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Molecular Characteristics of Neuroblastoma with Special Reference to Novel Prognostic Factors and Diagnostic Applications

by

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To the Angels

ABSTRACT

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Molecular Characteristics of Neuroblastoma with Special Reference to Novel Prognostic Factors and Diagnostic Applications

Department of Medical Biochemistry and Genetics
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Background: Neuroblastoma, which is the most common and extensively studied childhood solid cancer, shows a great clinical and biological heterogeneity. Most of the neuroblastoma patients older than one year have poor prognosis despite intensive therapies. The hallmark of neuroblastoma, biological heterogeneity, has hindered the discovery of prognostic tumour markers. At present, few molecular markers, such as *MYCN* oncogene status, have been adopted into clinical practice.

Aims: The aim of the study was to improve the current prognostic methodology of neuroblastoma, especially by taking cognizance of the biological heterogeneity of neuroblastoma. Furthermore, unravelling novel molecular characteristics which associate with neuroblastoma tumour progression and cell differentiation was an additional objective.

Results: A new strictly defined selection of neuroblastoma tumour spots of highest proliferation activity, hotspots, appeared to be representative and reliable in an analysis of *MYCN* amplification status using a chromogenic *in situ* hybridization technique (CISH). Based on the hotspot tumour tissue microarray immunohistochemistry and high-resolution oligo-array-based comparative genomic hybridization, which was integrated with gene expression and *in silico* analysis of existing transcriptomics, a polysialylated neural cell adhesion molecule (NCAM) and poorly characterized amplicon at 12q24.31 were discovered to associate with outcome. In addition, we found that a previously considered new neuroblastoma treatment target, the mutated c-kit receptor, was not mutated in neuroblastoma samples.

Conclusions: Our studies indicate polysialylated NCAM and 12q24.31 amplicon to be new molecular markers with important value in prognostic evaluation of neuroblastoma. Moreover, the presented hotspot tumour tissue microarray method together with the CISH technique of the *MYCN* oncogene copy number is directly applicable to clinical use.

Key words: neuroblastoma, polysialic acid, neural cell adhesion molecule, *MYCN*, c-kit, chromogenic *in situ* hybridization, hotspot

TIIVISTELMÄ

Miikka Korja

Neuroblastoomien molekulaariset ominaisuudet ja erityisesti uudet ennustetekijät ja diagnostiset menetelmät

Lääketieteellinen biokemia ja genetiikka
Annales Universitatis Turkuensis, Medica-Odontologica, 2009, Turku, Suomi
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Tausta: Neuroblastooma, joka on yleisin ja tutkituin lapsuusiän kiinteä syöpä, on kliinisesti ja kasvainbiologisesti huomattavan heterogeeninen kasvain. Suurimmalla osalla yli vuoden ikäisistä neuroblastoomapotilaista on huono ennuste huolimatta intensiivisistä hoidoista. Neuroblastooman tunnusmerkki, biologinen heterogeenisyys, on hankaloittanut ennusteellisten kasvainmerkkitekijöiden löytämistä. Kliinisessä käytössä on tällä hetkellä vain muutama molekyylimerkkitekijä, kuten esimerkiksi *MYCN*-syöpägeenimääritys.

Tavoitteet: Tutkimuksen tavoitteena oli parantaa nykyisiä neuroblastooman ennustearviomenetelmiä, erityisesti neuroblastooman kasvainbiologinen heterogeenisyys huomioiden. Lisäksi tavoitteena oli löytää uusia molekyylibiologisia ominaispiirteitä, jotka ovat osallisena neuroblastooman etenemisessä ja syöpäsolujen erilaistumisessa.

Tulokset: Täsmällisesti määritelty, neuroblastooman korkeimman solunjakautumisaktiivisuuden omaavan kasvainkohdan, hotspotin, valinta *MYCN* -geenimonistuman määritykseen kromogeenisella *in situ* hybridisaatiolla (CISH) vaikuttaa olevan edustava ja luotettava uusi geenimonistuman määritysmenetelmä. Polysialyloidun neuraalisen soluadheesiomolekyylin (NCAM) ja huonosti karakterisoidun geenimonistuma-alueen 12q24.31 havaittiin assosioituvan syövän lopputuloksen kanssa. Nämä tulokset perustuivat hotspot-kohtiin pohjautuvien kasvainkudoskokoelmien immunohistokemiaan, korkearesoluutioisen vertailevan genomisen hybdridisaation löydöksiin sekä olemassa olevan *in silico* -datan hyväksikäyttämiseen. Lisäksi totesimme uudeksi neuroblastooman hoitokohdemolekyyliksi ehdotetun mutatoituneen c-kit -reseptorin olevan neuroblastoomanäytteissä mutatoitumaton, ja näinollen huono hoitokohdemolekyyli.

Päätelmät: Tutkimuksemme osoittaa, että polysialyloitu NCAM ja 12q24.31 geenimonistuma-alue ovat uusia molekulaarisia merkkitekijöitä, joilla on tärkeä merkitys neuroblastooman ennustetta arvioitaessa. Lisäksi kuvattu hotspot kasvainkudoskokoelma yhdessä CISH-tekniikan kanssa soveltuu suoraan kliiniseen käyttöön *MYCN*-syöpägeenin kopioluvun määrittämiseksi.

Avainsanat: neuroblastooma, polysialohappo, neuraalinen soluadheesiomolekyyli, *MYCN*, c-kit, kromogeeninen *in situ* hybridisaatio, hotspot

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ABBREVIATIONS

aCGH	Array-based comparative genomic hybridization
BAC	Bacterial artificial chromosome
CGH	Comparative genomic hybridization
CISH	Chromogenic in situ hybridization
CT	Computed tomography
DFS	Disease-free survival
EFS	Event-free survival
EST	Expressed sequence tag
FISH	Fluorescent *in situ* hybridization
GIST	Gastrointestinal stromal tumour
HPLC	High performance liquid chromatography
HSR	Homogeneously staining region
HVA	Homovanillic acid
INPC	International Neuroblastoma Pathology Committee
INRG	International Neuroblastoma Risk Group
INRGSS	INRG Staging System
INSS	International Neuroblastoma Staging System
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
MIBG	Meta-iodobenzylguanidine
MRD	Minimal residual disease
MKI	Mitosis-karyorrhexis index
MRP	Multidrug resistance-associated protein
MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NCAM	Neural cell adhesion molecule
NSE	Neuron-specific enolase
OS	Overall survival
PCR	Polymerase chain reaction
PET	Positron emission tomography
PI	Proliferation index
PolySia	Polysialic acid
PolySia-NCAM	Polysialylated NCAM
RT-PCR	Real-time polymerase chain reaction
SNS	Sympathetic nervous system
TMA	Tissue microarray
Trk	Tyrosine kinase receptor
VMA	Vanillymandelic acid

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following three publications (I-III) and one unpublished manuscript (IV).

I. Korja M, Finne J, Salmi TT, Kalimo H, Karikoski R, Tanner M, Isola J, Haapasalo H. Chromogenic in situ hybridization-detected hotspot MYCN amplification associates with Ki-67 expression and inversely with nestin expression in neuroblastomas. **Mod Pathol.** 2005;18: 1599-605.

II. Korja M, Finne J, Salmi TT, Haapasalo H, Tanner M, Isola J. No GIST-type c-kit gain of function mutations in neuroblastic tumours. **J Clin Pathol.** 2005;58: 762-5.

III. Korja M, Jokilammi A, Salmi TT, Kalimo H, Pelliniemi TT, Isola J, Rantala I, Haapasalo H, Finne J. Absence of polysialylated NCAM is an unfavorable prognostic phenotype for advanced stage neuroblastoma. **BMC Cancer.** 2009;9: 57.

IV. Wolf M, Edgren H, Kilpinen S, Korja M, Haapasalo H, Kallioniemi OP. High-resolution array-CGH integrated with gene expression and in silico analysis of existing transcriptomics data suggest a novel oncogene at 12q24 in neuroblastoma. **Manuscript**.

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1 INTRODUCTION

Neuroblastoma is a malignant tumour of undifferentiated neuroectodermal cells, which are derived from the neural crest. These neural crest precursor cells are committed to differentiate into cells that make up sympathetic ganglia or the adrenal medulla; therefore, neuroblastoma can be considered to be a tumour of the sympathetic nervous system (SNS). Neuroblastoma is the most common extracranial solid tumour of childhood, comprising 8-10% of all childhood cancers (Bernstein et al., 1992). Approximately 30% of all neuroblastoma cases are diagnosed during the first year of life, nearly half of the newly diagnosed patients are between the age of one and four years, roughly 80% of patients are younger than four years (Brodeur and Castleberry, 1997), and the median age at diagnosis is 22 months (Brodeur and Castleberry, 1997). The overall incidence is estimated to be one case per 10,000 live births (Gurney et al., 1995), which corresponds roughly to six new neuroblastoma cases per year in Finland. Despite the relatively low incidence, neuroblastoma accounts for 15% of all childhood cancer deaths, indicating the poor prognosis of the disease (Grovas et al., 1997; Maris and Matthay, 1999; Cotterill et al., 2000).

Neuroblastoma is often described as an enigmatic cancer with characteristics of heterogeneous pathology and diverse clinical behaviour that ranges from spontaneous regression to rapid malignant progression (Brodeur and Castleberry, 1997). Interestingly, the incidence of spontaneous regression in neuroblastoma is between 10 and 100 times greater than in any other human cancer (Pritchard and Hickman, 1994). Due to the diverse spectrum of clinical behaviour and biological characteristics, there is a strong possibility that biological and morphological features together with evident genomic alterations can be used as clinically significant biomarkers to stratify neuroblastoma patients into well-defined risk groups. However, only one biomarker, *MYCN* oncogene copy number, is currently in widespread clinical use. Therefore, knowledge of new prognostic markers is needed to improve current treatment strategies and to develop new treatment options. New clinically significant biomarkers could also help to understand the genesis of this disease.

Currently, neuroblastomas are classified into five clinical stages, ranging from the localized stage 1 neuroblastoma to stage 4 neuroblastoma with extensive dissemination (Brodeur et al., 1993). Patients with stage 1 and 2 neuroblastomas are usually treated with surgery alone, whereas patients with stage 4 tumours receive advanced radiation together with chemotherapy. Some neuroblastomas fall within the intriguing stage 4s category, in which tumours can regress spontaneously. Stage 4s is characterized by neuroblastoma dissemination to liver, skin and/or bone marrow. These neuroblastomas occur mainly in infants and, despite their disseminated disease, the overall survival (OS) rate of stage 4s patients is approximately

80%, in contrast to the 20% survival rate of stage 4 patients (Grovas et al., 1997; Cotterill et al., 2000) In brief, patients with stage 1, 2, or 4s disease, or presenting in the first year of life, normally have a good prognosis, whereas most patients over the age of one year at diagnosis have metastatic (stage 4) disease with poor survival rates.

Despite current multimodal cancer therapies and extensive neuroblastoma research, neuroblastoma has remained a treatment challenge with an unpredictable clinical course and dismal overall prognosis for advanced-stage disease. Currently, prognostic and therapeutic risk stratification is based primarily on age (dichotomized around 18 months), stage, and *MYCN* amplification status. These evaluations often overestimate the number of patients in need of chemotherapy, because the evaluation cannot define patients with stage 1–3 tumours that are capable of spontaneous or easily induced regression. The prognostic and therapeutic evaluation of stage 2-4 patients with the normal *MYCN* copy number is often challenging, too.

In order to facilitate screening, diagnosis, prognosis and monitoring of neuroblastoma patients, a vast number of biological tumour characteristics have been investigated in recent years. In addition to *MYCN* oncogene copy number, studies have identified new prognostic tumour markers, such as tumour ploidy (the number of homologous sets of chromosomes in a cell), deletion or loss of heterozygosity (LOH) of chromosome 1p and gains of chromosome 17q, all of which have been associated with OS or disease-free survival (DFS). However, due to the tremendous biological heterogeneity of neuroblastoma, it has proven difficult to achieve congruent and repeatable study results of new prognostic tumour markers. Since one single neuroblastoma sample has multiple different tumour components, it would be of the utmost importance to unify and standardize the protocols and methods of neuroblastoma sample analysis. Before a standardized methodology in neuroblastoma research is reached, large, controlled, prospective and multicentre clinical biomarker studies are probably groundless.

The present study was conducted to establish new prognostic markers and a strictly defined methodology with clinical value for screening, diagnosis, prognosis, and monitoring of neuroblastoma. At their best, the study results should facilitate the development of future research strategies by establishing the importance of the new methodology and markers studied.

2 REVIEW OF THE LITERATURE

Neuroblastoma represents a serious challenge to researchers and clinicians who have a passion for understanding paediatric tumours. As shown by the great number of published papers over the past 30 years, neuroblastoma serves as a peculiar research model with a wide range of research applications and future perspectives.

2.1 General aspects of neuroblastoma

The clinical as well as biological hallmark of neuroblastoma is its variability. Stage 4 neuroblastomas are aggressive, chemoresistant, and in practice incurable. Children younger than one year have often low-stage neuroblastoma, *i.e.* stages 1, 2, and exceptional 4s. Stages 1 and 2 are generally chemosensitive and thus relatively often curable. Stage 4s neuroblastoma, defined as a small primary tumour in the abdomen or thoracic cavity accompanied with metastases in the liver, skin or bone marrow but not in the cortical bone, almost always regress spontaneously without any treatment. D'Angio and colleagues were the first who described children with the clinical phenotype of stage 4s (s = special) disease (D'Angio et al., 1971), which occurs in about 5% of cases. Although spontaneous regression is most commonly a unique phenomenon of stage 4s neuroblastoma, it has been described as also occurring in stage 1–3 neuroblastomas, even in patients older than one year (Fiorillo et al., 1982; Eklof et al., 1983).

2.1.1 Epidemiology

Neuroblastoma accounts for approximately 8-10% of childhood cancers (Bernstein et al., 1992), as mentioned. The median age at diagnosis is 18-22 months, and more than 95-98% of patients are diagnosed before the age 10 of years (Castleberry, 1997; Brodeur and Maris, 2002). The overall incidence is estimated to be one case per 7,000-10,000 live births, and there are about 700 new cases per year in the United States (Gurney et al., 1995; Brodeur and Maris, 2002). The incidence is fairly uniform throughout the world, at least for industrialized countries. There appears to be slight sex predominance with a male-to-female ratio of 1.2:1 (Cotterill et al., 2000).

2.1.2 Pathophysiology

Neuroblastoma is an embryonic tumour of neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system. These precursor or immature cells of the postganglionic neurons (or their precursors) locate in the paravertebral ganglia of the sympathetic trunk, in

the preaortic ganglia of plexuses surrounding the main branches of the abdominal aorta, or in the adrenal gland (a modified sympathetic ganglion). Also, paraganglia, discrete encapsulated neuroendocrine structures adjacent to sympathetic ganglia, may provide a site for tumour origin, especially in the prenatal period.

As in embryonic tumours, neuroblasts of neuroblastoma are histologically indistinguishable from developing neuroblastic cells in the embryo. The precise etiology of neuroblastoma is unknown, but it seems unlikely that environmental exposures or hereditary factors play an important role. However, a family history of neuroblastoma has been identified in 1–2% of cases (Shojaei-Brosseau et al., 2004). The concordance for neuroblastoma in twins during early childhood and the discordance in older twins indicate that hereditary factors might be predominant especially in familial neuroblastoma of infancy (Kushner and Helson, 1985). Indeed, familial cases are often diagnosed at an earlier age (Kushner and Helson, 1985). In brief, neuroblastoma is a complex embryonic tumour, in which multiple sporadic genetic aberrations are probably needed for tumourigenesis.

2.1.2.1 Normal SNS development

The development of the sympathetic nervous system (SNS) originates from the emergence of pluripotent neural crest cells from the neural tube. These pluripotent cells migrate and aggregate adjacent to the dorsal aorta to form the primary sympathetic chain (Francis and Landis, 1999). This step involves two distinctly different neural crest populations, from which the sympathoadrenal and sympathoenteric lineages are derived (Sieber-Blum, 2000). Next, precursor cells in the primary sympathetic chain form the paravertebral ganglia and also migrate to generate the primordial (earliest recognizable stage) of the prevertebral ganglia in the celiac and mesenteric plexuses, which locate next to the main branches of the abdominal aorta. A sub-population of migrating cells respond to glucocorticoids and enter the adrenal primordium, when they lose neuronal traits and begin to express endocrine markers (Sieber-Blum, 2000). These so-called chromaffin cells are the major sympathetic cell type of the adrenal gland and the paraganglia, but also sympathetic neuroblasts are also frequently seen in developing human foetal adrenal glands (Hoehner et al., 1996). During the neuronal development of the SNS, neurons are initially overproduced, as in the central nervous system, and cell survival is dependent on the establishment of synaptic connections and the production of appropriate neurotransmitters (Francis and Landis, 1999). Thus, there is a significant level of programmed cell death during the development of the SNS.

2.1.2.2 Neuroblastoma development

Tumourigenesis and tumour progression are thought to be a multi-step process in which genetic aberrations accumulate sequentially, eventually leading to the neoplastic phenotype (Fearon and Vogelstein, 1990). Previous data suggests that instead of a simple linear progression model, a more complicated genetic evolution pattern takes place in neuroblastoma (Westermann and Schwab, 2002). The embryonic nature of neuroblastoma suggests that an early disruption of normal developmental processes plays a role in tumour initiation. The extreme heterogeneity of neuroblastoma has raised questions regarding different subtypes of neuroblastoma represent more than one distinctly different disease. However, as a common sympathoadrenal precursor is thought to give rise to three distinct sympathetic neuronal/neuroendocrine lineages: 1) the neuronal/ganglionic, 2) the small intensely fluorescent (SIF), and 3) the chromaffin lineages (Patterson, 1990), it is more likely that immature sympathetic tumour precursors are arrested at various stages of differentiation, thus explaining at least partially the heterogeneity of the disease. The relatively high incidence of spontaneous regression and differentiation of neuroblastoma resembles normal neuronal differentiation and programmed cell death (apoptosis) during the development of the nervous system. This similarity indicates indirectly that neuroblastoma cells may retain the genetic programme of their normal counterparts. Indeed, comparison of marker genes expressed in developing human SNS and neuroblastoma has revealed that neuroblastoma has immature neuronal characteristics, which suggests that it truly is derived from precursors or immature cells of the SNS lineage (Hoehner et al., 1996).

