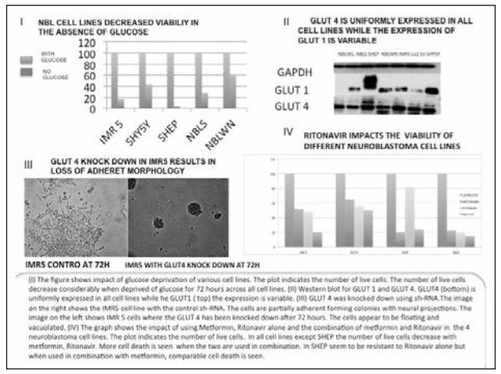

POT056

Hsp90 Inhibition is a Viable Therapeutic Strategy for High-Risk Neuroblastoma

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Background: Hsp90 is a validated molecular target in several adult malignancies, which has resulted in the clinical use of second-generation inhibitors. This follows the hypothesis that oncogenic signalling within malignant cells depends upon Hsp90 for stabilisation of essential client proteins. In high-risk neuroblastoma gene amplification of MYCN is strongly associated with poor-outcome. Furthermore, mutations in anaplastic lymphoma kinase (ALK), the most common mutation in neuroblastoma, occur at a higher frequency in tumours with MYCN amplification, which is associated with an even worse prognosis. Due to the reliance of both MYCN and ALK protein stability on Hsp90, we sought to investigate in vitro and in pre-clinical models in vivo whether Hsp90 inhibitors would be efficacious in the treatment of this type of neuroblastoma.

Methods: Firstly,we undertook in vitro experiments, including proximity ligation assays, to show that there is an intracellular association of mutant ALK with Hsp90. Subsequently, pre-clinical evaluation of NVP-AUY922 in our MYCN-driven and ALK(^{F1174L})/MYCN-driven transgenic models of high-risk neuroblastoma was performed.

Results: The intracellular association of mutant ALK with Hsp90 in vitro can be reversed by the addition of the small molecule Hsp90 inhibitor, NVP-AUY922. This treatment leads to a reduction in the total amount of ALK and/or MYCN protein and cytotoxicity. Treatment of tumour-bearing animals with NVP-AUY922 resulted in a statistically significant survival benefit. Ex-vivo treated tumours have less total ALK as seen through both western blotting of snap-frozen samples and immunohistochemistry of paraffin-fixed sections, when compared to controls.

Conclusion: Clinically, second-generation, small molecule inhibitors of Hsp90, including NVP-AUY922, have shown benefit in the treatment of adult malignancies, without significant toxicities. Their role in paediatric oncology has yet to be fully explored, but as concluded from our work, they could provide a valid therapeutic strategy for neuroblastoma with known high-risk molecular aberrations.

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POT057

The Histone Methyltransferase Activity of EZH2 in Neuroblastoma: Friend or Foe

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Background: Whole gains of chromosome 7 are observed in 50% of high-risk neuroblastoma. Local gains of the chromosome 7 region encompassing the EZH2 locus was identified in three tumours. Previous studies have shown that EZH2 induces the methylation of histone 3 (H3K27me3) of CpG islands in promoter regions of known tumour suppressor genes. This would raise the possibility that EZH2 overexpression could have an oncogenic role in neuroblastoma. We therefore tested the in vitro efficacy of two EZH2 histone methyltransferase inhibitors; GSK126 and EPZ6438.

Methods: MTT assays, Western blotting, Facs analysis, soft agar assays, migration assays

Results: The IC50's of neuroblastoma cells treated with GSK126 and EPZ6438 ranged from 570nM-8μM. We analysed concentrations required for H3K27me3 inhibition to measure EZH2 function. Both inhibitors induced a dose-dependent decrease of H3K27me3 levels. A full functional EZH2 block was observed at much lower concentrations than the IC50's of the cell lines. Mild G1 arrest was observed with both inhibitors at concentrations required for complete functional EZH2 inhibition. Colony-forming capacity of neuroblastoma cells treated with both inhibitors decreased at high concentrations but this did not correspond to pathway inhibition. Our observations suggest that inhibiting the histone methyltransferase activity of EZH2 does not result in significant phenotypic changes in neuroblastoma cells. Therefore, growth inhibition observed at higher concentrations of these compounds might be due to off-target effects. In contrast, lentiviral knockdown of EZH2 causes cell cycle arrest and reducted in colony-forming capacity in neuroblastoma cells. Profiling these cells after knockdown showed strong inhibition of cell cycle regulatory genes.

Conclusion: Targeting EZH2 histone methyltransferase activity in neuroblastoma might not induce growth inhibition in neuroblastoma cells. Nevertheless, EZH2 is frequently gained and over-expressed in neuroblastoma and targeted knockdown of EZH2 results in significant cell cycle inhibition. These observations suggest a function of EZH2 on cell cycle regulation independent of its histone methyltranferase activity.

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POT058

Toll-Like Receptor 3 Agonist Augments 2-deoxyglucose-induced Suppression of Neuroblastoma Cell Growth

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Background: Toll-like receptor 3 (TLR3) agonist polyinosinic-polycytidylic acid [poly(I:C)] has been shown to induce neuroblastoma (NB) cell apoptosis through mitochondrial pathway. 2-deoxyglucose (2DG), as a glycolytic inhibitor, is effective to suppress NB cell growth either alone or as a complementary agent to cisplatin. We conducted the study to verify if TLR3 agonist could augment 2DG suppression of NB cell growth.

Methods: Two NB cell lines without (SK-N-AS, SK-N-FI) and 2 with (SK-N-DZ, IMR-32) MYCN amplification were treated with 2DG without or with poly(I:C) in different order for a total of 48h. Cell proliferation and cell death were evaluated by WST-1-based calorimetry assay and lactate dehydrogenase (LDH) release. Protein expression was measured by using Western blot.

Results: Poly(I:C) was additive to 2DG in suppression of NB cell proliferation, which was significant in 3 of the 4 cell lines, and which was associated with significant release of LDH. Pretreatment with poly(I:C) was more effective than pretreatment with 2DG in TLR3 expressing SK-N-AS and SK-N-FI, while the reverse was true for IMR-32. There was no significant induction of active caspase 3, but significant up-regulation of autophagosomal marker LC3-II. While protein kinase R (PKR) and interferon regulatory factor 3 (IRF-3) was activated during the process in TLR3 expressing SK-N-AS cells, mitochondrial dynamic protein mitofusins (MFNs) were involved in suppression of MYCN-amplified SK-N-DZ by poly(I:C)+2DG. The latter was confirmed by siRNA targeting MFN1 or MFN2, which significantly augmented the combined treatment effect of poly(I:C)+2DG on the cell death in SK-N-DZ.

Conclusion: The results suggested that TLR3 agonist poly(I:C) could augment 2DG suppression of NB cell growth. The effects depend on different pathways and on the order of treatment, which are determined by molecular characteristics of NB cells.

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POT059

Identification of Novel Candidate Compounds Targeting TrkB to Induce Apoptosis in Neuroblastoma: In Silico Screening Utilizing a Grid Computing Technology

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Background: Neuroblastoma (NB) is one of the most frequent solid tumors in children and its prognosis is still poor. The neurotrophin receptor TrkB, expressing at high levels in high-risk tumors, is involved in defining the bad prognosis of the patients. However, the TrkB targeting therapy has never been real in the clinic.

Methods: We performed an in silico screening procedure utilizing an Autodock / grid computing technology (courtesy of IBM Co.Ltd.) in order to identify novel candidate compounds targeting the BDNF binding domain of TrkB.

Results: A library of synthetic compounds including three million molecules was screened in silico. The top-ranked 60 compounds were further screened functionally for cytotoxicity by using NB cell lines. We have finally identified 7 low molecular weight compounds to kill NB cells by the IC-50 values of 0.05 to 5.0 μM. The candidate compounds and BDNF demonstrated a synergistic effect on cell growth, possibly suggesting the competition at the BDNF binding site of TrkB. Indeed, we showed that the candidate compounds could inhibit the phospho-activation of TrkB, which was mediated by BDNF, further supporting the notion above. The TUNEL assay showed that these molecules induce apoptosis accompanied by p53 activation in NB cell lines. Tumor xenograft study utilizing scid mice demonstrated that the compounds could significantly decrease the size of tumors in mice, suggesting the anti-tumor activity of the candidate compounds in vivo. Interestingly, the candidate compounds decrease the phosphorylation levels of mitogen-activated protein kinases, suggesting that these compounds inhibit the signaling pathway downstream of TrkB.

Conclusion: In this study, we demonstrated that novel candidate compounds exerting anti-tumor activity were effectively identified by an in silico screening, followed by in vitro and in vivo assays. We propose that this approach could help developing a novel treatment and cure for childhood cancers including neuroblastoma.

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POT061

Metabolic characteristics of 13-cRA and anti-tumor activity of the 13-cis retinoic acid metabolite 4-oxo-13-cis retinoic acid in neuroblastoma

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Background: Isotretinoin (13-cis-retinoic acid; 13-cRA) is a differentiation inducer effective in treating minimal residual disease after myelobative therapy for high-risk neuroblastoma. Approximately 40% of children develop recurrent disease during or after 13-cRA treatment. Plasma concentrations of 13-cRA in earlier studies were considered subtherapeutic while 4-oxo-13-cis-RA (4-oxo-13-cRA), a metabolite of 13-cRA assumed in studies by other investigators to be inactive, were > 3-fold higher than 13-cRA. We sought to define the metabolic pathways of 13-cRA and to define the antitumor activity of 4-oxo-13-cRA.

Methods: 13-cRA metabolism was determined using tandem mass spectrometry in human liver microsomes and in patient samples. Activity of 13-cRA and 4-oxo-13-cRA in neuroblastoma cells was assessed by DIMSCAN, cell proliferation by flow cytometry, MYCN down-regulation by RT-PCR and immunoblotting, and neurite outgrowth by confocal microscopy.

Results: Seven major metabolites of 13-cRA were identified in 28 patient samples; 4-oxo-13-cRA was the most abundant. 4-oxo-13-cRA glucuronide and 13-cRA glucuronide, were also detected suggesting that the major metabolic pathway of 13-cRA was oxidation to 4-oxo-13-cRA followed by glucuronidation. In liver microsomes CYP3A4 played the major role in catalyzing 13-cRA to 4-oxo-13-cRA. Both 4-oxo-13-cRA and 13-cRA were equally active in inducing neurite outgrowth, inhibiting proliferation, decreasing MYCN and increasing retinoic acid receptor-β mRNA and protein expression in neuroblastoma cells, showing that 4-oxo-13-cRA is as active as 13-cRA against neuroblastoma cell lines. In 137 patients enrolled in a COG phase III study (ANBL0032), 4-oxo-13-cRA levels (mean: 6.8±4.9 μM) were 4-fold higher than 13-cRA (1.8±1.6 μM). Fifty-two percent (72/138 patients) had levels of both 4-oxo13-cRA and 13cRA less than 5 μM.

Conclusion: Our data indicate that for neuroblastoma 4-oxo-13-cRA and 13-cRA plasma levels are equally important in pharmacokinetic (PK) studies aimed at defining the relationship of drug exposures to outcome and/or defining PK-guided dosing strategies.

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POT062

The Influence of Chelator- and Radiolabelling on the in Vivo and in Vitro Binding Characteristics of the aAnti-GD2 Antibodies ch14.18, hu14.18K322A and ch14.18-Δch2

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Background: Radiolabelled anti-GD_2-antibodies may constitute highly specific agents for diagnostic imaging and targeted radiation therapy of Neuroblastoma (NB). Here we study the influence of chelator- and Copper-64-labelling to the binding characteristics of the GD_2-specific antibodies ch14.18, hu14.18K322A and ch14.18-Δch2 (Δch2). Furthermore, we track the biodistribution of the labelled antibodies in vivo to evaluate their benefit for diagnostics and radioimmunotherapy (RIT).

Methods: Radiolabelling of the antibodies was done using the complexing agents NHS-DOTA or NHS-NOTA and the radioisotope Cu-64. The labelling efficiency and the stability of the Cu-64-antibody complex were compared among the chelators. The influence of chelator- and Cu-64-labelling to the binding and specificity of the antibodies was evaluated in FACS and γ-counter experiments. Small animal PET was used to analyze the in vivo biodistribution of the radiolabelled antibodies in a subcutaneous NB mouse model over 3 days.

Results: DOTA and NOTA show very high labelling efficiencies, the stability of the radiolabelling is comparable among the chelators. Chelator-labelling reduces the immunoreactivity of the antibodies by around 50%. However, binding and blocking studies approve specificity of the antibodies after chelator- and Cu-64-labelling. The Δch2 shows the highest immunoreactivity among the antibodies. In vivo, the accumulation of the radiolabelled antibodies in GD_2 expressing tumors is significantly increased in comparison to muscle tissue and higher than the uptake of an isotype antibody.

Conclusion: It is the first time that chelator- and radiolabelling of anti-GD_2-antibodies were studied in detail. Our in vitro data demonstrate a significant reduction of immunoreactivity after labelling but verify the specificity of the labelled antibodies. Labelling yield and stability were shown to be independent of the chelator. In vivo the labelled antibodies accumulate in the tumour region, which confirms their potential for NB imaging or RIT. Especially Δch2 with its small size and low immunoreactivity is a promising candidate for this approach.

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POT063

New Synthetic Bismuth Zinc Compound Could Reduce Cisplatinuminduced Nephrotoxicity without Compromising the Anticancer Effect

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Background: Cisplatinum is an important drug for advanced stage neuroblastoma but it hassignificant nephrotoxicity. We developed a novel compound bismuth zinc citrate (BiZn) with potential protective effect against cisplatinum induced nephrotoxicity.

Methods: Cytotoxic effect of BiZn+/-cisplatinum on a panel of normal [human mesenchymal stromal cells, human liver cells (MIHA), rat neural stem cells (C17.2), human non-malignant kidney cells (HK-2)] and neuroblastoma cell lines were tested by XTT assay. Orthotopic neuroblastoma (luciferase transduced SKN-LP) xenograft mouse in-vivo model wasused.The treatment groups were PBS-treated (n=6), BiZn-treated (n=6), cisplatinum-treated (n=6) and BiZn+cisplatinum-treated (n=6). BiZn (0.14mmol/Kg) was administered orally24 hours before and just prior tocisplatinum administration and then every two days thereafter. Bioluminescence imaging was performed on all of the mice weekly after tumor implantation. For protective effect of BiZn against cisplatinum lethal doses, normal mice were used.

Results: BiZn did not have anti-cancer effect on its own but it did not affect the therapeutic effect of cisplatinum in-vitro and in-vivo. The protective effects of BiZn on cisplatinum treated neuroblastoma cells were insignificant in the in-vitro setting as compared to thein-vivosetting, suggesting BiZn acted indirectly in-vivo. Significant tumor shrinkage but no overt toxicity were noted in cisplatinum+/-BiZn treated mice and BiZncould significantly reduced the levels of blood urea nitrogen in cisplatinum treated mice. Under the lethal dosage of cisplatinum, pre-treatment with BiZn significantly improved the survival. We postulated that the mechanism of protection may be related to BiZn induced soluble factors released by the liver and kidney.

Conclusion: Pre-treatment of BiZn can decreasethe cisplatinum induced nephrotoxicity without affectingthe antitumor activity of cisplatinum in-vivo. Since BiZn can beadministeredorally with good absorption, itcan readily applicable as a drug to minimize the adverse effects of cisplatinum.

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POT064

The MRN Complex: A Potential Target to FIght against MYCN Amplified Neuroblastoma

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Background: MYCN amplification (MNA) is the most relevant negative prognostic marker occurring in about 25% of neuroblastoma (NB) cases. Due to the absence of specific pharmacological strategies to target it, MNA is still associated with early tumor progression and poor outcome in most patients.The high rate of replication stress in Myc-addicted cancer cells makes them more sensitive to inhibition of ATR and CHK1, the main kinases activated in response to replication stress-associated DNA Damage Response (DDR). A prominent role of the MRE11/RAD50/NBS1 (MRN) complex in replication stress response is also emerging.We have shown that MYCN induces a replication stress dependent DDR and relies on the induction of the MRN complex to limit its deleterious effects.

Results: We report that a pharmacological inhibitor of MRE11 nuclease activity, mirin, selectively kills MNA NB cells, but not MYCN single copy (MNSC) NB cells or non-NB cancer cell lines. p53 defective (LAN1 or SK-N-BE2c) cell lines are far less sensitive to mirin dependent apoptosis, but a full response can be obtained by p53 reconstitution. Interestingly, while mirin induces a sudden, but transient, p53 accumulation in both MNSC and MNA cells, p53 is S15-phosphorylated and further stabilized later in time in MNA cells only. The initial p53 accumulation still awaits molecular characterization. On the contrary its subsequent activation is associated to a very prominent activation of a replication stress associated DDR (i.e. 53BP1 foci formation and H2AX, p53 and CHK2 phosphorylation) and by the accumulation of DNA double strand breaks, all of which selectively occurs in mirin treated MNA cells. Although its poor solubility in aqueous solution delayed mirin usage in animal models so far,our data indicate that pharmacological inhibition of the MRN complex might be exploied for therapeutic purposes in MNA NBs.

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POT065

Noradrenaline Transporter Gene Transfer via Oncolytic Virotherapy Enhances 131I-MIBG uptake: A Novel Combination Viroradiotherapy Approach to High-Risk Neuroblastoma

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Background: Outcomes for children with recurrent/refractory neuroblastoma remain dismal and novel therapies are needed. Pharmacologic stimulation of noradrenaline transporter (NAT) expression is being studied as MYCN amplified tumors have low NAT. HSV1716 is an oncolytic herpes virus genetically altered to selectively replicate in tumor cells that is currently in clinical trials. HSV1716/NAT was constructed to deliver cDNA for NAT to cancer cells. We sought to determine if exogenous expression of NAT and increased sensitization to ^{131}I-MIBG is feasible in the context of oncolytic virotherapy in neuroblastoma cells.

Methods: We mined the expression database of the Pediatric Preclinical Testing Program and utilized qRT-PCR to determine endogenous NAT expression in 12 neuroblastoma cell lines and in xenograft tumors grown in nude (nu/nu) mice. We tested the cell lines' response to HSV1716/NAT via gene transfer, viral production, and MTS analysis. Cells were exposed to ^{131}I-MIBG +/- HSV1716/NAT to determine both cytotoxicity and ^{131}I-MIBG uptake.

Results: We found wide variations in endogenous NAT mRNA expression and HSV1716/NAT susceptibility and permissivity . The combination of virus and 131I-MIBG showed Increased cytotoxicity than with either treatment alone. Neuroblastoma cells infected with HSV1716/NAT showed increased NAT mRNA expression, increased specific uptake of ^{131}I-MIBG, and increased cytotoxicity with 131I-MIBG.

Conclusion: ^{131}I-MIBG uptake and neuroblastoma cytotoxicity increase when treated with both HSV1716/NAT and ^{131}I-MIBG. Additionally, mouse model studies are underway to evaluate the animal survival and neuroblastoma tumor regression with HSV1716/NAT, ^{131}I-MIBG, and the combination therapy. If these studies are successful, oncolytic viroradiotherapy may represent a new therapeutic approach to high-risk neuroblastoma.

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POT066

Spermidine-Dependent Posttranslational Activation of Hypusinated eIF5A is Blocked by DHPS Inhibitor GC7 and Induces Rb-Mediated Inhibition of Neuroblastoma Tumor Growth

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Background: Neuroblastoma (NB) is an aggressive pediatric malignancy that typically occurs in infants and children until the age of 10 years (N Engl J Med, 2010, 362:2202-11). Relapse and refractory patients eventually succumb to this devastating cancer, thus calling for the development of new drugs that suppress tumor growth and prevent relapse. We previously showed that polyamines such as spermidine play an essential role in NB tumorigenesis (Oncogene, 2005, 24:5606-18) and that ODC inhibitor DFMO is effective in vitro and in vivo which spurred our efforts to move DFMO into the clinic. Despite this progress, the specific molecular action of polyamines remains poorly defined.

Methods: Spermidine and the deoxyhypusine synthase (DHPS) are essential components in the hypusination process required for activation of eukaryotic initiation factor 5A (eIF5A). Because hypusine formation is the most specific spermidine-dependent molecular event, we were interested in assessing the role of DHPS in NB and the impact of specific DHPS inhibition by N1-guanyl-1,7-diaminoheptane (GC7) on tumor cell growth using biological assays and Affymetrix DNA micro-array analysis.

Results: We found that blocking the production of hypusine through GC7 inhibits NB cell growth in a dose-dependent manner and hypophosphorylates Rb, suggesting Rb-mediated cell cycle arrest. In a cohort of 88 human NB tumors, we found that high DHPS mRNA expression correlated significantly with poor survival prognosis using Kaplan-Meier analysis, proposing an oncogenic role for DHPS in NB tumorigenesis.

Conclusion: These findings postulate that polyamines and DHPS are key contributing factors in NB tumor proliferation through regulation of the hypusine-dependent eIF5A/Rb axis.

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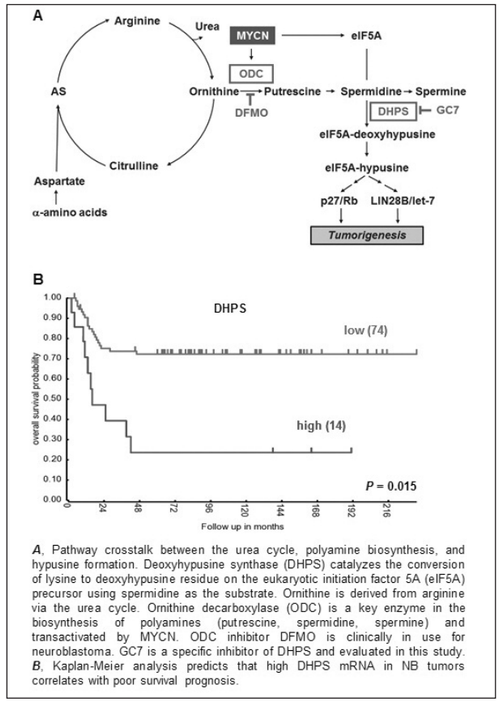

A, Pathway crosstalk between the urea cycle, polyamine biosynthesis, and hypusine formation. Deoxyhypusine synthase (DHPS) catalyzes the conversion of lysine to deoxyhypusine residue on the eukaryotic initiation factor 5A (eIF5A) precursor using spermidine as the substrate. Ornithine is derived from arginine via the urea cycle. Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines (putrescine, spermidine, spermine) and transactivated by MYCN. ODC inhibitor DFMO is clinically in use for neuroblastoma. GC7 is a specific inhibitor of DHPS and evaluated in this study. *B*, Kaplan-Meier analysis predicts that high DHPS mRNA in NB tumors correlates with poor survival prognosis.

POT067

Structure-Based Docking of Transcription Factor STAT3 to Withaferin A, a Natural Product That Impedes Neuroblastoma Tumor Cell Growth

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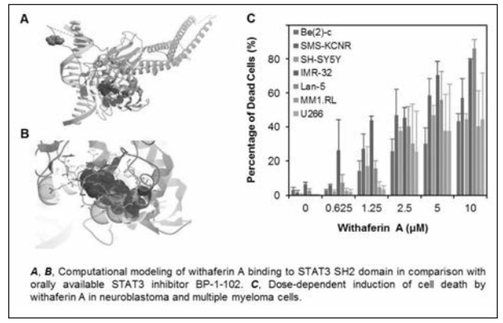
Background: STAT3 is an oncogenic transcription factor implicated in a large number of human cancers including neuroblastoma (NB) and therefore has emerged as an ideal target for cancer therapy (Cancer Res, 2013, 73:3852-64). Withaferin A (WFA) is a natural product with promising anti-proliferative properties through its action on a number of molecular targets. However, the physical interaction and direct binding of proposed targets, including STAT3, has not been demonstrated. Importantly, the potency of WFA in pediatric neuroblastoma has not been studied.

Methods: Our data provide compelling evidence that WFA directly binds STAT3. We defined the amino acid residues of STAT3 that participate in this ligand-protein interaction using computational docking simulations and biological assays.

Results: A total of 100 different docking scenarios were screened using all possible combinations of the five ligand conformers and various STAT3 protein conditions. We found that WFA binds to the protein near the Y705 phospho-tyrosine residue of the STAT3 SH2 domain and prevents STAT3 dimer formation. Strikingly, the thermodynamically stable structure shares multiple binding sites with the orally administered STAT3 inhibitor BP-1-102 (PNAS, 2012, 109:9623-28), thus suggesting a similar binding mechanism and inhibitor potential.



A, *B*, Computational modeling of withaferin A binding to STAT3 SH2 domain in comparison with orally available STAT3 inhibitor BP-1-102. *C*, Dose-dependent induction of cell death by withaferin A in neuroblastoma and multiple myeloma cells.

Indeed, WFA effectively induced dose-dependent cell death in high-risk and drug-resistant NB and multiple myeloma tumor cells, prevented IL-6-mediated and persistently activated STAT3 phosphorylation at Y705, and blocked the transcriptional activity of STAT3.

Conclusion: Our findings suggest that the antitumor activity of WFA is mediated in part through inhibition of STAT3 and provides a rationale for further development and potential clinical use in NB tumors.

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POT068

Resistance to Alisertib (MLN8237) in Xenograft Derived NeuroblastomaCcell lines is Mediated through Cell Cycle Arrest

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Background: Alisertib (MLN8237) is an investigational compound that inhibits Aurora A kinase, and has shown promising results in both Phase I and II clinical trials in the treatment of relapsed/refractory neuroblastoma. It plays a critical role in the regulation of spindle assembly and chromosome alignment during mitosis and has been shown to lead to improper chromosomal alignment and disruption of spindle organization in tissue culture. This improper chromosomal alignment results in a transient mitotic delay and eventual apoptosis. The Pediatric Preclinical Testing Program (PPTP) examined Alisertib in varying pediatric tumor models and noted that in the neuroblastoma xenograft models, three out of the six models maintained a complete response irregardless of Aurora A expression. These results begged the question, "What are the possible mechanisms of resistance to Alisertib in neuroblastoma?"

Methods: To answer this question, we evaluated four of the xenograft derived neuroblastoma cell lines from the PPTP, two sensitive (NB-EBc1 and NB-1643) and two resistant (SKNAS and NB-1691) at their own defined inhibitory concentrations (IC) of IC100, IC60, and IC40 at 24, 48 and 72hrs. We evaluated protein expression, mRNA expression, and immunofluorescence of Aurora A, TPX2 (which regulates Aurora A kinase activity), and CDC25b.

Results: Our data showed that in the most resistant cell line (SKNAS which is derived from bone marrow disease) protein expression of Aurora A, TPX2, and CDC25b was not affected, where as in the sensitive cell lines expression decreased with increasing IC at their corresponding time points. In addition, immunofluorescence showed improper chromosomal alignment and disruption of spindle organization in the sensitive cell lines, but in SKNAS, our results indicated cell cycle arrest.

Conclusion: Thus we hypothesized that SKNAS is able to maintain resistance to Alisertib through cell cycle arrest and that resistance to Alisertib maybe further pronounced in metastatic disease.

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POT069

Cytosolic Melanoma Differentiation-Associated Gene 5 functions to Complement Toll-Like Receptor 3 Agonist–Induced Neuroblastoma Cell Apoptosis

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Background: Cytosolic melanoma differentiation-associated gene 5 (MDA5) is a viral sensor belonging to retinoic acid-inducible gene-I-like receptor (RLR) family. Activation of MDA5 has been implicated in mitochondrial apoptosis of melanoma. As Toll-like receptor 3 (TLR3), another viral sensor, has been shown to inhibit cell growth in neuroblastoma (NB) in our previous study, we hypothesized that MDA5 might share a role with TLR3 in inhibition of NB growth.

Methods: Archival NB tissues from 92 patients were available for immunohistochemical staining. MDA5 expression was scored and correlated with biological characteristics of NB. In vitro studies were conducted on 2 NB cell lines without and 3 with MYCN amplification to verify the role of MDA5 in the presence of absence of TLR3 and another cytosolic RLR member retinoic acid-inducible gene-I (RIG-I) under the treatment of TLR3 agonist polyinosinic-polycytidylic acid [poly(I:C)].

Results: MDA5 was present in the cytoplasm of ganglion and neuroblastic cells in ganglioneurobastoma and differentiated NB, but not in the neuroblastic cells of undifferentiated or poorly differentiated NB. In vitro studies showed that MDA5 and RIG-I were absent in the three NB cell lines with MYCN amplification, but present in the two NB cell lines SK-N-AS and SK-N-FI without MYCN amplification, after treatment with poly(I:C). Single and double knockdown of TLR3, MDA5 and RIG-I with siRNA targeting these genes revealed that combined knockdown of TLR3 and MDA5 was most effective to rescue poly(I:C)-induced NB cell apoptosis. The latter was accomplished with suppression of mitochondrial antiviral signaling protein (MAVS), interferon regulatory factor 3 (IRF-3) and active caspase3.

Conclusion: Our results indicate that MDA5 functions to complement TLR3 in poly(I:C) -induced NB cell apoptosis, at least in part through MAVS-IRF3-caspase3 signaling pathway.

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POT070

SMARCA4/BRG1 is a New Epigenetic Target for Neuroblastoma Therapy

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Background: Neuroblastoma (NBL) is an extracranial neoplasm originated in the neural crest lineage of the sympathetic nervous system, and is the most common solid tumor of infancy. NBL patients are assigned to three different risk groups according to clinicopathological variables such as age at diagnosis, MYCN amplification, tumor histology and DNA ploidy. While prognosis is good for low (>95% survival) or intermediate-risk (70-90% survival) cases, high-risk patients, however, have poor prognosis (30-40% survival) and need intense therapeutic regimens. Despite the intense multimodal therapy, high-risk patients frequently relapse and present progression of the disease. Owing to the diverse mechanisms that are responsible of NBL resistance to therapy, we aimed to target epigenetic factors that control multiple pathways to bypass therapy-resistance.

Methods: The mRNA expression of epigenetic genes was analyzed in multiple neuroblastoma data sets. Epigenetic genes that showed differential expression in the high-risk NBL group with the same trend in the 3 independent data sets were selected for expression validation by immunohistochemistry. For functional characterization, NBL cell lines were infected with shRNA lentiviral vectors and effects on proliferation and viability were evaluated in vitro and in mouse NBL xenografts.

Results: According to multiple available neuroblastoma expression datasets, several epigenetic genes were found to be deregulated in high-risk tumors. Among them, (SMARCA4/BRG1) was consistently upregulated in advanced stages of NBL patients in multiple NBL data sets as well as in our independent cohort of patients. Moreover, patients with higher levels of SMARCA4 had shorter relapse-free survival and worse overall survival. Loss of function experiments in NBL cell lines showed that SMARCA4 knockdown reduced cell proliferation and increased apoptosis in vitro and in vivo.

Conclusion: Our results support that SMARCA4 is essential for the proliferation and viability of NBL cells and its inhibition may represent an attractive and novel therapeutic target for high-risk NBL.

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POT071

Changes in Phosphorylation of Aurora Kinases A, B, and C, and Expression of Natural Aurora A Substrates MYCN, P53, PHLDA1, in Neuroblastoma Cells Double Hit with an Anti-GD2 Ganglioside Antibody 14G2a and a Novel Aurora A Inhibitor MK-5108

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Background: High-risk neuroblastoma patients await new treatments improving their survival. Therefore, we tested in vitro strategies to enhance neuroblastoma killing.

Methods: We used cytotoxicity tests, western blot analyses, and flow cytometry to characterize effects of dual targeting of two vital molecules, namely GD2 ganglioside (GD2), and aurora A kinase in neuroblastoma.

Results: We showed that the GD2-specific mouse monoclonal antibody 14G2a (mAb) exhibits time- and dose-dependent cytotoxic effects on MYCN-amplified IMR-32, LA-N-1, CHP-134, HTLA-230, LA-N-5, KELLY cells (Horwacik et al., Cancer Letters, 2013), with IC50 values from 64±1 to 249±39 μg/ml. This correlated with statistically significant down-regulation of both phosphorylated and unphosphorylated aurora A, B and C kinases in IMR-32, CHP-134 and LA-N-1. , Then, we investigated expression and localization patterns of selected aurora A kinase substrates, MYCN, P53 and PHLDA1, in IMR-32 and CHP-134 cells treated with the 14G2a. We measured statistically significant increase in total PHLDA1, nuclear P53 accumulation that peaked 6 h after the treatment, and decrease in nuclear MYCN levels (that were the lowest for 24 h and 48 h time points). We also tracked in time HSF1, HSP40, HSP70 proteins, known to regulate expression or pro-apoptotic function of PHLDA1. , Additionally, we tested an aurora A inhibitor MK-5108, and showed that it decreased cell viability of all six cell lines with IC50 values form 0.16±0.1 to 6±0.1 μM. The combination of MK-5108 and 14G2a mAb further enhanced the effect (1.8 – 3.1 fold for three cell lines). This was compared to 13-cis retinoic acid used alone or together with the mAb. Finally, phosphorylation of aurora kinases A, B, C, localization, and levels of P53, MYCN, HSF1, and total PHLDA1, HSP70, HSP40 proteins were analyzed in the inhibitor- and double-agent treated cells.

Conclusion: The results widen our knowledge on effects of GD2-specific antibodies on neuroblastoma, and have implications on development of novel therapies.

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POT072

Implications of the Down-Regulation of Stemness/Reprogramming Factor Expression by Ibuprofen and Biguanides

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Background: Cancer stem cells (CSCs) are thought to have the capacity to renew indefinitely, to initiate tumor formation, and to give rise to multiple non-tumorigenic progenies via asymmetric cell division. As a result of this phenotypic drift, an established tumor would consist of a mixture of CSC and non-CSC. Based on the current view, the CSC population is likely responsible for distant metastases, drug resistance and recurrence. However, the process by which this cell population is generated in a tumor mass has continued to be unclear. Based on our previous study (PNAS 110: 6097-6102, 2013), this process may involve nuclear reprogramming, namely the elevated expression of stemness/reprogramming factors (SOX2, OCT4, NANOG, LIN28, KLF4, MYC/MYCN, and those with equivalent functions). If so, it is conceivable that destabilization of these proteins would prevent the generation of CSC compartment(s) within a tumor mass. Recently, we reported that anti-inflammatory Ibuprofen and anti-diabetic biguanides destabilized MYC and MYCN in neuroblastoma cells.

Methods: In this study, we have investigated the effects of Ibuprofen and Phenformin on stemness phenotypes of cancer cells (i.e., the expression of stemness factors) using the teratocarcinoma cell line NT2. NT2 cells were chosen as a model because they retain the expression of reprogramming factors, including MYC and MYCN.

Results: Our data showed that high-dose/short-term treatment of NT2 cells with Phenformin and Ibuprofen significantly down-regulated the expression of MYC/MYCN, OCT4, SOX2, and NANOG. Moreover, the anti-stemness effect of Ibuprofen in the low-dose/long-term treatment was also evident (0.15 mM at Day 7 of the drug-treatment or 0.15 mM and 0.25 mM at Day 4). We are also investigating a change in global gene expression in the drugs-treated NT2 cells, with the emphasis on stem cell-related genes and pathways.

Conclusion: Results of these studies will provide insight into how these common drugs can be used as anti-CSC therapeutics.

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POT073

The Bcl-2 Selective Antagonist, ABT-199, Restores Apoptosis and Chemotherapy Response in High-Risk Neuroblastomas (HR NB)

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Background: Many HR NB cell lines and primary tumors depend on anti-apoptotic Bcl-2 for survival. Bcl-2 dependent xenografts derived from aggressive NBs (ALK mutated and MYCN amplified) can be cured with short courses of cyclophosphamide and ABT-737, a Bcl-2/Bcl-xl/Bcl-w antagonist (Goldsmith, 2012). The oral analogue to ABT-737, ABT-263 (Navitoclax, Abbvie), causes an immediate drop in patient peripheral platelet counts as mature platelets depend on Bcl-xL for survival. ABT-199 (Abbvie) is a Bcl-2 selective inhibitor that does not target Bcl-xL. A Phase I trial of ABT-199 in CLL showed stable platelet counts and remarkable anti-tumor activity. In HR NBs, Bim is not sequestered by Bcl-xl, suggesting ABT-199 would be as effective as ABT-263.

Methods: HR NB cell lines were exposed to ABT-199 in vitro and assessed for cell death by WST-1, and apoptosis by Parp-1 cleavage. Established NB murine xenografts (250 mm3) were treated with 14-days of ABT-199 (75 mg/kg/day), cyclophosphamide (75 mg/kg biweekly x 2), vehicle or ABT-199/cyclophosphamide at monotherapy dosing and response measured by tumor regression.

Results: Bcl-2 dependent NB1643 was very sensitive to ABT-199 (IC50 2nM) where Mcl-1 dependent IMR5 and SK-NAS were resistant (IC50 > 1000nM). Bcl-2 dependent SMS-SAN exhibited Parp-1 cleavage following ABT-199, confirming apoptosis. Co-immunoprecipitation showed ABT-199 completely displaces Bim off of Bcl-2 in SMS-SAN. Mcl-1 dependent IMR5 xenografts continued to grow in the ABT-199 and vehicle arms and briefly regressed to cyclophosphamide and ABT-199/cyclophosphamide (day 20), but ultimately showed no difference in time to progression/sacrifice. Ongoing Bcl-2 dependent xenograft treatments show significant tumor regression so far to ABT-199 and more so to ABT-199/cyclophosphamide compared to vehicle or cyclophosphamide alone ($p < 0.05$).

Conclusion: Our data confirms that Bcl-2 selective inhibitors are as potent as ABT-263/ABT-737 pre-clinically and safeguard against untoward platelet effects that are concerning in heavily pretreated or relapsed patients. Our results support ABT-199 should be translated to treat Bcl-2 dependent pediatric tumors like HR NB.
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POT074

Combined Therapeutic Effects of Irinotecan and Cyclophosphamide Chemotherapy in Neuroblastoma

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Background: Neuroblastoma is the most common extracranial solid tumor of childhood. While treatment response remains poor for high-risk clinical phenotype. Therefore, development of new treatment regimen has been the focus of many NB studies. Irinotecan (IRN) has demonstrated significant preclinical and some clinical activity against NB. We wanted to determine if the combination of cyclophosphamide (CP) and IRN had synergistic therapeutic efficacy in neuroblastoma both in vitro and in vivo.

Methods: KCNR cells were treated with IRN and/or CP both in vitro and in vivo. Cell survival and tumor growth were investigated in KCNR inoculated nude mice treated with IRN and/or CP. Animal body weights were observed for toxicity. Apoptosis was detected by TUNEL staining in vivo.

Results: Compared with control group, both IRN and CP had anti-proliferative cytotoxicity against KCNR cells. Combination of these two drugs has synergistic effect in vitro. Both IRN and CP could inhibit tumor growth as single agent in KCNR subcutaneous animal model. Combination of IRN and CP led to reduced tumor growth and increased survival in KCNR tumor bearing model. This drug combination induced more apoptosis of tumor cells than single agent in vivo.

Conclusion: Clinical achievable concentration (CAC) IRN and CP have anti-tumor effect when treating NB cell line KCNR and combination of these two drugs synergized in vitro .The combination delayed tumor growth and improved survival compared with single agents in xenografts and this was probably induced by increasing apoptosis of tumor cells.

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POT075

Taurolidine-induced Extrinsic and Intrinsic Induction of Apoptosis in Neuroblastoma

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Background: Neuroblastoma is the most common extracranial tumor in childhood. Outcome of high-risk and late-stage disease remains poor despite intensive treatment regimens including megatherapy followed by blood stem cell rescue, differentiation therapy, or immunotherapy. The development of novel therapeutic approaches is thus urgently needed. Taurolidine (TRD), originally invented for avoidance of infections in central venous catheters, has been shown to exhibit anti-neoplastic activity in various cancers. In a recent study the inhibitory effect of TRD on proliferation of neuroblastoma cell lines was demonstrated. More detailed research was therefore warranted to elucidate TRD's possible use for neuroblastoma therapy.

Methods: Cell lines served as an experimental model for in vitro treatment of NB. Taurolidine was used alone or in combination with the cytotoxic drugs doxorubicin and vincristine used for standard neuroblastoma therapy. Effects on proliferation and apoptosis induction were evaluated. Caspase activation and induction of the intrinsic and extrinsic pathways of apoptosis was determined.

Results: The negative effect of TRD on proliferation could be confirmed in four neuroblastoma cell lines. Apoptosis was induced in a time-dependent manner mediated by a simultaneous activation of the intrinsic and extrinsic pathways. This was confirmed by cleavage of caspases-3, -8 and -9 and abrogation of apoptosis by caspase inhibition. Application of the classic cytotoxic drugs doxorubicin and vincristine in combination with TRD resulted in significant increase of apoptosis.

Conclusion: TRD showed remarkable potency in inducing cell death in neuroblastoma cells if used as single treatment. Simultaneous application of chemotherapeutic drugs resulted in significant cooperation with TRD thus making it a promising candidate to be included in neuroblastoma therapy regimens in the future. Further research on TRDs impact on neuroblastoma treatment in vivo using tumor-bearing animal models is needed to survey its possible clinical use.

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POT077

Screening of Okinawan Natural Resources for Antitumor Activities Using Midkine as an Indicator

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Background: We previously found that the growth factor midkine (MK) is highly expressed in human neuroblastoma (NB), and its blood levels work as a prognosis factor (Ikematsu et al., Cancer Sci 99, 2008; Br J Cancer 88, 2003). The purpose of this research is to search for active antitumor ingredients from Okinawan natural resources. We did this by using MK expression in NB cells as an indicator to identify functional molecules contained in libraries of Okinawan natural resources.

Methods: We used a library of marine resources, microalgae, Okinawan vegetables

and fruits extracted from Okinawa, Japan. First, ingredients for the library were added to SK-N-SH cells, an NB cell line. The culture medium after 24 h- and 48 h-incubation with ingredients were collected. Next, MK in the culture medium was measured by MK-ELISA, which we had developed. Ingredients which showed the reduction in MK production was applied to the second screening. In the second screening, MK production as well as cell viability was evaluated.

Results: We obtained 147 candidates of 1210 after the first screening. The secondary screening hit 8 ingredients which significantly suppressed MK production. Among them, two molecules (MW 415 kDa and MW 859 kDa) are currently subjected to structure analysis.
Conclusion: Our approach of screening natural compounds targeting MK would open a new avenue for the therapy of neuroblastoma. We plan to apply the candidate compound to the therapy of MYCN transgenic mice.

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POT122

The Effects of Hypoxia on BER Inhibitor-induced Radiosensitisation in Neuroblastoma Cells In Vitro

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Background: Tumour cells adapt to low oxygen status (hypoxia) by modulating genes which control metabolism, proliferation, apoptosis, angiogenesis and DNA repair, which can lead to treatment failure and increased incidence of cancer relapse. In this study, we investigated the activities of PARP-1 and APE1, which are involved in repair of single-strand DNA damage through the break excision repair (BER) pathway, in a panel of neuroblastoma cell lines exposed to hypoxia.

Methods: SK-N-BE(2c), SK-N-DZ, IMR32 and SHSY5Y cells were maintained at 37oC in an atmosphere containing 5% CO_2 and either 20% O_2 (normoxia), or 1% O_2 (hypoxia). Cells were exposed to 2Gy X-irradiation either alone, or in combination with PJ34 (PARP-1 inhibitor) or CRT0044876 (APE1 inhibitor). Cytotoxicity was then evaluated by long-term MTT assay. PARP-1 and APE1 expression levels in normoxic and hypoxic cells were assessed by Western blot.

Results: In long-term MTT assays, inhibition of PARP-1 significantly increased the efficacy of 2Gy X-irradiation in all cell lines treated under normoxic conditions. However, this effect was inhibited in SK-N-BE(2c) cells exposed to identical chemo-radiation treatments under hypoxic conditions. In Western blot analysis, exposure to hypoxia increased PARP-1 levels in untreated cells. Treatment with X-irradiation also increased PARP-1 expression, but this was more evident in hypoxic SK-N-BE(2c) cells. Inhibition of APE1 reduced cellular sensitivity to X-irradiation under normoxic and hypoxic conditions, however this effect was only significant in cells exposed to hypoxic conditions.

Conclusion: Inhibition of the BER factor PARP-1 can enhance radiosensitivity under normoxic or hypoxic conditions. In contrast inhibition of APE1 induces increased radioresistance in neuroblastoma cells.

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POT125

miRNA-Deep Sequencing Identifies miR-10b-5p as the most Abundant microRNA in Retinoic Acid Treated Neuroblastoma Cells

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Background: Retinoic acid (RA) treatment is used clinically in combination with other chemotherapeutics to treat neuroblastoma. Several lines of evidence suggest miRNA involvement in retinoid signaling, including miR-10b-5p. Unlike other studies, we used miRNA-deep sequencing to profile the miRNome changes after retinoid treatment.
Methods: miRNA signatures were identified by Next Generation Sequencing (NGS) in MYCN amplified neuroblastoma-derived BE2C and IMR-32 cell lines. Microarray analyses were used to identify miR-10b-5p and/or RA-induced changes in gene expression.

Results: NGS analyses revealed that miR-10b-5p was the only microRNA commonly, upregulated in both analyzed cell lines. Additionally, we confirmed by qPCR that miR-10b-5p induction by retinoic acid, was both time and dose dependent. Exogenous expression of pre-miR-10b modestly enhanced RA-dependent effects on cell proliferation and apoptosis. To further understand miR-10b's role in neuroblastoma and identify putative downstream targets, changes in gene expression were determined by microarrays in cells treated with RA or stably transduced with miR-10b-5p. This identified various candidate target transcripts of miR-10b-5p including the proto-oncogene PIM1. PIM1 expression was downregulated by miR-10b-5p overexpression as well as RA-treatment. As both miR-10b-5p and PIM1 appear to be important factors in neuroblastoma retinoid therapy response, we investigated the expression of these factors in a tumor cohort. Interestingly, for 30 untreated neuroblastoma tumors, miR-10b-5p was the most abundant microRNA on average, however, there was no clear associations with clinical parameters. On the other hand, PIM-1 was lowly expressed in localized tumors, with significantly higher expression in Stage 4, indicating a previously unreported role for PIM1, in neuroblastoma disease.

Conclusion: Our investigation identified miR-10b-5p as a possible mediator of RA-treatment in neuroblastoma-derived cell lines. Although we have found a spectrum of changes modulated by miR-10b overexpression, PIM1 is a strong candidate involved in its function and the current focus of our ongoing work.

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POT128

Development of Robust Pharmacodynamic Biomarker Assays for MYCN and the PI3K/AKT Pathway and Isolation of Bone Marrow-Resident Neuroblasts as a Substrate for Clinical Trials-Based Biomarker Analysis

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Background: Robust pharmacodynamic (PD) biomarker assays are essential for the development of early phase clinical trials in neuroblastoma, and provide direct or surrogate evidence of target-suppression in patients enrolled on such trials. To address the need for PD biomarkers in upcoming clinical trials, we aimed to develop assays to measure MYCN protein expression and PI3K pathway activation in patient samples.

Methods/Results: Using Meso Scale Discovery® assays for phosphorylated/total AKT, GSK3β and p70S6Kinase we confirmed that these biomarkers could be detected in platelet-rich plasma (PRP) from paediatric patients. Treatment of PRP or neuroblastoma cells with the TORC inhibitor AZD8055 resulted in decreased phosphorylationof these biomarkers. Surrogate tissues such as PRP often provide a rich source for measurement of particular PD biomarkers, however, for certain biomarkers such as MYCN, expression istumour cell-specific. We therefore developed methodology to isolate neuroblastoma cells from bone marrow for biomarker analysis. Healthy volunteer blood supplemented with Kelly neuroblastoma cells was assessed via fluorescence-activated cell sorting (FACS). Positive and negative selection using three cell surface markers (CD45, GD2 and CD56) was used to achieve maximal recovery and purity of neuroblastoma cells and conditions were optimised to preserve protein expression for PD biomarker analysis. Next, a quantitative immuno-assay for MYCN protein in neuroblastoma cell lysates was developed. We used this assay to measure reduction in MYCN protein levels in isolated neuroblastoma cells following pre-treatment with AZD8055. To clinically validate these methods we are nearing completion of a pilot study in which MYCN levels are being measured in neuroblastoma cells isolated from patient bone marrow samples.

Conclusion: We have developed a FACS-based technique to isolate neuroblastoma cells from bone marrow and have validated robust PD biomarker assays for detection of PI3K/AKT signalling and MYCN protein expression that are suitable for implementation in upcoming early clinical trials in neuroblastoma.

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Translational Research: Molecular Markers

POT079

The Entire Polyamine Gene Pathway is Coordinately Regulated by the MYCN Oncogene to Sustain Polyamine Levels and Support Neuroblastoma Progression

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Background: We have previously shown that the MYCN target ornithine decarboxylase (ODC1), rate-limiting for polyamine biosynthesis, is a therapeutic target for neuroblastoma (Cancer Res 68:9735, 2008) and an international Phase I NANT trial testing ODC1 inhibition with difluoromethylornithine has opened. We have now evaluated the expression of all polyamine pathway genes in neuroblastoma and their regulation by MYCN.

Methods: Gene-expression profiles of 650 primary untreated neuroblastomas were analyzed for 11 polyamine pathway genes, and related to clinical outcome. Gene expression was evaluated by qPCR, while activation and repression of polyamine genes by MYCN were analyzed by chromatin immunoprecipitation (ChIP). Gene promoters were cloned into luciferase reporters and tested as a function of MYCN expression. Histone modifications and DNA methylation of gene promoters were evaluated by ChIP and Methyl-ChIP, respectively.

Results: High levels of each polyamine biosynthetic gene and low levels of each catabolic gene strongly predicted poor outcome. Multivariate analysis showed 6/11 genes retained independent prognostic significance following adjustment for MYCN, age and stage. qPCR analysis demonstrated a direct correlation between MYCN and biosynthetic polyamine gene expression, but an inverse correlation between MYCN and catabolic polyamine gene expression. ChIP assays confirmed that MYCN associates with biosynthetic gene promoters by binding to E-box sites in order to activate transcription. In contrast, MYCN binds the promoters of the catabolic genes by interacting with the Sp1 protein in order to repress transcription. The findings were confirmed using luciferase reporter assays.

Conclusion: These results provide an unprecedented demonstration of an oncogene coordinately regulating the expression of every gene in a metabolic module (polyamine homeostasis) to support oncogenesis. The findings highlight the critical importance of polyamines in neuroblastoma and suggest that targeting polyamine pathway genes in addition to ODC1 may be a valuable therapeutic approach.

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POT080

Individual Patient Risk Stratification of High-Risk Neuroblastomas Using a Four-Gene Ratio Score Suited for Clinical Use

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Background: Several gene expression based prognostic signatures have been described in neuroblastoma, but none have successfully been applied in the clinic. We decided to develop a clinically applicable prognostic gene signature based on both clinical stratifications and biological annotations in combination with backwards-conditional cox regression. The aim was to develop a simple signature with regards to both number of genes and analysis platform, as well as to determine a signature score that does not require comparison between patients.

Methods: Two independent gene expression arrays consisting of 251 and 88 neuroblastomas, respectively, were used to define and validate a prognostic signature. Applicability of the score using Taqman-based qPCR analysis was examined in two additional cohorts consisting of 202 and 344 neuroblastomas, respectively.

Results: Evaluation of the 4-gene score in a training dataset (n=251) displayed significant prognostic relevance in the global tumour set and in high-stage (stage 4 and/or MYCN-amplified) tumours alone (logrank p=$1.3x10^{-12}$ and $7.2x10^{-5}$ respectively). Validation of the signature score in an independent dataset (n=88) further supported its prognostic relevance. qPCR-based analysis in matched cDNAs confirmed the cross platform (array-qPCR) and clinical transferability of the signature score (Pearson correlation, r = 0.878). We also defined a fixed score value that identified a subset of ultra-high risk patients amongst stage 4 and MYCN-amplified patients in two independent patient cohorts (logrank p=0.005 and 0.012, respectively).

Conclusion: In summary, this first truly clinically applicable gene expression signature identifies patients on an individual basis that are in need of new/additional treatment regimens.

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POT081

An Extensible Data-Model for Neuroblastoma Information Management

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Background: Neuroblastoma is characterized by its remarkable biological heterogeneity and the variety of clinical outcomes of the disease. Clinicians and researchers alike require to integrate clinical, biological and genomic data to better characterize the disease, identify proper prognostic factors, and design personalised treatments. Such a multidisciplinary information require management systems with extensive metadata support. The heterogeneity of the collected metadata grows as research is evolving in to international collaborations promoting data sharing among institutions. Single standardization is not feasible and it becomes crucial to develop digital repositories with flexible and extensible data models, as in the case of modern integrated biobanks management.

Results: We developed a data model in JSON format todescribeheterogeneousdata inabiomedicalscenario. The model is built on two hierarchical entities: processes and events, roughly corresponding to research studies and analysis steps. A number of sequential events can be grouped in a process building up a hierarchical structure to track patient and sample history. Each event can produce data. Data are described by a set of user-defined metadata, and may have associated files. We integrated the model in a web based digital repository with a data grid to manage large datasets located in geographically distinct areas. A graphical interface allows users to define new data types dynamically, according to their requirements. Operators compose queries based on metadata fields using a flexible search interface and run them on the database and on the grid. We applied the digital repository to the integrated management of Neuroblastoma patients in the BIT-Gaslini biobank.,

Conclusion: This is a novel approach to track the full clinical history of Neuroblastoma patients integrated with sample management and genomic profiling. Our platform currently manages 1200 tissue and blood samples of 620 Neuroblastoma patients.The system is equipped with data integration capabilities with other bi obanks for worldwide information sharing.

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POT082

Segmental Chromosomal Aberrations of Disseminated Neuroblastoma Cells in Stage M Patients with and without Relapse

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Background: More than 90% of all stage M neuroblastoma patients display disseminated tumor cells (DTCs) in the bone marrow (BM) at diagnosis and the frequency at relapse being less, is still high. However, so far only very little is known about the genomic profile of DTCs at these two time points.

Methods: To allow ultra-high-density SNParray analyses, enrichment of DTCs was done by magnetic beads technique in 50 fresh or DMSO frozen BM samples with initially low DTC-infiltration rate (0.06%-50%) resulting in 39 samples with a DTC content of 30-90%. In 35 cases with >30% DTCs, DNA was extracted from BM cytospin slides or BM smears.

Results: Samples were categorized into three groups: i) 18 diagnostic BM and tumor samples from patients in complete remission, ii) 16 diagnostic BM samples from patients who experienced relapse, iii) 14 relapse BM samples. The average number of chromosomal breakpoints (n=21) in relapse samples was twice as high as in the other two groups. 17q gain was the most frequent aberration in all three groups (88.9%/75%/92.9%) followed by 11q- (38.9%/37.5%/64.2%), 1p- (61.1%/50%/42.8%), +2p (27.8%/25%/21.4%), 3p- (22.2%/12.5%/42.8%), and MYCN amplification (33.3%/50%/35.7%). However, the frequencies of +1q (5.5%/37.5%/42.8%), 4p- (5.6%/12.5%/21.55) and 6q- (11.1%/25%/57.1%) increased strongly among the three groups. Interestingly, three out of eight 6q losses in the relapse samples were acquired in the BM during disease progression, possibly being already present in a sub-clone at diagnosis.

Conclusion: We identified gain of 1q and losses of 4p and 6q specifically associated with relapse samples but also in diagnostic BM samples of patients who underwent relapse, making them possible markers for an ultra-high risk group of stage M patients.

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POT083

Characterization of Li-Fraumeni Syndrome Associated Germline TP53 Mutations R248W, R158H and E286K in Neuroblastoma

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Background: Somatic TP53 mutations are uncommon in neuroblastoma (NB) at the time of diagnosis. However, TP53 mutations and aberrations of the p53/HDM2/ARF pathway are more frequently detected at relapse. In Li-Fraumeni syndrome (LFS), germline TP53 mutations are associated with early onset of tumours, most commonly sarcomas, breast cancer, CNS malignancies, and adrenocortical carcinoma. Germline TP53 missense mutations have only rarely been detected in NB patients. The four previously reported LFS NB-associated mutations are R248W, R273G, R282W and R219S.

Methods: We screened our database for LFS families with NB. LFS NB-associated p53 mutant proteins were studied for the ability to transactivate target genes, induce apoptosis, bind p73, and promote invasion in vitro and tumor growth in vivo.

Results: We identified three NB cases in families with known TP53 germline mutations including R248W, a TP53hotspot mutation that has been previously reported in NB and two novel LFS NB-associated TP53 mutations- R158H, E286K. The R248W and R158H mutations occurred in two infants with high risk NB that were resistant to chemotherapy. Functional studies of 248W and 158H mutant proteins revealed defects in activation of canonical p53 target genes and induction of apoptosis in response to chemotherapy. P53 R248W and R158H bind to and inactivate the p53 paralogue p73 and promote colony formation as well as invasion and migration.

Conclusion: We identified three additional LFS –NB associated TP53 mutations and hypothesize that these mutations contributed to the chemoresistant progressive NB observed in the patients with the 158 and 248 mutations, both of whom died of disease. Functional studies of the E286K mutation, which was detected in a patient with intermediate risk NB, are ongoing. It remains to be determined whether increasing surveillance screening in LFS will detect additional NB cases and whether NB in the setting of LFS occur only in the context of specific TP53 missense mutations.

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POT084

Genomic Background of Neuroblastomas with Intra-Tumor Heterogeneity of MYCN Amplification

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Background: MYCN amplification (MNA) is the most powerful therapy-stratifying marker in neuroblastoma (NB). Approximately 20% of NBs with MNA show intratumoral heterogeneity (hetMNA) ranging from few amplified cells to up to the majority (~70%) of all tumor cells. The clinical meaning of hetMNA is still unclear, compromising the patients' assignment to specific treatment strategies. To characterize the genomic background of hetMNA tumors, we looked for segmental chromosome aberrations (SCA), allelic imbalances, whole chromosome uniparental disomies (wcUPD), chromothripsis and expression of the favorable NB marker (CD44).

Methods: Ultra-high density SNParray analyses (2.7 million markers) and interphase-FISH on tumor and bone marrow samples from 20 hetMNA, 22 homMNA and 110 nonMNA NB patients were performed. Median age for hetMNA patients was 13.5m (range 6-168, 13 patients below, 7 above 18m). CD44 staining was done by immunofluorescence on cryo-sections.

Results: Besides hetMNA, seven tumors showed no SCA, four were heterogeneous concerning both, MNA and SCA (hetSCA), one had two SCAs and eight exhibited a high number (>7) of SCAs (highSCA). In the latter group, chromothripsis (n=1) and deletions within the ATRX1 gene (n=2) were observed. WcUPD occurred in 15/20 (75%) of hetMNA tumors, in 4/22 (18.2%) homMNA and in 38/110 (34.5%) of nonMNA NBs. WcUPD 11 was predominantly found in the hetMNA group (10/15) and decreased with age (UPD11: 9/13 infants and 1/7 patients >18m). By contrast, SCAs increased with age (no/hetSCA: 11/13 infants and highSCA: 7/7 patients >18m). Unlike homMNA tumors, hetMNA tumors were frequently CD44+.

Conclusion: HetMNA tumors differ in their genomic make up from fully amplified and non-amplified tumors. The high frequency of wcUPDs, especially UPD11, possibly representing a hallmark of hetMNA, has not yet been described as well as the genetic background, which either totally lacks SCAs or bears a multitude of them, thus contrasting with homMNA.

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POT085

Targeted Analysis of TP53 Pathway SNPs in Neuroblastoma Patients

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Background: Neuroblastoma is a pediatric cancer that exhibits a wide clinical spectrum ranging from spontaneous regression in low-risk patients to fatal disease in high-risk patients. The identification of single nucleotide polymorphisms (SNPs) may help explain the heterogeneity of neuroblastoma and assist in identifying patients at higher risk for poor survival. SNPs in the TP53 pathway are of special importance, as several studies have reported associations between TP53 pathway SNPs and cancer. Of note, less than 2% of neuroblastoma tumors have a TP53 mutation at diagnosis. We selected 21 of the most frequently studied SNPs in the TP53 pathway and evaluated their association with outcome in 500 neuroblastoma patients.

Methods: We used TaqMan allelic discrimination assay and Sanger sequencing to genotype 21 SNPs in 15 p53 pathway genes in 500 neuroblastoma patients.

Results: This study represents the first targeted SNP analysis of TP53 pathway genes in a large cohort of neuroblastoma patients. We investigated the impact of 21 SNPs in 15 TP53 pathway genes on overall and event-free survival, age at diagnosis, MYCN status, and disease stage in 500 neuroblastoma patients. Our results show that a missense SNP in exon 10 of the CASP8 gene, SNP D302H, is associated with worse overall and event-free survival in neuroblastoma patients with MYCN amplification. We also examined the effect of CASP8 SNP D302H on caspase 8 mRNA expression in two independent neuroblastoma cohorts. The presence of CASP8 SNP D302H did not alter caspase 8 mRNA expression levels.

Conclusion: A missense SNP in exon 10 of the CASP8 gene SNP D302H was associated with worse overall and event-free survival in patients with MYCN-amplified neuroblastoma tumors. Functional analysis is needed to investigate the effect of CASP8 SNP D302H on caspase 8 activity.

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POT086

Primary Anatomic Location of Neuroblastomas Correlates with Genomic Profile: A Retrospective Study of 133 Tumors

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Background: Neuroblastomas (NBLs) originating from extra-abdominal sites are known to be associated with a better outcome than those located in the abdomen. However, the relationship between the genomic profile and the anatomic location of tumors according to their sympathetic origin has not been investigated.

Methods: Patients treated in our institution for histologically-proven NBL and with availability of a genomic profile performed by array-CGH analysis, CT/MR imaging and surgical data, were eligible. Anatomic data derived from diagnosis and preoperative imaging and surgical reports were determined, blinded to genomic information. Tumors were categorized according to the primarily involved compartment (neck, chest, abdomen and pelvis) and their presumed origin along the sympathetic system (cervical, paravertebral (thoracic-lumbar), peri-arterial and adrenal). Genomic profiles were classified as numerical (whole-chromosome copy number variations only), segmental (segmental chromosome alterations with or without numerical alterations) or MYCN-amplified.

Results: 133 (73M/60F) patients treated between 1998 and 2012 were included. Median age at diagnosis was 15 months (0-151). Tumor stage distribution according to INSS was 1 (10.5%), 2 (13.5%), 3(25%), 4(47%) and 4s (4%). Genomic profile showed numeric-only alterations in 37%, segmental alterations in 35% and MYCN-amplification in 28%. Tumors located in the cervical (n=4) and pelvic (n=8) compartments displayed numerical profile in all cases, whereas tumors originating from the abdomen mostly showed segmental alterations (40/95) or MYCN-amplification (35/95) ($p < 0.0001$). No significant difference was observed for thoracic tumors. In contrast, according to sympathetic anatomy, NBLs arising from the cervical and paravertebral chains were more frequently associated with a favorable biological profile than adrenal NBLs ($p<0.0001$).

Conclusion: This study demonstrates a significant correlation between the anatomic location of NBLs according to their neural crest origin and their biology as defined by aCGH, suggesting that NBLs' behavior depends on the location of neural crest cells they arise from.

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POT087

Deep microRNA Sequencing Reveals Downregulation of miR-29a in Neuroblastoma Central Nervous System Metastasis

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Background: The central nervous system (CNS) is an increasingly common site of metastasis for patients with stage 4 neuroblastoma and radioimmunotherapy directed at B7-H3 showed clinical potential in maintaining long term remission after such catastrophic events (J Neurooncol 97:409, 2010). The microRNA (miRNA) profile of this metastatic process has not been fully explored.

Methods: To identify miRNA sequence families with differential expression, tumor pairs (pre-CNS primary and CNS metastasis) from 13 patients with stage 4 neuroblastoma were analyzed by miRNA sequencing. miRNA and mRNA dysregulations were independently confirmed by quantitative reverse transcription-PCR (qRT-PCR). Further validation using additional samples from patients with stage 4 neuroblastoma was performed.

Results: Seven miRNA sequence families had distinct ($p<0.01$) expression in CNS metastases versus their corresponding pre-CNS primaries. miR-7 was up-regulated (3.75-fold), and miR-21, miR-22, miR-29a, miR-143, miR-199a-1-3p, and miR-199a-1-5p were down-regulated (3.5-6.1-fold). miR-29a, shown previously to be downregulated in a broad spectrum of solid tumors including neuroblastoma, was the most significantly changed miRNA ($p=0.001$). Consistent with the regulatory function of this miRNA, its known onco-targets including B7-H3 (an inhibitory ligand for T cells and natural killer cells), CDC6, CDK6, and DNMT3A were found to have higher expression in CNS metastases versus pre-CNS primaries. Moreover, miR-29a expression was significantly lower among pre-CNS primaries compared to those from patients who did not relapse in the CNS, irrespective of MYCN amplification status. Cell lines from patients with MYCN amplified CNS relapse had lower miR-29a expression compared to MYCN amplified cell lines from patient without CNS relapse ($p=0.02$).

Conclusion: Downregulation of miR-29a may have prognostic value and biologic implication for CNS metastasis independent of MYCN amplification status. This may also explain, in part, the clinical efficacy of radioimmunotherapy directed at B7-H3.

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POT088

Genome-Based Sub-Classification of Neuroblastoma: A Retrospective Study by Using 573 Neuroblastoma Samples Obtained in Japan

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Background: Neuroblastoma (NB) is known to exhibit wide ranges of clinical behavior. We have examined molecular profiles in approximately 300 tumors to construct risk classification system for NB and indicated that global copy number variations in tumors are strongly correlated with patient prognosis. In this study, we further conducted clinical validation of the genome-based classifier by adding new samples including those from the patients enrolled in the Japan Neuroblastoma Study Group (JNBSG) phase II clinical trial.

Methods: Array CGH data were obtained for 573 NBs (sent to Chiba Cancer Center from 1995 to 2011; stage 1:74; stage 2:48; stage 4s:41; stage 3:102; stage 4:296; unknown:12) including 44 pairs of tumors obtained at diagnosis and after chemotherapy. We use three genomic groups (GGs): silent (S), partial (P) and whole (W), which were further segregated by MYCN amplification (a;MYCN-amp), 1p-loss, 11q-loss and 17q-gain.

Results: CGH signature showed reproducible correlation to patient survival: 8-yr survival rates (SR) of Ws (s:MYCN single copy) and Ss were 89%, whereas patients with Pa exhibited poor prognosis (8-yr SR:39%). 11q-loss was observed in 25% of our cohort, and significantly correlated with poor prognosis (loss: SR:45% vs. retain:70%, logrank-test, p=0.0002). As previously observed, 3.8% and 5.6% of samples had both 11q- and 1p-losses as in the "P2s" (SR:45%) and "P2a" (SR:25%) subgroups, respectively, whose prognoses tend to be worse than tumors with only 1p-loss in "P1s" (SR:71%) and "P1a" (SR:39%) subgroups, respectively. Comparison of GGs in the 44 sample pairs indicated that most of them were in the same GG-types, however, a few pairs exhibited additional 11q rearrangements in the later counterparts, such as from "P1a" or "Wa" to "P2a".

Conclusion: These results suggested that the genomic classifier can be applied to primary as well as post-chemotherapy samples to follow. Other markers such as gene expression signatures and gene mutations will be also discussed.

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POT089

Study of Inflammatory Cells in Neuroblastoma

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Background: It has been demonstrated that the immune system plays a central role in the progression of different malignancies. Nevertheless, the role of the different lineages of immune cells in the tumor environment, and their relationship with the survival of neuroblastic tumor patients remains unclear. We aim to characterize the importance of some different immune cells in neuroblastoma (NB).

Methods: We analyzed two representative cylinders of 1 mm of 78 primary NB included in tissue microarrays. Serial sections were immunostained using anti-CD7, CD4, CD8, CD20, CD11b, CD11c, CD163, granzyme b and CD45, for detecting lymphocytes T and B, macrophages, and leucocyte common antigen. The stained tumor sections were digitized with Panoramic Midi (3D Histech) and the positive cells quantified with Panoramic viewer (3D Histech) provided by an image analysis module customized for each marker. The measurements were validated by independent subjective assessment by two pathologists.

Results: The amount of CD7 positive cells is related to clinical and genetic features which are currently used for patient stratification. It is increased in tumors from patients with long survival (OS) with MYCN not amplified and age under 18 months (in all cases $p<0.05$). Common segmental chromosome aberrations are related with the expression of CD20, CD45 and CD11c ($p<0.05$). It has also been seen that the state is related with CD4, CD20 CD45 and CD163 ($p<0.05$).

Conclusion: The potential importance of the background inflammatory cells, especially CD7 positive cells, previously reported to be found on the majority of T cells and natural killer cells, have an influence on OS and event-free survival and, therefore, their inclusion as markers for patient stratification should be considered., GRANTS: ISCIII (FIS PI10/15), ISCIII (RD12/0036/0020).

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POT090

Prognostic Role of Immunosuppressive Cell Molecular Markers in High-Risk Neuroblastoma Patients

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Background: It is generally assumed that efficacy of anti-cancer immune-therapy is hampered by immune-suppressive mechanisms. In patients with metastatic NB, the presence of bone marrow (BM)-infiltrating and of circulating tumor cells may contribute to the establishment of tolerogenic microenvironment in different anatomical compartments.,

Methods: The expression of FOXP3, as marker of regulatory T cells (T_{reg}), of CD163, as marker of tumor-associated macrophages, and of the immune-suppressive cytokine IL-10 mRNAs were evaluated in primary tumors, BM and peripheral blood (PB) samples taken at diagnosis from children with metastatic NB aged more than 18 months. Event-free and overall survival (EFS and OS) analyses were then performed by log-rank test.

Results: Surprisingly, in primary tumor specimens, high expression of FOXP3 associated to a better EFS and OS ($p=0.0180$ and $p=0.0331$, respectively), and a direct correlation between IL-10 and CD163 expression was found. In BM samples, low CD163 mRNA expression significantly associated to better EFS and OS ($p=0.0434$ and $p=0.0077$, respectively), whereas in PB samples no significant association was found. However, in these latter samples a significant inverse correlation between FOXP3 and IL-10 expression was observed. In both BM and PB samples, IL-10 mRNA expression was significantly higher than in healthy children ($p=0.001$ and $p=0.018$ respectively). Similarly, IL-10 protein levels measured in plasma from high-risk patients were significantly higher ($p<0.001$).

Conclusion: In a cohort of high-risk NB patients, high FOXP3 mRNA levels in primary tumors and low CD163 levels in BM samples significantly associated to better EFS and OS. Taken together, our data point out to a potential role of FOXP3 as a marker of T cell activation rather than of immune-suppressive T_{reg} cells, whereas immune-regulatory macrophages present in BM appear to affect the natural history of high-risk neuroblastoma.

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POT091

GSTP1 Gene Status in 11q Loss NB

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Background: 11q23 loss of heterozygosity is associated with poor survival and high genetic instability in neuroblastoma (NB). High expression of the detoxifying enzyme glutathione transferase gene (GSTP1), located in 11q13, is involved in multidrug resistance in a broad range of tumors. Recent NB studies have focused on GSTP1 status. Increased gene expression has been found in MYCN amplified tumors (MNA) and conversely high hypermethylation of the gene has been described in high risk tumors with 11q loss.

Methods: From a cohort of 264 primary NB analyzed we selected the 11q loss cases using SNP arrays (Illumina, HumanCytoSNP-12/GenomeStudio software and/or Affymetrix GeneChip Human Mapping 250K/CNAG software). Identification of GSTP1 status and other chromosome aberrations (numerical -NCA- and segmental -SCA-) were taken in consideration. Association with clinicobiological characteristics was described.