2.1.3 Localization and clinical presentation

Due to its neural crest origin, neuroblastoma may occur anywhere along the sympathetic chain. Hence, presenting signs and symptoms are highly variable and commonly a manifestation of tumour location. About half of all neuroblastomas arise in the adrenal medulla, and the remainder originate in paraspinal sympathetic ganglia in the chest, neck or abdomen, or in pelvic ganglia (Brodeur and Maris, 2006). The intraabdominal neuroblastoma, especially when retroperitoneal, tends to present as an incidental finding detected by parents or during routine clinical visits. Similarly, thoracic neuroblastoma presents often as an incidental mass on chest X-rays. Pelvic masses may produce urinary retention or constipation due to compression of the bladder or rectosigmoid colon. Primary or metastatic cervical neuroblastoma can occasionally compromise sympathetic innervations leading to Horner's syndrome, *i.e.* miosis, ptosis and anhidrosis on the affected side of the face, or cause cervical lymphadenopathy. Around 40% of patients will present with localised disease, most of which have favourable biological features and are successfully treated with surgery alone (Matthay et al., 1989; Evans et al., 1996; Kushner et al., 1996; Alvarado et al., 2000; Perez et al.,

2000). About half of all patients present with evidence of haematogenous dissemination at diagnosis. Unlike patients with localised disease, who often have only few minor symptoms at diagnosis, patients with metastatic neuroblastoma have more extensive tumour burdens and are therefore in poorer health at presentation. Dissemination in the central nervous system is a rare event (Ahdevaara et al., 1977), but can occur especially with progression or relapse of the disease. In general, severe symptoms occur when the tumour has reached a critical size and/or developed metastases.

The most common clinical symptoms are typically nonspecific symptoms, such as pain, fever, weight loss, and general malaise. If the tumour bleeds spontaneously, an acute onset of abdominal pain may occur. Bone metastases present usually with local bone pain and sometimes with sudden changes in the activity level of children. In the case of skull involvement, periorbital ecchymoses and/or proptosis may occur. Paraspinal tumours may extend through the vertebral foramina and compress the spinal cord, thereby generating motor or sensory deficits of spinal origin. During infancy, blueberry muffin syndrome, *i.e.* painless and bluish subcutaneous nodules, is a relatively common feature in stage 4s disease. Blueberry muffin syndrome patients have a favourable prognosis with frequent spontaneous tumour regression. Paraneoplastic syndromes are rare, but a neuroblastoma that secretes vasoactive intestinal peptide may typically cause intractable secretory diarrhoea with subsequent dehydration and hypokalemia (El Shafie et al., 1983). Opsoclonus-myoclonus syndrome, another neuroblastoma induced paraneoplastic syndrome, consists of involuntary jerking limb movements, ataxia, and rapid conjugated eye movements. These symptoms are probably caused by autoantibodies against the cerebellar and/or cerebral neural tissue. Symptoms of opsoclonus-myoclonus syndrome tend to persist despite successful treatment of neuroblastoma, and they may result in neuro-developmental delay (Hiyama et al., 1994).

2.1.3.1 Metastasis

Since neuroblastoma is intratumorally heterogeneous, it contains numerous subpopulations of cells with different biological characteristics. Only a small subpopulation of cells in a primary tumour has the potential to disseminate (Fidler, 1970), and it has appeared difficult to identify these cells in neuroblastoma. Metastasis, which is the leading cause of death in neuroblastoma, has remained considerably less well understood. The discovery of new metastasis-specific pheno- and genotypes of neuroblastoma would help to define appropriate treatment strategies.

Approximately 75-80% of patients over the age of one year have widespread metastases (Brodeur and Castleberry, 1993), *i.e.* stage 4 disease. Metastasis in neuroblastoma occurs in 70% of the cases in the bone marrow and in 56% of the cases in the bone itself (DuBois et al., 1999). Other common sites of

metastasis, which occurs via lymphatic and haematogenous routes, are the lymph nodes, liver and lung (Brodeur and Castleberry, 1993).

The first step of metastasis is local invasion to surrounding tissues, which requires that malignant cells lose cell–cell adhesions and become motile. Interestingly, overexpression of the MYCN protein in neuroblastoma has been shown to diminish the levels of the neural cell adhesion molecule (NCAM) protein dramatically, and contribute therefore to the increased metastatic activity of *MYCN*-amplified neuroblastomas (Akeson and Bernards, 1990). NCAM molecules can have a covalently attached polymer, polysialic acid, on their cell surfaces. Polysialylation of NCAM molecules has been reported to reduce the adhesive properties of cell-cell adhesions (Cunningham et al., 1983; Edelman, 1983), and subsequently increase the metastatic activity of *e.g.* small cell lung carcinomas and Wilms' tumours (Roth et al., 1988; Tanaka et al., 2000).

2.1.4 Routine clinical assessment of disease

Diagnosis of neuroblastoma is based on the presence of characteristic histolopathological features of a tumour sample, which includes tumour phenotypes from undifferentiated to more differentiated neuronal (sympathetic nervous system) or neuroendocrine characteristics. Tumour-specific histopathological assessment is a crucial determinant of treatment planning, especially for children younger than 18 months. For example, variable tumour growth activity from regressive to actively proliferating status has an effect on treatment decisions.

In addition to surgically-achieved histopathological samples of neuroblastoma, which can be discovered and localized by various imaging modalities, raised concentrations of urinary catecholamines support the diagnosis of neuroblastoma. The routine clinical assessment of the extent of neuroblastoma also includes bone marrow aspirates or biopsies, which have to be of sufficient size to provide material for possible genetic analyses, such as for the *MYCN* amplification status.

2.1.4.1 Imaging modalities

A number of imaging modalities are often utilized in diagnostic and follow-up studies. Ultrasonography is rarely but occasionally used in determining the consistency of neuroblastoma, especially of abdominal neuroblastoma. Chest X-ray may aid in detecting tumour calcification, and in differentiating neuroblastoma from other tumours. However, contrast-enhanced computed tomography (CT), is the gold standard of imaging studies of neuroblastoma and provides information on tumour consistency, calcification, localization and

distant organ involvement. Therefore, CT is the preferred modality for the assessment of neuroblastoma in the abdomen, pelvis or mediastinum.

Magnetic resonance imaging is beneficial for paraspinal lesions and for assessing the degree of tumour extension into the spinal canal, particularly when intra-foraminal lesions with the potential for spinal cord compression are suspected. CT and magnetic resonance imaging are both used in follow-up studies to control residual or residive neuroblastoma. Radiolabelled meta-iodobenzylguanidine (MIBG) scans dramatically enhance sensitivity and specificity for detecting bone metastases and bone marrow disease (Andrich et al., 1996). Since MIBG also accumulates in soft tissue disease, more than 90% of primary and metastatic neuroblastomas can be specifically detected with MIBG scintigraphy (Geatti et al., 1985; Voute et al., 1985). MIBG scintigraphy is a recommended imaging modality both during and after therapies (follow-up imaging) in high-risk patients (Kushner et al., 2003), in whom MIBG positive residual disease after therapy is a significant unfavourable prognostic factor (Schmidt et al., 2008). However, MIBG scanning may be negative for a subset of highly undifferentiated neuroblastoma. Since a technetium 99m methylene diphosphonate scan reduces the number of false negatives (Gordon et al., 1990), this scanning modality is recommended when MIBG is negative or unavailable. The sensitivity and specificity of positron emission tomography (PET) for detection and follow-up of neuroblastoma has been assessed and compared to MIBG scintigraphy (Kushner et al., 2001b; Scanga et al., 2004), but currently PET is utilized mainly for research purposes.

2.1.4.2 Urinary catecholamines VMA and HVA

The adrenal gland and sympathetic nerve ganglia normally secrete catecholamines, especially noradrenalin. In the majority of neuroblastoma patients, there is an abnormality of catecholamine excretion. Most consistently, there is an increase in the urinary levels of vanillymandelic acid (VMA) (Gitlow et al., 1970), which is a metabolite of noradrenalin. An assessment of urinary catecholamines and their metabolites is an important adjunct to differentiate neuroblastoma from small round cell tumours, and to detect primary tumours or early neuroblastoma relapses. However, reviewing the literature has provided evidence that the poor quality of previously published results prevents a quantitative evaluation of the frequently used practice to measure VMA (Riley et al., 2004). In addition, the single parameters of catecholamine metabolites, such as VMA, homovanillic acid (HVA) and dopamine, have low sensitivity in detecting low-stage neuroblastoma, probably as a consequence of low tumour burden (Strenger et al., 2007). However, high VMA:HVA ratios have been seen in patients with a favourable prognosis; therefore, a VMA:HVA ratio has been proposed as a parameter to identify high-risk and low-risk patients (Brodeur and Castleberry, 1993; Strenger et al., 2007). Since no better non-invasive predictive parameters are available, elevated urinary catecholamine

metabolites, especially HVA and VMA (Laug et al., 1978; LaBrosse et al., 1980), are routinely used in new and follow-up screenings of neuroblastoma.

2.1.4.3 Bone marrow samples

Bilateral bone marrow aspirates and biopsies together with standard histological analyses should be used for the detection of neuroblastoma cells in bone marrow, as these analyses are a prerequisite for correct pretreatment staging and monitoring of response to therapy (Brodeur et al., 1988b; Brodeur et al., 1993). The identification of cell clumps, neuroblastoma rosettes (Mills and Bird, 1986), or accompanying stromal changes (Reid and Hamilton, 1988) has been the gold standard of the morphology-based screening of bone marrow aspirates (Head et al., 1979) and trephine biopsies (Bostrom et al., 1985).

It has appeared that morphological techniques alone are insufficiently reliable and sensitive to monitor minimal residual disease (MRD), seeing that contaminations of 0.1% in conventional cytology and 1% in a histological screening are often not detectable (Cheung et al., 1997; Mehes et al., 2003), and even a 10% tumour cell infiltrate can be overlooked in conventional cytology of bone marrow smears (Mehes et al., 2003). However, conventional cytology remains the only accepted procedure for detecting disseminated neuroblastoma cells in bone marrow, according to the exclusively used International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993).

There is a large body of published papers regarding the use of immunocytochemical stainings, polymerase chain reaction (PCR)-based technologies and flow cytometry in detection of neuroblastoma cells and neuroblastoma-specific transcripts in bone marrow, but whether increased sensitivity in detecting primary bone marrow dissemination or MRD provides additional prognostic information is obscure. For the reason that alkaline phosphatase-based antibody detection systems are not specific, since some hematopoietic cells express alkaline phosphatase causing false positive results (Borgen et al., 1998), high sensitivity fluorescence-based detection techniques have been widely tested, too. However, even though fluorescence-based techniques are used in a number of laboratories for a quick and sensitive detection of many different types of tumour pheno- and genotypes, there are no routine fluorescence-based techniques used in neuroblastoma diagnostics. In brief, the clinical utility of improvements in the detection of molecular markers remains still unclear. This may be partly explained by the small number of patients in most of the reported studies, the inconsistency in methodology, and discrepancies in reporting the results.

2.1.5 Current prognostic risk stratification

In an effort to develop an International Neuroblastoma Risk Group (INRG) classification system, in 2005 a representative team of major paediatric groups around the world reviewed data of 11054 neuroblastoma patients treated in Europe, Japan, USA, Canada, and Australia between 1974 and 2002. According to the consensus decision, age (dichotomized around 18 months), stage (assessed before treatment), and *MYCN* status were considered to be significant risk factors for neuroblastoma (Cohn et al., 2009). Unfortunately, these risk factors cannot stratify patients with (spontaneously) regressing stage 1–3 tumours, and therefore the number of patients in need of chemotherapy can be easily overestimated. Therefore, histological category, grade of tumour differentiation, 11q aberrations and tumour cell ploidy were included in the INRG classification system (Table 1) (Cohn et al., 2009), since all these factors appeared to be statistically significant and clinically relevant in prognostic evaluations (Cohn et al., 2009). Currently, however, only some groups and hospitals include tumour histology and genetic aberrations in a risk group assessment. Very recently, a new INRG Staging System (INRGSS) for pretreatment risk stratification was reported (Monclair et al., 2009). The new INRG classification system (Cohn et al., 2009) (Table 1) and INRGSS (Monclair et al., 2009) has not been in long-term clinical use as yet.

Table 1. International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification schema (table from Cohn et al., 2009). GN = ganglioneuroma, GNB = ganglioneuroblastoma.

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

2.1.5.1 Stage

In an effort to facilitate comparison of the results of clinical trials performed throughout the world, the International Neuroblastoma Staging System (INSS) was published in 1993 (Brodeur et al., 1993), and it has been in use in numerous European and North American countries ever since. The INSS stratification is based partially on surgical result (Table 2). Stages 1, 2 and 4s are commonly considered to be favourable, but infants (under one year of age) belong to the favourable group regardless of the INSS group (Castleberry et al., 1994). Since surgical approaches differ from one institution to another, and not all neuroblastomas are treated with surgery, the INSS definitions for patients with locoregional disease vary substantially between institutes. Thus, the staging system is not highly reliable. The 4-year OS rate for infants with the INSS definitions 1, 2, 3 and 4s is 98.5%, whereas the OS for the INSS definition 4 patients is 73.1% (Ikeda et al., 2002). Interestingly, the 4-year OS rate for patients over one year of age is 100% in stage 1, 2, 3, and 4s tumours, but only 48.5% for stage 4 tumours (Ikeda et al., 2002). In the proposed INRG staging system (Monclair et al., 2009), the extent of neuroblastoma is defined by pretreatment imaging studies and bone marrow morphology. Radiological features distinguish locoregional tumours involving local structures (INRG stage L1) from locally invasive tumours (INRG stage L2), and stages M and MS are proposed to categorise neuroblastomas that are widely disseminated or have an INSS 4S pattern of disease (Table 2), respectively (Monclair et al., 2009). In terms of documenting stage and extent of the disease, the generally utilized method is still the INSS definition (Table 2) (Brodeur et al., 1993).

Table 2. International Neuroblastoma Staging System (INSS).

Stage	Definition
1	Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive)
2A	Localised tumour with incomplete gross excision; representative ipsilateral non-adherent lymph nodes negative for tumour microscopically
2B	Localised tumour with or without complete gross excision, with ipsilateral non-adherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically
3	Unresectable unilateral tumour infiltrating across the midline, with or without regional lymph node involvement; or localised unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement
4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S)
4S	Localised primary tumour (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver and/or bone marrow (limited to infants <1 year of age)

2.1.5.2 Age

Age has usually been analysed as a dichotomized variable in neuroblastoma risk stratifications. Despite extensive clinical research, there is currently no consensus on age grouping for stratifying neuroblastoma patients, in particular for INSS 4 neuroblastoma patients. There is emerging evidence that the suggested cut-off age of 18 months may be less predictive in prognostic evaluations than previously thought. Moreover, neuroblastomas in adolescents and adults indicate that age categories have a number of limitations that should be noted when designing therapeutic protocols for older patients. Analysis of data for 3666 patients (the same patients as in the INRG classification system analysis) with neuroblastoma suggests that a cut-off of 460 days (around 15 months) might be more reliable than the cut-off age of 18 months (London et al., 2005). The 4-year event free survival (EFS) rate for patients younger than 460 days was 82%, whereas the same EFS rate for patients older than 460 days was 42% (London et al., 2005). There are ongoing clinical trials which are testing the safety of therapy reduction in children between 12 and 18 months of age with INSS 3 or 4 neuroblastoma and favourable biological characteristics (no *MYCN* amplification).

2.1.5.3 *MYCN* amplification

In 1983, neuroblastoma cell lines and a primary tumour were shown to have an amplified domain with sequence homology to the human cellular oncogene *c-myc* (Schwab et al., 1983). The amplified gene in the short arm of chromosome 2 was termed *N-myc (MYCN)* (Kanda et al., 1983; Kohl et al., 1983; Schwab et al., 1984). *MYCN* amplification results in high concentrations of a nuclear phosphoprotein Myc, but the detrimental mechanism by which Myc protein contributes to a more aggressive phenotype is still uncertain. However, *MYCN* is an oncogenic transcription factor regulating proliferation, differentiation, transformation and apoptosis of cells (Luscher and Larsson, 1999; van Noesel and Versteeg, 2004).

MYCN amplification is detected in roughly 20-30% of primary neuroblastomas, and it strongly correlates with rapid disease progression, treatment failure and poor clinical outcome (Brodeur et al., 1984; Seeger et al., 1985; Brodeur et al., 1986; Nakagawara et al., 1987; Maris and Matthay, 1999; Ribatti et al., 2002). The risk of death is over five times greater and the risk of disease recurrence over four times greater for patients with *MYCN* amplification compared with those without amplification (Riley et al., 2004). The significance of *MYCN* in neuroblastoma is unquestionable. No other prognostic biological factors than *MYCN* amplification (Seeger et al., 1985) are consistently used for risk-group stratification and therapeutic decisions, and there is no evidence for activation of any other oncogene in human neuroblastomas.

MYCN amplification has been associated with other unfavourable prognostic indicators including chromosome 1p deletion and increased expression of multidrug resistance-associated protein (MRP) (Bordow et al., 1994; Norris et al., 1996). In addition, fluorescent *in situ* hybridization (FISH) techniques have shown that 17q may be a preferential recombination site for *MYCN* amplicons (O'Neill et al., 2001). Remarkably, the amplified *MYCN* gene does never locates at the 2p23–24 resident site of the gene itself, but is found in homogeneously staining regions (HSRs) on other chromosomes (Kanda et al., 1983; Emanuel et al., 1985), especially in 17q (O'Neill et al., 2001), or in extrachromosomal double minutes (Schwab et al., 1984).