Results: 11q loss was present in 63 tumors (24%). Two types were segregated in relation to the region containing the GSTP1 gene: type 1 (GSTP1 not alterated, n=37) and type 2 (GSTP1 gained, n=26). In addition, SCA at 11p arm were seen in both GSTP1 related types of 11q loss (22% for type 1 and 42% for type 2). Significant differences were observed related to presence of more than 9 SCA (type 2) and average number of CN-LOH (1.6 type 1 and 3 type 2). When excluding the MNA plus 11q- tumors (n=12) a tendency to poor median tOS (45 versus 25 months) and to older age (35 versus 48 months) was seen.
Conclusion: We identified 2 subgroups of 11q loss NB related to GSTP1 status. This observation requires confirmation in a larger cohort and detailed studies of cooperation between the GSTP1 gene and tumor suppressor genes/oncogenes to shed more light on the 11q deleted NB. Grants: RD12/0036/0020 (ISCIII & ERDF), FIS10/0015 (ISCIII).

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POT092

Discovering Robust Survival signatures from Neuroblastoma mRNA Expression Profiles

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Background: Identifying relevant signatures for clinical patient outcome is a fundamental task in high-throughput studies. However, hitherto identified prognostic signatures for NB as well as for other diseases are largely non-overlapping, mostly due to the fact that the sample sizes are far smaller than the numbers of features to be considered. Thus, many signature selection methods suffer from statistical limits.

Methods: We propose a robust signature selection framework that enhances the selection stability of existing algorithms for predicting survival outcomes using neuroblastoma exon resolution mRNA expression data (Affymetrix Human Exon ST; n=176). Our method is based on an aggregation of multiple signatures obtained by considering subsamples of a given cohort data, where the aggregated signature is shrunken by a simple threshold strategy. The resulting method, RS-PrLasso, is conceptually simple and relies only on such parameters that are automatically tuned by cross validation. We provide a means to report a precise estimate of prediction performance, and to measure the selection probabilities of individual features. The software is available as an R package rsig in CRAN.

Results: RS-PrLasso based prediction robustly identified features predictive of neuroblastoma outcome independent of sampling. The method performed significantly better than LASSO-only based tests and using clinical covariates. Genes present in the most robust signature included known neuroblastoma genes, NTRK1, CHD5 and RGS9, indicating that relevant biological processes were mapped.

Conclusion: , Since RS-PrLasso uses generalized linear models that are capable of handling both continuous features (e.g. gene expression) and discrete features (e.g. SNPs), an extension of this method to other data types is feasible and bears the potential to truly enhance predictive power of signatures obtained from high-throughput studies.

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POT093

Establishment of Pharmacodynamic Biomarkers of MDM2-P53 Inhibitor Activity in Neuroblastoma Cells Using the Amnis Imagestream

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Background: Neuroblastoma is predominantly p53 wild-type (wt.) therefore MDM2-p53 antagonists which activate p53 offer a novel and potentially less toxic therapeutic strategy for patients with high-risk disease. RG7388 and other MDM2-p53 antagonists are currently undergoing early phase clinical trials in adults. The establishment of suitable pharmacodynamic (PD) biomarkers of response are needed for the clinical evaluation of novel targeted agents. Aim: To identify PD biomarkers in neuroblastoma cell lines following treatment with the MDM2-p53 inhibitor RG7388, using the Amnis ImageStreat imaging flow cytometer.

Methods: p53 wild-type SHSY5Y, SHEP, NGP, IMR32 and p53 mutant IMR32-KAT100 neuroblastoma cells were treated with 1**X** and 5**X** their GI_{50} values for RG7388 or DMSO for 24hours, prior to staining with DAPI and antibodies against p53 and p21 and then analysed on the ImageStream. Western blotting for p53 pathway activation was performed in parallel to confirm the results of the ImageStream.

Results: ImageStream analysis demonstrated that all p53 wt cells showed a concentration-dependent upregulation of p53 and p21 in response to RG7388 together with a G1 cell cycle arrest and/or apoptosis, the latter shown by an increase in sub-G1 fraction or nuclear fragmentation. In contrast, p53 mutant IMR32-KAT100 cells failed to upregulate p21 and did not undergo apoptosis.

Conclusion: Our results show that the ImageStream can be used for the detection of PD biomarkers of MDM2-p52 antagonist activity in neuroblastoma cells. Future work will focus on expanding the panel of PD biomarkers and establishing a protocol for analysing patient samples.

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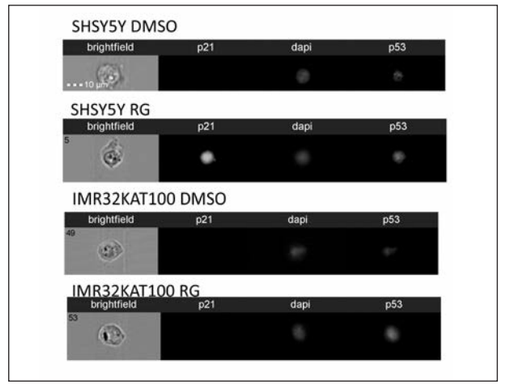

POT094

Clinical Characteristics and Outcome of Patients with Neuroblastoma Presenting Genomic Amplification of Loci other than MYCN

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Background: Somatically acquired genomic alterations are a key feature of neuroblastoma (NB). Little is known about the frequency, clinical characteristics and outcome of NB harbouring genomic amplification(s) distinct from MYCN.

Methods: Between 1996 and 2011 tumour samples from 1100 patients with NB from French centres were studied by array-CGH. Their profiles were re-examined specifically to identify regional amplifications. Patients were included in the study if amplifications distinct from the MYCN locus were seen. A subset of patients treated at Institut Curie, whose tumours harboured a MYCN amplicon as determined by array-CGH without other amplification, were studied as a control group. Clinical and histology data were retrospectively collected.

Results: A total of 56 patients were included and categorised into 3 groups. Group 1 (n=8) presented regional amplification(s) without MYCN amplification, with the 12q13-14 locus being the most recurrent amplified region (4/8 cases). In this heterogeneous group, various INSS stages, primary locations and histology were observed with atypical clinical features. Group 2 (n=26) had MYCN amplification as well as other amplicons. These patients shared clinical features of those of a control group (Group 3, n=22) of NBs MYCN amplified. Outcome was globally poor for the 3 groups but overall survival for group 1 was better than that of groups 2 and 3 (5 year OS : 87.5% ± 11% vs 34.9% ± 7%, log-rank $p<0.05$).

Conclusion: NBs without MYCN amplification but harbouring other regional genomic amplification(s) are rare (1%) and seem to show atypical features in clinical presentation and genomic profile. Further high resolution genetic explorations are justified in this heterogeneous group of NB, especially when considering these alterations as predictive markers for targeted therapy.

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POT095

Genomic Profiling in Low and Intermediate Risk Neuroblastoma to Refine Treatment Stratification and Improve Patient Outcome - LINES: a SIOPEN Trial

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Background: In neuroblastoma, a genomic profile characterized by segmental chromosome alterations is associated with higher risk of relapse and poorer outcome.

Methods: SIOPEN has recently launched the LINES trial (Low and Intermediate Neuroblastoma European Study). In order to maintain or improve the excellent outcome inlow risk patients, whilst diminishing overall treatment burden whenever possible, treatment is stratified according to the genomic profile in addition to clinical parameters. In intermediate risk patients, the genomic profile is studied prospectively whenever possible. NB samples that contain >60% of tumor cells are analyzed by a multi-locus or pangenomic technique such as MLPA, array-CGH or SNP-arrays. The genomic profile results are centrally reviewed, the result entered in the SIOPEN-R-NET database, and the clinically relevant conclusion is returned to the treating clinician within six weeks after diagnosis. In tumors without MYCN amplification, genomic profiles are classified into two large classes: those harboring numerical chromosome alterations (NCA) only, versus those harboring segmental chromosome alterations (SCA) known to frequently occur in NB, with or without numerical alterations.

Results: To date, for 65 enrolled patients (45 LR, 20 IR), a genomic profile could be determined in 44 patients (34 NCA, 10 SCA). For 7 patients, the obtained genomic profile could not be classified according to the previously defined criteria, either because no copy number changes were seen (n=3), or because atypical segmental chromosome changes were observed, for which the prognostic relevance has not been clearly established yet (n=4). The latter included a small interstitial deletion of chromosome 8p, a small distal deletion of chromosome 3p, a chromosome 17p deletion and a focal amplification of 12q14 encompassing the CDK4/MDM2 genes. For 14 other cases, no up-front genomic profiling was performed.

Conclusion: In SIOPEN's LINES trial, in which a genomic profile is used to stratify treatment in all low-risk patients, genomic profiling in a diagnostic, real-time setting is proving feasible.

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POT097

Chromothripsis in Neuroblastoma

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Background: Chromothripsis is defined by the presence of tens to hundreds of genomic rearrangements acquired in a single catastrophic event. This phenomenon is observed in at least 2%-3% of all cancers. In neuroblastoma (NB) it has been recently shown to occur in 18% of high-stage NB associated with a poor prognosis.

Methods: 264 NB samples containing >60% of tumor cells from 243 patients were analyzed by SNP arrays. MYCN status and 1p36 and 11q22 region integrity were evaluated by FISH. Ploidy was assessed by static cytometry.

Results: Chromothripsis was observed in 18 biopsies (14 from primary tumors, 1 from relapse, 2 from metastases, 1 post-chemotherapy sample) from 15 patients (6,8%). Most patients suffered stage 4 disease (64.3%) and were over 18 months of age (92.85%), with a median of 63±44 months (from 14 to 154). Twelve patients were included in SIOPEN HR-NBL1 and 2 in other national studies. Four patients died of the disease. The median tEFS was 21±33.8 months (from 9 to 83) and the median tOS was 49±43.9 months (from 2 to 131). By FISH, nine samples presented MNA, two MYCN gain and seven 11q deletion. Analyzing by aSNP, two samples had amplifications in 2p (one in ALK), one in19p and another in 20q. Eleven samples presented more than 10 segmental chromosomal alterations; the most frequent were 1p deletion (10), 2p gain (7), 2q gain (5), 11q deletion (7) and 17q gain (10). Chromosomes affected by chromothripsis and number of samples were: 5(8), 11(3), 2(2), 7(2), 9(2), 1(1), 4(1), 6(1), 12(1) and 19(1). Three patients presented chromothripsis in 2 chromosomes simultaneously. Six samples had focal segmental chromosomal alterations. ATRX and PTPRD were deleted in 1 and 2 samples respectively.

Conclusion: Further studies with high number of samples with chromothripsis are needed to elucidate if this single catastrophic event makes tumors vulnerable to therapeutic strategies that specifically target regions of genomic instability. Grants: RD12/0036/0020 from ISCIII & ERDF and FIS10/0015 from ISCIII.

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POT099

Prognostic Significance of Non-Cellular Extracellular Matrix Elements in Neuroblastic Tumors

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Background: The study of extracellular matrix (ECM) elements in neuroblastoma (NB) through the objective morphometric analysis of histologic sections stained with histochemistry techniques can shed light on their influence on tumor homeostasis, dynamics and growth, and could provide novel therapeutic targets as well as enhance patient stratification. We present the relationship of non-cellular ECM elements with current stratification factors and prognosis.

Methods: We constructed 19 tissue microarrays including at least two representative cylinders of 459 primary NB. Serial sections were stained to detect reticulin fibers (RetF), collagen type I (ColIF) fibers and glycosaminoglycans (GAGs) with Gomori, Masson's trichrome and alcian blue pH 2.5, respectively. The stained tumor sections were digitized with Aperio Scanscope XT (Aperio technologies). We used different image analysis systems to detect and characterize the quantity, size, shape and orientation of RetF and the area occupied by ColIF and GAGs. Non-parametric Mann-Whitney and Kruskal-Wallis tests, Cox-regression and Decision tree statistical analysis were used for the comparison between ECM and current stratification elements and survival analysis, respectively.

Results: Quantity (number of RertF and percentage of stained area), size (area, length and width) and shape (aspect, roundness, fractal dimension and perimeter-ratio) of RetF are strongly related to all current factors used for NB pre-treatment stratification. The amount and shape are related to EFS and OS. ColIF quantity is related to age, histopathology, MYCN, genetic profile and percentage of 10-year EFS and OS. GAGs abundance is related to stage, histopathology, MYCN state, frequent SCA, and to OS. In all cases p-values were <0.05. Decision tree analysis shows that current risk stratification could benefit from the inclusion of some of the markers of ECM.

Conclusion: Objective data to improve the classic histopathologic parameters and new elements for patient evolution prediction and therapeutic stratification could arise from the study of the ECM in neuroblastic tumors. Grants: RD12/0036/0020 (ISCIII & ERDF), FIS10/0015 (ISCIII).

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POT100

Telomere Biology in Neuroblastoma: FOcusing on ATRX/DAXX Genes and Alternative-Strengthening of Telomere (ALT)

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Background: Neuroblastoma (NBL) shows remarkable biological heterogeneity, resulting in favorable or unfavorable prognosis due to aggressive growth or chemo-resistance. Previously, we reported ALT and telomere binding proteins (TBPs) may be biomarkers for chemo-sensitivity in neuroblastoma. Recently, ATRX/DAXX gene alterations were found in NBLs and might be correlated with telomere biology, especially alternative lengthening of telomere (ALT).
Methods: To evaluate the correlation between telomerase activity, telomere length, and the expression levels of TBPs in 145 NBLs, we already selected 11 NBLs with long telomeres（ALT-activated NBLs）(J. Pediatr. Surg 44:2258,2009). In this study, alterations of ATRX/DAXX gene and TBP (TERF1, TERF2, TIN2, Tank1, Tank2, Rap1 and POT1) gene were examined using next-generation sequencing.

Results: In 11 ALT-activated tumors, telomere lengths were ranged between 15 and 40 kb and the lengths of 3'-overhang were also elongated (1.54-3.85 folds). Three of them showed high telomerase activity and one had MYCN amplification. ATRX/DAXX gene alterations were detected in all 11 ALT-activated cases including 8 ATRX deleted cases. Moreover, the TBP genes such as DDX11, XRCC5, HNRNPA3, TNKS, TERF1, and WRN were frequently mutated or deleted. Clinically, 7 were detected more than 18 months old and 8 were INSS4. Among them, all cases detected at more than 18 months old had INPC unfavorable tumor and were alive more than 24 months but deceased (relapse or recurrence).

Conclusion: ATRX/DRXX alterations were associated with ALT activation in NBLs with elongated telomeres. Clinically, these tumors were usually detected in elder patients and showed slow-growing but high-chemoresistance. Therefore, ALT-activated NBLs might be an inherent entity and ALT and ATRX/TBP alterations might be biomarkers for this NBL entity. Thus, a better understanding of telomere biology and developing of therapeutic strategies for ALT- activated NBLs may help to improve the outcome of NBL patients.

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POT101

Genetic Variants in the Calcium-Sensing Receptor are Associated with Clinical Outcome of Neuroblastoma

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Background: We have previously reported that the calcium-sensing receptor (CaSR) is expressed in benign, differentiated neuroblastic tumors, but silenced by genetic and epigenetic events in unfavorable neuroblastomas. We have now analyzed three functionally relevant polymorphisms (rs1801725, rs1042636 and rs1801726) to assess if variants reducing CaSR activity are associated with more aggressive disease course. Also, mutations in coding exons and regulatory regions of the CaSR gene have been analyzed in primary tumors.
Methods: Genotyping was performed in DNA samples uninvolved with disease from 65 patients. Mutation analysis was carried out in 24 primary neuroblastomas.
Results: Mildly inactivating variant rs1801725 was associated with clinical stage 4 (P = 0.002) and undifferentiated histology (P = 0.046). Patients harboring this polymorphism had lower overall (P = 0.022) and event-free survival (P = 0.01) rates than those homozygous for the most common allele among Caucasians. However, this genotype was not independently associated with outcome. Conversely, the tri-locus haplotype TAC was independently associated with an increased risk of death in the entire cohort (Hazard Ratio = 2.45; 95% Confidence Interval [1.14 -5.29]; P = 0.022) and also in patients diagnosed with neuroblastomas (Hazard Ratio = 2.74; 95% Confidence Interval [1.20-6.25]; P = 0.016). In primary tumors, several polymorphisms were identified, but no mutations were found.

Conclusion: The TAC haplotype includes the moderately inactivating variant rs1801725 and absence of the gain-of-function induced by rs1042636. Thus, its association with metastatic disease and poor outcome would add to our previous data and further support that inactivation of the CaSR is a mechanism associated with neuroblastoma malignant behavior. These results would also increase the growing body of evidence indicating that benign and malignant forms of neuroblastic tumors might arise as a result of different initiating genetic events.

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POT102

Modulation of Radiation Biomarkers in Children with Neuroblastoma Treated with 131I-mIBG

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Background: ^{131}I-mIBG is a targeted radiopharmaceutical for advanced neuroblastoma and is one of the most active agents in this treatment resistant population. We aimed to identify potential biomarkers of response and toxicity in patients treated with ^{131}I-mIBG.

Methods: Patients with advanced neuroblastoma treated with ^{131}I-mIBG at UCSF were eligible. Patients had serial blood drawn at baseline and hours 72 and 96 after ^{131}I-mIBG infusion. We quantified a panel of biomarkers shown to be altered in patients receiving other forms of radiation, including: serum amylase; plasma Flt-3 ligand; CDKN1A mRNA in mononuclear cells; and gamma-H2AX. Extent of modulation of each marker was evaluated using ANOVA. We assessed potential differential modulation between groups based on response (PR/CR vs SD/PD), toxicity (grade 3 non-hematologic toxicity; grade 4 neutropenia or thrombocytopenia) or administration of 131I-mIBG with or without a radiosensitizing agent (vincristine/irinotecan or vorinostat) using unpaired t-tests.

Results: 36 patients (25 male; median age 7 years) participated. 23 patients received ^{131}I-mIBG with a radiosensitizer. We observed robust modulation of amylase (median maximal fold change of 2.4; p <0.001), Flt-3 ligand (median maximal fold change of 4.4; p<0.001), and CDKN1A mRNA (median maximal fold change of 7, p=0.003). No differences were noted in gamma-H2AX in the 9 patients with data available to date for this marker (p=0.35). Patients receiving a radiosensitizer with ^{131}I-mIBG had more robust modulation of Flt-3 ligand (mean difference between groups of 338 pg/mL; p=0.02) compared to single agent ^{131}I-mIBG. No correlations with clinical response or toxicity were found in this initial pilot study.

Conclusion: Amylase, Flt-3 ligand, andCDKN1A mRNA all show robust modulation after ^{131}I-mIBG treatment. Patients receiving ^{131}I-mIBG with a radiosensitizer demonstrated the largest changes in plasma Flt-3 ligand. In this preliminary analysis, no associations were found between response or toxicity and extent of modulation of the proposed biomarkers.

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POT104

Chemotherapy-Induced Upregulation of CHD5 Expression in Neuroblastoma is Associated with Patient Outcome: A Potential Marker for Early Assessment of Response to Treatment

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Background: Neuroblastoma (NB) tumors with unfavorable clinico-biological features (INSS stage 4 and MYCN-amplified) show low expression levels of CHD5 at diagnosis. Our previous findings highlighted the existence of a subset of high-risk NBs that acquire CHD5 expression after chemotherapy. We investigated whether therapy induced CHD5 expression in high-risk NB was associated with patient outcome.

Methods: Primary tumor biopsies from 21 high-risk patients managed at our institution between 2003 and 2010 and with more than 3-years follow-up, were analyzed by quantitative real-time PCR and immunohistochemistry to assess CHD5 expression both at diagnosis and following 3 cycles of induction chemotherapy. Estimation of survival curves was performed using the Kaplan-Meier method and compared using the log-rank test.

Results: At diagnosis all tumors showed low CHD5 mRNA expression and negative immunostaining. At response evaluation, upregulation of CHD5 expression (mRNA >3-fold change) was observed in 12 (57%) NB tumors. These patients remained event-free at a median follow-up of 32 months, whereas patients where low expression levels persisted, median event-free follow-up was 8 months (log-rank, P=0.001). Similarly, higher CHD5 expression at response evaluation was significantly associated with longer median overall survival rates of patients (93 months vs. 21 months; log-rank, P=0.004).

Conclusion: These results confirm and extend our previous findings and suggest that upregulation of CHD5 expression in primary high-risk NB at early response evaluation is associated with longer overall and event-free survival of patients. CHD5 expression levels could thus be a surrogate marker for early assessment of response to treatment that can be useful to identify patients who do not benefit from conventional treatment. These results warrant further investigation in larger cohorts of patients.

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POT105

The Role of FGFR4 in Neuroblastoma

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Background: Neuroblastoma is the most common extra-cranial solid tumor of childhood. Many children present with high-risk disease characterized by rapid tumor growth, resistance to chemotherapy, and widespread metastasis, and novel therapies are needed. A germline polymorphism in the FGFR4 gene is associated with increased incidence, treatment resistance, and poor outcomes for many cancers. Recent studies have also shown that this FGFR4 variant protein demonstrates both reduced degradation and sustained activation and signaling.

Methods: We screened DNA from 129 neuroblastoma patients collected through an IRB-approved protocol for the FGFR4 genotype using RT-PCR analysis. Allele frequencies were determined and compared to the allele frequencies in a representative control population as reported in HapMap (release 27, NCBI build 36). In order to evaluate the degradation rate of FGFR4 in neuroblastoma tumor cells, neuroblastoma tumor cell lines were starved in serum-free media and then treated with ligand to induce receptor endocytosis. Western blots using antibodies against individual receptors were performed and relative expression levels were determined.

Results: The frequency of the A allele in neuroblastoma patients was increased compared to the control population, suggesting an association of the FGFR4 genotype with neuroblastoma incidence. Furthermore, logistic regression analyses suggested a strong association between having the "A" allele and having neuroblastoma ($p<0.05$), with a 3.3-fold increase in patients with the AA genotype having neuroblastoma compared to those with the GG genotype. Additionally, the FGFR4 protein does not undergo ligand-induced degradation as seen with EGFR, consistent with increased FGFR4 stability.

Conclusion: We have shown that neuroblastoma patients disproportionately possess the FGFR4 variant allele and that neuroblastoma tumor cell lines have reduced FGFR4 degradation. Ongoing studies are underway to determine the association of the FGFR4 polymorphism with neuroblastoma patient clinical features and outcomes and the association of the FGFR4 genotype with the responses to FGFR kinase inhibitors.

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POT106

Paired Analysis of Primary Tumor and Metastatic Relapse of Localized Neuroblastoma Using FISH and aSNP

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Background: It is known that the presence of segmental chromosomal aberrations (SCA) in neuroblastoma (NB) carries a worse prognosis, being associated with relapse, even in localized stages, especially when the number of SCA is >7 in primary tumors (PT). Metastatic relapse (MR) is a rare event in children with localized MYCN non amplified (MNNA) NB (4%). We aimed to investigate histological, MYCN status and genomic profile (GP) changes, between PT and MR samples of three localized NB.

Methods: SNP arrays (Illumina HumanCytoSNP-12 and/or Affymetrix GeneChip Human Mapping 250K) were used. We established a final diagnosis of the MYCN status, taking into account the results of FISH plus aSNP techniques. PTs were classified as stage 2. Age at diagnosis was 33, 41 and 45 months, OS ranged from 29 to 60 months, only one case died.

Results: Histopathologically, nGNB (2 samples) and pdNB PT changed to pdNB at MR. At diagnosis, distinct genomic profiles were present, MNNA (2 samples) and MYCN heterogeneous amplified. All cases had segmental GP with less than 7 SCA (2, 3 and 6). The GP at MR did not show a common pattern except the presence of evident copy neutral LOH (CN-LOH). CN-LOH events differed in length and chromosome location (4Mb/6p22.3-22.1, 54Mb/7p11.1-ter,35Mb/17pter-q12 and 7.4Mb/19p13.2-13.12). In one case no modification of the genomic profile was observed apart from a CN-LOH at 6p. The second case increased the number of SCA, from 2 to 11. The last case preserved 5 of the 6 SCA, losing a 2p gain and modified a 19p deletion, acquiring the CN-LOH.

Conclusion: Efforts aimed at detection of potential relapse localized NB are necessary. Localized PT and MR MNNA NB requires in-deepth genetic studies to highlight the presence of CN-LOH, in addition to SCA, to improve the prognostic value of the genetics. Grants: RD12/0036/0020 from ISCIII & ERDF and FIS10/0015 from ISCIII.

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POT107

Copy Number Variations of Chromosomal Regions are Associated with Unfavorable Outcome in Neuroblastoma Patients

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Background: 1p, 11q deletions, MYCN amplification (MNA) are known to be adverse prognostic markers while significance of many other copy number variations (CNV) is less clear.

Methods: We analyzed 108 primary neuroblastomas with MLPA for loci 1p,2p,3p,11q,17q and 100 tumor samples for 4p,7q,9p,12q,14q. Prognostic significance was estimated by event-free survival (EFS) with median of follow-up time 28 months (range 1-166 months).

Results: MNA was revealed in 19 patients (17.6%) and their EFS was significantly low (0.20±0.11 vs. 0.69±0.06, $p<0.001$). 2p23-24 gain was observed in 15 patients (13.9%) and showed negative prognostic value in patients below 1 year old (EFS 0.53±0.25 vs. 0.96±0.04, $p=0.047$). In 29 patients (26.9%) 1p deletion was revealed. It had prognostic impact in thewhole group (EFS 0.33±0.10 vs. 0.67±0.06, $p=0.002$) and in MYCN non-amplified patients (EFS 0.40±0.14 vs. 0.71±0.06, $p=0.029$). 17q gain was detected in 54 patients (50.0%) and led to decreased EFS in all patients (0.42±0.08 vs. 0.71±0.07, $p<0.001$) as well as in MYCN-negative subgroup (0.47±0.10 vs. 0.77±0.07, $p=0.002$). 4p gain detected in 8 patients (8.0%) decreased EFS in patients below 1 year of age (0.00 vs. 0.88±0.06, $p=0.055$). Gain of both arms of chromosome 7 observed in 6 patients (6.0%) led to reduced EFS in MYCN non-amplified patients: 0.40±0.22 vs. 0.64±0.06, $p=0.044$., In 8 patients (8.0%) 9p deletion was found and associated with dramatic decreasing of EFS in all evaluated patients (0.00 vs. 0.60±0.06, $p=0.035$) and MYCN non-amplified group (0.00 vs. 0.68±0.06, $p=0.047$). Neither interstitial (n=16, 14.8%) nor terminal (n=9, 8.3%) 11q deletions had prognostic significance in our series: EFS 0.28±0.14 and 0.78±0.14 vs. 0.60±0.06, $p=0.164$ and $p=0.477$ respectively. 3p deletion (n=30, 27.8%), 12q gain (n= 20, 20.0%) and 14q aberrations (n=14, 14.0%) had no influence on survival.

Conclusion: Thus, in our cohort of patients, treated according GPOH NB2004 protocol, MNA, 1p, 9p deletions and 17q gain had negative influence on EFS. Significance of 2p23-24, 4p and chromosome 7 gains was demonstrated in the subgroups of patients.

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POT108

Characterization of Chromosomal Rearrangements Involving the Anaplastic Lymphoma Kinase (ALK) Gene in Neuroblastoma Tumors through Targeted Re-Sequencing

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Background: Alterations of ALK are involved in the pathogenesis of both familiar and sporadic neuroblastoma. The oncogenic potential of ALK was first identified in ALCL from a translocation causing the NPM–ALK fusion protein. Subsequent studies shows additional ALK chimeras generated with different fusion partners in various cancers. However, to date no ALK containing rearrangement has been reported in primary neuroblastoma tumors although two studies describes neuroblastoma cell lines with intragenic deletion of ALK leading to constitutive activation. This led us to screen for and further investigate possible ALK containing translocations in our material.

Methods: Previous SNP microarray profiling of 350 samples indicated ALK rearrangement in four primary tumors and two cell lines which were further analyzed through targeted re-sequencing of the common MYCN amplicon region and the ALK gene. Briefly, detection of rearrangements was performed through solution-based enrichment followed by pair-end sequencing of 500bp libraries. Junction sites were identified by manual viewing on IGV and validated through PCR and Sanger sequencing.

Results: In one tumor ALK was fused with GAB2 (11q14) although in opposite transcription directions and thus, unlikely to cause a chimeric protein. In another tumor ALK were fused with the 4q-subtelomeric region. Samples with both MYCN and ALK amplification showed high level complexity of respective amplicon with multiple rearrangements although at subclonal level for the primary tumor. IMR32 show involvement of at least six different 2p-regions. CLB-Bar has also multiple 2p rearrangements including an ALK intragenic alteration ultimately resulting in an ALK protein with truncated extracellular domain.

Conclusion: Here we show that ALK translocations could be deduced from targeted re-sequencing without previous knowledge about fusion partner. However, no translocations resulting in chimeric protein were detected. Patient specific MYCN amplicon junctions could also be characterized and thereby used for detection of minimal residual disease.

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POT109

An 18-gene Myc Activity Signature for Neuroblastoma and Other Myc-Driven Cancers

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Background: MYCN oncogene amplification is a powerful adverse prognostic factor in neuroblastoma. Expression profiling studies indicate that tumours with aberrant up-regulation of the Myc transcriptional program in the absence of MYCN amplification are also associated with particularly poor outcome. Such "Myc activity" signatures are based on large numbers of Myc target genes. A prognostic Myc signature derived from a smaller set of genes may be more practical for identification and monitoring of these cases, both in neuroblastoma and in other Myc-driven malignancies.

Methods: Selection of 18 Myc target genes was based on previous studies and on analysisofpublicly available neuroblastoma data. Taqman real-time quantitative PCR assays, arrayed in low density format, were used to determine Myc activity scores for a panel of 36 cell lines derived from neuroblastoma and other cancers, for which MYCN/MYC gene copy number and expression had been determined. Myc activity scores were similarly derived for a cohort of 42 primary neuroblastomas and tested for association with event-free and overall survival (EFS, OS) using Kaplan-Meier analysis and Cox regression.

Results: In cancer cell lines, MYCN amplification was restricted to neuroblastoma, while MYC amplification was observed in a range of cancer types, including one neuroblastoma cell line. The 18-gene Myc activity score was positively correlated with MYCN mRNA expression in neuroblastoma cell lines and with MYC in cells derived from other cancer types. In neuroblastoma, high Myc activity was associated with poor outcome, both overall ($P=0.000$) and in tumours with single-copy MYCN ($p=0.000$). The 18-gene signature was also associated with poor outcome in microarray datasets of medulloblastoma ($P=.012$, OS) and a "high MYCN" subtype of ovarian cancer ($P=.04$, EFS).

Conclusion: An 18-gene signature can be used as a measure of MYCN/MYC deregulation in neuroblastoma, and in other cancers where Myc is a driver of malignancy.

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POT110

The High Incidence of 2p Gain in Polish Patients with Neuroblastoma

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Background: Neuroblastoma is a childhood cancer with considerable morbidity and mortality. Tumor-derived biomarkers may improve risk stratification. Structural chromosomal aberrations (SCA) and MYCN gene amplification have unfavorable prognosis. SCA increase risk of tumor progression with bone marrow metastases and relapse. The co-attendance of SCA and NCA (numeric chromosomal aberrations) reduces a patient's outcome.

Methods: We screened 16 tumor samples of neuroblastoma (collected from October 2010 to August 2013) using interphase-FISH with probes: N-MYC (Cytocell Ltd.), SRD(CHD5)(Cytocell Ltd.), ONMLL(11q23)/SE11(Kreatech), LSITop2a/CEP17(Abbot Lab.). Results were evaluated according the INRG Biology Committee guidelines and were confirmed by MLPA (P252 probemix; MRC-Holland).

Results: Amplification of the MYCN gene was identified in 2/16 tumors (one was heterogeneous) and 2p gain in 6/16. Deletion at 1p36 was found in 2/16 tumors, LOH at 1p36 in 2/16. 11q23 deletion was presented in 1/16 tumors, LOH at 11q23 in 1/16 and 17q gain in 7/16 tumors. The 2p gain subgroup was unexpectedly numerous. Cases with 2p gain had additional unfavorable factors: age > 18 month (4/6), co-occurrence of SCA (17q gain 4/6; 1p and 11q deletion 2/6). All of them presented polysomy at least one of the four chromosomes, usually trisomy (5/6). The only one patient from this subgroup had recurrence. The association of SCA and NCA with relapse and survival were not determined because of very short observation time (3-24 months).

Conclusion: In our material highlights the high incidence of 2p gain (37,5%) coexisting with NCA and 17q gain and also age. Results should be confirmed on a larger number of patients with longer follow-up.

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POT111

Fluorescence in Situ Hybridization Evaluation of MYCN, 1p36, 11q22-23 in Neuroblastoma Patients. Single Center Experience in Russia

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Background: MYCN amplification, del(1p36) and del(11q22-23)-the genetic aberration that are important for risk stratification of patients with neuroblastoma. We report results of defining status of MYCN, 1p and 11q from 01.2012 to 11.2013.

Methods: Tumor samples of 131 patients with neuroblastoma were investigated using FISH DNA-probes: LSI N-MYC (2p24)SG/CEP2 SO, LSI N-MYC (2p24.1)SO, LSI 1p36/1q25 DC, LSI MLL(11q23) and LSI ATM (11q22)/CEP11 (Abbott Molecular). The results were assessed according to recommendation of NB2004-Protocol and INRG Biology Committee. Analysis was performed on FFPF in 73 cases, touch preparation in 36, bone marrow – in 22 cases. Patients median age was 24,9 months (range 0,5-128 months). Male: female ratio was 1,1 :1. 54 patients were under one year.

Results: MYCN amplification was found in 26(19,8%) cases, in one of them intratumoral heterogeneity was found. "Gain" pattern was found in 7 cases. Incidence of MYCN amplification in patients under 12 months were 3,7%. In patients older than 12 month – 31%. , Del(1p36) was observed in 19(16%) of 119. In combination with MYCN amplification - 12(63,1%). Imbalance was occurred in 9 (7,5%) cases. Incidence of del(1p36) in patients under 12 months were 5,7%. In patients older than 12 month – 24,2%. , Del(11q22) was found in 5(9,6%) of 52 samples. 68 cases was investigated for del(11q23) and 8(11,7%) were positive. In 6 cases we applied both DNA-probes. In 3 cases negative for 11q22 deletion we found deleted 11q23 region. Incidence of del(11q22-23) in patients under 12 months were 6% . In patients older than 12 month –15,6%. In 9 of 13 patients with del(11q22-23) bone marrow was involved.

Conclusion: Frequency of MYCN amplification consisted 19,8%, that corresponded to literature data. Incidence of del(1p36) and del(11q22-23) in evaluated group was low and consisted 16% and 11% respectively. For del(11q) analysis DNA-probe for 11q23 region should be applied.

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POT112

Recurrent 14q32.33 Gain Including the Genes: ADAM6 and KIAA0125 in Neuroblastoma Tumors

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Background: We evaluated the chromosomal alterations using SNP array analysis and correlated with clinical characteristics of NB patients.

Methods: High resolution SNP array analysis (Cytoscan HD, Affymetrix) was performed on DNA isolated from 26 NB primary tumors. Fourteen patients (54%) were above 18 months. Staging according to INSS: 5 stage 1, 2 stage 2, 4 stage 3, 15 stage 4. Five patients harbored the amplification of MYCN (MNA). Progression occurred in 9/26 patients.

Results: We confirmed several common copy number chromosomal alterations. Chromosome 1p deletion was identified in 11 cases (42%), 3p deletion in 10 cases (38%), 9 of them in the non-MNA group. Eight cases (30%) exhibited deletion of 11q, all from the non-MNA group. Gain of 17q was present in 13 cases (50%). We discovered a previously unknown focal copy number gain on chromosome 14q32.33 (14:106329183-106525210) in 18 cases (69%). The region of gain spanned from 196-1134 kbp length. This region of gain harbors 2 genes: ADAM6 and KIAA0125. The ADAMs consists of a family of multidomain transmembrane and secreted proteins and belongs to a super family of zinc-based proteinases. ADAM function has been associated with various physiological processes including fertilization and development, and has also been implicated in pathological conditions such as inflammation, Alzheimer disease and cancer progression. KIAA0125 has been suggested to be involved in neurogenesis.

Conclusion: Our work identified a new region of copy number gain harboring the ADAM6 and KIAA0125 genes that may be involved in neuroblastoma tumorigenesis.

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POT113

High-Resolution Array CGH Profiling Identifies Na/K Transporting ATPase Interacting 2 (NKAIN2) as a Predisposing Candidate Gene in Neuroblastoma

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Background: Neuroblastoma (NB) is a childhood solid tumor that originates from neural crest cells of the sympathoadrenal lineage during development and represents the third leading cause of cancer-related mortality in children accounting for 10% of all pediatric cancer deaths. Approximately 1-2% of NB cases have a positive family history, with patients either distributed along three generations or clustered in the most recent generation. Germline mutations in the ALK and PHOX2B genes have been found in a subset of familial NBs, however, because some individuals harboring mutations in these genes do not develop this tumor, other predisposing genes remain to be discovered. Herein, we studied an Italian family with three NB patients, two siblings and a first cousin, carrying an ALK germline-activating mutation R1192P, which was inherited from their unaffected mothers and with no mutations in the PHOX2B gene.

Methods: A comparative genomic hybridization (CGH) array performed in the somatic and germline DNA contents of the two affected siblings. Data were confirmed by qPCR analysis.

Results: aCGH analysis revealed a gain at NKAIN2 (Na/K transporting ATPase interacting 2) locus in one of the sibling, which was inherited from the parent who does not carry the ALK mutation. Surprisingly, NKAIN2 was expressed at high levels also in the affected sibling that lacks the genomic gain at this locus, clearly suggesting further mechanism of gene regulation., High levels of NKAIN2 were detected in the MYCN-amplified NB cell lines, in the most aggressive NB lesions as well as in the peripheral blood of a large cohort of NB patients. Consistent with a role in NB development, NKAIN2 was down-regulated during all-trans retinoic acid differentiation in two NB cell lines.

Conclusion: Taken together, these data indicate a potential role of NKAIN2 gene in NB growth and differentiation.

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POT114

MYCN/MYC Protein Expression in Neuroblastoma, Undifferentiated and Poorly Differentiated Subtype - C-myc Activation is a New Marker for Aggressive Tumor Behavior: A Report from the COG Neuroblastoma Committee

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Background: MYCN amplification and subsequent MYCN protein (MYCNP) expression is a strong indicator for poor prognosis in neuroblastoma patients. Little is known about the role of MYC (C-myc) protein (MYCP) expression in neuroblastoma.

Methods: During 2009, 357 COG cases were diagnosed with neuroblastoma, undifferentiated (20) or poorly differentiated (337) subtype (82 MYCN Amplified-A, 272 Non-Amplified-NA, 3 data missing; 176 Favorable Histology-FH, 181 Unfavorable Histology-UH; 159 Low Mitosis-Karyorrhexis Index-MKI, 103 Intermediate MKI, 81 High MKI, 14 data missing; and 110 with prominent nucleoli-PN, 247 without PN). MYCNP/MYCP expression was analyzed immunohistochemically.

Results: MYCNP staining: 279 tumors were negative (-), 10 showed sporadic/weak (+/-), and 68 had diffuse/strong (+) positivity. MYCP staining: 297 tumors were negative (-), 21 showed sporadic/weak (+/-), and 39 had diffuse/strong (+) positivity. Both proteins were detected in 3 tumors (1 + and 2 +/-). MYCNP(+)tumors were significantly associated with UH (100%; p<0.0001), MYCN-A (99%; p<0.0001), (+)PN (99%; p<0.0001) and High MKI (82%; p<0.0001). MYCP(+)tumors were significantly associated with UH (77%; p=0.0024), MYCN-NA (97%; p=0.0002), and (+)PN (74%; p<0.0001). Prognosis of patients with MYCNP(+)tumors (3-year EFS 47.4+11.5%; OS 63.8+11.6%) was significantly worse than those with MYCNP(-)tumors (3-year EFS 75.2+6.2%; OS 87.9+4.7%) and MYCNP(+/-)tumors (3-year EFS 77.8+36.7%; OS 88.9+21.0%) (EFS: p=0.0003, OS: p<0.0001). Similarly prognosis of patients with MYCP(+)tumors (3-year EFS 46.5+19.6%; OS 65.3+17.2%) was worse compared to those with MYCP(-)tumors (3-year EFS 72.5+5.9%; OS 85.9+4.6%) and MYCP(+/-)tumors (3-year EFS 81.0+25.0%; OS 90.5+19.7%) (EFS: p=0.0157, OS: p=0.0841).

Conclusion: MYCP(+)tumors comprise a unique subset in neuroblastoma that are not associated with MYCN-A, but often classified as UH or (+)PN. Survivals for patients with MYCP(+)tumors and those with MYCNP(+)tumors are similar, suggesting that MYC-driven tumors link to a poor prognosis even in the absence of MYCN amplification. Further investigation on the role of C-myc in neuroblastoma pathobiology is warranted.

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POT121

Modeling the Influence of MYCN on the p53-Mdm2 Pathway in Neuroblastoma

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Background: MYCN amplification occurs in 20% of neuroblastomas and is associated with treatment failure and poor prognosis. MYCN play a paradoxical role in cells: promoting cellular survival and sensitizing cells to apoptosis through p53 activation. Our aim is to understand the influence of MYCN on an imbalance in expression and function of p53, Mdm2 and p21.

Methods: We used MYCN-regulatable, p53-wild type neuroblastoma cell line SHEPTET21N for data generation. We first treated cells with doxorubicin in MYCNon and MYCNoff conditions. Then we collected the quantitative, time-resolved mRNA and protein data on the activation status of key players in the p53 pathway. Finally, we established a simple ODE-based mathematical model for MYCN dependence of p53-Mdm2 pathway by means of mass action kinetics. The interaction of main regulatory proteins (p53, Mdm2, p21, Bax and Puma) and transcriptional activation were modelled.

Results: A simple mathematical model can capture the time series pattern for the key players, such as p53, Mdm2, p21 (for cell cycle arrest/senescence) and Bax/Puma (for apoptosis) in response to MYCN influence after the doxorubicin treatment. Model results shows that p21 expression is quite similar between both MYCN situations within 24 hours after the treatment. This should indicate that cell first tries to repair DNA damage. Once DNA damage cannot be repaired, cell will make decisions: senescence, apoptosis or proliferation, in response to chemotherapy. This hypothesis is also nicely confirmed by Bax/Puma time-series expressions. More importantly, the related experiment on the analysis of cell death after the treatment strongly supports this finding.

Conclusion: Although the developed mathematical model is only for the key players of p53-dependent signalling pathway, results can still well indicate experimental observations. As the next step we will perform, parameter sensitive analysis and system robustness analysis, to explain in details the influence of the MYCN and p53 combination for different cellular decisions.

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POT123

High Expression of the Histone Demethylase JARID1C Correlates with Aggressive Tumor Stages Independently of MYCN and Controls Proliferation and Apoptosis in Preclinical NB Models

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Background: In the absence of MYCN amplification, few molecular risk factors have been found so far determining the prognosis and outcome of high-stage NB. We here used mRNA expression data to identify risk-associated genes with independent prognostic power in high-stage NB with single copy MYCN.

Methods: Exon-resolution microarrays were used to generate mRNA expression profiles from 113 primary NBs. Rank-based statistics and machine learning algorithms were used to define candidate markers associated with patient outcome independent of known risk factors. Target validation was achieved using immunohistochemistry and western blotting. Functional screens included siRNA-mediated knock-down or enzymatic target inhibition with subsequent viability or flow-cytometry-based assays.

Results: Expression of the JARID1C histone demethylase was significantly elevated in relapse tumors independent of MYCN amplification status. Interference with JARID1C using siRNA or chemical inhibition of JARID1C functions significantly decreased cell viability and induced morphological changes indicative of apoptosis, which could also be confirmed by flow cytometry. In addition, a shift in H3K4 methylation status affecting both trimethyl and dimethyl H3K4 was observed.

Conclusion: Our results suggest that high expression of the histone demethylase JARID1C is a marker for aggressive neuroblastoma independent of MYCN status. Functional consequences of epigenetically reprogramming NB cells by interference with JARID1C should be further explored in in vivo NB models.

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POT124

High Frequency of Subclonal ALK Mutations in Neuroblastoma

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Background: In neuroblastoma (NB), activating ALK receptor tyrosine kinase point mutations are detected in 8–10% at diagnosis using conventional sequencing. To explore the potential occurrence of ALK mutations at a subclonal level, we studied ALK variation frequencies using targeted deep sequencing.

Methods: A clinically representative series of 269 diagnostic NB samples was analyzed, focusing on the exon 23 hotspot containing the F1174 mutation. DNA was amplified via a two-step PCR approach, the second step consisting of addition of sample-specific barcodes for targeted resequencing in a single experiment. Amplicon sequencing (Illumina HiSeq2500) achieved an extremely high depth over the relevant hotspot (80,000X). The background base variability (error rate) in 32 control samples was 0.017%+/-0.010 at the studied position. Given the mean coverage and error rate, a base frequency >0.06% is significantly different from background noise (Fisher's exact test).

Results: At the F1174 hotspot, mutations were observed in 26/269 samples. For 5 cases, these mutations, observed with base frequencies of >20%, were confirmed by Sanger sequencing. In 21 additional cases mutations were observed at a subclonal level (range of mutation frequencies: 0.113%-12.858%). In one case, a subclonal ALK mutation (0.101%) was observed at relapse, with no mutation seen at diagnosis. A strong correlation between ALK mutations and MYCN amplification was observed (p=0.0001). Kaplan–Meier analysis showed a significantly poorer overall survival in patients with ALK mutations, whether clonal or subclonal, compared to those without (5-year OS : 48%+/-9.9 versus 77%+/-2.9, p<0.002, logrank test).

Conclusion: Our study documents a high frequency of subclonal F1174 ALK mutations at diagnosis (7.8%) as compared to clonal events (1.8%). These findings are of utmost clinical importance given the potential role of ALK mutations in clonal evolution and relapse (see abstract number A-0020/POT078) and increasing significantly the number of patients who might potentially benefit from ALK-targeted therapy.

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POT127

The Role of Trk Receptor Expression in Checkpoint Activation and DNA Double Strand Break Repair in Neuroblastoma Cells

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Background: High expression of the receptor tyrosine kinase TrkA is associated with a favorable outcome in neuroblastoma, while TrkB expression has been linked to aggressive disease and an unfavorable outcome. Enforced TrkA expression in human SY5Y neuroblastoma cells causes induction of differentiation and enhanced DSB repair capacity, while TrkB expression in the same model cell line increases proliferation, chemoresistance and sphere formation in vitro.

Methods: Response to ionizing radiation (IR) was analyzed in SY5Y cells engineered for conditional expression of TrkA or TrkB by monitoring effects on DNA repair capacity, cell viability and protein expression levels. To analyse the effect of Trk expression in a DNA repair deficient background, SK-N-AS neuroblastoma cells harboring a mutation in the ATR gene were engineered to express TrkA or TrkB and tested for survival in a clonogenic assay.

Results: When irradiated with 2 Gy, proliferation of TrkA-expressing cells was significantly enhanced upon activation by the TrkA-ligand, NGF, in comparison to cells without TrkA-expression. TrkB-expressing cells could be partially rescued from IR-induced cell death by treatment with the TrkB-ligand, BDNF. Interestingly, DNA repair kinetics were not significantly altered upon induction of Trk expression. However, expression of NHEJ-factors PARP1, XRCC4 and DNA-Ligase III were upregulated in TrkA-expressing and to a lesser extent also in TrkB-expressing SY5Y cells. Clonogenic survival of ATR-deficient SK-N-AS cells was not increased upon enforced expression of TrkA.

Conclusion: Taken together, these results suggest a protective effect of TrkA against IR-induced proliferation arrest, possibly by enhancing the efficiency of DNA repair. However, TrkA-expression does not seem to rescue cells from DNA damage-induced cell death in ATR-deficient cells.

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Translational Research: Minimal Residual Disease

POT115

Neuroblastoma (NB) Gene Expression in Bone Marrow (BM) of High-Risk (HR) Patients at the Conclusion of anti-GD2 Antibody and Retinoic Acid Therapy Is Associated with Disease Progression. A Children's Oncology Group Study

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Background: Event-free survival (EFS) for patients with HR NB was improved by addition of anti-GD2 antibody ch14.18, IL-2, and GM-CSF to isotretinoin, but 40% still relapsed. We quantified expression of five neuroblastoma-associated genes in BM of patients before and at the end of therapy with a TaqMan® Low Density Array (TLDA) assay to determine if expression predicted EFS.

Methods: Patients enrolled on ANBL0032 60-100 days after myeloablative chemotherapy. Disease evaluations, including BM mononuclear cells evaluated by immunocytology, occurred before and after 6 cycles of therapy. When >5 X 106 cells were available, total RNA was prepared from viably frozen cells or from cells in RLT buffer. The TLDA assay, which quantified expression of CHGA, DCX, DDC, PHOX2B, and TH, was performed when RNA quantity and quality (RIN≥5.5) were sufficient. Results are reported as detectable/non-detectable and as ΔCT = geometric mean (GM) of 5 detection genes minus GM of 4 housekeeping genes (CT = Cycle Threshold). DCT is inversely related to mRNA quantity.

Results: NB-associated RNA was detected in BM from 75% (120/160) and 64% (117/183) of patients before and after 6 cycles of therapy respectively. The TLDA assay did not identify subgroups with different EFS before therapy. In contrast, patients whose BM had detectable vs. non-detectable disease at the end of therapy had 46±9% vs. 75±7% 5-year EFS (logrank P=0.005), and stronger RNA signal was associated with lower EFS (P<0.001, trend). Within 124 patients in clinical complete remission (CCR) after 6 cycles, 59% had detectable disease by TLDA. The 5-year EFS was 52±9% for detectable (n=73) vs. 74±8% non-detectable (n=51) disease (p= 0.022) and the signal strength/EFS association was significant (P=.002, trend).

Conclusion: The 5-gene TLDA assay is prognostic for outcome in patients at the conclusion of anti-GD2 antibody and retinoic acid therapy, even for those in CCR.

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POT116

Quantifying Expression of Five Neuroblastoma-Associated Genes in Bone Marrow (BM) and Blood of Patients with Refractory or Relapsed Neuroblastoma (NB) Improves Assessment of Disease Status and of Disease Progression Risk. A New Approaches to Neuroblastoma

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Background: Disease evaluation provides risk and response data for assigning and evaluating therapy respectively. We determined if a TaqMan® Low Density Array (TLDA) assay that quantifies expression of five NB-associated mRNAs (NB-mRNA) in BM and blood improves assessment of disease status and of risk for disease progression.

Methods: mRNA of CHGA, DCX, DDC, PHOX2B, and TH was quantified with the TLDA assay and reported as detectable/non-detectable or as ΔCT (geometric mean [GM] of 5 detection genes minus GM of 4 housekeeping genes). CT and ΔCT are inversely related to mRNA quantity. BM (n=184) and blood (n=142) obtained at clinical disease evaluations from 106 high-risk NB patients (94 with relapsed/refractory disease) were analyzed. Tumor longest diameter (LD) from CT/MRI (n=262), Curie score from MIBG (n=122), tumor cell percent in BM biopsies by morphology (mBM) (n=226), and overall response were determined (central review).

Results: Clinical disease evaluations showed Currie>0 in 71%, LD≥1cm in 57%, and mBM>0 in 46%.NB-mRNAwas detectable in 82.5% of BM (137/166) and 62% of blood specimens (78/126). Among positive mBM, MIBG, and CT/MRI evaluations, NB-mRNA in BM was detected in 100% (79/79), 92% (49/53), and 82% (73/89) respectively. Among negative mBM, MIBG, and CT/MRI evaluations, NB-mRNA was detected in 66% (54/82), 70% (14/20), and 82% (50/61) respectively. BM and blood ΔCTs correlated with mBM (r= -0.66 and r =-0.42; p<0.0001) and with Curie (both r=-0.63; p<0.0001). Blood but not BM ΔCT correlated with LD (r=-0.34; p=0.0001). BM and blood ΔCTs were correlated (r=0.63, p<0.001), but BM NB-mRNA was 5.0±0.5 CT stronger. For patients with relapsed/refractory NB, change of ΔCT in BM correlated with clinical response (p<0.05) and correlated with progression-free survival even when mBM was negative (p<0.01).

Conclusion: This five-gene assay for NB-mRNA improves definition of disease status, and serial analyses may provide a new biomarker for predicting outcome.

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POT117

Different Prognostic Impact of Bone Marrow Tumor Cell Content and Response Pattern in MYCN Amplified (MNA) and MYCN Non-Amplifeied (nMNA) Stage 4 Neuroblastoma

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Background: The impact of the tumor cell content in bone marrow at diagnosis and in response to chemotherapy on survival is still controversial.

Methods: Inclusion criteria for this retrospective study were stage 4 neuroblastoma ≥18 months of age, diagnosis between 01.11.2002 and 31.12.2010, and central assessment of MYCN tumor status and tumor cell content in bone marrow. Anti GD2 immunocytochemistry was performed on 1.5-2.0x106 bone marrow cells pooled from 2-4 aspiration sites. The prognostic impact of tumor cell content was evaluated by logrank tests and time-dependent Cox regression analysis.

Results: Of the 249 included patients, 74 had MNA and 175 nMNA tumors. At diagnosis the tumor cell content in bone marrow was not different between MNA and nMNA patients (30-100% tumor cells in 47.3/52.6% of MNA/nMNA patients, 10-<30% in 5.4/8.6%, 1-<10% in 14.9/13.1%, >0-<1% in 21.6/22.3%, and 0% in 10.8/3.4%) (n.s.). Bone marrow clearing was faster and more pronounced in MNA cases: 56.8% after 2 cycles of chemotherapy, 80.8% after 4 and 88.7% after 6 cycles compared to 20.4/56.9/77.3% in nMNA cases. Patients with nMNA neuroblastomas had a better outcome (EFS,OS) if the tumor cell content was low (0-<1%) at diagnosis. The cut-off at 1% discriminated better than a cut-off at 10% (p_{EFS}=0.003, p_{OS}=<0.001). In nMNA patients, clearing of bone marrow (0%) after 4 cycles of chemotherapy was associated with better EFS (p=0.010), but had no impact on OS. In MNA cases, no prognostic impact of the amount of bone marrow infiltration was detected (neither at diagnosis nor in response to chemotherapy).

Conclusion: A considerable number of patients has <1% of tumor cells in bone marrow at diagnosis. ≥1% was associated with worse outcome in nMNA cases only. The 1% cut-off discriminates better than the 10% cut-off. The clearing of bone marrow is more pronounced in MNA cases.

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POT118

Prognostic Significance of Flow Cytometric Tumor Cells Detection in Bone Marrow of Children with Neuroblastoma

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Background: Bone marrow (BM) micrometastases detection in children with neuroblastoma (NB) is crucial for correct patients staging and risk group stratification. Flow cytometry (FC) is widely available, fast and easy-to perform approach for finding NB cells among normal BM hematopoietic cells. Aim of the study was to investigate prognostic significance of flow cytometric tumor cells' detection in BM of children with NB at the time of diagnosis.

Methods: 51 patients (24 boys and 27 girls) aged from 6 days to 15 years (median age 1 year 3 months) with NB were included in the study. BM samples at the time of diagnosis were obtained from 1-5 aspiration sites per patient (median 3 samples per patient). 4-5-color FC was applied for CD45(-)CD56(+)CD81(+)GD2(+)CD9(+)-cells evaluation.

Results: NB cells were detected in BM by FC more frequently comparing to conventional cytomorphology (49.0% and 29.4% patients respectively, p=0.043). Patients with NB cells detected in BM by FC had significantly worse event-free survival, overall survival and progression-free survival (28.0±9.0%, 35.8±10.7% and 34.3±10.4% respectively) in comparison to children with negative result of immunophenotyping (83.5±7.6%, 87.7±6.7% и 86.8±7.1% respectively, p<0.001 in all cases). BM involvement detection by FC maintained its prognostic significance in following patients groups distinguished by other stratification criteria: patients without MYCN amplification, patients without BM lesion as assessed by cytomorphology, patients younger than 1 year, patients older than 1 year, patients with stages I-III and IVS, patients with stage IV, patients with localized tumor (stages I-III). In multivariate analysis immunophenotyping proved to be an independent prognostic factor when analyzed jointly with other risk factors such as age, disease stage and MYCN amplification.

Conclusion: Thus flow cytometric BM involvement detection could be used in combination with other parameters for the treatment strategy choice in patients with NB.

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POT119

Prognostic Value of the MMD and MRD in Patients with Neuroblastoma

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Background: To determine predictive value of neuroblastoma outcome from MMD existence at the diagnostics and MRD during therapy (in PBSC and in BM before autologous PBSC) detected by molecular biological (qPCR) and immunophenotyping methods (FC).

Methods: 72 patients (6 with stage 1, 4 – stage 2, 25 - stage 3, 33 - stage 4, 4 - stage 4s) who received treatment according to NB2004m protocol 2008 - 2013. MMD and MRD were detected in BM and PBSC, with FC and qPCR, the last was used for analysis of tyrosine hydroxylase (TH) relative expression by ΔCt method.

Results: 43% of patients (31/72) had morphologically verified BM involvement at diagnostics. According to FC data , BM involvement was found in 50% (31/62 pts) (2 with stage 1, 4-stage 3, 23 - stage4 , 2 - stage 4s). qPCR method revealed BM lesion (ΔCT ≥-10) in 43% of patients (31/72) (1 with stage 2, 2 -stage 3, 25 - stage 4, 3 - stage 4s). Spearmen correlation coefficient between two methods estimated r=0.59 (p<0.05). , At diagnosis PFS in FC-negative group was 0.75+0.08 vs 0.32+0.08 (p=0.018). PFS of qPCR negative pts was 0.80 +0.06 vs 0.22+0.07 (p=0.0042). MRD by FC was detected in PBSC in 9.3% (3/32), in BM before autologous PBSC in 4% (1/25). MRD by qPCR was detected in PBSC in 34% (11/32), before autologous PBSC in BM in 28% (7/25). Our data show that high MRD level detected in PBSC by qPCR correlated with decreased PFS (0.09+ 0.09 vs 0.63+0.11, p=0.004). MRD detected by FC didn't have prognostic value for PFS.

Conclusion: Positive MMD in BM detected by both FC and qPCR was associated with significantly decreased PFS. Positive MRD in PBSC by qPCR decreased PFS, while MRD in PBSC and BM by FC didn't correlate with outcome.

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POSTER EXHIBITION

CLINICAL RESEARCH

Clinical Research: Diagnosis and Prognosis

POC001

Computer-Assisted Curie Scoring of MIBG Scans for Neuroblastoma Patients

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Background: MIBG Curie score for neuroblastoma patients is predictive of survival, but calculation is subjective and variable. A standardized computer-assisted method would lead to more consistent scoring and facilitate longitudinal comparisons.

Methods: We developed an assistive interface to aid scoring. The radiologist identifies lesions through a point-and-click method and then assigns lesions to a region. Scores are automatically tallied and calculated. We conducted a pilot study in which two radiologists each scored 19 scans using either manual tallying or the assisted-scoring rubric. We compared the performance of each method for inter-reader reliability using Pearson's product-moment correlation.

Results: Using the unaided scoring method, the correlation between observers ranged from -0.10 to 0.97 (average 0.77) for the individual segment scores with a total score correlation of 0.96. For the assisted scoring method, the segment correlation between observers ranged from 0.24 to 0.92 (average 0.75) with a total score correlation of 0.92.

Conclusion: We have tested an assisted scoring method that maintains a high degree of correlation between radiologists, while preserving a record of the lesions and segments for further review. We have further developed and are testing a scoring method in which the software uses a segmentation based on the placement of nine anatomic points. We are collecting data for all methods on a total of 38 patients from three radiologists. An assistive method that maintains a high inter-observer correlation, while standardizing the scoring rubric and facilitating the recording of lesion markings would increase reliability of Curie scoring for MIBG scans while facilitating longitudinal comparisons for disease surveillance.

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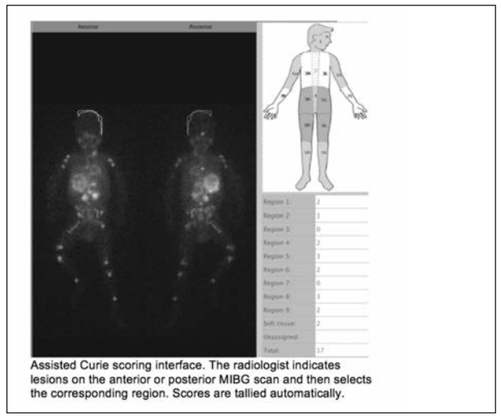

Assisted Curie scoring interface. The radiologist indicates lesions on the anterior or posterior MIBG scan and then selects the corresponding region. Scores are tallied automatically.

POC002

Identification of Plasma Complement C3 as a Potential Biomarker for Neuroblastoma Using a Quantitative Proteomic Approach

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Background: The majority of patients diagnosed with neuroblastoma present with aggressive disease. Improved detection of neuroblastoma cancer cells by biomarker following initial therapy may help in stratifying patient outcome and monitoring for relapse.

Methods: To identify potential plasma biomarkers, we utilised a liquid chromatography-tandem mass spectrometry-based (LC-MS/MS) proteomics approach, bioinformatics and pathway analysis to detect differentially-expressed proteins in plasma from TH-MYCN transgenic mice and the human neuroblastoma patient samples. The potential plasma biomarkers were then validated using 2D gel electrophoresis and ELISA.

Results: The abundance of plasma proteins was measured over the course of disease initiation and progression. Plasma collected from 10 TH-MYCN$^{+/+}$ and 10 wildtype (WT) mice at 2, 4 and 6 weeks of age was pooled and prefractionated using the electrophoretic ProteomeSep device. Datasets were analysed by Progenesis LC-MS/MS, which identified >200 proteins per size fraction and time-point differentially abundant in the plasma of TH-MYCN$^{+/+}$ and WT mice (± 5-fold relative peptide abundance and p<0.05). From our initial analysis, we removed abundant proteins including albumin, immunoglobulins, transferrin, alpha-1-antitrypsin and haptoglobulin. The list was further refined to include only proteins which were differentially expressed at more than one time point to generate a final list of 86 candidates. Pathway analysis identified significant association of these proteins with genes involved in the complement system. One candidate, complement C3 protein, was significantly enriched in the plasma of homozygous TH-MYCN$^{+/+}$ mice at both 4 and 6 weeks of age, and was found to be elevated in plasma samples from a cohort of neuroblastoma patients, compared to healthy subjects.

Conclusion: We have demonstrated the suitability of the TH-MYCN$^{+/+}$ mouse model of neuroblastoma for identification of novel disease biomarkers in humans, and have identified Complement C3 as a candidate plasma biomarker for measuring disease state in neuroblastoma patients.

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POC004

Risk Stratification Using Clinical Factors, For Treatment of Neuroblastoma Patients in Developing Countries: A Study from the International Neuroblastoma (NB) Risk Group (INRG) Database

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Background: Current methods for stratifying NB patients at diagnosis to risk/pre-treatment groups are based on prognostic clinical, genomic, and histologic factors [Cohn et al, JCO 2009]. In developing countries, testing tumors for genomic biomarkers or histologic features is not possible; however, clinical tests serum ferritin and serum lactate dehydrogenase(LDH) are likely available.

Methods: Retrospective analysis included INRG patients with sufficient data to be categorized by 5-year event-free survival (EFS); very low (>85%), low (>75-≤85%), intermediate (≥50-≤75%), or high risk (<50%) per INRG pre-treatment groups (IPTG), and had known ferritin and LDH. Survival tree regression was performed, considering only age (<18 months; ≥18 months), INSS stage (4;not 4), ferritin (<92; ≥92ng/mL), and LDH (<587; ≥587U/L). Patients were categorized into INRG-clinical pre-treatment groups (ICPTG) for use in developing countries. The concordance/discordance of four risk groups for IPTG versus ICPTG was determined. EFS time was calculated from diagnosis until first event (relapse/progression, second malignancy, death), or until last contact if no event occurred.

Results: From 8,800 INRG patients, 7,679 were able to be assigned an IPTG. Of 7,679, 3,509 (known LDH/ferritin) were able to be assigned an ICPTG. In 2,039 patients (58.1%; 95%CI: [56.5%,59.%]), IPTG and ICPTG risk group was the same. In 449 (12.8% [11.6%,14.0%]), IPTG and ICPTG risk group (per 5-year EFS) was clinically very similar. Based on 5-year EFS: In 663 (18.9% [17.5%,20.3%]), IPTG overestimated risk but ICPTG correctly assigned risk; in 130 (3.7%; [3.1%,4.3%]), ICPTG overestimated risk but IPTG correctly assigned risk; in 228 (6.4%; [5.6%,7.2%]), ICPTG underestimated risk but IPTG correctly assigned risk.

Conclusion: In 89.9% of patients, clinical factors (age, stage, ferritin, LDH) do as well or better than clinical, genomic, and pathologic factors currently used in INRG risk/pre-treatment group assignment. The INRG-clinical pre-treatment risk stratification shows promise for developing countries to assign treatment intensity.

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POC005

Immunocytological GD2 Expression on Neuroblastoma Cells in Bone Marrow is Overestimated and May Have Implications for α-GD2 Immunotherapy

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Background: AntiGD2 immunotherapy is becoming standard in the treatment of children with high risk neuroblastoma. Parallel cytological and immunocytological investigations of bone marrow demonstrated that the tumor cells do not uniformly stain for GD2. This study examines the frequency of GD2 negativity at diagnosis and during treatment and the pattern of visible antigen loss.

Methods: Bone marrow samples of patients with newly or recurrent neuroblastoma stage 4 and 4S diagnosed between November 1st, 2002 and August 31st, 2013 were investigated by cytology and GD2- immunocytology in parallel. 1-2x106 cells each were reviewed cytologically (slides) and immunologiocally (cytospins, GD2 immunostaining according to consensus criteria Br J Cancer 2009). Negativity of tumor cells is defined as complete absence of immunostaining in all tumor cells, partial negativity as absence in a portion of tumor cells and/or atypically faint staining.

Results: Of 702 patients included in the trials (519 stage 4, 183 stage 4S), 519 had unequivocal cytological bone marrow infiltration. At first diagnosis, 36/519 (6.9%) of patients had tumor cells with complete or partial negativity of GD2 staining. During high-risk chemotherapy GD2 negative neuroblastoma cells were detected in the bone marrow of 11 additional patients (2.1%), and at recurrence in additional 18 patients. Complete absence of GD2 staining was seen in 18, partial negativity staining in 47 cases. Change of GD2 staining during therapy or at relapse occurred in 42 cases: 39 cases from positive to negative staining and 3 cases from negative to positive staining. Three patients with negative staining at recurrence had preceding αGD2 immunotherapy. Survival estimation (EFS, OS) comparing negative vs. positive staining at diagnosis did not show differences neither in stage 4 nor in stage 4S patients.

Conclusion: GD2 staining on neuroblastoma cells in bone marrow is not always present. The results may have implications for the use and evaluation of αGD2 immunotherapy.

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POC006

The New Guideline from the International Neuroblastoma Risk Group (Inrg) Project Has Profound Effects on Clinical Trials Which Employed Image Defined Risk Factors

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Background: International Neuroblastoma Risk Group (INRG) recently propounded a new pretreatment staging system (INRGSS) according to Image defined risk factors (IDRFs). Japan Neuroblastoma Study Group (JNBSS) has been started low / intermediate protocol which employed IDRFs as therapeutic decision since 2010. In 2011, the new guideline (NG) for assessing IDRFs precisely was published (Brisse et al, Radiology). In NG, \\\'even if the tumor is in contact with renal vessels only', should be considered IDRF present which used to be diagnosed as IDRF not present. This statement may alter the results of IDRFs in a considerable number of patients. In this study, we conducted a preliminary single institutional reevaluation of IDRFs by NG in localized neuroblastoma patients, in order to assess the effects of NG on clinical trials which employed IDRFs.

Methods: Eighty-four localized NBs diagnosed between 1991 and 2012 were entered in this study. Of these, 27 patients were diagnosed IDRF present before NG. All images at diagnoses were re-evaluated by a single radiologist (MN).

Results: Thirty-two patients turned IDRF not present to present according to NG. Therefore, totally 59 patients were considered IDRF present according to NG respectively. Regarding with kidney complications (KC), 3 of 19 patients (16%) with IDRF, and 3 of 42 patients (7%) without IDRF encountered KCs before applying NG, respectively. On the other hand, after the application of NG, 6 of 46 patients (13%) with IDRF encountered KCs, and all 15 patients without IDRF did not encountered KCs. Then, sensitivity for KCs increased from 50% to 100%, and specificity decreased from 71% to 27% after introduction of NG.

Conclusion: NG increased the ratio of patients with IDRFs from 32% to 70%. NG improved the sensitivity of IDRF for KCs but less specific. We concluded that NG has profound effects on clinical trials which employed IDRFs.

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POC007

Characterisation and Evaluation of a GD2-Specific Peptide for Hybrid Imaging Using PET/MR

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Background: Due to its tumor-specific expression pattern, GD2 serves as a target for immune therapy in neuroblastoma. We here set out to explore the potential of GD2-directed peptides for in vivo imaging purposes.

Methods: To obtain GD2-binding peptides, we performed a combined in vivo and in vitro phage display screen using a recombinant phage display peptide library. Peptides specifically binding to GD2 were tested for their tumor-homing capability in vivo and the specificity of radiolabeled GD2-binding oligopeptides was evaluated using PET/MR imaging in tumor bearing mice.

Results: Phages specifically binding to GD2, but not to the related ganglioside GD1b were identified in vitro. Specific binding of GD2-phages to tumor tissue was confirmed in vivo. The corresponding peptides were identified and specificity of peptide binding to tumor cells was verified by permutation of amino acid residues. Biodistribution of In111- or Ga68-labeled peptides as well as tumor homing was analysed and compared to I124-MIBG using PET/MR using NB xenografts. While tumor-specific enrichment of the identified peptide and I124-MIBG were comparable, the thyroid was not affected by the GD2-specific peptide identified here. Enrichment of the GD2-specific peptide in tumor-free kidneys could be reduced in part by pretreatment with gelofundin.

Conclusion: This novel GD2-binding peptide could be a useful tool for diagnostic and/or therapeutic purposes via coupling to magnetic nanoparticles, radionuclides or cytotoxic agents.

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POC008

PET Imaging at Diagnosis as a Predictor of Gross Total Resection and Treatment Outcome in Neuroblastoma

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Background: Gross total resection (GTR) of neuroblastoma (NB) could be predicted by imaging-defined risk factors (IDRFs) on CT/MR images present at diagnosis. This study aims to investigate the complementary role of positron emission tomography (PET) scans in predicting GTR and treatment outcome of NB.

Methods: From 2008 to 2012, NB patients who received diagnostic PET scans with 18F-fluorodeoxyglucose (FDG) and ^{18}F-fluoro-dihydroxyphenylalanine (FDOPA) at National Taiwan University Hospital, Taipei, Taiwan, were included. The extent of tumor resections and patient survival was correlated with clinical features and imaging findings.