2.1.6 Treatment

Treatment of children, especially older ones, with widely disseminated neuroblastoma (INSS 4) remains one of the greatest challenges for paediatric oncologists. Currently, treatment options for neuroblastoma comprise combined modalities of surgery, chemotherapy, radiotherapy and biotherapy, as well as observation alone in carefully selected circumstances. Every option is individually tailored depending on current prognostic risk assessments, which basically include biological features, disease stage and patients' age at diagnosis. The acquiring of biological data is often crucial in facilitating treatment selections for any residual tumour, since in many circumstances the presence of macroscopic residual tumour in primary or metastatic sites does not warrant any additional treatment. On the other hand, despite gross-total resection of some localised tumours, unfavourable biological features may cause a need for intensive adjuvant therapy.

2.1.6.1 The surgical approach

The general objectives of surgical interventions of neuroblastoma are 1.) extirpation (total resection) of tumour, 2.) exploration of the tumour nest surroundings for staging (INSS), and 3.) to obtain tumour samples for diagnostic purposes. Low-stage tumours (INSS 1 and 2) are resected completely, if possible. However, there is no scientific evidence that the extent of resection has any prognostic value in either low- or high-stage neuroblastoma risk stratification. For this reason, the INSS definitions have been frequently criticized. For advanced-stage tumours (stages 3 and 4), surgery is frequently limited to an open biopsy or partial resection, after which tumour samples can be analysed for biomarkers. Chemotherapy usually fails to eradicate the advanced-stage disease, but it often reduces the tumour burden, allowing residual tumours to be surgically resected after adjuvant treatment. Postponement of surgical resection is justified, since preoperative adjuvant therapy increases the success rate of complete excisions, and may result in decreased surgical morbidity. Surgical resection of stage 4S tumours is frequently unnecessary, since a significant number of these tumours regress

spontaneously. Postoperative diagnostic imaging studies are used to determine the extent of resection. Local recurrences of low-stage neuroblastoma can typically be treated with surgery and/or radiation therapy. Metastatic recurrences of low-stage neuroblastoma are rare and the majority of the time treated successfully with chemotherapy. Recurrences of high-stage tumours are frequently treated non-surgically.

2.1.6.2 Chemotherapy

The current standard treatment for high-risk neuroblastoma patients is comprised of initial induction chemotherapy, attempted surgical resection of the primary tumour, myeloablation, local radiation to the primary tumour bed, and differentiation biotherapy with 13-cis-retinoic acid. Bone marrow stem cells are harvested during induction therapy, and they are prepared for the consolidation phase of therapy. The goal of consolidation is to eliminate any MRD, usually with myeloablative chemotherapy. Chemotherapy has also been successful in patients with refractory or metastatic neuroblastomas. The most commonly used chemotherapeutic agents include topotecan, cisplatin, cyclophosphamide, vincristine, iphosphamide, doxorubicin (adriamycin), carboplatin, etoposide (VP-16) and melphalan, which are used in various combinations. For example, a recent study of the European Neuroblastoma Group (ENSG 5) used alternating courses of two chemotherapy regimens (vincristine, cisplatin, etoposide, cyclophosphamide, and vincristine, carboplatin, etoposide, cyclophosphamide), which were given using a standard (seven alternating courses over 18 weeks) or rapid (same courses over 10 weeks) protocol for INSS 4 patients (Pearson et al., 2008).

Despite improvements in long-term survival with more intense combination therapies, bone marrow-ablative therapy (myeloablation) with melphalan followed by bone marrow transplantation is the most effective treatment option to improve outcome for patients with high-risk neuroblastomas (Matthay et al., 1999). Therefore, the majority of patients with high-risk neuroblastomas receive myeloablation, and for example all INSS 4 patients who had undergone tumour resection in the study of Pearson et al. received myeloablation (Pearson et al., 2008). The treatment of INSS 3 patients is currently inconsistent throughout the Western world. The toxicity of chemotherapy diminishes the benefits of chemotherapy, and has remained a major problem. INSS 3 patients without *MYCN* amplification are treated with surgery alone or with surgery and following adjuvant therapy, whereas INSS 3 patients with *MYCN*-amplified neuroblastomas undergo dose-intensive induction, tumour resection, local radiotherapy, chemotherapy or myeloablative chemotherapy and occasionally immunotherapy. In cases when neuroblastoma is refractory to chemotherapy, INSS 3 and 4 patients may be treated with even more aggressive treatments, such as the combined therapy of 131I-MIBG with myeloablative chemotherapy (Matthay et al., 2006). However, these harsh procedures commonly have severe adverse side effects. That said, for stage

4s neuroblastoma, chemotherapy can be used, especially in infants younger than two months of age with rapidly progressing abdominal disease causing a compromise in vital functions (Nickerson et al., 2000).

Imatinib mesylate, which inhibits Bcr-Abl tyrosine kinase and tyrosine kinase receptors c-kit and platelet-derived growth factor, was originally designed to block abnormal tyrosine kinase activity, which was caused by a so-called Philadelphia chromosome in chronic myelogenous leukaemia. Later, a Finnish finding showed that imatinib has a drastic antitumorous effect in a patient with a recurrent and metastatic gastrointestinal stromal tumour (GIST) (Joensuu et al., 2001), and a number of new studies were conducted to find new treatment targets for imatinib. Subsequently, it was shown that imatinib inhibits neuroblastoma cell growth *in vitro* (Vitali et al., 2003; Beppu et al., 2004). High-level expression of the c-kit protein, a target of imatinib, was preferentially detected in unfavourable neuroblastoma, though only in 13% of all studied neuroblastoma samples (Uccini et al., 2005). Based on observed preclinical studies on neuroblastoma and clinical studies on other tumours, a phase II trial of imatinib in children and adolescents with recurrent or refractory solid tumours, such as neuroblastoma, was conducted (Bond et al., 2008). According to the study, imatinib had no beneficial effects in children with neuroblastoma (Bond et al., 2008). However, there are studies suggesting that c-kit expression associates with favourable prognosis in neuroblastoma (Krams et al., 2004; Shimada et al., 2008), whereas others have found contrary results (Vitali et al., 2003; Uccini et al., 2005). On the basis of current knowledge, further development of imatinib-based treatment strategies for neuroblastoma does not appear warranted.

Temozolomide is a DNA-methylating cytotoxic agent, which is used in the treatment of *e.g.* malignant gliomas. After DNA replication, the temozolomide-induced methylation of DNA can be repaired by the action of the nuclear protein methylguanine-DNA methyltransferase, MGMT, which removes placed methyl adducts from DNA. Thus, resistance to temozolomide correlates with high activity of MGMT in tumour cells. The expression of MGMT is generally lower in gliomas than in other brain tumours, which may explain the relatively high sensitivity of gliomas to temozolomide. Epigenetic silencing of the MGMT DNA-repair gene by promoter methylation (hypermethylation) compromises DNA repair, and a recent study showed that adult patients with glioblastoma (the most malignant glioma) containing a methylated MGMT promoter benefited significantly from temozolomide administration (Hegi et al., 2005). MGMT is expressed in all neuroblastomas (Wagner et al., 2007), and a phase II study of temozolomide in relapsed or refractory high-risk neuroblastoma showed some benefit from temozolomide in heavily pretreated patients (Rubie et al., 2006). Currently, temozolomide together with the topoisomerase I inhibitors topotecan and irinotecan is sometimes used clinically in the treatment of refractory and relapsed neuroblastoma.

2.1.6.3 Radiation therapy

Preoperative radiation therapy is frequently used in conjunction with chemotherapy in order to ease surgery and improve the resectability of high-risk neuroblastoma. Radiation therapy after surgery has been shown to be beneficial in decreasing the local relapse rate in high-risk neuroblastoma (Kushner et al., 2001b; Haas-Kogan et al., 2003; Gillis et al., 2007); therefore, postoperative external radiotherapy to the primary site and to major metastatic sites are commonly provided in high-risk disease. The exact optimal radiation indications, technique and dose are not clarified, and, for example, the Children's Oncology Group evaluates dose escalation in high-risk patients to a dose of 36 Gy, and in patients with MRD to a dose of 21.6 Gy. For low-stage tumours, even with local residual disease, the potential post-treatment complications associated with radiotherapy outweigh the potential benefits of the therapy. Radiation-related post-treatment complications include hypertension and vascular stenosis (aortic, mesenteric, renal artery etc.), which can lead to mesenteric ischemia and bowel necrosis, for example (Gillis et al., 2007). Radiation therapy is contraindicated for intraspinal tumours, except for rare cases with spinal cord compression symptoms due to intraspinal neuroblastoma, when radiation therapy has proven to be of value (De Bernardi et al., 2001). Radiation therapy of the spinal region may cause vertebral damage in children, leading to growth arrest and scoliosis. Radiation therapy together with chemotherapy may also be used in stage 4s disease (Nickerson et al., 2000), especially when tumour mass causes a compromise in vital functions, such as is the case in hepatomegaly-induced respiratory symptoms. After the introduction of targeted MIBG treatment (De Kraker et al., 1995), 131I-MIBG has been an option for both high-risk and refractory neuroblastomas (Kang et al., 2003; Mairs et al., 2005; De Kraker et al., 2008). Unfortunately, severe complications such as secondary malignancy or thyroid dysfunction due to the treatment have been described in literature (van Santen et al., 2002; Garaventa et al., 2003a).

2.1.6.4 Biological therapies

Biological therapies are a growing area with a potentially high clinical impact. Natural and synthetic vitamin-A -related retinoids have been the most commonly used biotherapy adjuvants, although the mechanism of retinoid-induced differentiation of neuroblastoma cells is not fully known (Reynolds et al., 2003). In addition to induction of differentiation, retinoids modulate the major histocompatibility class I presentation pathway to sensitize neuroblastoma cells to cytotoxic lymphocytes (Vertuani et al., 2003). Pulse administration of high dose synthetic 13-cis-retinoic acid is beneficial for patients with MRD (Reynolds et al., 2003), but retinoids do not seem to have an effect in patients with solid tumours, possibly due to acquired resistance mechanisms (Freemantle et al., 2003). Moreover, according to phase II clinical trials, retinoids do not show any therapeutic benefit in children with recurrent or

refractory neuroblastomas (Finklestein et al., 1992; Adamson et al., 2007). Regardless, the intermittent use of highdose 13-cis-retinoic acid in the treatment of high-risk neuroblastoma patients in combination with autologous bone marrow transplantation has been shown to improve EFS in a randomised phase III trial done by the Children's Cancer Group (Matthay et al., 1999). Thus, retinoid-based biotherapy has now become standard practice in the management of high-risk neuroblastoma patients treated with bone marrow transplantation. Currently, synthetic atypical retinoids, whose mechanism of action differs from the classical retinoids, are to be tested in treating RA-resistant neuroblastomas (Garaventa et al., 2003b; Di Francesco et al., 2007). Since relapse is a frequent occurrence after autologous transplantation, biological therapy with or without retinoids combined with other pharmacologic options will probably be a future treatment protocol for persistent MRD.

Numerous non-retinoid-based biotherapies have also been investigated with promising results, but the breakthrough has yet to come. Increased angiogenic capacity is an important determinant of growth and poor prognosis in neuroblastoma (Meitar et al., 1996; Ribatti and Ponzoni, 2005), and an angiogenic regulator, vascular endothelial growth factor, correlates with unfavourable histology and aggressive behaviour in neuroblastoma (Langer et al., 2000; Fukuzawa et al., 2002). Recent animal studies with bevacizumab, an antibody against vascular endothelial growth factor, demonstrated reduced angiogenic activity and decreased growth of neuroblastoma in a xenograft model (Segerstrom et al., 2006; Dickson et al., 2007). Biotherapeutic inhibition of MYCN expression, or post-transcriptional silencing of *MYCN*, has been shown to decrease proliferation and induce differentiation *in vitro* (Negroni et al., 1991; Whitesell et al., 1991; Kang et al., 2006; Nara et al., 2007). Disialoganglioside GD2, which is a glycoconjugate, is expressed on neuroectodermal tumour cells, and anti-GD2 immunotherapy has been shown to eradicate some recurrent high-risk neuroblastomas after failed intensive chemotherapy (Kushner et al., 2001a).

2.1.7 Population-based screening programs

Population-based screening of urinary catecholamine metabolites, HVA and VMA (Laug et al., 1978; LaBrosse et al., 1980), was a standard practice for all infants in Japan between 1984 and 2003, whereas HVA and VMA measurements have been only selectively utilized in other countries. The Japanese government stopped mass-screenings in 2003, since there appeared to be no effect on the neuroblastoma mortality rate. Scientific evidence is still lacking, or is highly controversial, conserning whether population-based screening for neuroblastoma is advantageous and cost-effective. Moreover, uncertainty of the optimal screening age, strategy and method suggest that population-based screening programs are not justified, yet. Indeed, previous literature suggests that there is currently insufficient evidence to support a population-based screening program for infants younger

than six months of age (Brodeur et al., 2001). Surprisingly, a very recent retrospective population-based Japanese cohort study again raised the question of the advantages of neuroblastoma screening, as the results suggested a drop of almost 50% in mortality (Hiyama et al., 2008). Unfortunately, major methodological shortcomings were observed regarding the representativeness of the study cohorts. Moreover, the study cohorts were not validated for major confounding factors, such as for the intention-to-treat analysis (patient transition between study cohorts) and the actual cause of death.

Extensive screening studies in Europe, Japan, and North America suggest that the incidence of neuroblastoma in a screened population is increased roughly two-fold over that seen in unscreened populations (Schilling et al., 2002; Woods et al., 2002; Hiyama et al., 2008). However, population-based screening for neuroblastoma rarely detects unfavourable neuroblastoma (Schilling et al., 2002; Woods et al., 2002), and does not improve the overall mortality (Schilling et al., 2002; Woods et al., 2002). It is widely believed that screening detects low-stage and favourable neuroblastoma of early childhood, which probably leads to unnecessary testing, surgery and even chemotherapy of patients with a high likelihood of spontaneous regression. Therefore, it seems unlikely that a significant proportion of advanced stage tumours, which often evolve after one year of age, could be detected with population-based HVA and VMA screenings, which have been conducted at the age of six or twelve months (Schilling et al., 2002; Woods et al., 2002; Hiyama et al., 2008).

2.1.8 OS and prognosis

The likelihood of cure after treatments varies significantly from patient to patient, depending on age at diagnosis, extent of disease, and most of all, the highly heterogeneous tumour biology. A subset of low-stage and low-risk tumours undergoes spontaneous regression, while many high-stage and high-risk tumours progress relentlessly. Roughly half of all cases are currently classified as high-risk neuroblastomas with a high likelihood of disease relapse. Considering the impact of tumour biology, very recent data shows that patients with *MYCN*-amplified low-stage (INSS 1 and 2) tumours have a less favourable OS rate than patients with nonamplified tumours (72% versus 98%, respectively) (Bagatell et al., 2009).

Despite the progress in treatment options, clinical prognosis of aggressive neuroblastomas, especially in older patients, has remained dismal (Brodeur, 2003). The well-established clinical factors of unfavourable prognosis are age over 1 year at diagnosis and advanced tumour stage (Cotterill et al., 2000). For INSS 4 patients over one year of age, Pearson et al. reported a 10-year OS of 20-28% and 5-year OS of 21-32% (Pearson et al., 2008). There are no recent reports of an OS for INSS 4 patients under one year of age, but children under

18 months have an OS rate of 68%, whereas patients older than 18 months have an OS rate of 31%, regardless of *MYCN* amplification status (Cohn et al., 2009). Especially for patients younger than 18 months with INSS 4 disease, *MYCN* status has the most powerful prognostic value (Cohn et al., 2009). In a small retrospective analysis of 69 patients, the 10-year OS for INSS 3 patients with or without *MYCN* amplification was 85-97% (Modak et al., 2009). The 10-year OS for INSS 3 patients equals roughly that of INSS 1 and 2 patients (Bagatell et al., 2009), or is even slightly better. These minor differences might be explained by more aggressive treatment strategies in the group of INSS 3 patients in comparison to INSS 1 and 2 patients. Moreover, these two cited studies have different patient characteristics and follow-up times (Bagatell et al., 2009; Modak et al., 2009).

2.2 Molecular characteristics and risk factors of neuroblastoma

The identification of clinically significant markers has been complicated not only by acknowledging the problems relating to a representative sample selection and analysis of highly heterogeneous neuroblastoma, but also by the existing large variability of methods used in data analyses for various neuroblastoma markers. Indeed, a systematic review of relevant literature between 1966 and 2000 revealed a total of 195 different neuroblastoma markers in 428 papers (Riley et al., 2004). A list of the 31 most frequently reported markers are listed below (Table 3). The 13 most commonly studied prognostic markers were selected for a detailed study to establish their individual prognostic value (Riley et al., 2004). Analysis of these 13 markers, which were reported in 211 (81%) of all papers, was restricted largely due to variability in both clinical and statistical factors relating to the markers (Riley et al., 2004). For example, *MYCN* analyses included 9 different cut-off points to dichotomize the marker, 9 different stage groups, 4 different age groups, 17 adjusted and 77 unadjusted estimates, and 2 different outcomes (OS and DFS). If the type of treatment and methods of marker measurement had been recorded, they would have added yet more variability to that already noted (Riley et al., 2004). However, the pooled results of the systematic review identified *MYCN*, VMA:HVA ratio, chromosome 1p, DNA index, TrkA, NSE, lactate hydrogenase (LD), ferritin, MRP, and CD44 as potentially important prognostic tools which should be considered in the development of future prognostic evaluations (Riley et al., 2004). Except for *MYCN* and VMA:HVA ratio, which have been discussed in previous contexts, these prognostic variables are reviewed in following sections in more detail. For future research, a universal guideline for sample selection and analyses is necessary to characterize clinically significant new molecular risk factors.