Results: Twenty-six patients (median age, 1.8 [0.5–6.9] years; male:female, 18:8) were eligible for analysis. Six patients (23%) had stage 3 disease; 14 (54%) had stage 4 disease; and 4 (17%) had MYCN amplification. GTR removing more than 90% of the primary tumor was undergone at first operation in 7 (27%) and at best operation in 13 (50%) patients, while the other 6 patients (23%) only had partial resection. Based on the primary tumors' maximal standard uptake value (SUV_{max}) on PET scans, we defined a group of "PET high-risk" patients (PET-HR; n=10) with the SUV_{max} ratio between FDG and FDOPA equal to or higher than 1.6, the median value of this cohort. The GTR rate of these PET-HR patients was significantly lower than that of the others (54% vs. 100%; P=0.005), and remained to be lower when only considering patients with IDRFs (n=17; 65% vs. 100%; P=0.01). GTR had no impact on event-free survival (EFS; P=0.54) and overall survival (OS; P=0.71); neither did IDRFs (EFS, P=0.08; OS, P=0.39). However, PET-HR patients had significantly worse 3-year EFS (23% vs. 73%; P=0.02) and OS (35% vs. 92%; P=0.04).

Conclusion: PET imaging at diagnosis may provide a complementary role to IDRFs and help surgical planning. Primary NB tumors with FDG-high and FDOPA-low uptake were less likely to be totally resected and had a worse outcome.

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POC009

Diffusion-Weighted Imaging in Neuroblastoma and Ganglioneuroma - A Pilot Study

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Background: Diffusion-weighted imaging (DWI) is increasingly used in oncologic imaging, because it might be able to differentiate benign from malignant tissues, and/or to predict response to therapy. The purpose of this study was to analyse the value of DWI for the differentiation of ganglioneuromas and neuroblastomas. In addition, the change in apparent-diffusion coefficient (ADC) values during and after therapy was assessed for the neuroblastomas.

Methods: The MRI studies of patients with a ganglioneuroma or a neuroblastoma, acquired between June 2009 and July 2013, were retrospectively analysed. The tumours were segmented by drawing an ROI around it on every slice. Cystic areas were excluded. The median ADC was calculated for the whole tumour volume.

Results: Fourteen patients were included: three patients with a ganglioneuroma and eleven patients with a (ganglio)neuroblastoma.
The median ADC of the ganglioneuromas was significantly higher than the median ADC of the neuroblastomas at diagnosis (p=0.005).
Three patients with a neuroblastoma underwent MRI during chemotherapy. In these patients, the tumours showed an increase in median ADC compared to the ADC at diagnosis.
Five patients with a neuroblastoma underwent preoperative MRI (including two patients who underwent MRI during chemotherapy). Two tumours showed a decrease in ADC after therapy compared to the ADC at diagnosis. Both tumours showed no vital tumour at histology. In contrast, the other three tumours showed an increase in ADC after chemotherapy compared to the ADC at diagnosis; these tumours showed vital tumour at histology.

Conclusion: Ganglioneuromas in this study had a higher ADC than neuroblastomas.
A difference in change in ADC values was found between tumours with complete response to chemotherapy and tumours with incomplete response.
Larger prospective studies are, however, needed to evaluate if ADC values can be used to predict tumour response to chemotherapy.

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POC010

High-Risk Neuroblastoma Recurrence after High Dose Chemotherapy (HDC) and Autologous Stem Cell Transplantation (ASCT)

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Background: This study aimed to analyse hi-risk neuroblastoma recurrence after high dose chemotherapy (HDC) and autologous stem cell transplantation (ASCT). To this purpose, we focused on clinical presentation, treatments performed, and prognostic factors.

Methods: Between 2000 and 2010, at Gustave Roussy, 67 out of 134 children affected with high-risk neuroblastoma, who had received HDC and ASCT, presented tumour recurrence.

Results: Out of 67 patients, 40 had a progression and 27 a relapse. There were 35 males and 32 females. Median age at diagnosis was 39.5 mos (3% <1 year; 74.6% between 1-5 years; 15 >5 years). 65 patients presented metastatic disease and 2 had localized disease with MYCN amplification. 17 patients (26%) presented MYCN amplification. HDC consisted of busulfan and melphalan in 62 patients (92%). Median time from transplantation to first relapse was 13 months (1-48). Median survival after recurrence was 9 months (0-28 months). All patients died but 3 (5.5%) who are alive (11 months+, 14 months+ and 50 months+). Patients (N=35) treated with temozolomide alone or in combination with topotecan had the longest time to progression, median 122 days (4-632+). Oral etoposide (40 patients), even if administered late, increased survival with a good quality of life, median 65 days (9-393). Prognosis factors, influencing life expectancy after recurrence, were: age at diagnosis <18 months, MYCN amplification, and time <1 year between diagnosis or transplantation and recurrence.

Conclusion: Outcome after recurrence post HDC and ASCT is poor. However, factors involved in life expectancy duration can be identified. These factors should be taken into account in trials evaluating new treatment strategies as well as stratification criteria in randomized studies to avoid bias and wrong conclusions.

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POC011

Characteristics of UK Patients with Relapsed, Intermediate Risk Unresectable, Non-MYCN Amplified Neuroblastoma: A Pilot Study

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Background: Intermediate risk neuroblastoma is a heterogeneous disease requiring minimal therapy to cure in some cases, whereas others relapse and are unsalvageable. This pilot study aimed to investigate clinical and biological factors associated with recurrence and length of survival following relapse in UK patients with intermediate risk neuroblastoma.

Methods: All cases of relapsed neuroblastoma diagnosed during 1990-2010 were identified from 4 UK Children's Cancer and Leukaemia Group centres. Kaplan-Meier survival analyses were used to calculate overall survival (OS) time from diagnosis and post relapse overall survival (PROS).

Results: Out of 189 cases of relapsed neuroblastoma, 17 (9.0%) were intermediate risk stage 3, unresectable, non-MYCN amplified (non-MNA) and 7 intermediate risk, stage 4, non-MYCN amplified Out of 189 cases of relapsed neuroblastoma, 17 (9.0%) were intermediate risk stage 3, unresectable, non-MYCN amplified (non-MNA) and 7 intermediate risk, stage 4, non-MYCN amplified <12 months of age. For intermediate stage 3, unresectable, non-MNA cases, median age at diagnosis was 3.9 years (0-16), International Neuroblastoma Pathology Classification histology was unfavourable in 15/17, (86%) cases, 53% relapsed within 2 years of diagnosis and the median event free survival was 21.8 months (inter-quartile range (IQR) 10.1-38.6). 58% of patients relapsed at the primary site. Table 1 shows the treatments patients received at relapse. The median OS for this group was 72.9 months (IQR 19.5-99.0) and the median PROS was 11.8 months (IQR 9.0-51.6). 5-year PROS was 24% (95% CI 7%-45%).

Table 1: Treatment received at relapse for all relapsed neuroblastoma cases and for intermediate stage 3, unresectable, *non-MNA* neuroblastoma

Treatment	All relapsed cases N (%)	Intermediate risk N (%)
Second line Chemotherapy	72 (45.3)	9 (60.0)
mIBG Therapy	8 (5.0)	1 (6.7)
Palliative radiotherapy	23 (14.5)	1 (6.7)
Other (ex. Surgery or radiotherapy)	11 (6.9)	1 (6.7)
Supportive care	18 (11.3)	0 (0.0)
Combination of treatments (ex.	21 (13.2)	3 (20.0)
Phase I or II trials	6 (3.8)	0 (0.0)
Total	*159 (100)	**15 (100)

* Treatment at relapse was known for 159 (84.1%) of 189 relapsed cases.
** Treatment at relapse was known for 15 (88.2%) of 17 stage 3, unresectable intermediate risk cases.

Conclusion: This study shows that almost 50% of intermediate risk, unresectable, non-MNA neuroblastoma relapse >2 years from diagnosis. Although this group comprise <10% of all relapsed neuroblastoma, the failure to salvage these patients in over 75% of cases, even when given high risk type treatments at relapse, is concerning.

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POC012

Tumor Regression Rate in Neuroblastoma Stage 4S: Clinical, Biochemical and Radiological Parameters

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Background: Neuroblastoma stage 4S (NB4S) is characterised by spontaneous tumor regression and favourable outcome. However, the clinical regression pattern and rate have been poorly described.

Methods: Retrospective study of all NB4S patients (AMC/ EMC) (1980-2012), describing clinical, radiological and biochemical rate of regression and outcome, by reviewing of all medical files, imaging reports and urinary results.

Results: There were 33 patients, mean age at diagnosis 2.6 months (0-7), follow up 107 months (0-420). Nineteen out of 33 patients (58%) were treated. Four out of 33 (12%) patients suffered from rapid tumor growth and 5/33 (15%) patients had late progression to stage 4 NBL tumors n=4 (12%)/ relapse n=1 (3%). There was no correlation between regression rate and late relapse. Radiological residual lesions can be detected up to 168 months. The absolute levels of vanillylmandelic acid (VMA) and homovanillic acid (HVA) at diagnosis are significantly higher in the treated vs. non treated patients (p= 0.043 and p=0.04 respectively). The decline of VMA levels in the first 4 months after diagnosis is similar for the treated vs. non treated patients, but takes longer in treated patients. Table 1: median time to normalization of urine (VMA and HVA) and radiology (treated/ non treated and overall). The 5 years event free survival (EFS) and overall survival (OS) was 76 % and 85 %, respectively.

Conclusion: Biochemical regression rate in NB4S takes months, with a large range. Radiological residual lesions can be detected for a long time.

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Table 1: median time to normalization (months) of urine (VMA and HVA) and radiology in the treated and non treated patients and for the whole cohort.

Median (range) (months)	VMA	HVA	Adrenal gland/ sympathetic side chain	Liver size	Liver aspect
Treated	2.3 (0-64)	1 (0-13)	18.5 (0-77)	6 (0-40)	9.5 (0-40)
Non treated	0 (0-159)	1 (0-159)	16.5 (0-168)	0 (0-30)	7 (0-30)
Overall	1 (0-159)	1 (0-159)	17 (6-168)	2 (0-40)	8 (0-40)

POC013

^{123}I-MIBG scintigraphy and ^{18}F-FDG-PET(-CT) imaging for diagnosing neuroblastoma: a Cochrane diagnostic test accuracy review

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Background: Many studies reported on the diagnostic accuracy of Iodine-123-metaiodobenzylguanidine (^{123}I-MIBG)-scintigraphy in neuroblastoma patients, but they are very heterogeneous in number of included patients and performance of the imaging methods. Still, prognosis, treatment and response of patients are based on extension-scoring of ^{123}I-MIBG-scans. Therefore, we assessed its diagnostic accuracy and that of a possible add-on test: Fluorine-18-fluorodeoxy-glucose (^{18}F-FDG) positron emission tomography (-computed tomography) (PET(-CT)).

Methods: We searched databases of MEDLINE/PubMed (1945-September 2012) and EMBASE/Ovid (1980-September 2012), reference lists of relevant articles and reviews, conference proceedings and contacted experts. Inclusion criteria: cross-sectional studies comparing results of ^{123}I-MIBG-scintigraphy, ^{18}F-FDG-PET(-CT), or both with the reference standards or with each other; diagnostic design; children 0-18 years old; neuroblastoma of any stage at first diagnosis or at recurrence., Two review authors independently selected studies, extracted data and assessed methodological quality. Two-by-two tables were used to calculate sensitivity and/or specificity for each study and, if possible, forest plots were generated.

Results: Of 4693 references, we included 11 studies with 621 eligible patients. The pooled mean sensitivity of ^{123}I-MIBG-scintigraphy was 92.4% (95% confidence interval 84.6-96.4%; 95% prediction interval 63.0-98.9% (in 608 patients)). The specificity was described in one study: 85% in 115 lesions in 22 patients. The sensitivity of ^{18}F-FDG-PET(-CT) alone and compared to ^{123}I-MIBG-scintigraphy was reported in one study as 100%. The specificity could not be calculated. None of the studies provided outcome data on the diagnostic accuracy of ^{18}F-FDG-PET(-CT) in patients with negative ^{123}I-MIBG-scintigraphy. All studies had methodological limitations.

Conclusion: Analysis of the specificity was difficult, because only one study provided data on false positive and true negative results. As described in the literature in about 10% of all neuroblastoma patients ^{123}I-MIBG scans are negative. Although currently not enough evidence is available, a possible add-on test is ^{18}F-FDG-PET(-CT) for ^{123}I-MIBG negative tumours.

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POC014

Assessment of Bone and Bone Marrow Metastases on ^{123}I-MIBG Scintigraphy versus MRI-STIR in Patients with Stage 4 Neuroblastoma

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Background: To compare radionuclide 123-Iodide-metaiodobenzylguanidine (123I-MIBG) scintigraphy with magnetic resonance imaging (MRI) with short tau inversion recovery (STIR) to investigate bone and bone marrow metastases in neuroblastoma.

Methods: Diagnostic ^{123}I-MIBG-scans and MRI-STIR images from 10 patients with stage 4 neuroblastoma were evaluated to assess metastatic spread in 14 skeletal segments. Morphological characteristics of the lesions were qualified as either 'focal' (sharply demarcated, limited to one location in the skeletal segment) or 'diffuse' (indistinct margins, dispersed throughout the skeletal segment). We compared the scores of ^{123}I-MIBG-scans and MRI-STIR images.

Results: Eighty-nine skeletal segments were evaluated with ^{123}I-MIBG and MRI-STIR. In 36 segments, lesions were visible on both modalities. In 33 segments, lesions were only shown on one modality: 26 on MRI-STIR and 7 on 123I-MIBG-scintigraphy. In total, ^{123}I-MIBG-scintigraphy showed focal lesions in 12 segments, diffuse abnormalities in 30 and a combination of both in 1 segment. MRI-STIR showed focal lesions in 30 segments, diffuse abnormalities in 10, and a combination of both in 22. Concordant morphological findings were seen in 30 segments: 10 focal, 19 diffuse and in 1 segment both types of lesions. In 36 segments discordant findings were found. MRI-STIR^{pos}/ MIBG^{neg} lesions were: 22 focal and 4 both. Discordant ^{123}I-MIBG^{pos}/MRI-STIR^{neg} were diffuse lesions in 7 segments. Cortical destruction was seen in a total of eight affected segments (in 3 patients) on MRI-STIR. These lesions were all of the diffuse type on ^{123}I-MIBG-scans; on MRI-STIR 5 were of the diffuse type and 3 were focal.

Conclusion: MRI-STIR showed more affected skeletal segments than ^{123}I-MIBG-scintigraphy. Because all included patients were stage 4, these findings did not affect staging. MRI-STIR showed more focal and ^{123}I-MIBG-scintigraphy more diffuse lesions.

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POC015

Neuroblastoma in Children with Sotos and Weaver Overgrowth Syndromes

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Background: Sotos and Weaver syndromes are overgrowth syndromes with advanced bone age, psychomotor retardation, typical morphological features, and increased risk of neoplasia. The incidence of Sotos syndrome is 0.007% of newborns in Europe, of Weaver syndrome even lower. So far only five patients with neuroblastoma in Sotos (n=2) or Weaver (n=3) syndrome have been reported. We report a series of 11 neuroblastoma patients with these syndromes.

Methods: Data of patients registered in the German Cooperative Neuroblastoma trials were reviewed for evidence of overgrowth syndromes. Additional information was gained from a questionnaire asking for phenotypical findings in Weaver or Sotos syndrome.

Results: Out of 3280 patients registered between 1990 and 2012, Sotos syndrome was reported in eight, Weaver syndrome in three patients (n=11, 0.34%). Median age at diagnosis of neuroblastoma in these cases was 23.4 months (9 days – 117 months). Six patients presented with localized neuroblastoma, four with INSS stage 4, and one with stage 4S. One tumor showed MYCN amplification. At last follow-up, nine patients were in complete remission, two died of progressive disease. In all patients, overgrowth was described prior to the diagnosis of neuroblastoma. In five patients the diagnosis of Sotos or Weaver syndrome was clinically suspected or genetically investigated at that time. Clinical features of the patients with Sotos syndrome included big hands and feet (n=5), high arched palate (n=5), frontal bossing (n=5), and hypertelorism (n=4). The patients with Weaver syndrome presented with rectus diastasis (n=2), hoarse cry (n=1), and cutis laxa (n=1). Information on the characteristic mutation was availabe in four patients with Sotos syndrome, all of them showed NSD1-mutation. In patients with Weaver syndrome, the characteristic mutation (EZH2) was not investigated.

Conclusion: We found Sotos or Weaver syndrome in 0.34% of neuroblastoma patients. This proportion seems to be higher than estimated from previously reported cases.

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POC016

The Sense and Sensibility of Philadelphia Score" in Stage 4S Neuroblastoma Patients

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Background: Stage 4S NBL (NB4S) has a favourable outcome and frequently shows spontaneous regression, a wait and see policy is justified. The Philadelphia score (PS) a semi-quantitative scoring system, can be used to decide to start treatment (using a cut-off of PS ≥ 2) in patients that progress.

Methods: Retrospective, multi-centre, cohort study (AMC and EMC). We reviewed all medical charts from patients diagnosed with NB4S (1980-2012) noting patient characteristics, PS, treatment, response and outcome.

Results: The cohort consisted of 33 NB4S patients (mean age at diagnosis 2.6 months (0-7)). Twenty eight patients are alive (85%), 5 patients died of disease (15%), follow up 107 months (0-420). Four out of 5 (80%) patients that died of disease were younger than 2 months at diagnosis. Four had early progressive disease (PD) with tumor growth; four had late PD to stage 4 NBL and there was 1 (3%) relapse with stage 4 NBL., PS consists of 5 categories: feeding difficulties (21%) and tachypnea (18%) are most often abnormal, hepatic is only rarely abnormal (3%). Abdominal girth was measured in 14/33 (42%) patients, 11/14

(79%) of the treated patients. Abdominal distension (76%) and hepatomegaly (67%) were common presenting symptoms. Increase in abdominal girth > 10% was present in 9/11 (82%) of treated patients. Treatment (n=19): chemotherapy, radiotherapy, 131I-MIBG therapy and/or surgery. Treated patients had a higher PS before start of treatment mean 1 (0-6) compared to non-treated patients (n=14) mean 0.6 (0-5).

Conclusion: Early PD can be found in 12% of NB4S patients, measured in higher PS. Seventy-six percent of patients had abdominal distension and 67% hepatomegaly at diagnosis, measured using abdominal girth. We propose changing the PS by replacing the hepatic category (thrombocytopenia/ DIC platelet <50x10^9 L) with abdominal distension (measured using abdominal girth increase > 5% (mild), > 10% (severe).

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POC018

Lungs Involvement in Patients with Neuroblastoma at Diagnosis and at Therapy Failure

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Background: Neuroblastoma (NBL) is the most common extracranial solid tumor in children. Lungs involvement in the course of NBL is rare. It may cause diagnostic as well as therapeutic problems.

Methods: The aim of the study is evaluation of lungs involvement in 144 patients with NBL, age 0-14 years, treated in our Department in 1991-2013. All patients had chest imaging, including lungs CT. Lungs involvement was defined as isolated metastases to parenchyma and involvement of lungs and pleura by continuous infiltration. The presence of fluid in pleura was evaluated, although it was not defined as lungs involvement.

Results: Lungs lesions were found at diagnosis in 11/144 (7,6%) patients age 0 – 11 years (median 4,5). In 8 metastases to parenchyma were found, in 3 infiltration by mediastinal tumor. Two had also fluid in pleura. Six patients are alive, 1 after relapse (primary tumor and bone marrow). From 5 patients who died, in 3 metastases to lungs were found at relapse and 1 did not obtain lung remission. In 5 other patients lung parenchymal metastases were found only at relapse (2 stage 3 with MYCN amplification) and all of them died of disease. In 8/16 patients MIBG scanning were performed; it did not reveal lung disease in any case. Pleural effusion cytology was done in 4 of 5 cases – only in 1 case we found neoplastic cells.The symptoms of lungs involvement were present only in children with pleural effusion. In 1 patient lungs infiltration was found in post mortem evaluation – she died of respiratory insufficiency with positive Aspergillus culture, negative MIBG and no elevated catecholamines.
Conclusion: Although lungs are not typical place for NBL metastases, in each patient it is necessary to make chest imaging. In doubtful cases biopsy should be performed, especially because standard screening procedures may not reveal NBL lungs infiltration.

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POC019

Diagnostic and Prognostic Significance of Urinary Catecholamine Metabolites and n-myc Gene Amplification in Neuroblastoma

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Background: Elevated levels of urinary catecholamine metabolites and copy number variation (CNV) of n-myc gene are well known in neuroblastoma. Despite of multimodal therapy, patients with metastasis continue to have poor prognosis.

Methods: In the present study, 50 histologically confirmed neuroblastoma, from 2007-2012, were included. To check CNVof n-myc gene, relative quantification was done, by real-time PCR. Quantification of Vanillymandelic acid (VMA) and homovanillic acid (HVA) levels, was done by enzyme-linked immunosorbent assay (ELISA). Correlation of n-myc, VMA and HVA levels with age, sex, primary site, histological-category, response to neo-adjuvent chemotherapy (NACT) and stage, was done by Kruskal-Wallis and Fisher exact test.

Results: High levels of HVA were found in 94%(33/38), VMA in 76%(38/50) and CNV of n-myc gene in 34.7%(8/23) cases. In non amplified n-myc tumors, partial response to NACT was found in 92.3%(12/13) and complete response in 7.7%(1/13) while in n-myc amplified tumours, partial response was found in 42.86%(3/7), no response in 42.86%(3/7) and complete response in 14.29%(1/7) (p=0.013). A negative correlation was observed between HVA and age (p=0.043). HVA levels in INRG stage L2 patients (23/38), ranges from 6.17-525.9 (median 112.17), in stage M patients (11/38), ranges from 0.83-419(median 162.79) and in stage MS patients (4/38), ranges from 305-926 (median 593.22). A positive correlation was observed between VMA and tumor classification, neuroblastoma cases had VMA median 91.6 (range 3.2-736), in ganglioneuroblastoma had VMA median 16.7 (range 4.4-23.1) and ganglioneuroma had VMA median 7.93 (range 2.47-207) (p =0.024).

Conclusion: In 94.73% (36/38) patients at least one parameter (VMA&HVA) was above age related upper normal values, suggests that, it plays an important role in the diagnosis of the disease. On the basis of our results, we want to emphasize that catecholamines may have prognostic significance. N-myc amplified tumors have shown poor response to NACT, suggests its prognostic significance, in high-risk and low-risk group stratification.

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POC020

The Biological Characteristics and Treatment Results of Patients with Neuroblastoma in Belarus

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Background: Neuroblastoma is a unique disease in biological sense. The patients, having favorable biological characteristics, can recover without treatment. The disease forecast in the group with adverse biological lines is bad. To analyze the biological characteristics of pts with neuroblastoma in Belarus and estimate their influence on the illness forecast.

Methods: 111 pts with neuroblastoma revealed for the first time who received treatment according to NB-2004 protocol from 2008 till November 2013. The following biological parameters were estimated: Shimada histology type, MYC-N and 1ρ status , ploidy of tumor cells (by FISH). PFS rate for pts was estimated using the Kaplan-Meier method and survival function was compared using the long-rank test. Multivariate assessments of PFS were performed by Cox, s proportional hazards model.

Results: The Shimada histology type n = 105 pts: favorable n = 45 (42.8%), unfavorable n=60 (57.1%). PFS depending on Shimada histology type is 0, 86+0, 05 (favorable) vs. 0.40+ 0.06 (unfavorable) (p=0.0005). MYC-N status n = 111, positive n=23 (20.7%), negative n = 88 (79.3%). PFS depending on MYC-N status is 0.67+ 0.05 (negative) vs. 0.44 + 0.10 (positive) (p=0.019). The status 1ρ was determined in 106 pts: positive 10 (9.4%), negative n =96 (90.6%). 9 of 10 positive patients were also positive on MYC-N. The ploidy of tumor cells n=105 pts: near-triploidy n = 28 (26.6%), near-diploidy n =73 (69.4%), heterogeneous n =4 (3%). PFS depending on ploidy of tumor cells is 0.84+0.08 (3n) vs. 0.52 + 0.06 (2n-4n) (p =0.041). The multivariate assessments of PFS estimated influence: ploidy of tumor cells HR 1.89, Shimada histology HR 2.79, MYC-N status HR 1.2 (p=0.02).

Conclusion: All biological characteristics listed above have impact on PFS of patients with neuroblastoma, therefore they have to be considered at stratification of patients in risk groups.

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POC021

Is it feasible to improve quality of imaging studies in local sites for NB staging by central review nowadays? : The Spanish NB colllaborative network

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Background: Patients´risk stratification in neuroblastoma individualize therapy to cure with the most adequate intensity of treatment. Nowadays, staging assessment is the most important clinical risk-factor, being determined locally by Imaging studies (CT, MRI) before inclusion in SIOPEN trials. In Spain, the National Coordinating centre (NCC) supervises local centres for trial development, according to Good Clinical Practice (GCP). Since INGRSS is based on image-defined-risk-factors (IDRF), we tested the feasibility of evaluating prospectively the new staging system (INRGSS) in LINES, by an NCC expert panel, in a blinded quality control (QC) procedure.

Methods: A road-map was designed by NCC and disseminated to local ones. Those willing to participate were asked to follow Standard Operating Procedures (SOPs) with delivery of anonymized CDs with studies (CT, MRI) performed locally according to LINES guidelines. In NCC, images were coded and uploaded in the hospital image-system by the Quality-image team to be examined by two radiologists, in a blinded manner. They completed the IDFR-check-list and discussed problematic issues. A final report was issued and sent back to the local centres.

Results: 24 patients were registered in LINES between 2011 and 2013 from 16 centres. 17 cases had CDs sent for central review and 88% could be successfully reviewed, after uploading in the hospital-image-system. Failure to upload was due to lack of interoperability among health-image Information and Communication Technologies Systems (ICT). Quality of studies was not as good as expected. Difficulties encountered were due to either paediatric peculiarities (age, abdominal movements in MRI…) or technical ones.

Conclusion: 1/ Collaboration among local and reference centres for QC-Imaging review in Spain was feasible. 2/ SOPs were established to wide the experience i.e MIBG for all Spanish patients. 3/ The network and SOPs established will improve patient´s staging and treatment, and finally GCP in Spain. THIS WORK WAS SUPPORTED BY APU AND FIS09/02323. SPECIAL THANKS TO DESIREE RAMAL FOR HER WORK.

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POC022

Definitions of Bone and Bone Marrow Metastases Used in Diagnostic Imaging in Patients with Neuroblastoma: A Systematic Review

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Background: Since the presence of bone and/or bone marrow (BM) metastases correlates with bad prognosis, it is important to have clear and uniform definitions. The objectives of this review were thus: 1.To identify all definitions of bone and BM metastases used in imaging studies; and 2. To determine diagnostic accuracies for bone and/or BM metastases of all imaging tests.

Methods: We searched MEDLINE/PubMed (1945-April 2013) and EMBASE/Ovid (1980-April 2013) and bibliographies of relevant articles. Studies were included if they reported on diagnostic imaging of patients with suspected metastatic neuroblastoma and defined bone and/or BM metastases. Two review authors selected studies, extracted data and assessed methodological quality. Disagreements were resolved by discussion. Sensitivity and/or specificity were calculated using data in two-by-two-tables.

Results: Thirty of 400 identified studies were eligible for inclusion. The main reason for exclusion was not providing a definition for bone and/or BM metastases (n=52). Of the 30 included studies 9 defined bone, 13 defined BM and 8 defined both metastases (objective 1). Definitions of bone and BM metastases varied widely between included studies. Bone metastases were frequently defined as focal lesions or hotspots on scintigraphy and as osteolytic lesions with periosteal reaction on radiography; BM metastases as diffuse lesions on MRI and on scintigraphy (with or without focal lesions). BM metastases on MRI were additionally defined as: low-intensity on T1- and high-intensity on T2-weighted-images. Fourteen studies reported data on diagnostic accuracy (objective 2). Sensitivity and specificity values varied enormously between studies for both bone and BM metastases.

Conclusion: Despite the fact that many studies report on outcome data of patients with bone and/or BM metastases, the majority do not provide definitions. Furthermore, in the studies that do provide definitions, these are equivocal and the diagnostic accuracy varied so widely that no conclusions can be drawn.

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POC023

The Characteristics of Mediastinal Neuroblastoma in Childhood

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Background: To study the clinical characteristics of mediastinal neuroblastoma.

Methods: From 2008 March to 2012 September, the Forth Affiliated Hospital of China Medical University has Admitted 110 cases of neuroblastoma, including 26 cases of mediastinal neuroblastoma and 84 cases of other neuroblastomas. To compare the clinical manifestation, tumor markers, biological prognostic factors of mediastinal neuroblastoma with other neuroblastomas.

Results: The average age of mediastinal neuroblastoma group is 25.5M, which is very similar to other neuroblastomas.88.5% of mediastinal neuroblastomas had syndrome at newly diagnosis, and so did 60.7% in other neuroblastomas (P<0.05). The early stage cases of mediastinal neuroblastoma group were 34.6%, higher than that of other neuroblastomas which were 8.3% (P<0.05). 21.4% of serum NSE levels of Mediastinal neurobJastoma group risen more than 100 ng/ L, lower than 86.1% (P < 0.05) in other neuroblastomas . All the cases in mediastinal neurobJastoma had a N-myc copy number of less than 10 copies, while 23.1% in the other neuroblastomas' were more than 10 copies (P<0.05).The 4-year overall survival rate was 80% in mediastinal group and 44% in the other neuroblastomas. Of the cases whose primary tumors were in localized neuroblastoma, the 4-year survival rate was 100%, significantly higher than 82%in other neuroblastomas.

Conclusion: Majority of the mediastinal neuroblastoma cases perform early clinical stage and favorable biological prognostic factors.These may associated with the prognosis of mediastinal neuroblastoma.

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POC024

Muscle Involvement in Neuroblastoma with Spontaneous Regression

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Background: In infants, neuroblastoma with dissemination to the skin, liver, and minimal bone marrow involvement (stage 4S) has a favourable prognosis frequently showing spontaneous regression. The current definitions for stage 4S (INSS, Brodeur et al.) or stage MS (INRG, Monclair et al) exclude metastasis in other localisations, but we here report muscle involvement in two patients with metastatic patterns otherwise compatible with stage 4S.

Methods: Two infants presented with poorly differentiated neuroblastoma of the adrenal without MYCN amplification. The first patient was diagnosed at one month of age with INSS stage 3 and contralateral lymph node involvement. Muscle involvement emerged during disease progression at the age of 6 months (Musculus (M.) masseter, M. gluteus medius, and M. psoas). The second patient presented with stage 4S disease at the age of nine months with liver and skin metastases and involvement of the skeletal muscles including M. vastus lateralis, M. vastus medialis

M. vastus intermedius, M. semimembanosus and Mm. gastrocnemii. Both patients were closely monitored and - in the absence of threatening symptoms- received no cytostatic treatment. In both patients intermittent progression of tumor growth was observed, followed by spontaneous tumour regression resulting in very good partial remission at all sites involved at the age of 31 months (patient 1), and 41 months (patient 2), respectively.

Results: We present two infants with neuroblastoma developing muscle metastases.

Conclusion: We present two cases of infant neuroblastoma with atypical muscle involvement. Both patients were closely followed and despite intermittent tumour progression finally showed spontaneous regression over 2 years. These cases illustrate that a conservative approach with close monitoring may be appropriate for such patients to avoid over-treatment.

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POC025

Infant Stage4S Neuroblastoma with MYCN Amplification

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Background: Most of infants with stage4S neuroblastomas (NBs) have favorable biologic features, which is the favorable histology (FH) by International Neuroblastoma Pathology Classification (INPC) without MYCN amplification. MYCN amplification (MYCN-A), one of the strongest indicators of poor prognosis, is reported to be a powerful driving force preventing tumor maturation and increasing mitotic/karyorrhectic activities in NBs. Accordingly, MYCN-amplified tumors typically demonstrate the characteristic histology of NB, undifferentiated/poorly differentiated subtype with a high mitosis-karyorrhexis index (MKI), and are classified into the Unfavorable Histology (UH).MYCN-amplified tumors demonstrating FH are extremely rare. Recently, it is reported that this rare type of NBs could be divided into 2 prognostic groups by the morphology of nuclei and MYCN-protein expression, namely bull's eye (prominent nucleoli) tumors and conventional tumors. Patients with conventional tumors without MYCN-protein expression show an excellent prognosis comparable to patients having MYCN-nonamplified &FH tumors. Now, we report an infant with stage4S conventional NB showing MYCN-A without protein expression, who treated as intermediate risk. Case Report, A 3-month-old girl was diagnosed with stage4S NB based on imaging studies demonstrating left adrenal mass and liver metastases, and elevated urinary VMA /HVA. Irradiations to the liver and mild chemotherapy were done prior to biopsy for rescue from oncology emergency . The liver biopsy showed the morphology compatible with conventional NB without prominent nucleoli, though INPC was inapplicable due to post-treatment. Immunohistochemical staining was negative for MYCN-protein, despite 20 copies of MYCN-oncogene amplification detected by Real-time Polymerase Chain Reaction (RT-PCR). After combination chemotherapy, without autologos stem cell rescue, the primary tumor was resected. The patient, taking 13-cis-retioic acid, is free from disease for 6 months post resection.

Conclusion: We report a case of stage4S infant with MYCN-amplified NB that was treated less intensively according to a favorable conventional morphology without MYCN-protein expression.

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Clinical Research: Experimental Therapies in Patients

POC026

Accelerating Drug Development for Neuroblastoma: Summary of the New Drug Development Strategy Project Workshop from the Innovative Therapies for Children with Cancer (ITCC), European Network for Cancer Research in Children and Adolescents (ENCCA) and I

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Background: Neuroblastoma is one of the leading causes of death due to childhood cancer worldwide. There is an urgent need to develop new therapies to improve cure rates and reduce long term toxicities.

Methods: In December 2012, a workshop of European clinicians and scientists was held to prioritise new targets and drugs for neuroblastoma with the aim of elaborating a strategy to accelerate and improve the development of targeted drugs for this disease.

Results: The strategy covered the following areas:, 1)The population of children that should be considered appropriate for entry into early phase I/II studies includes high-risk patients with: a)progressive disease during frontline treatment, b)refractory disease after induction and second line chemotherapy and, c)relapsed disease (both early and late relapses). 2)New drugs for high-risk neuroblastoma should be developed in innovative phase I/II clinical trials incorporating predictive (patient selection) and pharmacodynamic (proof-of-target inhibition) biomarkers. Successful drugs should be transitioned seamlessly to larger randomized phase II/III trials and finally into standard clinical application, 3)Available data on more than 20 targets selected based on current biological knowledge and availability of appropriate drugs (not including immunotherapy and tumour microenvironment targeted drugs) were examined and ranked by the participants including: 1.presence of the target in patient samples, 2.molecular validation of target dependency, 3.in vitro and in vivo efficacy with pharmacological inhibitors, 4.availability of predictive biomarkers for patient selection, 5.combination data and 6.resistance mechanisms. With the existing data, six targets with most complete preclinical "proof-of-concept" data package were prioritised for 2013: ALK, Aurora kinase, BIRC5, CHK1, MDM2 and mTORC1/2, although this list will continuously evolve with emerging data.

Conclusion: The NDDS strategy will be used to influence drug development for neuroblastoma involving all stakeholders: ITCC, pharma and regulators, promoting a larger phase I portfolio, increased data in neuroblastoma preclinical models and transition of successful drugs to phase II/III trials.

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POC027

Phase I Trial of Anti-GD2 Humanized 3F8 Monoclonal Antibody (MAb) Combined with Subcutaneous Interleukin-2 (scIL2) in Patients with Relapsed Neuroblastoma or Other GD2-Positive Solid Tumors

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Background: Anti-G_{D2} murine-3F8 immunotherapy improves outcomes in high-risk neuroblastoma. Murine-3F8 was humanized to the IgG1 subclass (hu3F8), retaining nM affinity, with a K_{off} ~10-fold slower than other anti-G_{D2} MAbs. scIL2 is better tolerated compared to intravenous dosing and increases natural killer cell numbers and activity. Thus, combining hu3F8 with scIL2 may improve anti-tumor effect.