Table 3. List of some tumour markers of neuroblastoma that were identified by the systematic review by Riley et al (table from Riley et al., 2004).

Tumor Marker	Overall	Screening	Diagnosis	Prognosis	Monitoring
MYCN	201	7	148	151	9
VMA	125	44	78	45	18
HVA	105	38	64	35	16
DNA index/ploidy/diploidy/triploidy/aneuploid/hyperdiploidy	56	5	37	44	1
Chromosome 1p or chromosome 1p36	47	4	34	40	1
Ferritin or isoferritin	49	3	36	33	5
NSE	45	2	33	28	9
LDH	32	1	22	26	4
Dopamine	24	2	22	10	4
TrkA (nerve growth factor receptor)	25	0	16	16	0
Adrenaline/epinephrine	15	0	15	5	4
Multidrug resistance/associated protein/p-glycoprotein	16	0	7	16	0
Nonadrenaline/noradrenaline/norepinephrine	13	0	13	5	2
CD44	10	0	7	8	0
Neuropeptide Y	12	0	10	9	0
Tyrosine hydroxylase	12	0	11	3	3
Chromosome 17q	11	0	9	8	0
Ha-ras/P21/H-ras/c-ha-ras	11	0	8	6	1
Telomerase/Telomeric repeats	11	0	6	7	0
Chromosome 14q	8	0	6	7	0
GD2 ganglioside	8	0	7	5	2
S100	7	1	5	5	0
Chromosome 11q	6	0	5	6	1
Low affinity nerve growth receptor (LNGFR)	6	0	3	6	0
Metanephrine	6	0	6	1	1
TrkC	6	0	3	5	0
3-methoxy-4-hydroxyphenyl glycol	5	1	3	1	0
4-hydroxy-3-methoxymandelic acid	5	0	5	1	2
Dihydroxyphenylalanine	5	1	5	1	3
Dopamine ß hydroxylase	5	0	3	2	2
Proliferating cell nuclear antigen/proliferation index/Ki67/KiS5 protein	5	0	5	4	0

2.2.1 Neuroblastoma phenotype

A tumour phenotype describes observable tumour characteristics, such as histopathology and expression characteristics. Different phenotypes result from combined effects of acquired genetic code and environmental factors. A tumour phenotype, in general, is an important stratification factor in the evaluation and design of treatment modalities.

2.2.1.1 Histology

Evaluation of the degree of cellular differentiation within a neuroblastoma is the main role of histological analyses. Neuroblastoma is the commonly used term for all types of neuroblastic tumours, which are traditionally divided on the basis of differentiation and Schwannian stromal development into four different histopathological subgroups: neuroblastoma, intermixed ganglioneuroblastoma, nodular ganglioneuroblastoma and ganglioneuroma (Hicks and Mackay, 1995; Shimada et al., 1999a; Shimada et al., 1999b; Peuchmaur et al., 2003). These subgroups are further divided into more specific subtypes by the International Neuroblastoma Pathology Committee (INPC), which has published a widely accepted and used classification method of neuroblastomas (Shimada et al., 1999a; Shimada et al., 1999b).

In brief, neuroblastomas (undifferentiated, poorly differentiated, differentiating) are rich in neuroblasts with little Schwannian stroma. Ganglioneuroblastomas (intermixed, nodular) belong to an intermediary category possessing features of both the immature neuroblastomas and differentiated ganglioneuromas. Ganglioneuromas (maturing, mature) are well-differentiated benign tumours with mature ganglion cells, increased stroma compartments and sparse neuroblasts. The International Classification system distinguishes a favourable and unfavourable histology group on the basis of age and mitosis-karyorrhexis index (MKI) (Shimada et al., 1999a; Shimada et al., 1999b). Tumours in the favourable group include *e.g.* poorly differentiated subtype of neuroblastoma with either a low (defined as fewer than 100 mitotic or karyorrhectic cells per 5000 cells) or an intermediate (100–200 per 5000 cells) MKI (<18 months of age), differentiating subtype with a low MKI (<60 months of age), intermixed ganglioneuroma, and ganglioneuroma (Shimada et al., 1999a; Shimada et al., 1999b). In contrast, tumours in the unfavourable histology group include *e.g.* undifferentiated subtype (any age), poorly differentiated subtype (≥18 months), differentiating subtype (≥60 months), high (>200 per 5000 cells) MKI (any age), intermediate MKI (≥18 months), and low MKI (≥60 months) in the neuroblastoma category. According to the revised INPC system (Peuchmaur et al., 2003), the prognostic group for ganglioneuroblastoma is determined by evaluating the grade of neuroblastic differentiation and MKI of the neuroblastomatous nodule(s) using the same age-dependent criteria described above (Shimada et al., 1999a; Shimada et al., 1999b).

Even though the International Classification has been proven to be an independent prognostic factor in multivariate analyses (Schwab et al., 2000; Sano et al., 2006), this type of histological classification does not have an additional prognostic value in comparison to classifications based solely on age, stage and *MYCN* amplification status. However, in the previously presented INRG classification system (Cohn et al., 2009), the histopathological features of differentiation and mitosis-karyorrhexis index have been adopted for risk stratification. Furthermore, in the year 2000 the Children's Oncology Group was formed, which is a worldwide clinical trial co-operative group supported by the National Cancer Institute It incorporates tumour histopathology to assign patients to one of three risk-groups (low-risk, intermediate-risk, or high-risk) and to stratify treatment intensity accordingly. In brief, histology is currently used in different neuroblastoma risk stratification protocols, but the true independent value of histology in these protocols is questionable.

2.2.1.2 TrkA, TrkB and TrkC

A group of transmembrane tyrosine kinase receptors, TrkA, TrkB and TrkC, mediates the survival functions of the neurotrophins, which include nerve growth factor, brain derived neurotrophic factor, neurotrophin 3 and neurotrophin 4. Neurotrophin ligand pathways are implicated in many aspects of SNS development, and they are important regulators of neural survival, development, function and plasticity (Huang and Reichardt, 2001; Airaksinen and Saarma, 2002).

TrkA activation can lead to programmed cell death, depending in part on the phase of differentiation of the cell. High TrkA expression is seen in low-stage neuroblastomas, which tend to show a more differentiated neuronal phenotype with favourable outcome (Nakagawara et al., 1993; Tanaka et al., 1995; Nakagawara, 2001). Evaluation of TrkA expression together with *MYCN* copy number may be used as a prognostic tool (Azar et al., 1990; Nakagawara et al., 1993). Expression of a full-length TrkB receptor is predominantly seen in *MYCN*-amplified tumours with unfavourable prognosis (Nakagawara et al., 1994). Because these tumours also expressed the cognate TrkB ligand, brain derived neurotrophic factor, an autocrine or paracrine loop may provide some survival or growth advantage (Acheson et al., 1995; Matsumoto et al., 1995). TrkC has been shown to be expressed significantly more often in low-stage tumours (stage 1, 2, 4S) in comparison to high-stage neuroblastomas (stage 3, 4, $P < 0.04$), and to correlate positively with survival and negatively with *MYCN* amplification (Yamashiro et al., 1996; Svensson et al., 1997; Yamashiro et al., 1997). Thus, TrkC may be involved in the biology of favourable neuroblastomas.

Despite the interesting results, Trk receptors have not been adapted to frequent clinical use. This is mainly due to the lack of evidence showing any advantage for Trk-based prognostic evaluations in comparison to stage-, age- and *MYCN*-based evaluations. Therefore, there are no recent studies on the role of Trk receptors in neuroblastoma prognostic evaluations, and previous results should be repeated with much larger patient cohorts.

2.2.1.3 NSE

Neuron-specific enolase (NSE) is a cytoplasmic protein that is found in mature neurons and cells of neural origin. NSE is not specific for neuroblastoma, but increased serum levels of NSE have been shown to correlate with high-risk neuroblastoma and poor OS rates (Zeltzer et al., 1986; Berthold et al., 1991). Serial NSE measurements on an individual basis may reveal early changes in disease course (Massaron et al., 1998), but NSE is not routinely used for diagnostic screening or follow-up evaluations, partly because of the lack of a standardized methodology.

2.2.1.4 LDH

High serum levels of lactate dehydrogenase (LDH), a widely presented enzyme in plants and animals, are associated with rapid cellular turnover, large tumour burden and poor prognosis (Quinn et al., 1980; Woods, 1986; Shuster et al., 1992; Joshi et al., 1993). Although not specific for neuroblastoma, LDH levels have occasionally been used as a prognostic marker for neuroblastoma. Currently, LDH is not in routine use.

2.2.1.5 Ferritin

Increased serum levels of the iron-binding protein ferritin are detected in some high-risk patients with unfavourable outcome (Hann et al., 1985; Silber et al., 1991). Since increased levels of ferritin may simply be a cause of rapid tumour growth and/or large tumour burden, ferritin measurements are not currently in clinical use.

2.2.1.6 MRP

The multidrug resistance-associated protein (MRP), which is a membrane-bound glycoprotein, has been shown to correlate closely with *MYCN* amplification in neuroblastoma (Bordow et al., 1994; Norris et al., 2005). High expression of a member of the MRP family, MRP4, correlates with *MYCN* amplification, associates with poor outcome, and mediates resistance to cytotoxic agents *in vitro* (Norris et al., 2005). Despite the possible prognostic value of MRP4 in neuroblastoma, it has not emerged as a clinically utilised prognostic marker.

2.2.1.7 CD44

CD44 is a transmembrane glycoprotein which is involved in cell-cell and cell-matrix interactions, cell migration and tumour cell dissemination (Lesley et al., 1993; Jothy, 2003; Lee et al., 2008). The cell surface receptor CD44 binds hyaluronan, but also other glycosaminoglycans, collagen, laminin and fibronectin (Marhaba and Zoller, 2004). CD44 is involved in the progression of various malignant tumours and in metastasis formation (Jothy, 2003; Lee et al., 2008; Zen et al., 2008), whereas CD44 expression is reported to be low or absent in high-risk neuroblastoma (Kramer et al., 1997; Munchar et al., 2003). Indeed, CD44 expression in neuroblastoma has been found to be a significant factor of favourable prognosis (Christiansen et al., 1995; Combaret et al., 1997). However, the lack of publications showing the additional value of CD44 in prognostic evaluations has led to the fact that CD44 expression levels are not systematically determined in neuroblastoma patients.

2.2.1.8 c-kit

C-kit is a stem cell factor binding cytokine receptor, which is expressed on the cell surface of numerous cell types. After binding to the stem cell factor, c-kit activates signalling pathways involved in cell survival, proliferation and differentiation. High expression of c-kit is detected *e.g.* in hematopoietic stem cells, multipotent progenitors and common myeloid progenitors.

Mutations of the c-kit receptor, which is a proto-oncogene, are so-called gain-of-function mutations (Nagata et al., 1995; Hirota et al., 1998; Nishida et al., 1998), which lead to ligand-independent activation of the tyrosine kinase and promote tumourigenesis (Hirota et al., 1998). Tumours with c-kit mutations have the highest response rate to imatinib treatment and the most favourable outcome (Heinrich et al., 2003; Tarn et al., 2005). C-kit expression has also been detected in neuroblastoma tumour samples and cell lines (Cohen et al., 1994), and *in vitro* studies suggest that c-kit receptor expression plays a significant role in neuroblastoma growth (Cohen et al., 1994). Immunohistochemical stainings have revealed c-kit expression in 13-60% of neuroblastomas (Smithey et al., 2002; Vitali et al., 2003; Krams et al., 2004; Uccini et al., 2005; Shimada et al., 2008). Despite the expression of the c-kit receptor protein, no *c-kit*-activating gain-of-function mutations have been detected (Krams et al., 2004; Uccini et al., 2005), but these studies have examined only exon 11 of the *c-kit* gene. In GISTs, gain-of-function mutations have been detected in exons 9, 11, 13 and 17 (Lux et al., 2000; Rubin et al., 2001; Heinrich et al., 2003). Due to the negative results of the phase II trial of imatinib for neuroblastoma (Bond et al., 2008) and contradictory results relating to the prognostic value of c-kit in neuroblastoma (Vitali et al., 2003; Krams et al., 2004; Uccini et al., 2005; Shimada et al., 2008), c-kit has currently no value in prognostic evaluations concerning neuroblastoma.

2.2.1.9 NCAM

The neural cell adhesion molecule, NCAM, is a cell adhesion molecule which is expressed on various cell types, such as natural killer cells (NK cells) and cardiomyocytes, and on various tissues, including neuroendocrine glands as well as the central and peripheral nervous system. In neural tissues, NCAM appears to be involved in neuronal differentiation and synaptic plasticity (Walsh and Doherty, 1997), and in cell proliferation (Krushel et al., 1998; Amoureux et al., 2000; Shin et al., 2002; Povlsen et al., 2003). The biology of malignant cells can be influenced by NCAM expression. For example, rat glioma cells transfected with human NCAM showed significant reduction in tumour invasiveness *in vivo* (Edvardsen et al., 1994), and NCAM expression in a murine pancreatic tumour model abolished formation of metastases (Perl et al., 1999). In addition, loss of NCAM expression resulted in enhanced formation of lymph vessels leading to increased lymphatic metastases in a murine pancreatic tumour model (Crnic et al., 2004). Moreover, results from a study of NCAM expression in a murine fibrosarcoma model suggest that NCAM can reduce metastasis formation by stabilizing the vascular wall (Xian et al., 2006).

NCAM expression is detected in various malignancies, *e.g.* in neuroblastoma, rhabdomyosarcoma, small cell lung cancer and brain tumours, as well as in the majority of multiple myelomas and acute myeloid leukaemia (Hirano et al., 1989; Bourne et al., 1991; Ikushima et al., 1991; Mechtersheimer et al., 1991; Molenaar and Muntinghe, 1998; Tassone et al., 2004). Although NCAM is not solely restricted to tumour tissues, high-level expression in malignancies may allow the discrimination between normal and malignant tissues thus making NCAM an attractive target for immunotherapy. Animal and human *in vivo* experiments with huN901-DM1, a humanized variant of the murine monoclonal N901 (anti-NCAM) antibody (Roguska et al., 1994), provided the first proof of the validity of the concept of therapeutic anti-NCAM antibodies (Tassone et al., 2004; Smith, 2005). Currently, pharmaceutical companies are intensively promoting the clinical development of anti-NCAM immunotherapies (Tassone et al., 2004; Smith, 2005), and anti-NCAM treatments of small cell lung cancer, NCAM-positive solid tumours and multiple myeloma are in phase I/II clinical studies (http://www.immunogen.com/wt/page/IMGN901b).

In previous literature, NCAM expression has been detected in 100% of neuroblastoma samples (Figarella-Branger et al., 1990; Molenaar et al., 1991; Phimister et al., 1991; Wachowiak et al., 2008; Winter et al., 2008). Interestingly, the *NCAM* gene is located at locus 11q23, which is frequently deleted in neuroblastomas lacking *MYCN* amplification (Guo et al., 1999; Plantaz et al., 2001; Attiyeh et al., 2005). Indeed, loss of heterozygosity (LOH; loss of one allele) at 11q23 manifests in 34% of neuroblastomas (Attiyeh et al., 2005), of which the majority are high-risk neuroblastomas (Guo et al., 1999). Considering the nature of stochastic mutations in cancer cells, it seems rather probable that a loss of functional NCAM occurs in at least some of LOH 11q23

neuroblastomas. Overexpression of the MYCN protein in human neuroblastoma has been shown to cause a dramatic reduction in the levels of NCAM protein and mRNA, and as a result contributes to the increased metastatic activity of *MYCN*-amplified neuroblastomas (Akeson and Bernards, 1990). Therefore, it appears unlikely that all neuroblastoma focuses express NCAM. However, since NCAM expression has been reported to occur in 100% of neuroblastomas, it has currently no prognostic value in risk group stratifications.

2.2.1.10 Polysialylated NCAM

Of the different posttranslational glycosylations of all cellular proteins, glycosylation of NCAM with polysialic acid (polySia) appears to be a highly unique form of protein modification (Finne, 1982). PolySia is strongly expressed in all three germ layers during embryonal development, after which it is actively downregulated in the course of cell maturation and differentiation (Rutishauser and Landmesser, 1996; Kiss and Rougon, 1997; Kleene and Schachner, 2004). PolySia is virtually absent in the majority of adult tissues. It has been shown that polySia is an inhibitor of NCAM interactions at the intercellular level (Seidenfaden et al., 2003), and a number of studies suggests that polySia plays an important role in antagonizing native NCAM functions.

PolySia is reexpressed in some metastasis-prone malignant tumours, such as neuroblastoma, rhabdomyosarcoma, non-small cell lung cancer, Wilms' tumour and small cell lung cancer (Lipinski et al., 1987; Roth et al., 1988; Figarella-Branger et al., 1990; Moolenaar et al., 1990; Gluer et al., 1998; Tanaka et al., 2000), where it appears to increase metastatic behaviour (Moolenaar et al., 1990; Tanaka et al., 2000; Daniel et al., 2001; Cheung et al., 2006). More precisely, it has been suggested that polySia increases the motility of small cell lung cancer cells, and allows the cancer cells to detach from the primary tumour, thus causing metastatic dissemination (Scheidegger et al., 1994). Indeed, polySia-NCAM expression levels are significantly higher in high-grade than low-grade lung cancers, and the levels correlate with metastatic activity (Lantuejoul et al., 1998; Tanaka et al., 2000). *In vitro*, a significant reduction in cell proliferation has been demonstrated after removal of polySia from neuroblastoma cell surfaces (Hildebrandt et al., 1998; Seidenfaden et al., 2003). Therefore, polySia most probably promotes cell proliferation and motility during malignant processes, as well as during normal neural development.

Polysialylation is catalyzed by two polysialyltransferases, called STX and PST, of which STX plays a critical role in modulating the function of NCAM. In a recent small study, STX marker status (positive versus negative) in bone marrow samples of neuroblastoma patients associated strongly with an OS rate (Cheung et al., 2006). However, the results must be repeated in larger series, and a standardized methodology is necessary to evaluate the true role of polySia in neuroblastoma.

2.2.2 Neuroblastoma genotype

Regardless of the complexity of genetic abnormalities in heterogeneous neuroblastoma, numerous genetic features, including chromosome losses, chromosome gains and DNA index abnormalities (ploidy status), have been identified to correlate with clinical outcome. The key to understanding the aetiology of neuroblastoma lies in understanding genetics, but the primary as well as secondary genetic alterations responsible for tumourigenesis remain elusive. With the exception of *MYCN* amplification, major oncogenic pathways of human cancers, such as TP53, CDKN2A, and Ras pathways, do not seem to be altered in neuroblastoma. Despite extensive research on common genomic alterations in neuroblastoma, no outcome-associated target genes have been identified but *MYCN*. Future research on the genetic imbalance of neuroblastoma will most probably provide insights into the fundamental biology of origin and progression of neuroblastoma.

2.2.2.1 LOH 1p36

Recurrent deletions in the short arm of chromosome 1 in primary neuroblastoma and neuroblastoma cell lines were identified for the first time in 1977 (Brodeur et al., 1977), and subsequent studies confirmed the high frequency of genetic aberrations in 1p (Gilbert et al., 1982). LOH at 1p36 region has been reported to occur in over 70% of neuroblastomas (Brodeur et al., 1981; Gilbert et al., 1982), strongly correlating with unfavourable prognosis and poor outcome (Brodeur et al., 1988a; Christiansen and Lampert, 1988; Hayashi et al., 1989; Christiansen et al., 1992). Improvements in cytogenetic methodology have facilitated the identification of chromosomal regions with true gains and losses, and 1p LOH is currently believed to be present in 35% of neuroblastomas (Maris et al., 2000). Interestingly, 62% of neuroblastomas showing 1p LOH are *MYCN*-amplified, whereas only 3% of neuroblastomas with an intact 1p region have *MYCN* amplification (Fong et al., 1989).

The association between 1p36 LOH and *MYCN* amplification may partially explain the prognostic value of 1p36 LOH. Indeed, the unfavourable outcome of neuroblastoma patients with 1p loss was shown to be restricted to cases with coexistent *MYCN* amplification, and 1p LOH had no independent predictive value (Gehring et al., 1995). In two large studies of 238 and 898 cases, 1p36 had no independent prognostic impact on OS in any patient groups, when the confounding effect of *MYCN* amplification was eliminated (Maris et al., 2000; Attiyeh et al., 2005).

Rearrangements of 1p and losses of 1p regions are not pathognomonic to neuroblastoma, but found in a wide variety of other malignancies (Atkin, 1986; Schwab et al., 1996). It is conceivable that there is a putative tumour suppressor gene located in 1p36. Indeed, a tumour suppressor chromodomain helicase DNA-binding protein 5, CHD5 (Bagchi et al., 2007), has been found to

be the strongest candidate for tumour suppressor genes within the 1p36 region (Fujita et al., 2008). In neuroblastomas, CHD5 expression through the second allele is probably silenced by an epigenetic mechanism (for example by methylation) (Fujita et al., 2008).

2.2.2.2 17q gains

Another frequently detected genetic aberration in neuroblastoma is trisomy of the long arm of chromosome 17 (17q). Cytogenetic analyses of neuroblastoma cell lines (Biedler et al., 1980) and primary tumours (Gilbert et al., 1984) revealed chromosome 17 abnormalities as far back as in the early 1980s. These observations were put aside as genetic research interest began to focus on 1p deletion and *MYCN* amplification. Partial gain of 17q appears to be the most prevalent genetic abnormality in neuroblastoma, occurring in approximately 50-61% of tumours (Bown et al., 1999; Spitz et al., 2003). Although unbalanced gain of 17q can occur independently, it frequently occurs as part of an unbalanced translocation between chromosomes 1 and 17 (Fig. 1). Therefore, the importance of unbalanced translocation, with loss of distal 1p and gain of distal 17q (Caron et al., 1994; Savelyeva et al., 1994; Van Roy et al., 1994), as a mechanism for 1p loss must be highlighted. In general, 17q gains show a remarkably large number of translocation partner sites, and aside from 1p and 11q, which is the second commonest site, the extra segment may be found at least at 30 sites on 20 different chromosomes (Meddeb et al., 1996; Lastowska et al., 1997b).

In the largest study of 17q gains to date, 313 neuroblastoma cases with known 17q status (not measured with a single method) from six European centres showed that the OS rate at five years was 30.6% for the 168 cases with 17q gain, compared with an OS of 86.0% for 145 patients without the gain ($p < 0.0001$) (Bown et al., 1999). According to the same study, analyses of the subgroups of cases without 1p deletion and *MYCN* amplification showed that 17q status still provided significant prognostic information (both groups $p < 0.001$) (Bown et al., 1999). However, the first study using a single (fluorescence in situ hybridization *i.e.* FISH) technique to evaluate the presence of 17q gain in a large neuroblastoma cohort of 193 patients could not prove any prognostic value for 17q gain (Spitz et al., 2003). These contradictory results can be partially explained by differences in DNA techniques (FISH versus GCH, FISH and cytogenetic analyses), different cohort sizes, different 17q gain thresholds and different follow-up times (Bown et al., 1999; Spitz et al., 2003). Therefore, based on these preliminary studies, it is unclear whether unbalanced gain of distal 17q is a significant independent prognostic factor. 17q gain has also been seen in a variety of other neoplasms, but the prognostic function in other cancers is also obscure. In brief, the most important challenge at present is to identify an informative and reliable form of technology to analyse the prognostic usefulness of 17q gain in large prospective clinical studies.

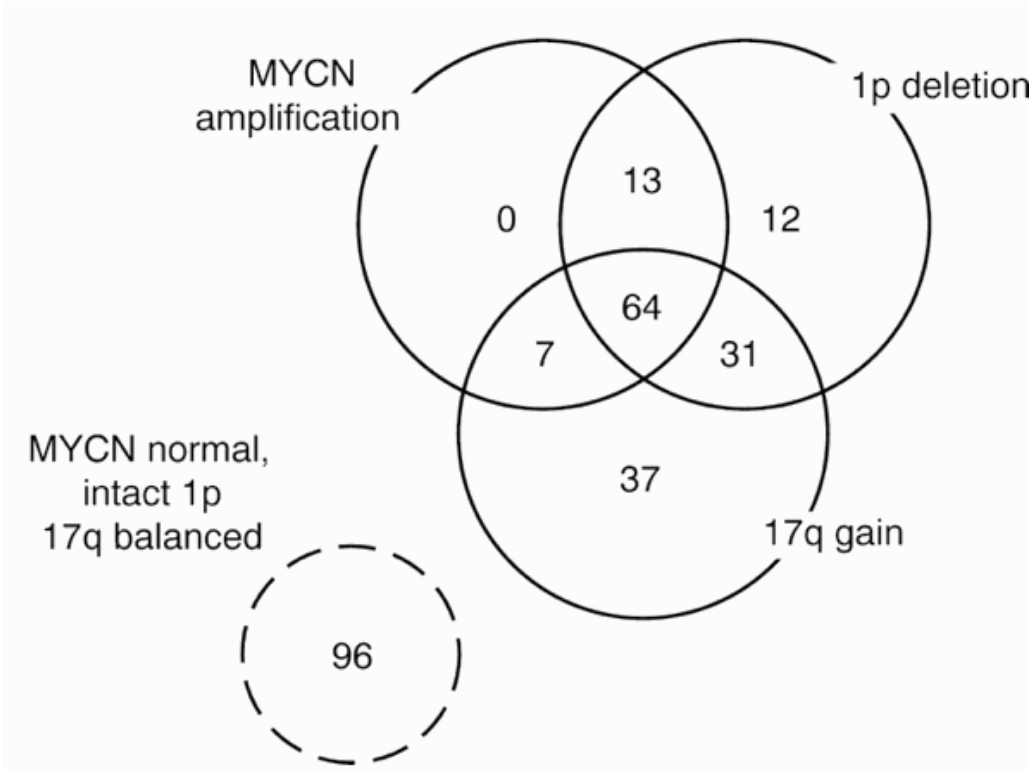

Figure 1. The frequency and association of LOH 1p, 17q gain and *MYCN* amplification in 260 primary neuroblastomas (data from Bown et al., 1999).

2.2.2.3 LOH 11q23

LOH at 11q23 occurred in 307 (34%) out of 913 studied neuroblastomas (Attiyeh et al., 2005). Whereas 1p36 LOH has been found to be associated with *MYCN* amplification (Fong et al., 1989), 11q23 LOH is rarely detected in tumours with this genetic aberration (Attiyeh et al., 2005). Given that 11q23 LOH occurs primarily in neuroblastomas without *MYCN* amplification, it is conceivable that 11q23 LOH could be a prognostic neuroblastoma marker. Indeed, particularly so-called unbalanced 11q23 LOH, where some genetic material is missing due to deletions, was strongly associated with OS, as patients with unbalanced 11q23 LOH had a three-year OS rate of 66±5% in comparison to 83±2% in the group without 11q23 LOH (Attiyeh et al., 2005). As mentioned earlier, the *NCAM* gene is located at locus 11q23. Whether NCAM antigen expression frequencies differ in those tumours with 11q23 LOH (especially with deletions) has not been studied. In conclusion, even though 11q23 LOH appears to be an independent prognostic marker, its prognostic power is far from *MYCN* amplification, age and stage in unadjusted study groups. Therefore, it has been mainly used as an adjunctive marker for research purposes. Whether 11q23 LOH could be a powerful marker in a subgroup of neuroblastoma patients remains to be studied.

2.2.2.4 Tumour ploidy

In addition to 1p LOH, *MYCN* amplification and 17q gains, recurrent ploidy changes are well established in neuroblastoma. The DNA index of neuroblastomas has been reported to associate with tumour chemosensitivity (Look et al., 1984), and to be a prognostic variable for patients younger than two years with disseminated disease (Bowman et al., 1997; Bagatell et al., 2005). The DNA content of neuroblastomas falls into two broad categories: (near-)diploid or hyperdiploid (often near triploid), as tumour DNA content is diploid in 34%, hyperdiploid in 65%, and hypodiploid in 1% of neuroblastomas (Look et al., 1991). Roughly two-thirds of advanced-stage neuroblastomas have diploid (two homologous copies of each chromosome) DNA content and are often chemoresistant (Look et al., 1984), whereas the presence of hyperdiploid DNA content is correlated with low-risk disease and improved prognosis in younger patients (Gansler et al., 1986; Kaneko et al., 1987; Brenner et al., 1989; Hayashi et al., 1989; Cohn et al., 1990; Look et al., 1991; Naito et al., 1991; Huddart et al., 1993; Muraji et al., 1993).

Unfortunately, ploidy loses its prognostic significance for patients who are older than 1–2 years of age (Look et al., 1991). In a large study of 648 neuroblastoma patients, hyperdiploidy was a significant prognostic factor of event-free survival (EFS) for children younger than 24 months with *MYCN*-nonamplified disseminated neuroblastoma (George et al., 2005). Particularly, children younger than 18 months with hyperdiploid neuroblastoma clearly had

a better EFS prognosis than older patients (4-year EFS of 93% in a 12- to 18-month-old subgroup versus 38% in a 19- to 24-month-old subgroup), whereas children with *MYCN*-amplified neuroblastomas and patients older than 24 months had a poor prognosis regardless of ploidy (George et al., 2005). Hyperdiploid and near triploid tumours in infants may have whole chromosome gains without structural rearrangements, whereas hyperdiploid and near triploid tumours in older patients most probably have several genetic aberrations. Taken together, flow cytometry and cytogenetic investigations suggest that hyperdiploid neuroblastomas are distinctly different from diploid neuroblastomas, and that ploidy is a significant prognostic factor in patients under 18–24 months of age.

2.2.3 Summary of prognostic molecular characteristics of neuroblastoma

Prognostic risk group stratifications on the basis of cancer biology may improve the accuracy of prognostic prediction by replacing or supplementing clinical prognostic factors. Minimizing treatment-associated morbidity by reserving the most heavy and toxic treatments for neuroblastoma patients with the worst prognosis has become an important objective, given that permanent morbidity is the factor that has the most profound effect on the quality of life of a cured child. Thus far, prognostic risk groups have not been optimized to maximize treatment success while minimizing morbidity, and there is an unquestioned need to identify and define more precise prognostic factors to stratify treatments.

To date, the number of published analyses including a considerable number of neuroblastoma patients (> 100 patients) is still limited, and only a relatively small number of studies have reported multivariate survival analyses of multiple clinical and molecular factors. Moreover, most of the prognostic molecular studies include patients treated with different protocols, which further causes a considerable confounding effect, in addition to the well-known heterogeneity of neuroblastoma itself. Therefore, the results of these analyses should be interpreted with caution.

MYCN amplification status is the only molecular characteristic of neuroblastoma with a definite independent value in treatment stratifications. Despite very intensive research efforts, no single genetic aberration has been found to be present in all neuroblastomas, and none has been identified crucial for tumourigenesis. The significance of the most commonly reported prognostic molecular markers, VMA:HVA ratio, TrkA, TrkB, NSE, LDH, ferritin, MRP, CD44, LOH 1p36, 17q gains and DNA index (tumour ploidy), has been identified in a number of univariate analyses. As mentioned earlier, only a few of these remain significant and powerful enough after multivariate analysis and correction for the most important variables, which are age, stage and *MYCN*

amplification. The pooled results, such as meta-analyses, must also be interpreted with caution given the reporting problems and large heterogeneity of clinical as well as statistical factors among studies. Large clinical trials with analysis of many variables are needed to determine if any of the suggested additional markers can substantially improve the current risk stratification.

In summary, none of the molecular markers but *MYCN* are used consistently at present to predict outcome or to select therapy. Novel markers discovered in small studies including less than 100 patients should be considered as potential markers, which can only have adjunctive value in prognostics when they are biologically relevant and also show significant power in large patient cohorts.

2.3 Some recent methods in assessing the molecular biology of neuroblastoma

Conventional molecular methods, including immunohistochemistry, (RT)-PCR, Southern blot analysis, Northern blot analysis, Western blot analysis, microarray technology, enzyme-linked immunosorbent assay, and flow cytometry, are compromised to some extent by the complexity and variability in the pattern of focus-specific molecular alterations in neuroblastoma. Furthermore, the conventional methods are often too expensive and time-consuming to be applied to the characterization of the tens or hundreds of proteins and genes associated with tumourigenesis. Thus, simple techniques that can facilitate research on large series of tumour samples are required.

2.3.1 Tissue microarray

The term multitissue array refers to all procedures that arrange multiple tissue samples, up to 1000, on one microscopic slide. Multitissue arrays allow for the examination of a large number of tissues, with a minimal use of material and technical resources. It also allows the direct comparison of tissue samples of different patients or tumour focuses on the same slide. Using multitissue arrays, the usability of archive samples can easily be lengthened.

The first multitissue array technique dates back to 1986, to the so-called “sausage block method”, in which single sections of 100 rods of different tissues are obtained (Battifora, 1986). A so-called “tissue core” technology was described one year later (Wan et al., 1987). Based on these methods, a tissue microarray (TMA) for tumour specimens was developed (Fig. 2) (Kononen et al., 1998). Currently, TMA is the most widely used array method. TMAs are assembled by taking core needle “biopsies” from specific locations in pre-existing paraffin-embedded tissue blocks and re-embedding them in an arrayed “master” block, using techniques and an apparatus developed by

Kononen et al (Kononen et al., 1998). Up to 200 consecutive sections (4–8 µm thick) can be cut from a single TMA block.

Research on heterogeneous tissues or tumour samples requires numerous samples of various regions in order to be representative enough for molecular analyses. In this sense, TMA does not have an obvious advantage over conventional methods. For example, in liver research, it is often necessary to evaluate staining patterns of larger samples than an area of about 3 mm^2 (2 mm diameter) or 0.27 mm^2 (0.6 mm diameter), which cannot include the minimum requirement of one portal tract and one central vein in the same sample. Therefore, for the use of TMAs in the research of heterogeneous tumours, defining a strict methodology to select best representative spots to be punched from a wide area of a donor tissue block could further facilitate the usage of TMAs.