Methods: A standard 3+3 design phase I study was initiated to define the maximum tolerated dosage of hu3F8 (dose level 1: 0.3mg/kg) combined with a fixed dosage of scIL2 ($6x10^6$ units/m^2/dose x5 doses/cycle) in patients ≥1 year old with neuroblastoma and other G_{D2}(+) solid tumors. Cycles consist of hu3F8 alone on day 1 (dose 1) and hu3F8+scIL2 on day 8 (dose 2); scIL2 was administered alone on days 9-12. Cycles were outpatient and repeated every 21 days x4 cycles.

Results: Eight patients have been treated without dose-limiting toxicities. Side effects were grade 1/2 pain, allergic reactions, and fever. Current hu3F8 dosage is 0.9mg/kg. Pharmacokinetic studies showed dose-dependent increases in Cmax and Cmin. In 6/6 patients treated on the first two dosage levels (0.3mg/kg and 0.6mg/kg), significant discrepancies in hu3F8 half-life ($T_{1/2}$) were seen between doses 1 and 2 of a cycle: $T_{1/2}$ of dose 2 of hu3F8 (given with scIL2) decreased by ~60% compared to $T_{1/2}$ of dose 1 (hu3F8 given without scIL2). However, for 2/2 patients treated at dosage level 3 (0.9mg/kg), $T_{1/2}$ of dose 2 of hu3F8 was decreased by only ~5% compared to dose 1. 4/8 patients developed human anti-human antibody (HAHA) despite no prior anti-G_{D2} therapy. There have been no objective responses to date but 1/3 neuroblastoma and 2/4 osteosarcoma patients completed 3+ cycles without disease progression.

Conclusion: Hu3F8+scIL2 is well tolerated. scIL2 may decrease $T_{1/2}$ of hu3F8 and may increase risk of HAHA. This dose-escalation study is ongoing.

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POC028

A Phase I Study of Buthionine Sulfoximine (BSO) and Melphalan (L-PAM) with Autologous Stem Cell Support for Recurrent/Resistant High Risk Neuroblastoma: A New Approaches to Neuroblastoma Therapy Study

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Background: Standard myeloablative therapy for high-risk neuroblastoma includes melphalan. Melphalan resistance may occur via glutathione (GSH). Buthionine sulfoximine (BSO), a selective GSH synthesis inhibitor, significantly enhances melphalan activity against neuroblastoma in vitro and in vivo.

Methods: Patients >9 months to 30 years age with recurrent/resistant high-risk neuroblastoma received BSO (3 gram/m^2 bolus, followed by 72 hour infusion of 24 grams/m^2 days -4 to -2), with escalating melphalan (20-125 mg/m^2 total) days -3 and -2, and autologous stem cells day 0, using standard 3 + 3 escalation. Eligibility included glomerular filtration rate ≥ 100 cc/min/1.73 m^2, Myeloablative therapy ≥ 3 months prior was allowed.

Results: Twenty-seven eligible patients received 20 to 125 mg/m^2 of melphalan. One dose limiting toxicity (DLT) occurred at 20 mg/m^2 melphalan (Grade 3 AST/ALT during BSO infusion requiring stopping therapy) and 80 mg/m^2 (alpha hemolytic streptococcal bacteremia, grade 4 hypotension, and grade 4 pulmonary/mechanical ventilation (resolved without sequelae). Ten other patients had grade 3 infections. Three evaluable patients received BSO + 125 mg/m^2 melphalan without DLT. Twenty-seven patients were evaluable for response. There was 1 partial response (PR) and 2 mixed responses (MR) among 11 patients with prior melphalan therapy; 1 PR, 4 MR among 15 patients with no prior melphalan; 1 non-responder with unknown melphalan history. Median progression-free survival was 3.2 (95% CI: 2.1-5.7) months. Median overall survival was 13.4 (95% CI: 7.8-22.8) months. L-PAM levels (available to date for 80 mg/m^2 = 10.9 ± 4.3 mM) were below levels needed for preclinical activity with BSO against multi-drug resistant neuroblastoma.

Conclusion: BSO 75 gram/m^2 with melphalan 125 mg/m^2 and stem cell support are tolerable in high-risk neuroblastoma patients. Partial plus mixed responses were achieved in 30% of evaluable patients, with similar response rates in patients who received prior melphalan versus no prior melphalan.

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POC029

Irinotecan and Temozolamide Chemotherapy as the Treatment of Progressive and Therapy Resistant Neuroblastoma - Experience of Polish Pediatric Solid Tumors Group

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Background: The aim of the study was evaluation of irinotecan/temozolamide (IT) chemotherapy (Kushner 2006) in progressive or therapy resistant neuroblastoma (NBL) in patients treated in the centers of Polish Pediatric Solid Tumors Group. The endpoints of the study were the best response to IT, overall and progression-free survival (OS, PFS), side effects and quality of life.

Methods: We evaluated 697 patients treated from 2000-2012. Therapy failure was diagnosed in 194 (28%). IT was employed in 46 patients (1 stage 1, 1 stage 2, 5 stage 3, 1 stage 4s and 38 stage 4 at diagnosis), age 1-189 months at diagnosis. All patients were treated with previous chemotherapy. Seven patients had primary resistant disease, 28 the first and 11 at least second therapy failure. Additionally, IT was given with vincristine in another 4 patients, analyzed separately.

Results: The objective response was observed in 38 patients (11 CR, 8 PR and 19 SD). The number of IT cycles in responding patients was 1-39 (median 6); 15 patients are alive (5 who obtained CR, 3 with PR and 7 with SD. All received other chemotherapy, 17 patients received HSCT (autologous in 9 and allogeneic in 8 cases, 7 of them with MIBG therapy), 10 – radiotherapy, 5 – 13-cis RA cycles and one immunotherapy. Means OS was 44 months (12-172,7) and PFS from IT employment – 8,3 months (0,2-54 months). In 31 cases (67%) adverse events were observed. It was diarrhea in 19 (41%) patients, cured with loperamid (only in 1 case atropine was necessary) and hematologic toxicities. Only 12 (26%) of patients required modifications in drugs dosage or frequency, all of them heavily pre-treated or with bone marrow involvement. The chemotherapy was accepted by all patients and parents. Generally, patients did not require hospitalization between the cycles.

Conclusion: IT is effective therapeutic option both as intensive and palliative treatment.

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POC030

Parvovirus H-1 Induces Oncolytic Effects in Human Neuroblastoma - First Clinical Experience in a Compassionate Use Application to a Neuroblastoma Patient

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Background: Based on recent safety data from a glioblastoma trial and pre-clinical in vitro and in vivo data demonstrating antineoplastic efficacy of the oncolytic parvovirus H-1 (H-1PV) in neuroblastoma, a palliative first-in-child compassionate use application was performed.

Methods: A 8 year old boy with second, multifocal relapse of neuroblastoma stage IV, who was still in good clinical condition experienced massive progression of a humeral metastasis under relapse treatment three months prior to virotherapy. Into this lesion two H-1PV doses (2.5 x 10^7 p. f. u. each) were injected at a 28 days interval. Prior to each virus injection, biopsies were taken from the injection site. The patient was monitored for viral excretion, viremia and anti-viral immune response. The patient's follow-up for safety and efficacy parameters included clinical, laboratory, tumor marker, imaging and immunological monitoring.

Results: In this individual case, we observed H-1PV administration to be safe to the patient as well as to contact persons. Infectious viral particles were detectable in the patient's blood within the first 13 days after infection. 14 days after H-1PV injection, vast necrotic areas within the infected humeral metastasis were detected by MRI. In the biopsy taken 28 days after the first H-1PV injection, neuroblastic cells were absent around the injection site. Intrametastatic H-1PV injection was followed by inflammation and subsequent necroses at local and distant metastatic lesions. Main adverse events included fever, local swelling and pain. Neutralizing antibodies occurred at day 10 and reached a maximum titer at day 55. At day 108 the patient died from progressive neruoblastoma infiltration into the bone marrow.

Conclusion: The local intratumoral injection of the oncolytic parvovirus H-1PV was tolerated well and induced systemic anti-viral immune response. H-1PV infection was followed by intra-lesional necrosis. First signs of local and systemic anti-tumoral immune response have been observed in this patient, indicating the need for further, systematic clinical research in a clinical trial setting.

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POC031

Feasibility of Delayed Local Control Treatment in Patients with High Risk Neuroblastoma: Report of a Pilot study from the Japan Neuroblastoma Study Group (JNBSG)

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Background: The progression-free survival rates of high risk neuroblastoma (HR-NB) patients are still unacceptable. The increase of time intensity and dose intensity are both key strategy of the treatment of neuroblastoma. In Japan it had taken so much time to restart the chemotherapy after surgery. Sometimes we needed 1 to 2months because of surgical adverse effects. The increase of time intensity for the chemotherapy is important problem to solve. The Japan Neuroblastoma Study Group (JNBSG) has examined the feasibility of time - intensive multimodal treatment with delayed local control treatment (DLCT) prospectively.

Methods: Between June 2006 and February 2008, 11 patients, four male and seven female, with newly diagnosed HR-NB patients were enrolled in the study. The median age at diagnosis was 22 months (range, 13 to 66 months). DLCT consisted of induction chemotherapy (IC) with cisplatin (100mg/m^2), pirarubicin (40mg/m^2), vincristine (1.5mg/m^2), and cyclophosphamide (2,400mg/m^2). After 5 courses of IC, all patients were treated immediately with myeloablative chemotherapy with carboplatin (1,600mg/m^2), etoposide (800mg/m^2), and melphalan (200mg/m^2). After these treatments, local tumor eradication with surgery and irradiation (21Gy) was performed.

Results: Four patients completed whole protocol and 7 patients discontinued due to progressive disease (PD) in 4 cases, regimen related toxicity (RRT) in 2 cases and withdrawal in 1 case. The myeloabrative chemotherapy were completed in 7 patients. The adverse effects were all under Gr. 3 in CTC ver. 3.0, except dead 2 cases of RRT.

Conclusion: 3 PD events had occurred in early period of protocol treatment. The time intensity of chemotherapy had kept in very good strength from the start of induction chemotherapy to DLCT. Adverse effects were all tolerable.These results suggest that this protocol is feasible. Based on the results of this study, we are now conducting a phase II study by the JNBSG.

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POC077

Pharmacokinetics & Pharmacogenetics of Isotretinoin in Indian Patients with High Risk Neuroblastoma and its Impact on Survival

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Background: To evaluate pharmacokinetics (PK) & pharmacogenomics of isotretinoin in Indian patients with high risk neuroblastoma and factors affecting it.

Methods: Children were enrolled after completion of induction/consolidation therapy for high risk neuroblastoma. They received isotretinoin 160mg/m2/day in two divided doses. Those who were unable to swallow capsules, the contents were extracted & mixed with yogurt prior to administration. PK samples were collected on days 1 and 14 of cycle 1(C1D1,C1D14) and cycle 4(C4D1,C4D14) at 0,1,2,4 and 6 hours after the morning dose. Plasma isotretinoin levels were determined by High Performance Liquid Chromatography (HPLC). Area under concentration-time curve from 0-6 hours (AUC_{0-6}) was estimated using non-compartment modelling. All patients were genotyped for *2,*3 and *4 variants of CYP2C8 by PCR-RFLP and for CYP3A5*3 and CYP3A7*2 using taqman assay.
Results: Thirty six children (M=27, F=9) were enrolled since April 2010. The median age was 5 (1-13) years. High inter-patient variability in pharmacokinetics was observed (AUC_{0-6} C1D14, CV=94.13%). AUC0-6 on day 1 was higher compared to day 14 in both cycles(p=NS).Children who swallowed the capsules (N=27) achieved higher AUC_{0-6} compared to those who could not (N=9) (median AUC (4.8) vs. (1.4) μM*hr/L, P=0.11). The median EFS is 8 months. Patients who were event free at one year had higher AUC_{0-6} on C1D1 compared to those who had event (p=0.08). Pharmacogenetic data is available for 22 patients. None of the patients carried CYP2C8*2/*3/*4 or CYP3A5*3 variants, whereas 50% (11/22) were heterozygous for CYP3A7*2 allele. AUC was not significantly different between homozygous wild-type and heterozygous states (P=0.74).

Conclusion: Method of administration affects PK of isotretinoin. Higher AUC on day 1 compared to day 14 suggests autoinduction of metabolism. Maximum concentration achieved and total exposure up to six hours can impact survival outcomes. No CYP2C8 and CYP3A5 variants were encountered. CYP3A7*2 heterozygous state do not affect PK of isotretinoin. Pharmacokinetic guided dosing strategy of 13-cisRA in high risk neuroblastoma can be explored for optimal therapeutic gains.

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POC078

Hypercalcemia is a Frequent Side Effect of 13-cis Retinoic acid Treatment in High Risk Neuroblastoma Patients

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Background: Treatment with cis-retinoic acid (RA) is considered standard consolidation treatment for high risk neuroblastoma. Hypercalcemia (HC) has been reported as side effect of RA treatment. We analysed frequency, symptoms and course of hypercalcemia after RA treatment. RA is given 160 mg/m2 in 2-(3) divided doses for subsequent 14 days followed by 14 day rest for a total of 6 cycles, then 3 months rest followed by 3 cycles.

Methods: Data of patients registered in the German Neuroblastoma trials treated with high intense induction regimes either followed by megatherapy or maintenance therapy and having received RA consolidation were retrospectively analysed. Hypercalcemia was defined as elevation of the serum calcium level above 2.9 mml/L (CTCAE ≥ grade 2).

Results: Out of 413 patients treated with RA between 2002 and 2010, 48 patients presented with hypercalcemia (11.6%). Hypercalcemia occurred in 47 out of 334 patients treated with megatherapy (14.1%), but only in one out of 79 patients treated with maintenance therapy. Additional data on hypercalcemia were available in 36 patients (48 RA cycles with hypercalcemia). The maximum serum calcium level was 3.45 ± 0,48 mmol/L. 29 patients (81%) developed hypercalcemia during the first RA cycle. Most patients (75%) showed hypercalcemia in only one cycle. In 30 cycles (63%), symptoms of hypercalcemia were reported (bone pain: 27%, stomach pain: 17%). Because of hypercalcemia, 11 RA-cycles (23%) were stopped, three continued with reduced dosing, and treatment was applied in 16 cycles (intravenous hydration 17%, diuretics 16%, bisphosphonate 2%). The RA consolidation was continued without dose reduction in 25 patients (69%), with reduced dosing in 10 patients (28%), and stopped completely in one patient.

Conclusion: In this cohort, hypercalcemia was observed more often than previously reported. Patients showed hypercalcemia mostly at the beginning of RA consolidation and after megatherapy. A substantial number of patients presented with symptoms of hypercalcemia.

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Clinical Research: Immunotherapy

POC032

Phase I Study of Anti-GD2 Humanized 3F8 (hu3F8) Monoclonal Antibody (MAb) Plus Granlocyte-macrophage Colonystimulating Factor (GM-CSF) in Patients with Relapsed High-risk Neuroblastoma (HR-NB)

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Background: Strategies to improve anti-G_{D2} immunotherapy include: avoiding sensitization (as can occur with murine or chimeric MAbs); augmenting affinity of MAb for target to enhance antibody-dependent cellular cytotoxicity (ADCC): and reducing pain partly a result of complement activation. Murine-3F8 anti-G_{D2} MAb is active against HR-NB but hu3F8 has greater ADCC and less complement activity in vitro. GM-CSF is well tolerated clinically and activates myeloid effector cells in ADCC.

Methods: In a phase I study (NCT01757626) (opened: 12/2012), patients with relapsed HR-NB receive cycles of hu3F8/GM-CSF in the absence of human antihuman antibody (HAHA) for up to 24 months. MAb dosing follows the standard 3+3 design, beginning at 0.9mg/cycle, to identify the maximum tolerated dosage (MTD). MAb is infused intravenously (30") on Mon-Wed-Fri (i.e., 3 days/cycle). Daily GM-CSF is administered subcutaneously, beginning 5 days pre-hu3F8, through the last dose of hu3F8. MAb pharmacokinetics (PK) are performed and pain is quantified.

Results: At study entry, the 18 patients enrolled to date were 4.1-31.3 (median 7.6) years old and 1.8-8.9 (median 4.5) years from diagnosis of HR-NB. Nine were in ≥2nd complete remission, nine had osteomedullary metastases, and 16 had prior anti-GD2 immunotherapyu with murine-3F8 (n=13), ch14.18 MAb (n=3), or both (n=1). PK studies showed dose-dependent increases in peak serum concentration, but not in terminal half-life (4.5±1.4 days). MTD has nor been reached; current hu3F8 dosage is 3.6mg/kg/cycle (100 mg/m2 - asd with standard-dose murine-3F8). Grade 3/4 toxicities were anaphylaxis (with 1st infusion of hu3F8 in one patient, and 1st infusion in 6th cycle in one patient). HAHA developed in three patients (two previously treated with ch14.18). Pain has been less than with equivalent daily dosage of murine-3F8. Treatment has been outpatient.

Conclusion: Hu3F8/GM-CSF is well tolerated in heavily prior-treated patients. PK studies support the alternate-day schedule. Assessing anti-NB activity requires longer follow-up.

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POC033

A Comprehensive Safety Trial of Chimeric Antibody 14.18 (ch14.18) with GM-CSF, IL-2 and Isotretinoin in High-Risk Neuroblastoma Patients Following Myeloablative Therapy: A Children's Oncology Group Study

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Background: In a Phase 3 randomized study (COG ANBL0032), we demonstrated that the addition of ch14.18 + GM-CSF + IL2 (ImmRx) to standard therapy isotretinoin for high-risk neuroblastoma (NB) patients in CR or VGPR after intensive induction and consolidation significantly improved outcome (Yu, NEJM, 2010). ANBL0931 study was designed to collect FDA-required comprehensive safety/toxicity data for ImmRx to support a new Biological License Application. Efficacy data were collected as a secondary endpoint.

Methods: Newly diagnosed high-risk NB patients who achieved ≥PR to induction therapy and received myeloablative consolidation with stem cell rescue then received isotretinoin x 6 with 5 concomitant cycles of ch14.18 combined with GM-CSF (cycles 1,3,5) or IL2 (cycles 2,4). Ch14.18 infusion time was 10 hrs. which was prolonged compared to 5.75 hrs for the initial 245 patients on ANBL0032. Toxicity data were collected.

Results: Of 105 patients enrolled (none ineligible), five patients developed protocol-defined unacceptable toxicities and came off study (four grade 4 allergic reaction/anaphylaxis, one sudden death -sudden onset of abdominal pain and arrest). The most common grade 3 or higher non-hematologic toxicities of ImmRx were neuropathic pain (cycles 1,2,3,4,5 were 30.9%,22%,13.3%,20%,17%, respectively), hypotension (9.6%,17%,3.1%,12.2%,5.7%), allergic reactions (2.9%,9%,3%, 6.6%,2.2%), capillary leak syndrome (1%,4%,0,2.2%,0), fever (21%,58%, 6.1%, 31.1%,4.5%). Toxicities occurred more frequently during IL-2 cycles compared to GM-CSF cycles. Dose modifications were reported in 73 patients (69%) most of which are thought to be prolongation of the ch14.18 infusion time beyond 10 hrs. The 2-year EFS and OS were 74+/-6% and 84+/-5%, respectively (n=105).

Conclusion: This study has confirmed the significant, but manageable treatment-related toxicities of the ImmRx including pain, allergic reactions, hypotension and

capillary leak syndrome. Decreased toxicity compared to ANBL0032 may be due to the 10 hr minimum ch14.18 infusion time for all patients in this study. EFS and OS appear similar to that observed on ANBL0032.

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POC034

Phase I Study of Anti-GD2 Humanized 3F8 (hu3F8) Monoclonal Antibody (MAb) in Patients with Relapsed or Refractory Neuroblastoma (NB) or Other GD2-Positive Solid Tumors

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Background: Anti-GD2-MAb immunotherapies contribute to improved outcomes in patients with NB, and might extend survival in patients with relapsed or refractory GD2(+) solid tumors. Murine-3F8 was humanized to the IgG1 subclass, retaining nM affinity and enhancing antibody-dependent cellular cytotoxicity (ADCC) in vitro. This improvement of ADCC may allow lower MAb dosing while retaining anti-tumor efficacy with less pain.

Methods: In this phase I study (NCT01419834) of standard 3+3 design (12 dosage levels, 0.06 to 3 mg/kg/cycle) patients ≥1 to 22 years with relapsed or refractory NB and other GD2(+) solid tumors were eligible. Hu3F8 was given outpatient intravenously over 30 minutes at 2 doses/cycle, 7 days apart and subsequently amended to 3 doses/cycle on days 1, 3 and 5; cycles were repeated every 3-6 weeks up to 2 years. Pain was measured with age appropriate scales to determine score (0-10) and by amount of opiate doses administered.

Results: Of 32 patients (30 NB, 2 osteosarcoma) treated thus far, 2 experienced a dose-limiting toxicity (DLT) of elevated liver transaminases (grade 3/4); MTD has not been reached. Although at equivalent doses hu3F8 was less painful than murine-3F8, multivariate analysis suggests that besides hu3F8 dose, subjective pain score >0 pre-hu3F8 treatment and age contributed to both subjective and objective pain measurements. Human anti-human antibody (HAHA) was measured before each cycle, and became detectable in 5/32, despite prior sensitization to murine-3F8 in 26 of 32, and despite multiple cycles (1-16) of hu3F8. Number of prior therapeutic regimens and pre-treatment extent of disease were variable. Best responses on hu3F8 treatment were: 5 (16%) complete remission (CR), 1 (3%) partial response (PR), 21 (66%) maintained CR or stable disease (range 1-23 months and median 7), and 5 (16%) progressive disease (PD). 25 of 32 patients are alive, 16 of 25 with disease.

Conclusion: Outpatient hu3F8 has been well tolerated and MTD not reached at 3 mg/kg/cycle. Pain was generally less than with murine-3F8. HAHA response was rare even with repeated cycles. Tumor response among these high risk patients was modest, but overall survival was encouraging despite disease.

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POC035

KIR Ligand Incompatible Cord Blood Transplantation for High Risk Neuroblastoma as a Salvage Treatment of Allogeneic NK Cell Based Immunotherapy

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Background: Venstrom et al. reported that superior survival was strongly associated with \"missing KIR ligand\" in patients with neuroblatoma (Clinical Cancer Research 2009). We evaluated KIR ligand incompatible allogeneic cord blood transplantation (CBT) as a salvage therapy in patients with high risk neuroblastoma.

Methods: We prospectively chose KIR ligand incompatible donor and planned tandem transplantation using high dose chemotherapy followed by autologus SCT and allogeneic CBT with reduced intensity conditioning regimens. Reduced intensity conditioning regimen for CBT were used either BU, Flu and L-PAM or Flu, L-PAM and low dose TBI 2Gy.Tacrolimus and methotrexate were used for GVHD prophylaxis. Eligibility criteria of this study were Stage IV neuroblastoma patients with 1) relapse, 2) MIBG positive metastases even after 4 courses of induction chemotherapy 3) over 10 years old at diagnosis or 4) MYCN amplification over 10. Donor derived single KIR positive NK cells which are not inhibited by patients' HLA were monitored by flowcytometry after CBT to access alloreactive NK cells.

Results: Thirty nine patients received KIR ligand incompatible CBT (30 tandem, 9 CBT alone). No patients received GD2 antibody treatment before or after this study. Only three patients developed grade III or more acute GVHD and only one patient develop extensive chronic GVHD. Eight patients died in total and three were because of disease progression and five were BU related toxicity. Donor derived single KIR positive NK cells significantly increased after CBT (p=0.0009). Surprisingly, no patients relapsed until 15 months after KIR ligand CBT in 27 patients who received planned tandem transplantation and whose CBT donor was KIR2DL1 positive. In this group, 3 year progression free survival was 63.9% ± 11.9%) with the median follow-up period of 33 (6-56) months.

Conclusion: Tandem transplantation with autologous SCT and allogeneic CBT from KIR ligand incompatible donor for neuroblastoma was feasible and increase of KIR mismatched NK cells may result in delayed relapse and better survival.

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POC036

Pharmacokinetics of ch14.18 in Pediatric Patients with High-Risk Neuroblastoma Following Myeloablative Therapy

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Background: Immunotherapy with ch14.18, GM-CSF, and IL-2 significantly improves event-free and overall survival in patients with high-risk neuroblastoma. This study evaluated the pharmacokinetic (PK) comparability and safety of ch14.18 manufactured by United Therapeutics Corporation (UTC) or the National Cancer Institute (NCI).

Methods: This was a multi-center, randomized, open-label, two-sequence, crossover study in subjects with high-risk neuroblastoma following successful completion of myeloablative therapy and autologous stem cell rescue. Subjects were randomized to receive ch14.18 manufactured by one manufacturer during Courses 1 and 2 followed by the other manufacturer during Courses 3-5. PK sampling was performed prior to, during, and for 25 days after 4 daily 10-20 hour ch14.18 infusions in Courses 1 and 3. ch14.18 plasma levels were measured with a validated sandwich immunoassay employing the Meso Scale Discovery electrochemiluminescence platform. PK parameters were determined using non-compartmental methods. The relative mean bioavailability (Frel) of UTC ch14.18 as compared to NCI ch14.18 was calculated using AUC_{last} which was calculated from the start of the first infusion in each course through the last time point with measureable ch14.18 concentrations.

Results: 28 subjects were enrolled. To date, the most commonly reported Grade 3+ AEs considered related to ch14.18 included: pyrexia (64%), anemia (43%), hypokalemia (43%), and decreased platelet count (32%). There were no notable differences in the number/severity of events reported between manufactures. Table 1 includes a summary of PK parameters using nominal infusion/sampling times from subjects who completed Courses 1-3., Table 1. Preliminary PK Analysis.

Conclusion: Preliminary data from the ongoing study supports a similar safety/PK profile between NCI and UTC manufactured ch14.18. Additional analyses will be performed once all subjects have completed the study.

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Treatment[1] N = 8		Cmax (mcg/mL)	Tmax (h)	AUClast (h*mcg/mL)	$t_{½}$ (days)	AUCinf (h*mcg/mL)	Frel	
NCI ch14.18 25 mg/m²/day	Mean	9.49	78.0	1356.40	5.42	1472.39		
	SD	1.73	12.4	407.32	1.94	519.76		
	Min	7.22	63.0	944.86	2.89	969.94		
	Median	9.15	87.0	1201.24	5.33	1260.00		
	Max	12.1	87.0	2142.74	8.75	2496.10		
	CV%	18.3	15.9	30.0	35.8	35.3		
UTC ch14.18 17.5 mg/m²/day	Mean	11.3	81.0	1330.28	4.63	1393.60	1.00	
	SD	1.72	11.1	309.26	1.80	356.51	0.32	
	Min	8.84	63.0	676.96	2.53	690.47	0.60	
	Median	11.7	87.0	1317.59	4.13	1360.00	1.03	
	Max	13.3	87.0	1684.47	7.54	1873.12	1.43	
	CV%	15.3	13.7	23.2	39.1	25.6	31.5	

[1] Doses of UTC/NCI ch14.18 contained the same total amount of active protein.

POC037

Pharmacokinetics of Humanized Anti-GD2 Monoclonal antibody Hu3F8 in patients with metastatic GD2-positive tumors

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Background: To determine the pharmacokinetics (PK) of intravenous Hu3F8 in a phase I dose escalation trial among patients with relapsed/refractory high-risk neuroblastoma (NB) or other metastatic GD2-positive tumors (NCT01419834).

Methods: From August 2012 to March 2013, 27 patients (25 NB and 2 osteosarcoma) received Hu3F8 at 2 doses (7 day interval) per cycle, with treatment repeated up to a total of 12 cycles. Seven dose levels (0.12, 0.3, 0.6, 1.2, 1.8, 2.4, 3.0 mg/kg) were tested using the standard 3+3 phase I design. For cycle 1 PK analysis, serum was collected pre-Hu3F8, 5 min, 3 h, 6 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 168 h post infusion; with similar sampling, if feasible, for cycles 4, 7, and 10. Cmax (peak serum concentration) for each cycle was obtained from pre-Hu3F8 and 5 min post-infusion samples.

Results: Seventeen of 27 patients completed at least 3 treatment cycles, with 6 patients completing all 12 cycles. Four patients with evidence of human anti-Hu3F8 antibody (HAHA) 7 days after treatment were excluded from PK analysis. The rest (n=23) remained HAHA-negative through subsequent cycles, despite having prior exposure to mouse 3F8 in 20/23 patients. Non-compartmental analysis of serum concentrations over time during cycle 1 revealed a dose-independent terminal half-life ($t_{1/2}$) of 3.26±1.09 days. Both Cmax (R=0.95) and area-under-the-curve (AUC, R=0.91) strongly correlated with dose level, while Cmax correlated with AUC (R=0.97). Six patients had dose escalation, and their $t_{1/2}$ and clearance remained statistically unchanged with subsequent treatment cycles. For patients receiving more than 1 cycle of Hu3F8, Cmax of subsequent cycles was comparable to Cmax of first cycle.

Conclusion: Twenty-three of 27 patients did not develop HAHA response, thereby allowing timely additional therapy without compromising $t_{1/2}$. At each dose level, Cmax was linearly correlated with AUC, irrespective of the number of treatment cycles given.

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POC038

Can Immunostimulatory Monoclonal Antibodies Be Used to Enhance the Efficacy of Anti-GD2 Immunotherapy?

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Background: Monoclonal antibody (mAb) therapy targeting GD2, a disialoganglioside expressed on the surface of neuroblastoma cells, has shown considerable promise in the minimal residual disease setting. In 2009, the U.S. Children's Oncology Group reported a significant survival benefit (68 vs 48% 2yr EFS) in children with high-risk neuroblastoma receiving the antibody in addition to standard therapy. The anti-GD2 was administered in combination with the cytokines IL-2 and GM-CSF, with the aim of augmenting Natural Killer (NK) cell effector function. Although these results are encouraging, the immunotherapy was associated with considerable toxicity and a large proportion of patients still died from their disease. An alternative approach may be to combine the anti-GD2 antibody with an immunostimulatory mAb. Rather than directly targeting tumour cells, such mAb enhance immune function by binding to co-stimulatory molecules within the immune system. Here we explore the effects of combining anti-GD2 with antibodies targeting the co-stimulatory molecule 4-1BB. 4-1BB is up-regulated on NK cells following Fc receptor engagement, and others have demonstrated that anti-4-1BB can be used to synergistically enhance the effects of other tumour targeting mAbs (Rituximab, Trastuzumab) in murine lymphoma and breast carcinoma models respectively.

Methods: To induce 4-1BB up-regulation on NK cells human PBMCs were co-cultured with anti-GD2 opsonised LAN-1 cells. The lytic activity of these cells following the addition of anti-GD2 +/- anti-4-1BB was assessed using a calcein-release based ADCC assay.

Results: Here we show significant up-regulation of 4-1BB on NK cells following co-culture, predominantly on the CD56dim NK cell subpopulation associated with most potent cytotoxicity. Furthermore we demonstrate that agonistic anti-4-1BB mAb enhances anti-GD2-mediated cytotoxicity in vitro. The effects of sequential administration of anti-GD2 and anti-4-1BB in vivo are being explored.

Conclusion: These results support a novel immunotherapeutic approach for the treatment of MRD in which antibodies can be used to target tumour cells and enhance the host immune response.

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POC039

Patient's Weight Influences Pharmacokinetic Parameters of Humanized Anti-GD2 Monoclonal Antibody Hu3F8 Administered to Patients with Metastatic GD2-positive Tumors

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Background: At Memorial Sloan-Kettering Cancer Center, patients with relapsed/refractory high-risk neuroblastoma (NB) or other metastatic GD2-positive tumors can enroll in phase I trials utilizing Hu3F8 (NCT01419834) alone (group 1) or Hu3F8 combined with recombinant interleukin-2 (rIL-2) (NCT01662804) (group 2). Thus far, dosage level at 0.3, 0.6, and 0.9 mg/kg can be compared.

Methods: In both trials, patients received intravenous bolus Hu3F8 at 2 doses (day 1 and day 8) per cycle. In group 2, rIL-2 was given subcutaneously from day 8 through day 12. For pharmacokinetics (PK) analysis, serum was collected pre-Hu3F8, 5 min, 3 h, 6 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 168 h post infusion. Non-compartmental analysis of serum concentrations over time in cycle 1 was used to compute Hu3F8 PK parameters including Cmax (peak serum concentration), Cmin (trough serum concentration), AUC (area under the curve), Cl (clearance) and $t_{1/2}$ (terminal half-life).

Results: By analyzing both groups at these dose levels, there was a strong positive linear correlation between patient's weight with AUC (R=0.95±0.11), Cmax (R=0.85±0.14), Cmin (R=0.88±0.14), and negative correlation with Cl (R=0.93±0.42). No correlation was observed between $t_{1/2}$ and patient's weight. However, within group 2, $t_{1/2}$ was consistently decreased during the IL-2 week by 62%±17% at 0.3 mg/kg Hu3F8 dose level, 58%±16% at 0.6 mg/kg, but only 7%±6% at 0.9 mg/kg.

Conclusion: We identified a strong influence of patient's weight on PK parameters, and a negative effect of rIL-2 on $t_{1/2}$, especially at low Hu3F8 dosage levels.

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Clinical Research: Late Effects

POC040

Second Malignancies in Patients with Neuroblastoma: The Effects of Risk-Based Therapy

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Background: To investigate if treatment modifications over the past 40 years for patients with neuroblastoma have influenced the incidence of second malignant neoplasms (SMN), we analyzed patients from the SEER database according to 3 treatment eras (1: 1973-1989, 2: 1990-1996, 3: 1997-2006) corresponding to the introduction of multi-agent chemotherapy, risk-based treatment, and stem cell transplant, respectively.

Methods: The SEER database was mined for all patients with neuroblastoma or ganglioneuroblastoma. Cumulative incidence rates of second malignancy were estimated with all-cause mortality as a competing event. The Mann-Whitney U test compared median latency from the primary neuroblastoma diagnosis to the development of a second cancer between eras.

Results: The analytic cohort included 2,801 patients. Thirty-four patients developed a SMN, accounting for 1.2% of all patients with a cumulative incidence at 35 years of 6.8% (95% CI: 3.6%-9.4%). While patient characteristics were similar between these two groups, those developing a SMN received radiation more frequently for their neuroblastoma (47.1% vs 25.1%; p=0.008). Although the incidence of SMN did not differ between treatment eras (p=0.5), the time to develop SMN was significantly shorter for patients treated in Era 3. Median latency from initial diagnosis to SMN for patients in Era 3 was 50 months compared to 158 and 214 months for patients diagnosed in Era 2 (p=0.007) and Era 1 (p=0.0003) respectively. Of the SMN, carcinomas (n=10) were more frequent in Eras 1 and 2 (p=0.017), whereas acute myelogenous leukemia (AML, n=6) was more frequent in patients treated in Era 3 (p=0.04).
Conclusion: In neuroblastoma patients, SMN often arise decades after diagnosis. Children treated after 1996 with intensive, multi-modality approaches, developed second cancers earlier, with a higher incidence of secondary AML. Further research investigating the genetic factors and the treatment exposures that account for these findings is needed to ensure that therapy is optimized for each child with neuroblastoma.

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POC041

Scoliosis as Late Effect in Neuroblastoma and Ganglioneuroma Results from Tumor itself and from Local Therapy

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Background: Scoliosis is considered a late effect of neuroblastoma. The contribution of the tumor itself, of neurosurgical and of radiotherapeutic intervention is not yet known. This study investigated scoliosis prevalence, time course and risk factors.
Methods: Data of neuroblastoma and ganglioneuroma patients registered registered within the German Neuroblastoma trials from 01.01.1990 to 31.12.2006 were analyzed. MRI images and plain x-rays were reviewed in scoliosis patients. 1703 patients were in the control cohort, the minimum follow-up was six years.