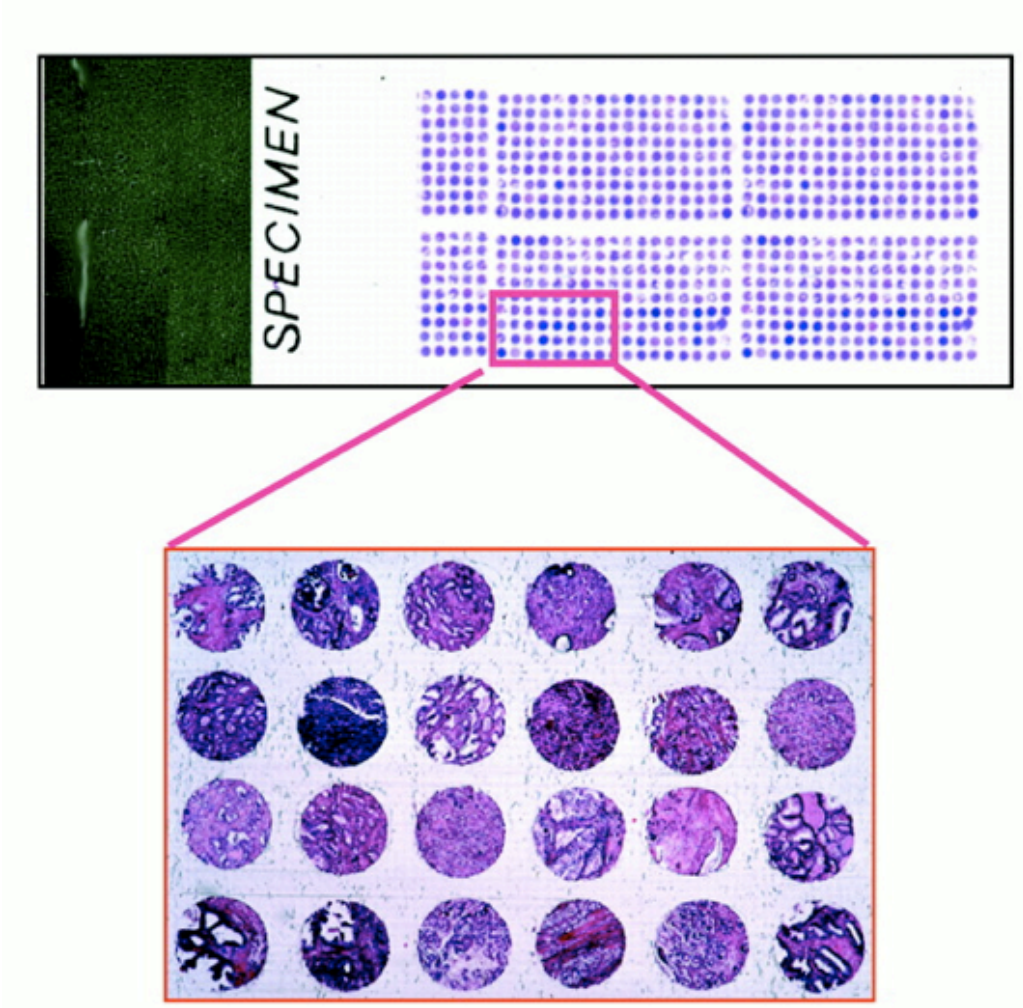

Figure 2. A tissue microarray microscopic slide showing numerous punched tissue samples on one microscopic slide. Thin sections of the paraffin-embedded tissue samples in a multitissue array block are cut and fixed on a microscopic slide for subsequent molecular analyses.

2.3.2 Gene amplification analyses

Two techniques predominate in the detection of *MYCN* amplification in clinical neuroblastoma samples: a.) time-consuming (1–2-week turnaround) Southern blotting (requires 5–10 µg of DNA), and b.) genomic PCR (requires only 50-100 ng of DNA). Both of these techniques provide semiquantitative information, *i.e.* yield an approximation of the number of *MYCN* gene copies, but the results presented as an average of a whole tumour sample do not represent the small proportion of highly malignant cells, and therefore the results may be

misleading. For example, in Southern blot analysis of *MYCN* copy number, the sample under evaluation may contain only a small subpopulation of *MYCN*-amplified cells, and therefore nonamplified cells may diminish the average copy number of *MYCN*. Together with a high cut-off value, this may result in a significant misinterpretation. Similarly, PCR-based methods for the detection of genetic aberrations or gene copy numbers are extremely sensitive to the amount of normal DNA "contaminating" the tumour sample.

An alternative approach for Southern blot analysis and PCR-based methods is FISH (Shapiro et al., 1993), which provides results that are more reliable than those from molecular analyses. Indeed, the FISH approach has several advantages. There is a minimal requirement for tumour material, since FISH is readily applicable to tumour touch imprint slides (Taylor et al., 1994), fine needle aspirates (Frostad et al., 1999), and paraffin wax embedded tissue sections (Leong et al., 1993). Most importantly, FISH enables the examination of tumour samples at an individual cell level, and hence it is clearly more specific and sensitive than Southern blot and PCR-based analyses. In other words, FISH can detect gene amplifications even in a few sample cells without difficulty (Lorenzana et al., 1997). The importance of this is especially evident in neuroblastoma, where a mixture of cells with copy numbers ranging from less than ten to many hundreds is commonly seen (Shapiro et al., 1993; Squire et al., 1996). The widespread use of FISH has been hampered due to its expensive (confocal microscopes and rapidly fading fluorescent probes) and laborious nature. To overcome these practical limitations, a chromogenic in situ hybridization (CISH) technique, in which the DNA probe is detected using a simple immunohistochemistry-like peroxidase reaction instead of fluorescent probes, has been developed (Tanner et al., 2000). However, even though CISH is routinely used in the detection of *HER-2* oncogene amplification in breast carcinoma, its applicability in evaluating *MYCN* amplification in neuroblastoma samples has not been widely studied.

2.3.3 CGH and array-CGH

Conventional comparative genomic hybridization (CGH) was developed as a new technique that allows measuring the copy number of altered DNA sequences (genomic aberrations) at several genomic positions (Kallioniemi et al., 1992). It maps these changes directly onto the sequence of the human genome (Kallioniemi et al., 1992). In brief, digested and fluorescence-labelled DNA from a tumour sample and reference DNA (normal diploid genome) are hybridized (FISH-like hybridization) to the metaphase (a stage of mitosis) chromosomes of the reference genome (normal diploid genome). The fluorescence-labelled DNA probes of the test and reference DNA give different fluorescence at different positions along the chromosomes, and digital image analysis technology is utilized to calculate the ratios of the signals. These ratios are used to determine the tumour karyotype. For example, if the tumour karyotype is normal, the observed fluorescence reflects an equal contribution

of both the red (test DNA) and green (reference DNA) fluorescence and thus the chromosome locations will appear yellow. Ratio profiles represent fluorescence hybridization signal intensities from test and reference DNA probes and are indicative of copy number changes. A ratio of 1.0 indicates that no copy number changes are present in the test sample. Ratios of 0.8-0.75 or less indicate loss or deletion of a whole chromosome or chromosomal sequence, whereas ratios of 1.2-1.25 or higher indicate chromosomal gains. A ratio of 2.0 or higher is an indication of high-level gene amplification. In the past few years, the high-resolution detection of DNA copy number aberrations with microarray-based formats for CGH (array-CGH) has been devised, and they are becoming more widely used than chromosome-based conventional CGH.

Many novel genomic alterations in neuroblastoma have been detected with conventional metaphase CGH analyses (Brinkschmidt et al., 1997; Lastowska et al., 1997a; Vandesompele et al., 1998), but this technique is hampered by a limited resolution (10–20 Mb in length) for identifying copy number changes. Array-based CGH (aCGH) on BAC (bacterial artificial chromosome), cDNA or oligonucleotide microarray has been developed, and aCGH can detect genomic alterations with a higher resolution and sensitivity than conventional CGH (Solinas-Toldo et al., 1997; Pinkel et al., 1998; Pollack et al., 1999; Snijders et al., 2001; Chen et al., 2004; Bilke et al., 2005). The clones spotted on the aCGH array contain 'sequence tags', which allow their positioning accurately to the genomic sequence and subsequent identification of genes mapping within genomic regions of copy number alterations (Fig. 3). aCGH with large clones, such as BACs, provides reliable copy number measurements on individual clones, but most clones comprise more than one gene, and it is not possible to do parallel examination of gene expression and copy number changes using the same array.

Oligonucleotide-based aCGH has been used for the detection of DNA single copy changes (Bignell et al., 2004; Brennan et al., 2004; Carvalho et al., 2004), but similarly to first generation microarrays containing cDNA clones, detection of single copy changes requires parameters which weaken genomic resolution. In other words, a substantial fraction of clones frequently has to be discarded simply because they do not produce adequate signals. Thus, in technical terms, the true genomic resolution to detect focal single copy changes is considerably less than implied by the so-called average genomic spacing of the clones on the array. The most significant advantage of aCGH is that it allows the investigation of the direct effects of genomic changes on gene expression by using the same microarray (Hyman et al., 2002; Pollack et al., 2002). Even though oligonucleotide-based aCGH offers enhanced design flexibility, full-genome representation of probes and the possibility to discover single copy number changes, it is a labour-intensive and expensive technique for the detection of chromosomal rearrangements.

Currently, the most commonly used platform contains short oligonucleotides that are synthesized on the microscopic slide itself. Microarray technology has multiple possibilities in genomic research, including highly multiplexed genotyping and polymorphism analyses, evolutionary studies and monitoring the binding of proteins to nucleic acids and other proteins. More sensitively detected genomic DNA alterations integrated with mRNA expression profiles may further facilitate important pathway discoveries, and transcriptional profiles can perhaps be used to define prognostic signatures. The most recent development in the array technology is a single nucleotide polymorphism (SNP) array that has greatly facilitated the detection of deletions.

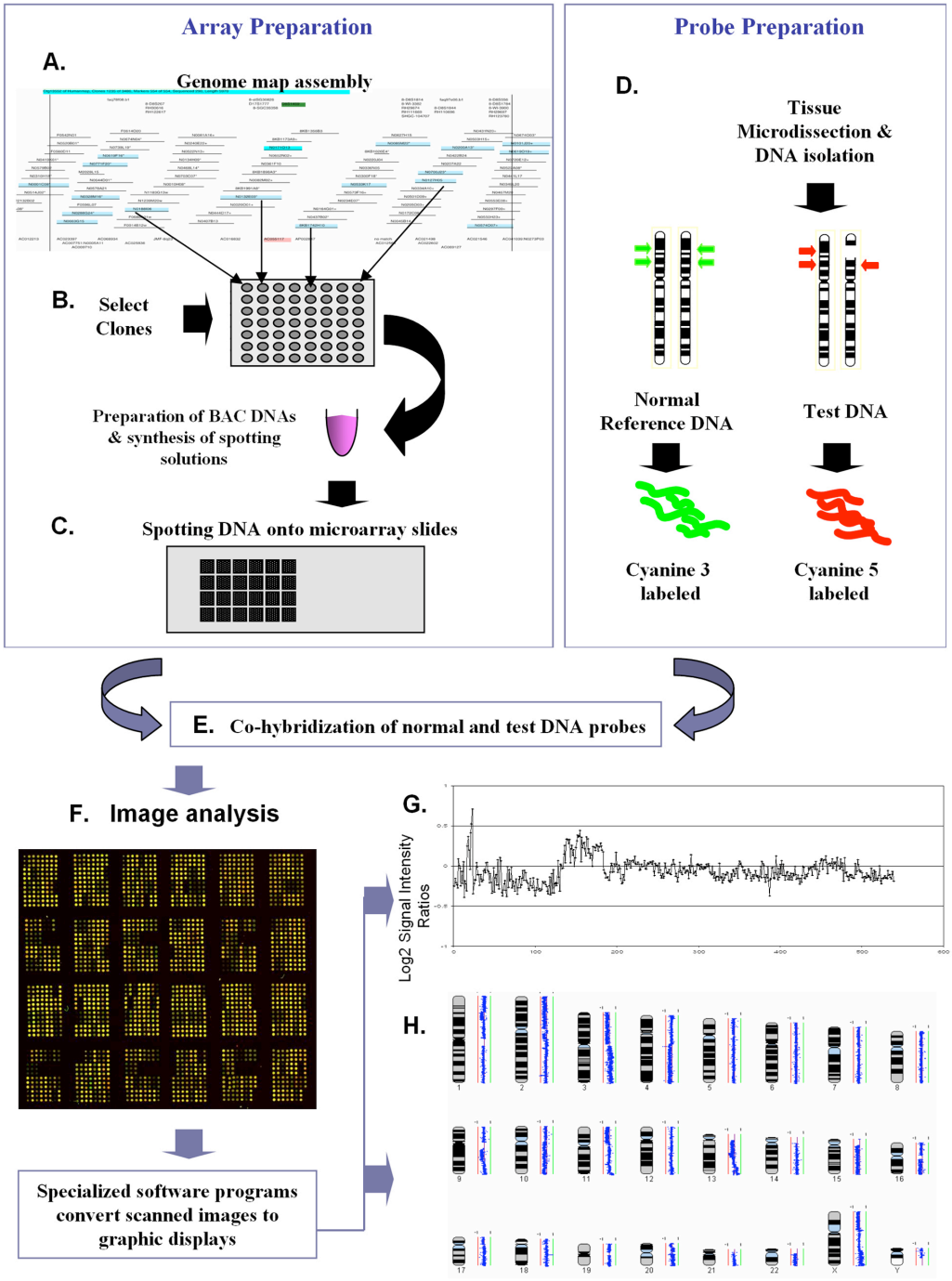

Figure 3. The figure shows the main principle of aCGH using BACs. (A and B) BACs can be selected and prepared based on *e.g.* genome maps. (C) Clones are automatically spotted on a microarray slide. (D) After preparing reference and test DNA samples, (E) labelled DNA probes are hybridized onto the previously spotted microarray slide (C). (F) Image analysis is performed using automated fluorescence scanners and a special software, which converts the scanned information to graphical illustrations (G and H) (figure from Garnis et al., 2004).

2.3.4 In silico screening

"*In silico"* is a Latin expression that translates into "performed on computer or via computer simulation." Gene discovery and analysis using *in silico* approaches is becoming a rapidly expanding and powerful tool in bioinformatics. Public databases, such as dbEST, GenBank, UniGene and Ensembl, are used for the collection, distribution and identification of, for instance, new genes and gene sequences. The largest human expressed sequence tag (EST) database is UniGene, which contains 6 000 000 ESTs (http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=9606). Ensembl is a software system project producing genome databases for vertebrates and other eukaryotic species, and the European Molecular Biology Laboratory, European Bioinformatics Institute and the Wellcome Trust Sanger Institute govern the data. ESTs originate from cDNA libraries prepared from specific cell types, tissues, or organs, and are usually short (typically 400–600 bp long) cDNA fragments representing a transcribed mRNA. If each cDNA library was a representation of all mRNA transcripts in that specific tissue, the frequency of each EST throughout the UniGene database, for example, would represent the level of that mRNA of a specific gene in the tissue studied. In other words, gene-specific expression could be directly quantified in relation to the total number of mRNAs in that tissue- or cell-specific library. Data mining of the human ESTs has been used to evaluate tissue-specific gene expression profiles (Vasmatzis et al., 1998; Hwang et al., 2000).

Construction strategies of cDNA libraries together with techniques used when obtaining ESTs may strongly bias the representativeness of libraries. For example, ESTs obtained from a random-primed cDNA library contain most probably more than one EST per mRNA template, whereas subtracted or normalized cDNA libraries represent only differentially transcribed gene transcripts. In contrast, if ESTs were obtained using oligo(dT) priming, an equal representation of all polyA-tailed transcripts in the target tissue is probable. In addition, each sequencing method of ESTs has its benefits and disadvantages. By using, for example, the UniGene Digital Differential Display tool (http://www.ncbi.nlm.nih.gov/Tools/), the comparison of EST-based expression profiles between several libraries in UniGene becomes possible, thus enabling the identification of genes that differ in transcription frequency in libraries of different tissues. Other useful tools are SAGEmap and the GEO Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/Tools/), which are databases designed for comparison of gene expression data from different experiments.

There is a great need for database integration. The enormous quantity of the data requires extensive use of bioinformatic tools to identify the genes with true tissue-specific expression patterns. For example, the use of only UniGene could provide data which is based on all available cDNA libraries. In this case, the prediction of tissue-specific gene expression and putative function of the

novel protein produced could be more reliable. Moreover, important information concerning sequencing strategy, specific tissue source and cDNA library titres are often hidden in specific supplemental databases, which makes the use and interpretation of *in silico* analyses even more complicated. In short, *in silico* analyses of gene expression profiles require specific knowledge of bioinformatics, and the standardization and integration of the various databases will probably ease these analyses in future.

3 AIMS OF THE PRESENT STUDY

The objective of the present study was to develop new, clinically-relevant methods and markers to improve the detection and prognostic evaluation of neuroblastoma.

The specific aims were:

1. To construct a feasible and strictly defined strategy to unify the detection of molecular markers in paraffin-embedded neuroblastoma samples (study I)

2. To construct a simple and reliable method for the assessment of *MYCN* amplification status (study I)

3. To uncover novel prognostic markers of neuroblastoma to further refine treatment decisions (studies I, III, IV)

4. To identify gene mutations for a new cytotoxic treatment (study II)

5. To study sites of genetic aberrations in order to provide insights into the mechanisms of malignant transformation and progression (study IV)

4 MATERIALS AND METHODS

4.1 Human tissue and tumour classifications

The study material consisted of a total of 37 archival paraffin-embedded neuroblastoma samples of 37 patients, who were diagnosed between the years 1967 and 2001 in the Turku University Central Hospital and the Tampere University Hospital, Finland. The local ethics committee approved our study. There were 34 primary tumours and three metastases, of which the primary tumours were not available. Even though intracranial primary neuroblastomas are rare, one primary tumour out of 34 was intracranial (Ahdevaara et al., 1977). Since two primary tumour samples were achieved after cytotoxic treatment, and one primary tumour was intracranial, there were 31 extracranial primary neuroblastoma samples taken before any treatments. All the tumours were immunohistochemically stained and microscopically re-evaluated by two experienced pathologists. For histological typing and grading (excluding the intracranial tumour and two cytotoxically pretreated tumours), the INPC definition was applied (Shimada et al., 1999b). The 34 classified tumours (31 primary tumours and three metastases) comprised 31 neuroblastomas (14 undifferentiated, eight poorly differentiated, and nine differentiating), two ganglioneuroblastomas (one nodular, one intermixed) and one ganglioneuroma. Of these 34 cases, nine (26%) were with favourable, and 25 (74%) with unfavourable histology. Follow-up of the patients was conducted by a review of outpatient medical records. The clinical stage of 22 cases could not be evaluated according to the INSS (Brodeur et al., 1993), because the tumours were diagnosed before 1993, and hence the clinical evaluation of dissemination status was inadequate. Therefore, dissemination status was evaluated on the basis of medical records, and the clinical stage of the disease was divided into four categories: 1) local tumour, extirpation (completely excised); 2) local tumour with regional lymph node involvement; 3) distant metastases; 4) tumours that met INSS Stage 4s definition (Brodeur et al., 1993).