Results: Of 2672 patients registered, 103 were documented with scoliosis (3.9%; 10/112 ganglioneuroma, 93/1591 neuroblastoma). Median age at scoliosis diagnosis was 6.5 years (range 0 - 17 years), the median Cobb-angle was 20° (8°-125°; n=28). 13 patients later underwent surgical scoliosis correction. 29 patients demonstrated scoliosis at tumor-diagnosis or after chemotherapy, but prior to surgery or radiation (range 0 days to 107 months, median 5.26 months; 10/29 ganglioneuroma, 19 neuroblastoma). Scoliosis developed before treatment in 9/10 ganglioneuroma. In 4 patients with ganglioneuroma scoliosis was the symptom leading to tumor-diagnosis. 74 patients developed scoliosis 3 days to 181 months (median 60.4 months) after tumor diagnosis, having had tumor-surgery (n=54), radiotherapy (n=3) or both (n=17). Compared to the control cohort, scoliosis patients more often had stage 3 (41% vs. 17%, p<0.001), thoracic tumors (56% vs. 14%, p=<0.001), and intraspinal involvement (55% vs. 9%, p=<0.001). Out of 196 patients with intraspinal tumor, scoliosis patients had undergone spinal surgery more often (33/41) than the control cohort (61/155; 81% vs. 39% p<0.001). Tumor location was at scoliosis convexity in 71/103 patients (69%; 10/10 ganglioneuroma, 61/93 neuroblastoma). In patients scoliotic prior to spinal surgery or radiotherapy 22/29 scolioses were convex at the tumor location. 55/58 thoracic, but only 16/44 (thoraco)abdominal tumors were located at scoliosis convexity. MRI images at diagnosis showed neural foramina extension in 34/44, muscular infiltration in 28 and increased intercostals spaces in 10 patients.

Conclusion: Scoliosis already presents at diagnosis or during chemotherapy in a significant number of patients. The tumor is mainly located at the convexity. Risk factors are ganglioneuroma, thoracic, intraspinal and stage 3 tumors. Spinal surgery and radiotherapy further contribute to scoliosis as late effect.

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POC042

Elevated TSH-Levels in Patients with Neuroblastoma and Ganglioneuroma after Diagnostic mIBG-Application

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Background: Radiolabelled metaiodine guanidine (mIBG), used in neuroblastoma patients for diagnostics (I-123-mIBG) and therapy (I-131-mIBG), is known to cause damage to the thyroid gland. We were interested in frequency and risk factors for hypothyroidism in patients after diagnostic and therapeutic mIBG-application.

Methods: Files of patients treated at our hospital were reviewed. Inclusion criteria were (1) diagnosis of neuroblastoma or ganglioneuroma of any stage between 1990 and 2012, (2) diagnostic and/or therapeutic mIBG application, and (3) thyroid function parameters (TSH, T3, T4) analysed. All patients received thyroid blockage according to hospital guidelines. To assess the efficacy of thyroid blockage, we developed a semi-quantitive score comparing the mIBG-uptake of the thyroid and the liver as reference (0: no uptake; 1: < liver uptake; 2: = liver uptake; 3: > liver uptake).

Results: Of 86 patients meeting the inclusion criteria, 39 patients had TSH-levels elevated above age-adapted cut-off levels (45.3%). One patient presented with elevated TSH-level already prior to first mIBG-application, and 38 developed elevated levels after therapeutic (n=9) or diagnostic mIBG-application (n=29). While 22 patients showed intermittent elevated levels without the need for substitution, 16 patients required thyroxine substitution. After mIBG-therapy (n=13), nine patients had elevated TSH-levels, with thyroxine substitution in eight. Of 72 patients with diagnostic mIBG-application only, 29 patients (40.3%) had elevated levels, eight of them required thyroxine substitution. Of 65 patients without undergoing MIBG-therapy, 258 mIBG-scans were scored (Score 0: 39.1%; 1: 55.8%; 2: 4.7%; 3: 0.4%). Median cumulative score per patients was 1 (range 0-7). Cumulative score of patients with elevated TSH-levels without substitution (Median 2, range 0-7) and patients with thyroxine substitution (median 3, range 1-7) was higher than that of patients with normal TSH-levels (median 0, range 0-7). However, the groups did not differ with respect to number of scintigraphies (elevated TSH-levels without substitution: median 3, range 1-9; thyroxine substitution: median 4, range 1-7; normal TSH-levels: median 2, range 1-13; p=0.23).

Conclusion: Even after diagnostic mIBG-application, elevated TSH-levels were observed and thyroxine substitution was required. We recommend assessment of TSH levels regularly during treatment and follow-up of patients with neuroblastoma to avoid underdiagnosis of hypothyroidism.

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POC043

Reduced Final Height after High-risk Neuroblastoma Therapy

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Background: Impaired growth is a common late effect of cancer treatment in childhood. We were interested in the prevalence of growth impairment in survivors after treatment of high-risk neuroblastoma.

Methods: ata of patients registered in the German neuroblastoma trial NB97 were retrospectively reviewed. Inclusion criteria were: high-risk neuroblastoma (stage 4 > one year of age or MYCN-amplification in any stage), minimum survival of two years after diagnosis, body height at diagnosis of neuroblastoma known, and at least one measurement during or after treatment. Body height was converted to the age- and sex-adapted percentile. A body height < 3rd percentile was defined as abnormal.

Results: A total of 163 patients were included. Median time between diagnosis of neuroblastoma and last body height measurement was 5.2 years (0.25-16.2 years). At diagnosis, body heights ranged from the 1st to > 99th percentile (median 51.0). Median percentile in the third year after diagnosis was 25.0 (<1-98, n=82), and in the sixth year 26.0 (1-98, n=29; p=0.002). 34 patients (21%) had a body height < 3rd percentile, diagnosed in 23 patients during follow-up and in 11 after neuroblastoma recurrence. Median age at first abnormal height was 5.9 (0.7-15.1) years. Median duration from diagnosis of neuroblastoma to body height below the 3rd percentile was 2.0 (0.4-8.0) years for the whole cohort and 0.7 (0.4-8.0) years for patients not experiencing neuroblastoma relapse. Growth hormone treatment was started in 3/34 patients. Body heights above the 3rd percentile were reached in 15 patients with longer follow-up without growth hormone substitution. Kaplan-Meier-estimates for time from diagnosis to first abnormal height resp. last follow up revealed height at first diagnosis (< 50th vs. > 50th percentile; p<0.001), but not treatment (megatherapy yes vs. no; ch14.18 antibody vs. retinoic acid) or age at diagnosis as risk factor.
Conclusion: Survivors of high-risk neuroblastoma are at increased risk of impaired final height. Similarly to other chronic diseases, small height at neuroblastoma diagnosis seems to present a risk factor for impaired growth.

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POC044

Neuroblastoma with Symptomatic Epidural Compression in the Infant. The AIEOP Experience

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Background: Symptoms of epidural compression (SEC) in children with neuroblastoma may escape early recognition, and this may lead to delay diagnosis, especially in infants. Treatment of SEC in these children remains undefined. Furthermore, all therapeutic modalities are associated with short- and long-term morbidities.

Methods: Clinical, imaging and follow-up data of 34 infants with neuroblastoma and SEC diagnosed between 2000-2011 in the Italian paediatric oncology centres participating in the Italian Association for Paediatric Haematology-Oncolgy (AIEOP), were retrieved and reviewed.

Results: Median age at initial SEC was 104 days (IQR 47-234). Main symptoms included motor deficit (85.3%), pain (38.2%), bladder and bowel dysfunctions (20.6% each). In the symptom-diagnosis interval (S-DI) (median, 12 days; IQR 7-34), the frequency of grade 3 motor deficit increased from 11.8% to 44.1% and that bladder dysfunction from 20.6% to 32.4%. S-DI was significantly longer (P =.011) for patients developing grade 3 motor deficit. First treatment of SEC was neurosurgery in 14 patients, and chemotherapy in 20. SEC regressed in 11 patients (32.3%), improved in 9 (26.5%), and remained stable in 14 (41.2%), without treatment-related differences. Median follow-up was 82 months. At last visit, 11 patients (32.3%) were sequelae-free while 23 (67.7%) had sequelae, including motor deficit (55.9%), bladder disfunction (50.0%), bowel dysfunctions (28.4%), and spinal abnormalities (38.2%). Sequelae were rated severe in 50% of patients. A severe sequelae score was more frequent in patients presenting with spinal canal invasion >66% (P = .039) and grade 3 motor deficit (P =.084).

Conclusion: Both neurosurgery and chemotherapy provide inadequate results once paraplegia has established. Sequelae developed in the majority of patients and were severe in a half. Greater awareness by parents and physicians regarding SEC is warranted.

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POC045

Neuroblastoma with Intraspinal Extension: Late Adverse Events in Long Term Survivors

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Background: Intraspinal neuroblastoma (NBL) is a childhood tumor growing through the intervertebral foramina into the spinal canal. We evaluated the prevalence of adverse events (AE's) in survivors with intraspinal NBL.

Methods: Retrospective, single centre cohort study (AMC) of patients treated for a childhood intraspinal NBL (1980-2007) who survived ≥ 5 years after diagnosis. AE's were graded according to the Common Terminology Criteria for Adverse Events (CTCAEv.3.0).

Results: All 19 patients received a combination of neuro-(surgery), radiotherapy,

131I-MIBG therapy and/or chemotherapy, with 100% survival. Median age at diagnosis and follow-up: 1.2 (0.5- 10.8) years and 15.5 (7.2- 29.5) years respectively. At presentation the neurological symptoms ranged from asymptomatic (n=8, 42%), moderate (n=9, 47%) to severe (n=2, 11%). Overall AE's: grade 1 n=23 (32%), grade 2 n=31 (43%), grade 3 n=13 (18%) and grade 4 n=5 (7%). AE's: 19/72 (26 %) orthopaedic (grade 3 AE's n=2),18/72 (25%) neurological (grade 3/4 n=5). Ninety-five percent of survivors have ≥ 1 AE's and 48% ≥ 4 AE's with a mean of 3.8 AE's/ survivor. Fifty-three percent of survivors have AE's ≥ grade 3. Top 5 AE's see Table A. These percentages are higher compared to a previous study from our centre including all childhood cancer diagnosis (JAMA 2007; 297:2705-15 Geenen et al). Currently in a prospective, multi-disciplinary follow-up clinic, concentrating on AE's in patients with intraspinal NBL (children and adults) 15 patients have been investigated.

Conclusion: Ninety-five percent of survivors treated for a childhood intraspinal NBL have AE's; specialized long term follow-up is needed.

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Top 5 AEs
1. Scoliosis (17%)
2. Neuropathy-motor (8%)
3. Neuropathy-sensory (7%)
4. Urinary tract infecti on (6%)
5. Sterility/inferti lity (6%)

Clinical Research: Phase 2 and 3 Trials

POC046

Influence of Age and Stage on Outcome in the High Risk Neuroblastoma HR-NBL1/SIOPEN Trial

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Background: Evaluation of age and stage influence the unselected patient cohort of the HR-NBL1/SIOPEN trial including all non-randomised patients.

Methods: Since 02/02/2002, the trial accrued in 21 countries (175 centres) 2242 patients (pts) of whom 2022 had INSS stage 4 (eligibility <1year only pts with MycN amplification (MNA)) and 220 stage 2&3 with MNA. The median age is 3.6yrs (range,1 day-20yrs). After Rapid Cojec or modified N7 induction, pts with insufficient response received additional 2nd line treatments (i.e. TVD 2- 4 courses) to proceed to myeloablative therapy (MAT; BUMEL, CEM or mIBG containing regimes). Local control aimed at complete surgical resection (achieved in 76%) and radiotherapy of 21 Gy only to the primary tumour site. Till 2007 maintenance treatment was 13cis RA alone. In 2007 ch14.18/CHO mAb based immunotherapy (IT) was introduced with a modification towards a randomised IL2 question in 2009. To date 428pts received ch14.18/CHO based IT by the 8 hour infusion scheme. Three randomised questions were embedded in this trial (R0/R1/R2).

Results: In stage 4 pts the following MNA frequencies were observed: 66% in 1-1.5 yrs (116/177, 66%), 44% in 1.5-5yrs (449/1108) and 22% (79/363) > 5yrs.The 5-yrs EFS&OS for all pts is 0.31±0.01/0.41±0.01 with rates of 0.28±0.01/0.38±0.01 for stage 4, but 0.63±0.04/0.68±0.04 in MNA stages 2&3 with lower rates in 24 infants (0.44±0.12/0.43±0.12). In stage 4 pts prognosis declines with age: infants and pts 1-1.5 yrs showed comparable outcomes (n=186, 0.39±0.07/0.47±0.08), followed by pts of 1.5-5yrs (n=1234, 0.30±0.02/0.40±0.02) and pts>5yrs (n=402, 0.15±0.02/0.28±0.03). In addition, response of at least PR including VGPR and CR was more frequently observed in younger children. Response rates are 90%, 84%, 82%, 66%, 56% and 41% for age groups of 1<yr, 1-1.5, 1.5-5, 5-10, 5-14 and >14 yrs of age.

Conclusion: Stage and age remain major prognostic factors whilst MNA pts clearly benefit from intensification.

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POC047

Non High-Risk Neuroblastoma European Strategy: SIOPEN LINES Protocol Implementation, Challenges, and Achievements

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Background: The Low and Intermediate Risk Neuroblastoma SIOPEN study (LINES), opened in 2011, stratifies patient's treatment according to biological and clinical markers in order to: i) minimize the treatment burden in those low-risk patients who in previous studies were shown to have an excellent long-term outcome, ii) intensify treatment in those patients with biologically unfavourable but not MYCN amplified neuroblastoma to improve outcome.

Methods: LINES (EudraCT: 2010-021396-81, ClinicalTrials.gov Identifier: NCT01728155) includes ten separate therapeutic groups, one of them randomised. After the trial has opened in a SIOPEN country following the National and European legislation, patient data is entered into the Siopen-r-net database following registration of the patient with check-points to control the quality of prospectively entered staging data, including real time central review for biology and histology. Neonatal adrenal masses (NAM) in infants below 3months are also registered.

Results: In July 2011, the LINES trial with an international sponsor of IISLaFE (Spain) obtained approval from the Spanish Competent Authority and Ethic Committees and opened at 28 Spanish sites. Since then the trial has been opened in Italy (21 sites), Austria (5 sites), Denmark (3 sites), France (29 sites), Norway (4 sites), Israel (1 site) and Belgium (4 sites). Since December 2011, 100 patients have been enrolled. Distribution according to groups is as follows: 52 low risk, 24 intermediate risk and 24 NAM.
Conclusion: 1/ Recruitment is rapidly increasing in countries where the trial has been opened. 2/ Quality control check-points are functioning in a timely manner. 3/ It has become evident that it is essential to review the current European directive to facilitate prompt and fast new trial implementation across all European countries.

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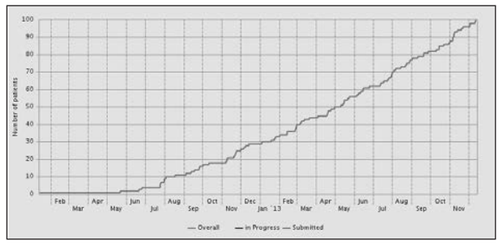

POC048

Treatment Results of Children with Low-Risk Neuroblastoma Observed and Treated According to Nb2004 Protocol: Data from Single Center

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Background: According to NB2004 Protocol stratification criteria the significant proportion of MYCN-negative patients should be allocated to low-risk arm and they have to be observed and receive chemotherapy only in case of life threating symptoms and/or progression of the tumor. Aim is retrospectivedetermination the treatment results in patients from observation group (OG) of NB2004 Protocol.

Methods: Among 90 children with primary neuroblastoma treated according to NB2004 in our clinic since December 2005 till October 2013 - 58(64,4%) patients aged from 10 days to 15 years (median 5 months) were stratified to OG. Patients' distribution by stage was as follow: stage I had 35(60,3%) children; stages II-4(6,9%), III-12(20,7%) and IVS-7(12,1%) patients. Genetic aberrations were determined in 46 patients. 17q gain was revealed in 22(47,8%) patients, 11q deletion in 6(13,0%), 9p deletion in 3(6,5%) and 4p gain in 1(2,2%) patient. Median of follow up is 44 months.

Results: In 13(22,4%) patients (10 with stage III, 3 with stage IVS) chemotherapy was started immediately after diagnosis because of the huge size of primary tumor or life threating symptoms. Also chemotherapy was given to other 4 patients during follow-up period due to relapse or progression. At present time 55(94,8%) patients are alive; 51(87,9%) are alive without progression. Unfavorable events were registered in 7(12,1%) cases: relapses – 2(3,4%); progressive disease – 4(6,9%); therapy-related death – 1(1,7%). 2(3,4%) patients died from tumor progression. 7-years event free survival is 84%±5% and overall survival is 92%±4%. The majority of unfavorable events were registered in children with stage IVS: 3 cases of tumor progression and 2 deaths – one from severe systemic infection and one from disease progression.

Conclusion: The prognosis of children with low-risk neuroblastoma is excellent. Existing NB2004 Protocol criteria of patients' stratification at observation group are demonstrating their effectiveness and in most cases can allow to reduce the intensity of treatment.

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POC049

MYCN Amplification is not Solely the Prognostic Factor in Treating of High-Risk Neuroblastoma: A Late Phase II Study of Japan Neuroblastoma Study Group (JNBSG)

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Background: Japan Neuroblastoma Study Group (JNBSG) prospectively enrolled patients with high-risk neuroblastoma according to COG risk group (2008) on the phase II clinical trial. Five courses of induction chemotherapy comprised of vincristine, cyclophosphamide, cisplatin and pirarubicin, and surgery, was followed by high-dose chemotherapy (HDC) comprised of melphalan, etoposide and carboplatin with autologous PBSC rescue, and radiation therapy. Our JNBSG protocol is characterized by the use of pirarubicin with less cardiotoxicity and no VP16 in the induction chemotherapy.

Methods: Between March 2007 and February 2009, 50 patients were enrolled. Forty-five patients were diagnosed pathologically according to the central review.

Results: Complete or very good partial response rate before HDC was 54.8% and the response rate including partial response account for 87.1%, which is comparable to COG or SIOPEN study. The 3-year OS and PFS for 50 patients were 69.5+/-6.6% and 36.5+/-7.0% respectively. MYCN amplification was observed in 20 patients, whose PFS was 45.0+/-11.1%, while the PFS of MYCN on-amplified cases was 29.9+/-8.7% (p=0.56). The 3-year PFS was 47.1+/-13.8% for 11q loss patients (n=14) compared with 47.4+/-11.5% for no 11q loss patients (n=19) (p=0.83). Twenty-six patients could complete the whole protocol therapy; 3 experienced disease progression before HDC. Treatment-related death occurred in 3 patients after HDC.

Conclusion: Our strategy revealed that MYCN amplification is not solely the prognostic factor in treating of high-risk neuroblastoma. In order to enforce the treatment intensity of induction chemotherapy, JNBSG are now conducting new protocol with delayed local control treatment, in which total resection of the primary tumor is postponed after HDC with autologous PBSC rescue.

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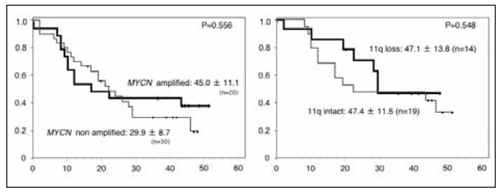

POC050

Outcome of Children with High-Risk Neuroblastoma Treated with Mega-Chemotherapy Consisting of Thiotepa and Melphalan

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Background: We retrospectively analyzed our institutional experience of mega-chemotherapy in using double-conditioning regimen comprised of thiotepa and melphalan with peripheral-blood stem-cell rescue (PBSCR) for patients with high-risk neuroblastoma, focusing on outcome and acute toxicities.

Methods: Eighteen patients with high-risk neuroblastoma were treated between 2002 and 2009 at our institution. Patients received induction chemotherapy with five cycles of standard agents in Japan, resection of the primary tumor and local irradiation, followed by mega-chemotherapy with autologous PBSCR. The double-conditioning regimen contains two cycles of thiotepa (320–480 mg/m2) and melphalan (90–140 mg/m2) with a 1-week interval.

Results: Nine patients have relapsed or died. Median follow-up time of the 9 patients who remain alive without progression is 7.2 years (range, 4.8 years to 11.0 years). Progression free survival (PFS) rate at 5 years from diagnosis was 56% (95% CI, 33% to 79%), and PFS rate at 7 years was 49% (95% CI, 25% to 73%). Overall survival rate was 72% (95% CI, 51% to 93%) and 63% (95% CI, 39% to 88%) at 5 and 7 years, respectively. Of note, 8 of 10 patients with MYCN amplified tumors remain alive without progression. One patient died from transplant-related toxicity (pulmonary hemorrhage with microangiopathy). None experienced hepatic veno-occlusive disease. Relapse occurred in 8 (44%) of 18 patients, all of which were at metastatic sites.

Conclusion: Mega-chemotherapy using thiotepa and melphalan with PBSCR is feasible and effective for treating high-risk neuroblastoma.

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POC051

Outcome of High-Dose Chemotherapy with Autologous Stem Cell Rescue for Patients with High-Risk Neuroblastoma: A Single-Institute Experience

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Background: The prognosis of high-risk neuroblastoma has improved since the introduction of single and tandem high-dose chemotherapy with autologous stem rescue (HDCT/ASCR). This study was to investigate the clinical effectiveness of single or tandem HDCT/ASCR and evaluate the prognostic factor for high-risk neuroblastoma.

Methods: We retrospective reviewed medical records of 33 patients who received single or tandem HDCT/ASCR from March 2001 to September 2012 in Asan Medical Center.

Results: The median age at diagnosis was 2.8 years (range, 0.6-10.0 years). Eighteen patients received single HDCT/ASCT, 15 patients tandem HDCT/ASCR, and 1 patient HDCT/ASCR followed by allogeneic haploidentical stem cell transplantation (SCT). The conditioning regimen of single HDCT/ASCR was CEM (carboplatin + etoposide + melphalan) (N = 14) or CEC (carboplatin + etoposide + cyclophosphamide) (N = 4), and those of tandem HDCT/ASCR was CEM followed by TC (thiotepa + cyclophosphamide) (N = 12) or TBI-thiotepa (N = 3). Three patients received 18 mCi/kg of ^{131}I-MIBG before second HDCT/ASCR. The 5-year overall survival (OS) rate and event-free survival (EFS) rate was 76.5% and 70.6% in a single group, and 77.1% and 69.3% in a tandem group (P = 0.995, 0.664). Three patients who received ^{131}I-MIBG-incorporated HDCT/ASCR are alive without significant adverse event. No patient died of transplant-related caused after HDCT/ASCR, and 1 patient, who received allogeneic SCT, died of CMV encephalitis. The 5-year OS rate was significantly better in the patients who had a complete response or a very good partial response compared with those who had not (94.1% vs. 53.3%, P = 0.017).

Conclusion: HDCT/ASCR was effective for improving survival in high-risk neuroblastoma. Also, ^{131}I-MIBG was successfully incorporated into tandem HSCT/ASCR. Further trials incorporating newer treatment strategies are needed to further improve the outcome, especially for poor responders to induction chemotherapy.

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POC079

Treatment of Neuroblastoma with Low Lose Chemotherapy

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Background: To study the outcome of neuroblastoma patients treated with low dose chemotherapy.

Methods: From 1992 to 2012, 155 patients were treated with low dose chemotherapy. Among them, 132 patients were in stage 3 or 4, 14 in stage 1 or 2, 9 in stage 4s. Treatment of VC or VAC was used in patients younger than 3 years old, while VCA or VACP plus traditional Chinese medicine was used in patients older than 3 years old. The doses for VCR, Ara-c and CTX were 0.04mg/kg, 3-5mg/kg, 5-10mg/kg, respectively. Bone marrow examination, tumor pathological examination, CT or MRI imaging were performed. The patients' response to treatment and the survival rates were analyzed.

Results: The 5-year and 10-year survival rate for all patients was 51.3% and 36.8%, respectively. 6 patients with stage 4 diseases had a complete remission with tumor disappearance after low dose chemotherapy; 3 patients with relapse disease after surgery received low dose chemotherapy and pathological examination showed a change from neuroblastoma to ganglioneurofibroma. We analyzed the responses of patients in 4s stage: without surgery or any other treatment, 2 patients died of brain metastasis and 1 survived but developed spinal cord metastasis; with surgery only, 3 patients survived and 1 died of brain metastasis; 2 patients with severe complications accepted only low dose chemotherapy and survived with tumor disappearance.

Conclusion: Our results indicated that infant neuroblastoma patients (including stage 4 patients) have a good response to low dose chemotherapy, and patients in stage 4s with complications may benefit from low dose chemotherapy.

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Clinical Research: Radiotherapy

POC052

Assessment of [131I]MIBG in Combination with DNA Repair Inhibitors for Neuroblastoma Therapy

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Background: ^{131}I-meta-iodobenzylguanidine (^{131}I-MIBG) is an effective treatment for neuroendocrine tumours. However, it is likely that maximal therapeutic benefit of ^{131}I-MIBG will be obtained by combination with chemotherapy. Activation of DNA repair pathways such as poly (ADP-ribose) polymerase-1 (PARP-1), follows genotoxic insult. We have previously shown that PARP-1 inhibition, following treatment with PJ34, enhanced the antitumour efficacy of ^{131}I-MIBG. However, administration of PJ34 is associated with substantial normal tissue toxicity. Therefore we determined the potential of the alternative PARP-1 inhibitors AG014699 and olaparib to enhance the efficacy of external beam X-radiation or ^{131}I-MIBG by assay of the survival of clonogens derived from noradrenaline transporter (NAT)-expressing SK-N-BE(2c) neuroblastoma cells and UVW glioma cells genetically engineered to express NAT.

Results: Single agent AG014699 or olaparib treatment (≤10μM) had negligible effect on clonogenic survival. However, both PARP inhibitors acted as radiosensitisers. In the absence of PARP inhibitors, 3.5Gy or 4.9Gy inhibited the survival of 50% clonogens derived from SK-N-BE(2c) or UVW/NAT cells respectively. In contrast, the IC50 was significantly decreased following 10μM AG014699 treatment, to 1.7Gy ($p<0.05$, SK-N-BE(2c)) or 2.8Gy ($p=0.07$, UVW/NAT). Similarly, following treatment with 50μM olaparib, the radiation dose required to inhibit survival of SK-N-BE(2c) or UVW/NAT clonogens by 50% was significantly reduced to 2.3Gy ($p<0.01$) or 2.0Gy ($p<0.01$) respectively. PARP-1 activity was also significantly inhibited in both cell lines by AG014699 in a dose-dependent manner ($p<0.05$).

Conclusion: Preliminary results suggest that the clonogenic cell kill resulting from external beam irradiation is enhanced following inhibition of PARP-1 by AG014699 or olaparib. We now intend to assess the effect of PARP inhibition in combination with ^{131}I-MIBG and to determine whether inhibitors of DNA repair, cell cycle progression or free radical generators have a beneficial effect in combination with radiation. By establishing the most effective use of ^{131}I-MIBG with synergising drug(s), we will improve the treatment of neuroblastoma.

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POC053

Radionecrosis in Children Treated with Conventional Radiation Therapy and Intrathecal Radioimmunotherapy for CNS Neuroblastoma: Is it a Concern?

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Background: Radionecrosis in children treated for brain tumors is a potentially severe and neurologically devastating long-term complication of external beam radiotherapy (XRT). Intraventricular compartmental radioimmunotherapy (cRIT) using ^{131}I-3F8 or ^{131}I-8H9 has been used to eradicate NB cells in the cerebrospinal fluid (CSF) space. The incidence of radionecrosis following both XRT and cRIT is unknown.

Methods: We retrospectively analyzed the incidence of radionecrosis in patients with radiographic and/or pathologically confirmed CNS NB who received both XRT and cRIT at MSKCC since 2003. Patients underwent MSKCC CNS salvage regimen incorporating conventional craniospinal XRT 1800-2160 cGy with parenchymal tumor boost 3060 cGy followed by cRIT, 10-70 mCi ^{131}I-3F8 or ^{131}I-8H9 as described1. CSF dosimetry by cRIT was based on post-injection serial intraventricular samplings over 48 hours. Disease evaluation included serial MR brain/spine, MIBG, CT, and bone marrows approximately every 3 months.

Results: 58 patients received both XRT and cRIT, median follow up 41.5 months (6.5-124.8 months). 5 patients received additional stereotactic XRT for persistent parenchymal nodules; 4 patients were treated with alternative craniospinal doses (1080 cGy, 1260 cGy, and 3600 cGy). Estimated median CSF dose by cRIT was 2595 cGy (1038-13143 cGy). One asymptomatic patient underwent resection of 0.6 cm hemorrhagic periventricular white matter lesion confirmed to be necrosis and granuation tissue, 2.5 years after XRT. No further evidence of radionecrosis occurred in the ensuing 5.5 years. No other clinical or radiographic evidence of radionecrosis was observed in the remaining 57 patients.

Conclusion: With current treatment recommendations, the risk of radionecrosis in children treated with XRT and cRIT for CNS NB appears minimal (<2%). No neurologic deficits secondary to radionecrosis have been observed in long term survivors of CNS NB.

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POC054

Comparison of 131i-Mibg Post-Therapy Dosimetry and 123i-Mibg Dosimetry in Patients with Neuroblastoma: Implications for Planning Repeat High-Dose 131 I-Mibg Treatment

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Background: ^{131} I-MIBG therapy including double infusions is being increasingly utilized for treatment of neuroblastoma. For double infusions, dosimetry planning has been restricted to post-therapy whole-body exposure-rate measurements but potentially therapy-limiting organ doses have not yet been evaluated.

Methods: Patients were enrolled on a single-institution phase II study (NCT00107289). We performed three serial whole-body gamma-camera scans for each patient 2-6 days after the first ^{131}I-MIBG therapy (18 mCi/kg) (131I-D) and ~0.5, 24 and 48hours after a diagnostic dose of 10mCi/1.7m^2 ^{123}I-MIBG to project ^{131}I-MIBG doses after second therapy (^{123}I-D). Regions of interest were drawn over liver, heart, kidney, lungs and whole body. The resulting conjugate-view time-activity data were fit to exponential functions and the ^{131}I-MIBG residence times for both ^{131}I-D and ^{123}I-D . Organ absorbed doses were calculated using the MIRD schema and OLINDA/EXAM . For ^{131}I-MIBG, blood dose was calculated using serial sampling.

Results: Data on 22 patients (age 2-21; median 4.5 years) were analyzed. For individual patients, overall dose estimations based on ^{123}I-D were higher than ^{131}I-D though not statistically significant ($p>0.5$ for all organs). Mean ratios for ^{123}I-D:131I-D were 1.21±0.94, 2.2±1.63, 1.96±1.66 and 1.13±81 for kidney, liver, lung and whole body, respectively. Hence ^{123}I-D provided a more conservative estimate for second infusion than ^{131}I-D.

Conclusion: For planning repeat ^{131}I-MIBG therapy, ^{123}I-D and ^{131}I-D provide whole-body and organ dose estimates for both infusions. However, despite significant variation among patients, ^{123}I-D provides a more conservative, safer estimate of maximum allowable second therapeutic ^{131}I-MIBG dose and is more practical to perform.

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	Administered activity in first ^{131}I-MIBG infusion (mCi)	Dose from ^{131}I-MIBG infusion (rad/mCi)					Predicted ^{131}I-MIBG dose based on ^{123}I-MIBG Dosimetry			
		Blood	Whole Body	Kidney	Liver	Lung	Whole Body	Kidney	Liver	Lung
Mean	411	0.2	0.65	0.65	2.01	1.7	0.52	0.63	3.28	1.68
SD	216	0.1	0.39	0.39	1.05	1	0.22	0.77	2.21	0.92
Minimum	208	<0.1	0.14	0.14	0.46	0.25	0.15	0.21	0.41	0.21
Maximum	1091	0.7	1.76	1.82	4.38	3.95	1.1	4.17	8.14	4.15

POC055

Radiationsafety Procedures Enabling the Administration of High-Dose 131i-Mibg Therapy (131i-Mibg) to Patients with High-Risk Neuroblastoma (Hr-Nb) Without Lead Lined Rooms

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Background: Although ^{131}I-MIBG is increasingly utilized for treating HR-NB, the lack of lead-lined rooms limits its wider use. Using rolling lead shields rather than dedicated lead-lined rooms, we implemented radiation safety policies and procedures to comply with local radiation-protection regulations for the use of therapeutic radioisotopes in patients receiving ^{131}I-MIBG.

Methods: Patients with HR-NB received 18 mCi/kg/treatment ^{131}I-MIBG (NCT00107289) via Graseby infusion pump. Radiation safety procedures included : (1) use of an appropriate private room with installation of rolling lead shields to maintain area dose rates <2millirem/hr outside the room; (2) patient isolation until dose rate <7mR/h at 1 m; (3) retention of a urinary catheter with urine collection in lead boxes. Primary caregivers were permitted to stay in the patient\\\'s room behind lead shields, trained in the principles of minimizing radiation dose (ALARA), and provided with real-time electronic radiation dosimeters. Medical staff were required to wear a radiation dosimetry badge, underwent radiation safety training prior to, and thyroid scans after each ^{131}I-MIBG. Contamination surveys were performed after patient release. Data were reviewed with IRB permission.

Results: Records on 11 ^{131}I-MIBG infusions in 7 patients (age 3-12 years) were audited. Radiation dose to all medical staff caring for the patient maintained ALARA. Thyroid bioassay scans were below limited detectable activity(<185Bq) and contamination surveys <200dpm/100cm2.

Conclusion: The use of rolling lead shields and implementation of specific radiation safety procedures allows administration of ^{131}I-MIBG while meeting radiation safety guidelines and may broaden ^{131}I-MIBG use in institutions without dedicated lead-lined rooms.

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Pt No.	No. days to discharge	No. of syringes	Total dose mCi (GBq)	Maximum dose rates (outside room including floors above and below) mrem/h	Cumulative exposure to parent/ caregiver(s) over entire treatment (mrem)/*: >1 caregiver]	Cumulative exposure to physician administering treatment (mrem)	Cumulative exposure to nurse present during treatment (mrem)
1	2	2	407 (15.06)	0.86	31.3	3.3	1.8
2	2	2	300.4 (11.12)	0.79	86.7*	9.7	4.5
3	2	2	284.4 (10.52)	0.48	68.4*	1	3.3
4	3	2	305.6 (11.31)	0.65	126.8*	3.3	9.3
5	3	2	334.2 (12.37)	0.8	108*	4.1	7.8
6	2	1	208 (7.70)	1.34	118	0.8	
7	2	1	169.3 (6.26)	1.59	25.5	0.5	1
8	3	4	804 (29.75)	0.34	47.5*	23	10.6
9	4	5	1061 (39.26)	1.74	154*	5.9	26.7
10	3	4	809 (29.93)	1.04	27.8	28.3	3.4
11	3	3	488.4 (18.07)	1.79	45.4	26.2	4.2

POC056

Single or Double High-Dose 131i-Mibg Therapy (131i-Mibg) Plus High-Dose Chemotherapy (Hdc) Followed By Autologous Stem

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Background: Patients with relapsed neuroblastoma generally have a limited number of unused cryopreserved stem cells, yet these are a prerequisite for ^{131}I-MIBG. To maximize therapy while conserving stem cells, and potentially to enhance response we treated patients with one or two doses of ^{131}I-MIBG plus HDC before ASCR.

Methods: Patients were treated on a phase II trial (clinicaltrials.gov NCT00107289) with an initial dose of 18mCi/kg ^{131}I-MIBG. They received a second dose ~6-8 weeks later if they had an objective response, persistent MIBG-positive disease and absolute neutrophil count (ANC) >1000/uL. After completing protocol requirements, patients received HDC followed by ASCR and were monitored for toxicity and response after ^{131}I-MIBG and after ASCR.

Results: 32 heavily prior-treated patients with relapsed neuroblastoma (mean# prior relapses: 3; 14 with active progressive disease [PD]) received one (n=22 patients) or two (nine with cumulative dose 36mCi/kg, one with 28.8mCi/kg) ^{131}I-MIBG doses. Mean±SD time to HDC and stem cell doses were 48±18 days and 4.1±2.7x106 CD34+cells/kg respectively. All patients engrafted (median time to ANC>500/uL: 11 days). Major grade 3 toxicities post-^{131}I-MIBG were central line infections (n=3) and emesis (n=1); and grade 4 myelosuppression (n=30). Major toxicities post-HDC (n= 30) were uncomplicated febrile neutropenia (n=15), bacteremia (n=3), fungal sepsis (n=3), transient ifosfamide encephalopathy (n=2), mucositis (n=2), and lethal pneumonitis (n=1, post-thiotepa). Overall responses (n=30) post-MIBG+HDC were: complete remission (n=1), objective but <partial responses (n=17), no change (n=3), PD (n=9). HDC was associated with further OR and PD in 5 and 2 patients who had responded to MIBG therapy. Median time to progression was 5.9 months; 2 patients survived progression-free >12 months post-^{131}I-MIBG.

Conclusion: ^{131}I-MIBG followed by HDC with a single ASCR can be performed safely and can preserve stem cells aliquots for future therapies. However, major response rates in multiply-relapsed patients were low and response durations short.

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POC057

Response in Soft Tissue Lesions Following Therapeutic [131I]MIBG in Children with Neuroblastoma

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Background: Impressive response rates to Meta-[^{131}I]iodobenzylguanidine ([^{131}I]MIBG) in patients with recurrent/refractory neuroblastoma have led to proposed trials in which [^{131}I]MIBG is administered earlier in therapy, when patients often have bulky soft tissue disease. Data regarding response to [^{131}I]MIBG specifically in soft tissue lesions are limited, particularly in patients receiving [^{131}I]MIBG early in the course of the disease. We evaluated response to [^{131}I]MIBG in patients with measurable soft tissue lesions, including those who received [^{131}I]MIBG after suboptimal responses to induction.

Methods: Medical records and imaging studies for patients who received [^{131}I]MIBG on single institution expanded access protocol were reviewed retrospectively. Subjects were included if soft tissue disease could be assessed on paired scans performed before and after therapy. Patients who received [^{131}I]MIBG due to relapse/progression or due to a Curie score >4 at end-induction were included. Response was defined as >50% reduction in volume of measurable lesions. Best response achieved after 1 or 2 cycles of [^{131}I]MIBG was used in analyses.

Results: Twenty-eight patients with measurable soft tissue disease treated with therapeutic [^{131}I]MIBG in 2007 or thereafter were evaluable. Median age was 3.8 (range 0.99-12.6) years. A >50% reduction in total lesion volume was observed in 11/28 (39%), all responders had total tumor volume ≤70 cm3 at enrollment. Among 11 patients who received [^{131}I]MIBG due to suboptimal response to induction, 2 had >50% reduction in soft tissue volume.

Conclusion: In a cohort of recently treated patients, the response rate in soft tissue disease was similar to overall [^{131}I]MIBG response rates previously reported. While partial and complete responses in soft tissue lesions were limited among patients receiving [^{131}I]MIBG due to suboptimal response to induction, there were children with >50% reduction in tumor volume in that group. The relationship between response and overall tumor size merits further study, as all responders had initial tumor volumes ≤70 cm^3.

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POC058

Administration of I-131 Metaiodobenzylguanidine (mIBG) Using a Peristaltic Infusion Pump

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Background: Syringe pumps are commonly used to administer therapeutic I-131 metaiodobenzylguanidine (mIBG). Here we describe our experience in the pediatric setting the use of a peristaltic infusion pump system for this purpose. This method can easily accommodate infusions from several vials simultaneously and this process is adaptable with various types of peristaltic pumps.
Methods: Connecting the vial to the primary infusion set is accomplished using a Male/Male Extension line and Aspirating Luer-Lock Needle. Using aseptic technique, the extension line is attached to the "Y\\\" connector closest primary IV line leading from the saline reservoir to the infusion pump. An A-clamp is attached to the primary IV line immediately before it enters the pump. Fluid purges air from the Extension Set, connector and the aspirating needle by gravity. Using aseptic technique insert the aspirating needle into the therapy vial. Program the pump with the desired infusion rate and volume.

Results: In the past year, 19 infusions have been performed using this method. Most infusions were with one vial, but 2 and 3 vials connected in tandem has been performed 7 times with success. Miscalculation of the volume in the vial, or the total infusion time can cause air to be pulled into the lines. To prevent this, the volume in the vial is equalized to 30 mL; additionally, observe the infusion of the last 2 or 3 mL and stop the pump when the air/fluid boundary is about ½ of the way up the extension.

Conclusion: The peristaltic infusion pump method is robust and easy way to infuse therapeutic radiopharmaceuticals. During infusion, the radiopharmaceutical remains in a well-shielded vial reducing exposure. Multiple vials can be connected in tandem to infuse the entire dose without interruption.

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POC059

Use of Pretherapy 124I-MIBG PET/CT Imaging to Perform Patient-Specific Tumor Dosimetry for 131I-MIBG Targeted Radionuclide Therapy

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Background: Accurate estimation of radiation dose of ^{131}I-metaiodobenzylguanidine (MIBG) targeted radionuclide therapy (TRT) in neuroblastoma has been a challenge. In this study, we used pretherapy ^{124}I-MIBG dynamic PET/CT imaging to perform patient-specific dosimetry for ^{131}I-MIBG TRT.

Methods: Serial ^{124}I-MIBG (0.695 mCi) PET/CT imaging of a 10-year-old girl with recurrent neuroblastoma was acquired before treatment with ^{131}I-MIBG (522 mCi). ^{131}I-MIBG residence times of source tissues were estimated from 124I-MIBG time activity curves. Gross tumor volume was approximated by the tumor isocontour with a threshold of 50% of maximum intensity. The identified tumor volume was added to University of Florida (UF) / National Cancer Institute (NCI) 10-year-old female computational phantom that matched closely to the subject's anatomy for simulation. Monte Carlo simulation was implemented using Geometry ANd Tracking (GEANT4) toolkit. Absorbed dose of the ^{131}I-MIBG TRT to organs and tumors was compared between GEANT4 method and OLINDA|EXM.

Results: The GEANT4 method estimated absorbed dose of 367.4, 170.1, 58.1, 36.1, and 35.0 Gy to bone metastases with volumes of 3.6, 0.52, 0.33, 1.4, and 0.36 mL from ^{131}I-MIBG TRT. The absorbed dose to salivary glands, liver, heart, thyroid, lungs, and spleen was 97.8, 34.3, 36.8, 22.6, 21.6, and 16.0 Gy. Up to 123.0 % difference (osteogenic cells target) in estimated absorbed doses between GEANT4 and OLINDA|EXM were observed (Figure 1).

Conclusion: Patient-specific tumor dosimetry of ^{131}I-MIBG TRT was feasible with ^{124}I-MIBG pretherapy PET/CT imaging and Monte Carlo simulation of a given clinical case. This method may be helpful to improve treatment planning of ^{131}I-MIBG targeted radionuclide therapy.

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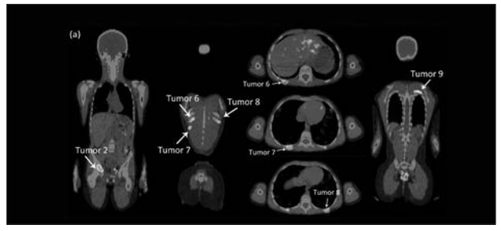

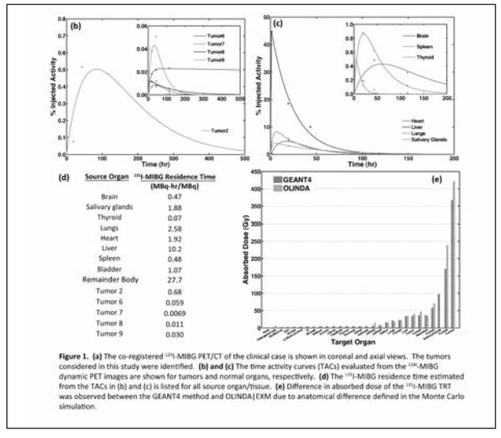

Figure 1. **(a)** The co-registered ^{124}I-MIBG PET/CT of the clinical case is shown in coronal and axial views. The tumors considered in this study were identified. **(b) and (c)** The time activity curves (TACs) evaluated from the ^{124}I-MIBG dynamic PET images are shown for tumors and normal organs, respectively. **(d)** The ^{131}I-MIBG residence time estimated from the TACs in (b) and (c) is listed for all source organ/tissue. **(e)** Difference in absorbed dose of the ^{131}I-MIBG TRT was observed between the GEANT4 method and OLINDA|EXM due to anatomical difference defined in the Monte Carlo simulation.

POC060

Utilizing Education to Enhance Nurses' Comfort Level in Caring for Patients Receiving 131I-MIBG Therapy

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Background: Access for Canadians to ^{131}I-MIBG therapy has been limited by the lack of a Canadian facility. As a result, families seeking ^{131}I-MIBG therapy travel to the US and need additional financial resources. Recently, our institution gained government and regulatory approvals for the construction and operation of a ^{131}I-MIBG therapy suite on the inpatient haematology/oncology unit. Establishment of a ^{131}I-MIBG therapy program requires the collaboration and active involvement of many different teams, as well as site visits to established ^{131}I-MIBG centres, in the design, planning and successful implementation of a such a program. An early learning needs assessment identified that nursing staff had significant concerns regarding radiation exposure. Therefore, a comprehensive education program was implemented led collaboratively by nursing, nuclear medicine and radiation safety.

Methods: Various modalities of knowledge translation methods were employed such as invited lectures by external experts, lunch &learns and in-class presentations. Nearly 100% of all inpatient haematology/oncology unit nurses attended the in-class sessions on "Radiation Safety", "^{131}I-MIBG Therapy" and "Care of the Patients Receiving ^{131}I-MIBG therapy."

Results: Post in-class instruction tests indicated that nurses' comfort level and understanding of "^{131}I-MIBG therapy" increased by 40%, "Radiation Safety" by 36% and "Nursing Care of Patients Receiving ^{131}I-MIBG Therapy" by 47%, from the initial learning needs assessment.

Conclusion: This further illustrated that a comprehensive education program is essential and effective in supporting staff in the implementation of a new therapy program. Future education projects include simulations of caring for the patients in the ^{131}I-MIBG suite, eLearning modules for annual review and utilizing the education modules for other key stakeholders.

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Clinical Research: Surgical Therapy

POC061

Decompressive Laparotomy with Temporary Abdominal Closure in Infants with Neuroblastoma stage 4S and Massive Hepatomegaly

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Background: Although neuroblastoma stage 4S is known for spontaneous regression, some patients develop massive neuroblastoma induced hepatomegaly causing life-threatening abdominal compartment syndrome. Here we report on 17 patients who, therefore, needed decompressive laparotomy (DL) as acute treatment.

Methods: Data of stage 4S patients registered between May 1997 and September 2013 and undergoing DL were analyzed regarding initial symptoms, treatment, complications, and outcome.

Results: Out of 299 stage 4S patients, 17 (5.7%) underwent DL with temporal artificial closure of the abdomen for massive hepatomegaly due to liver metastasis. Median age at diagnosis was 25 days (range 0-141) compared to 101 days of pa-

tients without DL (p=0.001). Three of 16 tumors analyzed were MYCN amplified. Prior to first DL 15/15 patients with available data showed respiratory failure, 14/15 impaired diuresis and 14/16 signs of liver insufficiency. Median time from initial hospitalization to DL was 10.5 days (range 3–40 days). Chemotherapy was given to 16/17 patients in median 4 days (range 1-64) prior to DL. In one patient, chemotherapy was started one day after surgery.The patients underwent in median 3 surgical procedures for decompression, revision, and closure. The course was complicated by infections in 7/12 patients with data available. The abdominal wall was definitively closed in 9/17 patients 25-141 days (median 73) after first DL. Eight patients died between 1 and 31 days (median 16.5) after DL. Causes of death were multifactorial including multi organ failure (n=7), tumor progression (n=2), infection (n=1), and surgery (n=1). One additional patient died 115 days after abdominal closure due to liver insufficiency and tumor progression.

Conclusion: The causes of death were primarily based on complications by the disease itself. To avoid irreversible organ failure caused by abdominal compartment syndrome, DL should be considered early in stage 4S patients with signs of increased intra-abdominal pressure.

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POC062

Surgical Intervention Strategies for Mediastinal Neuroblastic Tumors in Children

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Background: Mediastinal neuroblastic tumors (NBTs) are relatively rare and have more favorable prognosis compared to those arising from other sites. However, some of them may present severe clinical problems, and surgical intervention may often be invasive due to their anatomical features. In this study, we investigated the clinical characteristics and attempted to establish the surgical strategy for mediastinal NBTs in our institute.

Methods: From 1989 to 2013, 26 mediastinal NBTs were treated. Their median age at diagnosis was 1.0 year (2 months – 16 years). Medical charts were retrospectively reviewed regarding as surgical intervention and outcomes.

Results: The pathological diagnosis included 18 neuroblastoma (NBL), 2 ganglioneurblastoma (GNBL), and 6 ganglioneuroma (GN). In 20 NBL and GNBL, 2 cases presented with oncologic emergency such as respiratory failure and paraplegia due to spinal cord compression. MYCN amplification was not seen. Although most of them were assigned to low/intermediate risk group of JNBSG (Japan Neuroblastoma Study Group), only one case with stage 4 was high risk. Transthoracic surgery including postero-lateral thoracotomy or video-assisted thoracic surgery (VATS) was performed for 24 NBTs. Gross total resection was accomplished in 12 of 20 malignancies. In comparison between open surgery and VATS, VATS (n = 8) showed less blood loss and favorable wound appearance compared to open surgery (n = 16). No local recurrence was noted and the postoperative complications were Horner syndrome in 5, and progressive scoliosis in 4. There was one treatment-related death with high risk NBL except 5 lost-follow cases.

Conclusion: It is important to select surgical intervention for mediastinal NBTs based on tumor biology. Biopsy or partial resection by minimal invasive surgery using mini-thoracotomy or VATS is recommended for benign or low/intermediate risk tumors. While, in case of high risk tumors, gross total resection using open surgery is recommended for local control. The short-term surgical outcomes were excellent with prompt appropriate surgical procedure.

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POC063

Minimally Inavasive Surgery in Children with Neuroblastoma

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Background: MiniMinimally invasive surgery (MIS) is increasingly used in pediatric oncology. One of the promising area is MIS in children with neuroblastoma, especially in infants < 1 year. However, due to the lack of large number of observations, long follow-up data, the application of MIS requires the development of clear indications and structural analysis of the results in order to optimize surgical treatment of children with thoraco-abdominal neuroblastoma.

Methods: 169 patients with a diagnosis of \\\"neuroblastoma\\\" were observed and treated during the period 01.2012 - 12.2013. Patients were treated according to NB2004 protocol. Image-defined risk factors (IDRF) and size of the tumor were used to select patients for MIS. After initial work-up patients with the lack of IDRF and tumors < 6 cm in the largest dimension were eligible for MIS.

Results: During the study period definitive surgery was performed in 105 patients. MIS performed in 25 patients (23,8% of operated patients). Median age was 10,5 months (range 0,7-67,1). M:F ratio was 1:1.5. Distribution by stage according to INSS: stage 1 - 18 (72%) patients, stage 2 - 3 (12%), stage 4 - 3 (12%), stage 4S - 1 (4%). Laparoscopic tumoradrenalectomy performed in 18 (72%) patients, thoracoscopic resection in 5 (20%), endoscopic removal of retroperitoneal tumors in 2 (8%). The size of the tumor ranged from 1 to 6 cm. The mean duration of surgery was 105 minutes. Intraoperative complications occurred in 3 (12%) patients: 2 (8%) injury of major vessels required conversion to laparotomy, 1 - trauma of duodenum. In 1 (4 %) patient laparoscopic tumoradrenalectomy of the large tumor (6x6x6 cm) complicated by intestinal obstruction required the open re-surgery. Early discontinuation of mechanical ventilation, less severe pain, early activation, better cosmetic effect were observed after MIS compared to open procedures. No local relapses was observed. Median follow-up time was 5,7 months (range 0,4-16,9).

Conclusion: MIS of neuroblastoma may be the procedure of choice in children with localized form of the disease in the absence of contraindications and surgical risk factors (IDRF), especially in children < 1 year due to more favorable biology of the tumor.

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POC064

Outcome and Morbidity of Surgical Resection of Primary Cervical and Cervicothoracic Neuroblastoma in Children: a Comparative Analysis

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Background: Primary cervical (CN) and cervicothoracic neuroblastoma (CTN) is generally associated with good outcome however; surgical resection can be challenging and not without morbidity. The aim of this study is to assess the overall outcome and compare the clinico-radiological features, treatment and complications of CN and CTN.

Methods: Sixteen consecutive patients, (CN=9, CTN=7) treated between November 2006 and December 2012 were selected from the prospective database for this analysis. The median age of 10 boys and 6 girls was 20 months.

Results: The 2-year overall and event free survival of entire cohort is 100% and 72% respectively. Respiratory symptomsdue to compressionof airway and intraspinal extension were common in CTN.Gross total resection was feasible in all patients with CN, in contrast incomplete excision along with significantly longer duration of surgery and more blood loss occurred in CTN. Postoperative morbidity was seen in three patients with CN and only one patient with CTN. The extent of surgery did not affect the overall and event free survival of CTN (p=NS).

Conclusion: CN and CTN have characteristic clinico-radiological presentation and surgical specification. However, both have a favorable outcome, even though with a distinct but acceptable morbidity. The favorable outcome in CTN is unrelated to the extent of surgical excision.

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POC065

Surgical Treatment in Patients Enrolled in the Nationwide Phase Ii Study Nb-Hr07 for Advanced Neuroblastoma: A Report From Japan Neuroblastoma Study Group (Jnbsg)

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Background: Although primary tumor resection remains a mainstay of high-risk neuroblastoma (NB) treatment, the impact of the timing and radicality of surgery on the outcome of the patients remains unclear. We reviewed the surgical treatment of cases enrolled in the NB-HR07 study from this viewpoint.

Methods: Case report and surgical records of 50 high-risk NB cases enrolled in the study were reviewed. Three had mediastinal, and 46 had abdominal tumors. Primary tumor was undetermined in one.

Results: Forty-seven primary tumors were resected, while two remained unresectable. Surgery was performed after 3 or 4 courses of chemotherapy in 29 cases, following the protocol. Seven cases underwent upfront surgery. Surgery was performed after 2 courses of chemotherapy in one. In ten cases, surgery was postponed (until after HDC: high-dose chemotherapy in eight, timing unknown in two). Within the 45 cases with details, results of the surgeries were: gross total resection 32 (71.1%), subtotal resection 10 (22.2%), and partial resection 3 (6.7%). Locoregional recurrence occurred in 11.6% of the cases. Neither timing nor radicality of surgery had impact on the overall survival of the patients.

Conclusion: Radical surgery had been postponed until after HDC in a notable number of cases. The surgeries were performed safely without affecting the overall outcomes in these cases. In the currently ongoing high-risk NB study (NB-HR11), radical surgery is performed after HDC uniformly to evaluate the effect of postponed surgical treatment.

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POC067

Resectability and Morbidity in High-Risk Neuroblastoma with Encasement of Major Visceral Vessels

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Background: Gross total resection in high-risk neuroblastoma correlates with better local control and overall survival. However, in cases with encasement of great visceral vessels a radical resection is in debate.

Methods: 18 children with advanced neuroblastoma got ablative surgery in our institution from 2009 – 2013. Mean age was 5.3+- 4.9 years. According to INSS one child was stage II, 2 patients stage III, and 15 children were stage IV. Preoperatively, ablative surgery had been performed in 15 children, radiation therapy in 2 patients, and chemotherapy in all children. Actual follow-up time is 24.9+-17.6 months.

Results: At surgery, neuroblastoma tissue was found to encase the aorta, caval vein and renal hilus in nine children. Additional involvement of the hepatoduodenal ligament with liver infiltration was found in three, encasement of the celiac trunc in two and involvment of all vital abdominal vessels including the superior mesenteric artery in one child. The thoracic aorta was encased in two patients and additionally the abdominal aorta, azygos and caval vein in one child. We achieved removal of all tumor tissue macroscopically in 13 children by eliciting a separation layer between the tumor and the vessel wall. In five cases, only a partial tumor resection was possible. Complications consisted in one laceration of the thoracic aorta necessitating stent insertion and dialysis, and an abscess around the celiac trunc. All other children recovered uneventfully. A few months after total gross resection we obeserved one kidney shrinkage and two local recurrences.

Conclusion: The possibility to separate neuroblastoma tissue from the vessel wall completely can only be evaluated during surgery. Therefore, encasement of major visceral vessels is no contraindication to surgery in these high-risk patients. Surgery should be part of the interdisciplinary management.

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POC068

Liver Transplantation is a Potentially Life Saving Measure in Neuroblastoma Stage 4S

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Background: Neuroblastoma (NBL) is a common tumor among infants and young children. Arising from primordial neural crest cells, it is responsible for up to 15% of deaths due to cancer in infancy and childhood. Children aged <18 months in general have better outcome, while MYCN-amplification is known to deteriorate prognosis. Though prognosis has improved during the last years, there are patients succumbing to the disease as even aggressive therapy regularly fails or is unacceptable because of toxicities. Stage 4S neuroblastoma is defined as a special stage of neuroblastoma without MYCN-amplification in infants < 12 month with a localized ipsilateral tumor with or without ipsilateral lymphnode involvement, but with skin, liver and/or bone marrow involvement (< 10%) and is generally correlated with good outcome. However up to 20% of patients die of complications such as liver enlargement and consecutive respiratory failure.

Methods: We report a patient who initially presented with agitation. Staging exams and histology of an intrahepatic lesion confirmed suspicion of neuroblastoma stage 4S with multiple metastases infiltrating the liver and a primary in the right adrenal. Two courses of chemotherapy (N4 consisting of vincristine, doxorubicine and cyclophosphamide) and repeated plastic surgery of the abdominal wall could not alleviate the massive compression and eventual liver failure. As an ultimate measure liver transplantation was performed. As the primary graft failed a second transplantation became necessary.

Results: The patient has been free of disease since then and is, except for a global developmental delay, in a good general health condition.

Conclusion: To the best of our knowledge, this is the third reported patient suffering from NBL who required live-saving liver transplantation. Abdominal and respiratory complications, caused by hepatomegaly, are crucial in treating 4S NBLs. Our case demonstrates a conceivable problem-solving approach.

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POC069

Endoscopic Surgery for Neuroblastoma in Children Treated at a Single Institution

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Background: Endoscopic surgery has become the standard procedure for treating most benign adrenal and mediastinal tumors, even in children. However, evidence and indications are lacking with respect to endoscopic resection of adrenal and mediastinal neuroblastomas (NBs) in children. The aim of this study was to describe our indications for and our experience with endoscopic surgery for NB in children treated at a single institution.

Methods: We applied either laparoscopic or thoracoscopic surgery for five NBs in the low-risk group. We performed total extirpation of NB lesions with an Image Defined Risk Factor (IDRF) negative and biopsies of NB lesions with an IDRF-positive. If the tumor was too large to be removed via the umbilical port, we removed the lesion using a Pfannenstiel incision.We focused on early surgical outcomes.

Results: Three of the five patients were male, with a mean age of 24.3± 12.7 months. Four of the five cases involved NB and one case involved ganglioneuroblastoma. Regarding the primary site, four of the five cases were the adrenal glands and one case was the mediastinum. The mean tumor size was 4.4± 2.4 cm (1.8-7.5 cm). All five patients exhibited non-MYCN amplification. We performed total extirpation of four INSS stage 1 lesions with IDRF-negative and biopsies of the tumor and liver in one INSS stage 3 case with IDRF-positive. Two of the five patients required a small incision because the tumor was too large to remove from the port. The average surgical time was 139.8±46.8 minutes. No cases required conversion to laparotomy or a blood transfusion. In addition, all five patients resumed an oral intake on postoperative day 1. Only one patient treated with a biopsy received postoperative chemotherapy. There were no late complications or episodes of recurrence, and the mean follow-up time was 14.0±13.9 months.

Conclusion: Our endoscopic surgical indications for NB are appropriate in children with good outcomes.

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POC070

Results of Treatment of Patients with Neuroblastoma STAGES 4S

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Background: Results of treatment of patients with neuroblastoma STAGES 4S.

Methods: From 2007 to 2013 years we had been observing 98 patients with morphologically confirmed neuroblastoma at the mean age of 2,6 years (from 1 month till 15 years). There were 8 patients (8%) with IVS stage of the disease. In 4 patients, the primary tumor was located in adrenal, and others in the retroperitoneal space. In 3 patients the combined metastasis to the liver and bone marrow, 2 patients in the liver and soft tissues, in 3 patients only in the bone marrow.

Results: For the morphological diagnosis and risk on one stage of treatment in some patients performed adrnalectomy, liver biopsy and soft tissue. All the children had received chemotherapy for the treatment program intermedium risk neuroblastoma (COG A3961). None of the children had not received radiation therapy. With a median follow up of 4 years (range 9 months-5 years), all patients are alive and well. One patient at the age of 1 month with multiple liver metastases died from toxicity of chemotherapy. Two patients continue to have a residual abdominal mass.

Conclusion: Chemotherapy can get excellent results in children with 4S neuroblastoma. The indications for surgical treatment is diagnosis, removal of the primary tumor or the maximum amount of tumor tissue.

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Clinical Research: Trials in Countries with Limited Resources

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POC071

Impact of the Nurse-Led Case Management Program with Adherence to Care on Survival in Children with Neuroblastoma

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Background: Long-term adherence to care is emerging as an important factor for the survival among children with neuroblastoma. A nurse-led case management program was conducted for the children with neuroblastoma in 2009 to arrange their clinic visits, imaging examination and follow-up.

Methods: A longitudinal and comparative design was conducted at a medical center in northern Taiwan. A computerized data collection form was used to record demographic and clinical data through chart review. Follow-up ended at death or at the last clinic visits as of December 31, 2012. A number of 70 children who were diagnosed before 2008 is the group without case management. A number of 72 children is the group receiving case management service.

Results: Children who received case management were more likely adherence constantly to care than those who did not receive case management. We found the group without case management has higher irregular clinic visits or missed outpatient care, without chemotherapy, and loss to follow-up. There is a significant difference for the surgery-related complications during treatment, 36% for group without case management and 5% for the group with case management ($p = 0.025$). There was also a 10-month improvement of median survival, although it did not reach statistical significance.

Conclusion: Adherence to care is a key to increase survival, and case management may play a mediator affecting retention on survival. A nurse case manager can and need to identify high risk patients for irregular attendance and to retain them in neuroblastoma care in order to optimize their treatment outcomes.

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POC072

The Results of Treatment of Standard and High Risk Patients Group with Neuroblastoma. One Centre Experience

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Background: The most common and investigated prognostic factors of neuroblastoma include the child's age, stage, genetic markers (N-myc oncogeneamplification, DNA diploidy), some laboratory parameters. The purpose of this thesis was to study the effect of a combination of some biological signs of neuroblastoma to increase the accuracy for predicting the clinical course of disease.

Methods: In 2007-2013, 143 patients with neuroblastoma: 74 standard-risk (SR) patients and 69 high-risk (HR) patients received treatment in the Scientific and Research Department of Pediatric Oncology at the National Cancer Institute. All enrolled patients received treatment according to NB-2004 and HR-NBL-1/ESIOP protocols. During the late stage of treatment, 54 high-risk patients cured high-dose chemotherapy (HDHT) with autologous stem cell support, of these, 13 patients were cured with tandem HDHT.

Results: The 5-year overall survival (OS) was 67 % for SR patients and 30.4 % for HR patients. The following unfavorable prognostic factors for neuroblastoma were used in our study: the child's age at the time of making a diagnosis, stage, N-myc oncogene amplification. Depending on the child's age: the OS was 58.8 % for patients under 1 year of age, while the OS was 19.2 % for patients aged 1 year or over. We also analyzed the survival rate of high-risk patients based on whether the patient has N-myc gene amplification. The OS was 49.8 % for N-myc negative subjects and 24.3% for N-myc positive subjects. Currently, the OS is 69.2 % for patients whowere treated with tandem HDHT with autologous stem cell support.
Conclusion: The obtained results show that the survival in high-risk patients aged 1 year or over positive for N-myc oncogen was worst greatly than in patients with normal N-myc status. The results of treatment of HR patients which receive tandem HDHT with autologous steam cell support are encouraging.

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POC073

Stage 4S Neuroblastoma: Single Center Experience in Russia

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Background: Stage 4S neuroblastoma is a unique metastatic pattern of disease associated with high rate of spontaneous regression. The purpose of this study was to analyze a group of patients with stage 4S neuroblastoma treated in single center in Russian Federation.

Methods: 169 patients with a diagnosis of "neuroblastoma" were observed and treated during the period 01.2012 - 12.2013. 83 (49%) patients were aged < 1 year, of which stage 4S was diagnosed in 21 (25%) patients. Stage 4S was defined by the criteria of INSS, patients with the primary tumor infiltrating the midline and/or distant lymph nodes involvement were also included. Cytogenetic analysis done by FISH included MYCN, 1p and 11q status. Patients were treated according to NB2004 protocol.

Results: 21 patients were analyzed. Median age 3,1 months (range 0,9-9,7). Male: female ratio was 1,1:1. Diagnosis was confirmed by histology in 14 (67%) cases and was based on clinical data in 7 (33%). Adrenal gland was the most common location of the primary tumor - 13 (62%), followed by posterior mediastinum - 5 (24%), retroperitoneum - 3 (14%). 3 (14%) patients had bilateral adrenal neuroblastoma. The most common site of metastasis was liver - 15 (71%). Cytogenetic analysis revealed 1p deletion in 2 (9,5%) cases, 11q deletion in 1 (5%). 12 (57%) patients received chemotherapy (cyclophosphamide, doxorubicine, vincristine). Median number of courses was 2 (range 1-4). Surgery was done in 12 (57%) cases. Most common indication for therapy was life-threatening respiratory failure (24%). 2 (9.5%) received salvage radiation therapy to the liver. In 2 (9,5%) patients chemotherapy commenced by local physicians was stopped due to lack of indications. 20 (95%) patients are alive, 1 (5%) patient died due to infectious complications. Median time of follow-up was 10 months (range 0,8-23,7).

Conclusion: The management of patients with stage 4S disease requires a differentiated approach and in patients with massive hepatomegaly may require immediate commencement of specific therapy, including radiation therapy.

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POC074

Analysis of Mortality Risks in Infants with Unfavourable Stage 4S Neuroblastoma: A Single Institution Report

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Background: Despite overall good prognosis there is no standard approach to the treatment of stage 4S neuroblastoma. Most patients do not require chemotherapy unless bulk disease is causing organ compromise and risk of death. Significant amount of patients with stage 4S neuroblastoma present with massive dissemination, which leads to rapid deteriorating and is associated with a high mortality rate. The aim of treatment strategy is to identify infants with stage 4S disease who will benefit from chemotherapy to avoid toxicity.

Methods: A retrospective chart review of infants with stage 4S neuroblastoma treated and observed at surgery department of our institution from 2005 to 2013 was performed.11 cases with INSS stage 4S neuroblastoma with symptomatic disease where chemotherapy was administered were analysed. Diagnosis was confirmed by biopsy of the primary lesion or liver metastases. Bone marrow examination performed in all cases. Conventional CT, MRI, and mIBG scan were performed according to the site of involvement.
Results: The primary tumour was localised in the adrenal gland in 7 patients, mediastinum in 1 patient, and multifocal primary tumor was observed in 3 cases. Cyclophosphamide / doxorubicin / vincristine regimen has been used. 5 deaths were observed, 4 of them related to massive hepatomegaly and 1 case as the result of infection. Grade 4 bone marrow, liver and renal toxicities after chemotherpy occured in 9 cases (including all 5 lethal cases).

Conclusion: Infants diagnosed with INSS stage 4S neuroblastoma, particularly those with symtomatic disease (hepatomegaly), had the potential for rapid clinical deterioration. Chemotherapy should be administered only in the cases where threatening symptoms persist and in lower doses in order to avoid toxicity, which contributes to lower survival.

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POC075

Outcome and Clinical Risk Factors in 110 Chinese Neuroblastoma Patients

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Background: To study the clinical characteristics of mediastinal neuroblastoma.

Methods: From 2008 March to 2012 September, the Forth Affiliated Hospital of China Medical University has Admitted 110 cases of neuroblastoma. including 26 cases of mediastinal neuroblastoma and 84 cases of other neuroblastomas.To compare the clinical manifestation, tumor markers, biological prognostic factors of mediastinal neuroblastoma with other neuroblastomas.

Results: The average age of mediastinal neuroblastoma group is 25.5M, which is very similar to other neuroblastomas.88.5% of mediastinal neuroblastomas had syndrome at newly diagnosis, and so did 60.7% in other neuroblastomas ($P<0.05$). The early stage (**I?? II**) cases of mediastinal neuroblastoma group were 34.6%, higher than that of other neuroblastomas which were 8.3% ($P<0.05$). 21.4% of serum NSE levels of Mediastinal neurobJastoma group risen more than 100 ng/ L, lower than 86.1% ($P < 0.05$) in other neuroblastomas . All the cases in mediastinal neurobJastoma had a N-myc copy number of less than 10 copies, while 23.1% in the other neuroblastomas' were more than 10 copies ($P<0.05$).The 4-year overall survival rate was 80% in mediastinal group and 44% in the other neuroblastomas. Of the cases whose primary tumors were in localized neuroblastoma, the 4-year survival rate was 100%, significantly higher than 82%in other neuroblastomas.

Conclusion: Majority of the mediastinal neuroblastoma cases perform early clinical stage and favorable biological prognostic factors.These may associated with the prognosis of mediastinal neuroblastoma.

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POC076

Combined Treatment Strategy and Outcome of High Risk Neuroblastoma: Experience of the Children's Cancer Hospital-Egypt

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Background: Neuroblastoma (NB) is remarkable for its wide spectrum of clinical

behavior and biological characteris- tics in relation to outcome. The use of aggressive therapy, including autologous hematopoietic stem cell transplantation (HSCT) and the addition of isoretionin (cis-Retinoic Acid/cis-RA), has increased survival rates of patients with ad- vanced disease.

Methods: 271 newly diagnosed pediatric high risk NB patients were prospectively enrolled into the study. Patients received neoadjuvant chemotherapy of alternating cycles: [cyclophosphamide, doxorubicin, vincristine (CAdO)] and [etoposide, carboplatin]. Intensification courses of "ICE" (ifosfamide, carboplatin, and etoposide) regimen were administered to patients with bone marrow (BM) residual infiltration. Whenever safely feasible, complete surgical resection or debulking of the primary tumor was attempted for patients achieving partial response. Eligible patients underwent HSCT, while radiation therapy to the primary and metastatic sites, as well as maintenance with cis-RA was given for 6 months.

Results: The median age of our patients was 2.8 years with male to female ratio of 1.65:1. At 4 years, the overall and event free survivals were 33.7% and 23.3% for the entire group under study, with significantly higher rates (42.7% and 35.6%, respectively) for HSCT patients ($n = 94$; $p < 0.001$). The outcome was also significantly correlated with response to induction therapy, pathological subtype, as well as other variables.

Conclusion: Myeloabla- tive therapy followed by stem cell rescue is regarded as the most important goal of high risk NB treatment to improve survival till present. Each of consolidation HSCT, post induction disease status, as well as international neuroblastoma pathology classification (INPC) subtype was an independent predictive variable of survival. A collaborative effort with an emphasis on biologic characteristics of aggressive disease and tailored therapy needs to be strengthened to further our understanding of this disease.

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