A total of 19 paraffin-embedded bone marrow samples (range 1–7 samples per patient; median two samples per patient) from six patients were available. The original histopathological analysis of the samples revealed only two certain and one uncertain bone marrow involvements. These three samples, which were from the same patient at different time points (diagnosed in 2001), were selected for further analyses. The primary tumour was an undifferentiated neuroblastoma (stage 4) with unfavourable histology.

4.2 Hotspot selection with MIB-1 antibody

Tumour cell proliferation was determined with the monoclonal antibody MIB-1 against the Ki-67 protein (clone MIB-1, 1:110 dilution, DakoCytomation,

Denmark). Briefly, 5-mm-thick sections were cut from paraffin-embedded specimens, mounted on slides and dried overnight at 37°C. Immunoreactivity was restored by microwave pretreatment (2x7 min, 850 W) in Tris-EDTA (pH 9.0). The primary antibody was incubated on the sections for 25 min at room temperature, and immunostaining was performed in an automated immunostainer (TechMatet 500 Plus, DakoCytomation, Denmark) using the biotin–streptavidin–peroxidase procedure with diaminobenzidine (DAB) as the chromogen (ChemMate Envision Detection Kit, DakoCytomation, Denmark). Counterstaining was carried out using 0.4% ethyl green in acetate buffer for 15 min. The whole tumour specimen was evaluated for the number of MIB-1-stained cells with transmitted light microscopy (Zeiss), and hotspots, which were areas expressing quantitatively the highest number of immunopositive nuclei, were analysed with a computer-assisted image analysis as described (Sallinen et al., 1994). The proliferation index of the hotspots was the percentage expressing the ratio of brown and green nuclei in at least 1000 cells (mean 1945 cells, median 1796 cells) and at least 20 microscopic fields (x400 magnification).

4.3 Hotspot tumour tissue microarray

Tissue microarrays of neuroblastomas were constructed from a total of 37 formalin-fixed, paraffin-embedded blocks. The hotspots were determined as described above. After marking the hotspot on each slide, the donor block was positioned for sampling based on a visual alignment with the corresponding hotspot. The block surface was punched with a tissue arrayer (Beecher Instruments, USA), and the cylindrical tissue column (Ø 2mm) was transferred to a corresponding receiver pore of the recipient paraffin block with defined array coordinates. After the block construction was completed, 5-mm-thick sections of the resulting tumour tissue microarray block were cut with a microtome.

4.4 Chromogenic in situ hybridization

MYCN amplification status in neuroblastomas was determined by CISH analysis with a digoxigenin-labelled probe complementary to the *MYCN* gene (Spot-Light N-Myc Probe, Zymed, South San Francisco, CA, USA) as described (Rummukainen et al., 2001). Briefly, hotspot tumour tissue microarray slides and three bone marrow samples were hybridized after deparaffination, denaturation and dehydration with 10 ml of probe cocktail (2 ml of digoxigenin-labelled *MYCN* probe, 1 ml of 9.9mg/ml human placental DNA, and 1 ml of 1mg/ml Cot-1 DNA (Roche Molecular Biochemicals, Mannheim, Germany), and 6 ml of master mix (Rummukainen et al., 2001). Hybridization was carried out after codenaturation of the probe mixture in a humid chamber at 37°C for 16–24 h. After hybridization, the slides were washed, and the *MYCN* probe detected as described (Rummukainen et al., 2001). Microscopy

was performed after light counterstaining with haematoxylin with a transmitted light microscope (Zeiss). In every hotspot tumour tissue microarray sample, 100 nonoverlapping tumour cell nuclei were randomly scored to determine the number of *MYCN* signals. A tumour sample was considered to be *MYCN* amplified, when an average of 6.00 or more nuclear signals per cell were seen, or when tumour nuclei showed large clustered signals. No adjustment for potential hyperploidy was made.

4.5 Immunohistochemistry

Paraffin-embedded hotspot tumour tissue microarray slides, bone marrow core biopsy samples on slides, and bone marrow aspiration smears were used for conventional immunostainings. After the slides were deparaffinized, microwave pretreatment was performed in 0.01 mol/l citrate buffer (pH 6.0, 2x5 min, 750 W) for Id2, nestin and NCAM antigen retrievals, in Tris-EDTA buffer (pH 9.0, 3x7 min, 850 W) for c-kit protein, and also in Tris-EDTA (pH 9.0, 2x7 min, 850 W) for Ki-67 protein. For polySia-NCAM and simultaneous double-labelling with anti-NCAM antibodies, slides were deparaffinized, rehydrated in a descending ethanol series and stained conventionally.

Rabbit anti-human nestin IgG antibody at a concentration of 1 µg/ml (Immuno-Biological Laboratories, Japan), and rabbit anti-Id2 polyclonal antibody at a concentration of 2–4 µg/ml (Zymed Laboratories, San Francisco, CA, USA) were used as primary antibodies. PolySia-binding fluorescent fusion protein (EndoNA2-GFP) at a concentration of 10 µg/ml was used for polySia detection (Jokilammi et al., 2004). Mouse anti-human NCAM antibody (123C3) at a concentration of 4 µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody. All antibody incubations were carried out overnight at 4°C. In immunohistochemistry, antibody detection was carried out using the anti-rabbit HRP polymer (PowerVision; ImmunoVision Technologies, Daly City, CA, USA). The immunoreaction was visualised with 3-aminoethyl-carbazole (AEC) for 10 min (ready-to-use, LabVision, Fremont, CA, USA), or DAB as chromogen. Slides were slightly counterstained with haematoxylin, and mounted with Faramount (DakoCytomation). In immunofluorescence, the Alexa Fluor 594 chicken anti-rabbit secondary antibody and Alexa Fluor 594 chicken anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) were used, and slides were mounted with Immu-Mount (Shandon, USA).

Expression of the c-kit protein was detected using the polyclonal rabbit antihuman c-kit antibody (CD117; 1:250 dilution; DakoCytomation, Glostrup, Denmark). Immunostaining was performed in an automated immunostainer (TechMateTM 500 Plus; DakoCytomation) using the avidin–biotin complex method with diaminobenzidine as the chromogen. A GIST tumour served as a positive control. Mast cells served as internal positive controls and as a reference for positive immunolabelling. Immunostaining in more than 30% of the cells was considered to be a positive reaction (Vitali et al., 2003). A cut-off

value of 10% has also been used in one report (Krams et al., 2004), but this does not hamper the comparison of the results because normally more than 50% of the tumour cells were labelled in positive cases. Immunolabelling for c-kit was evaluated in a simple blind trial fashion by three independent observers

4.6 Bone marrow smears and flow cytometry

Bone marrow samples were prepared from an aspirate, obtained from an 18-year-old man, whose bone marrow was examined for non-malignant anaemia. For bone marrow smears, bone marrow cells were mixed with *in vitro* cultured human neuroblastoma SH-SY5Y cells (0, 50 and 100%), and the EndoNA2-GFP fusion protein at a concentration of 10 µg/ml was used for staining the smears. For flow cytometry, bone marrow cells (100 µl, 3.5×10^6) and polySia-NCAM-positive SH-SY5Y cells (100 µl, 3.5×10^6) were labelled with the EndoNA2-GFP (10µg/ml) fusion protein for 20 minutes at room temperature. Red cells were lysed with FACS lysis buffer (Becton-Dickinson, NJ, USA). The cell suspensions were analysed with a FACSCalibur flow cytometer (Becton-Dickinson).

4.7 Denaturing HPLC analysis of c-kit mutations

Exons 9, 11, 13, and 17 of the *c-kit* gene were evaluated for the presence of mutations. Genomic DNA was extracted and purified from paraffin-embedded tumour specimens using a QIAamp mini kit according to the manufacturer's recommended protocol for paraffin-embedded tissue samples (Qiagen, Hilden, Germany). Genomic DNA (50 ng) was amplified in a polymerase chain reaction (PCR) containing 0.6 ml of Platinium PCR buffer (Invitrogen, Carlsbad, California, USA), 1.4–2.4 mM $MgCl_2$, 160 mM dNTPs (Clontech, Palo Alto, California, USA), 0.3 mM forward and reverse primers, and DNA polymerases - AmpliTaq Gold (1.25 U; Applied Biosystems, Branchburg, New Jersey, USA) and Platinium Taq (1.25 U; Invitrogen) - in a 50 ml volume. Negative and positive (GIST tumour) controls were included in every batch of amplifications. Denaturing high performance liquid chromatography (HPLC) analysis was carried out using Agilent Technologies' series 1100 HPLC system (Agilent Technologies, Palo Alto, California, USA). The PCR product (5–10 ml) was injected into the Zorbax Eclipse double stranded DNA HPLC column (75 x 2.1 mm; Agilent Technologies) and eluted at a flow rate of 0.4 ml/minute within a linear acetonitrile gradient, consisting of a mixture of buffer A (100 mM triethylammonium acetate and 0.1 mM EDTA; Varian Inc, Walnut Creek, California, USA) and buffer B (100 mM triethylammonium acetate, 0.1 mM EDTA, and 25% acetonitrile; Varian Inc). The DYS271 standard, which consists of a 209 base pair fragment of heterozygous double stranded DNA, with an A to G mismatch at position 168, was used as a control. The elution temperatures for each amplicon were obtained from the denaturing HPLC Melt Program (http://insertion.stanford.edu/melt.html), and then optimised by

studying alterations in the elution profiles of the samples within a temperature range of 3°C under and above the suggested melting temperature. The temperature that best separated homoduplexes was used for the denaturing HPLC analysis. Samples with abnormal-like elution profiles in denaturing HPLC were subjected to automated sequencing. The PCR products were first purified using Montage DNA purification columns (Millipore, Bedford, Massachusetts, USA). Direct sequencing of PCR products was performed using BigDye3 termination chemistry (Applied Biosystems, Foster City, California, USA) and an ABI 3100 genetic analyser (Applied Biosystems), according to the instructions provided by the manufacturer.

4.8 Confocal microscopy

We performed confocal scanning microscopy for the TMA of the paraffin-embedded neuroblastoma samples using a Leica TCS SP MP confocal microscope equipped with a Spectra-Physics Tsunami Ti-sapphire laser and Leica confocal software. Sections were examined at two excitation wavelengths: 488 nm for the polySia-binding fusion protein and 546 nm for fluorescent secondary antibodies.

4.9 Neuroblastoma cell cultures and sample preparation

NGP and IMR-32 neuroblastoma cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 2 mM L-glutamine, and Minimum Essential Medium supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate, respectively. Poly-RNA was isolated from the samples using the FastTrack 2.0 mRNA isolation kit (Invitrogen, Carlsbad, CA). Genomic DNAs were obtained from the same samples by swirling a glass rod in the cell lysate, followed by standard phenol-chloroform purification.

4.10 Oligonucleotide array-based comparative genomic hybridization

A 95K high-resolution oligonucleotide array (Agilent Technologies, Palo Alto, CA) was used for the detection of copy number changes from neuroblastoma NGP and IMR-32 cell lines. Normal male reference DNA was used as a reference for both samples (Cat. # G1471, Promega, Madison, WI). Sample processing and hybridization was performed according to the August 2005 (version 2) protocol (Agilent Technologies), with minor modifications. Briefly, 10 µg of genomic DNA was digested overnight with AluI and RsaI (Life Technologies, Inc., Rockville, MD). Digested DNA samples were subjected to phenol-chloroform purification. 4 µg of digested tumour DNA and reference DNA were labelled with Cy5-dUTP and Cy3-dUTP (Perkin-Elmer, Wellesley, MA), respectively, in a random priming reaction using the BioPrime Array CGH

Genomic Labeling Module (Invitrogen, Carlsbad, CA.). After labelling, tumour DNA and reference DNA samples were pooled, cleaned and hybridization cocktails were prepared as instructed in the protocol. Hybridization and washes were performed according to the protocol. A laser confocal scanner (Agilent Technologies) was used to obtain signal intensities from targets, and Feature Extraction software (version 8.1.1.1, Agilent Technologies) was applied in image analysis using the manufacturer's recommended settings (44K_CGH_0605). To analyse the aCGH data we used CGH Analytics software (version 3.2.32, Agilent Technologies). Control hybridizations (male vs. male, male vs. female hybridizations) were used to estimate the baseline variation in the hybridizations, and hybridization quality metrics provided by CGH Analytics were evaluated to ensure good data quality.

4.11 Gene expression analyses

Gene expression levels from NGP and IMR-32 cell lines were measured using Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). In addition, a pooled sample composed of 16 different cancer cell lines was analysed as a reference sample. Sample processing and labelling were performed according to the protocol provided by Affymetrix. Briefly, 3 µg of messenger RNA was used for one-cycle cDNA synthesis using a T7-oligo(dT) promoter primer, followed by RNase H-mediated second-strand cDNA synthesis and *in vitro* transcription reaction with biotinylated ribonucleotide analogs. Biotin-labelled target cRNAs were fragmented and the quality of labelling procedures was assessed with test 3 arrays. Hybridizations to U133 Plus 2.0 arrays were performed for 16 hours at 45^{o}C, followed by automated array washing and staining procedures. Arrays were scanned immediately after staining using the GeneChip scanner (Affymetrix). Affymetrix Microarray Suite 5.0 (MAS 5.0) was used to normalize and calculate the expression values from the scanned images.

4.12 Integration of array-CGH and gene expression data

Expression ratios for NGP and IMR32 were calculated as the log2 ratio of the cell line divided by the reference pool hybridization. Expression and copy number data were integrated as follows: Affymetrix probe sets were mapped to Ensembl gene ids or the base pair position given by Affymetrix, if no matching Ensembl gene id was found. A copy number ratio for each probe set was then calculated as the median of CGH array oligos located within 50kb of the probe set's genomic position.

4.13 Hotspot FISH on neuroblastoma tissue microarray

Twelve BAC clones from 12q13-q14 (RP11-846E20, RP11-66N19), 12q15 (RP11-1024C4, RP11-77H17), and 12q24.31 (RP11-44F24, RP11-87C12,

RP11-94C5, RP11-512M8, RP11-152E17, RP11-679G17, RP11-1059L20, and RP11-486O12) were labelled with digoxigenin-11-dUTP (Roche Applied Science, Basel, Switzerland) using random priming. A spectrum Green-labelled, chromosome-12-specific centromere probe (Vysis Inc. Downers Grove, IL) was used as a reference. Hotspot FISH TMA was constructed as described. FISH on TMA containing 37 samples was performed as described (Andersen et al., 2001), except that the fixation of the slides was performed using 7% formalin for 10 min. The BAC probes were detected with anti-digoxigenin-Rhodamine (Roche Applied Science) and nuclei counterstained with 0.1 M 4',6-diamidino-2-phenylindole. The fluorescence signals were scored from non-overlapping nuclei using an Olympus BX50 epifluorescence microscope (Tokyo, Japan). The entire tissue area was evaluated and 20–60 representative non-overlapping nuclei were scored. A 1.5-fold increase in the test probe copy number relative to the chromosome 12 centromere was considered as gain in copy number.

4.14 In silico screening for deregulated genes at 12q24

In order to evaluate the *in vivo* effect of the 12q24.31 gain in gene deregulation, we performed *in silico* data mining for gene expression levels from data obtained from previously published studies / available datasets on samples with a nerve cell origin (Rickman et al., 2001; Pomeroy et al., 2002; Blalock et al., 2003; Nutt et al., 2003; Dangond et al., 2004; Freije et al., 2004; Su et al., 2004; Vawter et al., 2004; Yanai et al., 2005). Both normal and malignant tissues related to the nervous system were evaluated. Data were retrievable for 24 of 51 genes residing at the 12q24.31 amplicon based on Ensembl release 49.

4.15 Statistical analysis

Differences between two groups in categorical data were analysed by means of the Pearson's χ^2 test. OS analysis was computed by means of the Kaplan–Meier method, and the difference between the curves was compared with the log-rank test. Differences of mean values between multiple groups were analysed by using the one-way Anova test. Statistical analyses were performed with SPSS 12.0 for Windows or SPSS 16.0 for Apple, and *P* values of <0.05 were considered statistically significant.

5 RESULTS

5.1 CISH technique in the analysis of MYCN amplification (I)

CISH analysis of all 37 neuroblastoma specimens in hotspot tumour tissue microarrays was successfully performed, but two specimens (*MYCN*-nonamplified) were excluded from statistical analyses on account of cytotoxic pretreatment before biopsy. Eight (23%) neuroblastomas out of 35 were shown to contain *MYCN* amplification with CISH, and seven of these positive samples were primary tumours. In general, the *MYCN* unadjusted copy number ranged between 1.85 and 5.55, though the copy numbers were not countable for the strongly hybridized clusters in some neuroblastoma samples. Interestingly, the presence of *MYCN*-amplified tumour cells in two out of three paraffin-embedded bone marrow samples from a single patient with metastatic neuroblastoma was detected with CISH without difficulty. These two samples were the same, which showed clear bone marrow involvement in the original histopathological analysis. In the *MYCN*-nonamplified sample, there was no clear evidence of metastatic disease in the original histopathological analysis.

As the *MYCN* copy numbers for the strongly hybridized clusters were uncountable, the use of continuous variables in statistical analysis was not applicable. *MYCN* amplification status in 16 out of 35 tumours was measured by Southern blot hybridization at the time of diagnosis. Southern blot analysis detected *MYCN* amplification (≥10 copies) in three out of the 16 tumours (19%), and all *MYCN*-amplified tumours had strongly hybridized clusters in CISH analysis. All 13 nonamplified neuroblastomas had *MYCN* copy numbers below 6.00 in CISH analysis.

5.2 Significance of hotspot proliferation indices (I)

The PIs of hotspots were measured with the MIB-1 antibody against Ki-67, and the values ranged from 2.9 to 63.2, with a mean value of 28.5±17.9, among 34 histopathologically classified tumours (International Classification). A statistically significant (P = 0.040) association between PI and the International Classification was found, as undifferentiated neuroblastomas in particular had higher PIs. In addition, nine tumours with favourable histology according to the International Classification had a mean proliferation index of 18.5±15.9, whereas 25 prognostically unfavourable tumours had a higher mean proliferation index of 32.1±17.4 (P = 0.048). In contrast, an association between PI and INSS stages was not significant (P = 0.589) among those 15 tumours which were staged according to the INSS (Brodeur et al., 1993). However, despite the P value of 0.294, an obvious association between higher PIs and INSS stages was discovered, as stage 1 and 2 neuroblastomas (four

tumours) had a mean PI of 21.4±12.4 and stage 4 neuroblastomas (11 tumours) had a mean PI of 33.1±19.8. There were no stage 3 neuroblastomas. Furthermore, *MYCN*-amplified tumours had a higher mean PI (P = 0.047) than *MYCN* single copy tumours. Two cytotoxically pretreated primary tumours (*MYCN* single copy) were excluded from the analysis. In summary, the neuroblastoma growth fraction (PI) in hotspots, assessed using the monoclonal MIB-1 antibody, has a significant association with histological type, histopathological prognosis and *MYCN* amplification in neuroblastomas.

5.3 Id2, nestin and c-kit expression (I, II)

The Id2 antibody gave very faint and unreliable immunohistochemical staining results. Therefore, we used a fluorescent secondary antibody and confocal microscopy to detect Id2-positive signals. Id2 expression was detected in seven (22%) out of 32 neuroblastomas, when staining in more than 50% of the tumour cells was considered a positive case. Five samples could not be interpreted due to detached samples and unrepeatable staining patterns. In our series, Id2 expression did not associate with INPC, INSS, proliferation indices, histopathological prognosis, or outcome. None of the seven *MYCN*-amplified tumours expressed Id2 protein. Interestingly, six out of seven Id2-expressing neuroblastomas expressed nestin (P = 0.051).

Nestin immunohistochemical staining results were interpreted without difficulty, and detectable levels of nestin expression were observed in 19 (54%) of 35 neuroblastomas, when staining in more than 50% of the tumour cells was considered a positive case. Two samples could not be interpreted due to detached samples. The immunostaining pattern in all positive cells was cytoplasmic. Most interestingly, only one out of seven *MYCN*-amplified tumours showed nestin expression in hotspot analysis (P = 0.018). Nestin expression did not correlate with INPC, INSS, proliferation indices, histopathological prognosis, or outcome.

Tumour phenotyping with the anti-c-kit antibody detected expression of the c-kit receptor in four of the 37 tumours tested. More than 30% of the tumour cells were labelled in all positive cases. In general, the labelling intensity in positive neuroblastomas was much weaker than in GIST tumours, which served as positive controls. Only one out of eight *MYCN*-amplified neuroblastomas was c-kit-positive. The number of c-kit immunopositive samples in our study was too small to define a statistical relation between c-kit expression and common prognostic factors of neuroblastomas.

5.4 GIST-like c-kit mutations in neuroblastoma (II)

Because the mutational status has been shown to have a central role in GISTs, we analysed all our neuroblastoma samples for the presence of mutations in

exons 9, 11, 13, and 17 of the *c-kit* gene. No *c-kit* mutations were found in the 37 neuroblastoma samples. An abnormal-like elution pattern suspicious for a mutation in exon 11 was found in one tumour. However, bidirectional sequence analysis revealed a normal wild-type sequence, with no signs of mutation. This tumour was immunohistochemically c-kit-negative and had no *MYCN* amplification.

5.5 PolySia and NCAM expression in paraffin-embedded neuroblastoma samples (III)

PolySia expression proved to be positive in 17 (55%) out of 31 patients whose neuroblastoma samples were taken from primary tumours before any treatment (*i.e.* no treatment effect on antigen expression). An example of the staining pattern of polySia is given in Figure 4. PolySia-specific immunofluorescence was confirmed by pretreatment of neuroblastoma samples with endosialidase, which abolished polySia-positive immunofluorescent signals (data not shown). All three metastatic neuroblastoma samples from three patients were polySia- and NCAM-positive. The unselected and overall proportion of patients with polySia-positive neuroblastomas was 21 (58%) out of 36. In our analysis series, there were no polySia-expressing tumours which did not show concomitant and co-localized NCAM expression. NCAM is the carrier of cell surface polySia: co-localization of polySia-NCAM and NCAM is shown in Figure 4.

NCAM expression was positive in 20 (65%) out of 31 primary neuroblastoma samples, which were taken before any treatment. The NCAM staining pattern is shown in Figure 4. A negative NCAM staining control was obtained by omission of the primary antibody. Three out of 20 NCAM-positive primary neuroblastomas did not express polySia-NCAM. The unselected and overall proportion of patients with NCAM-positive tumours was 24 (67%) out of 36.

Bone marrow disease was previously evaluated in 14 out of 31 patients with untreated primary neuroblastomas, and 11 of these 14 patients had been reported (on the basis of medical records) to have bone marrow metastases at diagnosis. PolySia-NCAM and NCAM expression in untreated primary tumour samples (not metastatic samples from regional lymph nodes or distant organs) was positive in 9 out of 11 patients with bone marrow disease. One out of three patients without bone marrow involvement had a polySia-NCAM- and NCAM-positive primary neuroblastoma.

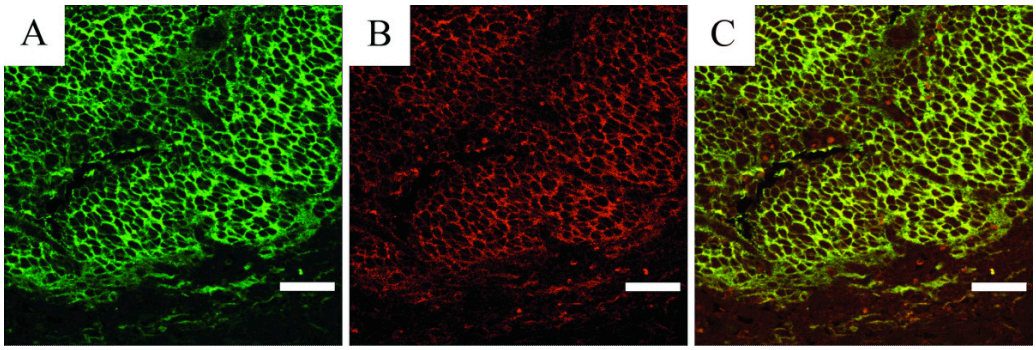

Figure 4. Fluorescence staining patterns of a primary neuroblastoma. (A) Expression of polySia NCAM (stained with an EndoNA2-GFP fusion protein), and (B) NCAM (stained with Alexa Fluor 594) at the same tumour site. (C) An overlay image of A and B identifying colocalized expression of polySia-NCAM and NCAM. Scale bar, 40 µm.

5.6 Detection of polySia-NCAM-positive neuroblastic cells in bone marrow with immunofluorescence and flow cytometry (III)

To investigate whether metastatic neuroblastoma cells in bone marrow express polySia-NCAM, as do their polySia-NCAM-positive primary tumour foci, 19 paraffin-embedded bone marrow biopsies from six different patients were labelled with the fluorescent fusion protein. PolySia-NCAM-positive tumour cell clusters were found in two different bone marrow samples, taken at different time points from the same patient with a polySia-NCAM positive primary tumour. Contrary to the primary tumours, polySia-NCAM-positive bone marrow neuroblastoma cells appeared to be in a nonproliferative state, when samples were double-labelled with anti-Ki-67 antibody.

Fresh bone marrow cells and *in vitro* cultured polySia-NCAM-positive SH-SY5Y neuroblastic cells were mixed together in different ratios, and the mixed cell suspensions were labelled with the polySia-binding fluorescent fusion protein. Interestingly, smears with added polySia-NCAM-positive SH-SY5Y cells appeared as cell clusters, as in fixed bone marrow biopsy samples, whereas smears without added SH-SY5Y cells did not show any cell clustering. Normal bone marrow samples were considered polySia-NCAM-negative, even though they contained a few single cells (approximately 1-3 individual cells per microscopic field), which were polySia-NCAM-positive, but significantly smaller in size than polySia-NCAM-positive tumour cell clusters.

In evaluating the possible applicability of flow cytometry to differentiate bone marrow metastases (polySia-NCAM-positive neuroblastoma cells or cell clusters) from normal bone marrow cells, our results revealed that polySia-NCAM-positive tumour cells produce distinct fluorescence emission wavelengths when compared to fusion protein-labelled normal bone marrow cells. As we had no permission to take fresh bone marrow samples from

neuroblastoma patients for research purposes, we had to use the above-described artificially created “stage 4 bone marrow samples” to test the applicability of polySia-based detection of neuroblastoma cells in fresh samples.

5.7 Association of polySia-NCAM and NCAM expression with clinical parameters (age and stage) (III)

Patients’ age did not associate with polySia-NCAM or NCAM expression, not even when groups were dichotomized at the ages of 12 or 18 months. However, polySia-NCAM expression in neuroblastomas did associate with clinical stage, as patients with polySia-NCAM-expressing primary tumours had advanced (*i.e.* metastatic) disease at diagnosis (P = 0.047). The association between NCAM expression and clinical stage was nearly significant (P = 0.053).

5.8 Association of polySia-NCAM and NCAM expression with histological parameters (INPC, proliferation index [PI] and MYCN amplification) (III)

Analysis of the correlation between polySia-NCAM expression and tumour differentiation (INPC) revealed that polySia-NCAM and NCAM were expressed more frequently in undifferentiated and in poorly differentiating than in differentiated neuroblastomas, but the P-value did not reach significance (P = 0.623 for polySia-NCAM, and P = 0.165 for NCAM). PolySia-NCAM- and NCAM-expressing primary tumours showed higher PIs than polySia-NCAM- and NCAM-negative tumours (P = 0.011 for polySia-NCAM, and P = 0.001 for NCAM). There was no association between polySia-NCAM or NCAM expression and *MYCN* amplification status. All patients with *MYCN*-amplified neuroblastomas (seven patients) had stage 4 disease (*i.e.* distant metastases) at diagnosis.

5.9 Genome-wide copy number alterations in neuroblastoma cell lines by array-CGH (IV)

An aCGH analysis revealed several copy number changes in IMR-32 and NGP cell lines. The most prominent copy number alterations of the IMR-32 cell line were found at 2p, where three clearly distinct high-level amplifications at 2p24.3, 2p14 and 2p13.3 (14.7-16.0, 66.7-67.8, 69.1-69.4 Mb from the p-telomere) were detected, including loci for *MYCN*, *MEIS1* (*myeloid ecotropic viral integration site 1 homolog*) and *ANTRX1/TEM8* (*anthrax toxin receptor 1/ tumour endothelial marker 8*), respectively. In addition, high-level microamplification at 2p23 affecting only two probes for the *ALK* (*anaplastic lymphoma kinase*) gene was observed.

NGP cells showed high-level amplification of *MYCN* at 2p24.2-p24.3 (16.0-17.4 Mb from the pter), as well as several other loci at 12q; 12q14.1, 12q15, 12q24.11 and 12q24.31 (56.4-59.5, 67.2-69.4, 108.0-108.3 and 119.9-122.5 Mb from the pter). Altered regions included previously known targets, such as *CDK4* (*cyclin-dependent kinase 4*) and *SAS* (*sarcoma amplified sequence*) at 12q14.1, as well as *MDM2* (*mouse double minute 2 homolog*) at 12q15. The previously unidentified microamplification at 12q24.11 involved *ACACB* (*acetyl-Coenzyme A carboxylase beta*) and *FOXN4* (*forkhead box N4*) genes, whereas several genes were included in the detected 12q24.31 amplification. Losses of copy number included, for example, those detected at 1p (0-50.7 Mb) and 16q (68.3-88.7 Mb) in the IMR-32 cell line, and chromosome 10 (121.6-135.4 Mb), 11 (98.9-134.4 Mb) and 19 (56.5-63.7 Mb) in the NGP cell line. In addition to these, putative loci with narrow homozygous microdeletions were observed at 11p15.4 (49.2-49.3 Mb) in both samples, at 3q26.1 (163.8-164.1 Mb) and at 10q26.13 (124.3-124.4 Mb) in the NGP cell line, involving *MMP26* precursor, an unknown gene, and *DMBT1* (*deleted in malignant brain tumours 1*), respectively.

5.10 Impact of copy number alterations on gene expression (IV)

Comparison between DNA and RNA level data showed that gene expression levels across the genome were very significantly influenced by copy number changes. The majority of the most highly amplified and overexpressed genes were located at the 2p- and 12q-amplicons. *MYCN* was identified as the target for 2p24 amplification in both cell lines. Other genes implicated in the IMR-32 cell line at 2p24 were *FAM84A/NSE1* (family with sequence similarity 84, member A) and *NAG* (neuroblastoma-amplified protein), as well as *MYCNOS* (*MYCN* opposite strand), and *FAM49A* in the NGP cell line. In the IMR-32 cell line, the 2p14 amplicon showed overexpression of both *MEIS1* and *ETAA16*, and *TEM8/ANTRX1* at 2p13.3.

At 12q, several genes were shown to be upregulated at distinct amplicons in the NGP cell line. At 12q14.1, amplification and overexpression of, for instance, *CENTG1/PIKE* (centaurin, gamma 1) and *AVIL* (advillin) was observed, in addition to the previously reported *SAS* gene. The 12q15 amplicon showed the characteristic involvement of *MDM2* as well as several neighbouring genes, such as *FRS2* (*fibroblast growth factor receptor substrate 2*), *CPM* (*carboxypeptidase M*) and *CPSF6* (*cleavage and polyadenylation specific factor 6*). At 12q24.11 microamplification, involving only *ACACB* and *FOXN4*, we observed moderately elevated expression of both genes when compared to the reference sample. The most distal 12q amplicon, located at q24.31, included, in addition to *RSN* (*restin*), several amplified and overexpressed genes whose role in neuroblastomas has not been demonstrated. The frequency and clinical significance of this previously poorly

characterized 12q24.31 amplification was explored and compared to the frequency of 12q14-q15 amplification using hotspot FISH in clinical neuroblastoma specimens in the hotspot tissue microarray format.

5.11 Copy number alterations at 12q and the clinical significance of the q24 gain (IV)

DNA copy number alterations at 12q14, 12q15 and 12q24.31 were analysed in the hotspot tumour TMA of neuroblastomas, and altogether 2 out of 31 (6%), 6 out of 32 (19%) and 14 out of 33 (42%) informative samples showed gain of copy number in the corresponding three chromosomal regions, respectively. High-level copy number increases of 12q24 were not seen in the clinical tumour samples (the mean ratio between test and centromeric probes was 1.7). Interestingly, 12q24.31 gains and *MYCN* amplifications were present in different subsets of tumours. Only 1 out of 14 (7%) of the 12q24-gained tumours showed *MYCN* amplification, which was detected in 7 out of these 33 tumours. The two genetic aberrations accounted for 64% of all neuroblastomas, which were analysed for gain of 12q24. Since gain of 12q24.31 was not significantly associated with any of the other obvious histological (INPC) and prognostic parameters (stage, age, PI) (results not shown), our data suggest that it may provide independent prognostic value.

5.12 In silico screening of 12q24.31 genes in primary neuroblastomas and healthy nervous system samples (IV)

In silico analysis of existing gene expression data suggested a putative effect for 12q24.31 gain in gene deregulation in neuroblastoma. The known deregulated oncogenes *MYCN*, *MEIS1* and *ALK* were used as positive controls. The expression of all three genes was highly elevated when compared to 445 healthy nervous system samples. Similarly, gene expression data evaluation for the 24 genes residing at the 12q24.31 region indicated *DIABLO* (*diablo homolog, Drosophila*) having statistically elevated expression specifically in neuroblastoma when compared to healthy nervous system samples.

5.13 Survival analyses (I, III, IV)

MYCN amplification (*MYCN* copy number ≥6.00) had strong prognostic significance (P = 0.0006), as shown in an OS plot in Figure 5 A. The *MYCN* copy number was between 3.00 and 5.99 in seven tumours, which did not differ prognostically from 20 nonamplified tumours (Fig. 5 B).

Negative polySia-NCAM expression was a strong unfavourable predictor of OS in advanced disease as all seven patients with negative polySia-NCAM expression and regional lymph node involvement or distant metastases died during the follow-up time (P = 0.0004). Negative NCAM expression was also an unfavourable predictor of outcome (P = 0.0088) in the same advanced disease group. When the prognostic value of polySia-NCAM (Fig. 5 C) and NCAM (Fig. 5 D) were evaluated solely for the 19 patients with distant metastases, they still reached significant P-values (P = 0.0019 and P = 0.0240, respectively). In comparison to the prognostic value of *MYCN* amplification in the advanced disease group (not significant, P = 0.0666), polySia-NCAM and NCAM absence were stronger predictors of unfavourable outcome.

Although high-level copy number increases of 12q24 were not seen in the clinical tumour samples (the mean ratio between test and centromeric probes was 1.7), our results provided evidence that a low-level gain of this genomic locus was associated with poor prognosis of patients with neuroblastomas. Patients without 12q24.31 gains and *MYCN* amplifications had the best OS, followed by those with only a gain of the 12q24.31 region, whereas patients with *MYCN*-amplified tumours had the worst prognosis (P = 0.0009) (Fig. 5 E).

In order to indirectly validate the value of the study cohort in prognostic analyses, prognostic significance of clinical stage and age were also analysed. Clinical stage was a significant risk factor in our study cohort, and patients having INSS 4s or a local disease with or without regional lymph node involvement had much better OS than patients with distant metastases (P = 0.0077) (Fig. 5 F). Similarly, age was a significant prognostic factor, too, when dichotomized at 18 and 12 months (P = 0.0020 and P = 0.0077, respectively) (Fig. 5 G and H). Therefore, age, stage and *MYCN* status are highly significant risk factors in the studied patient cohort, which is consistent with the previous consensus report (Cohn et al., 2009).

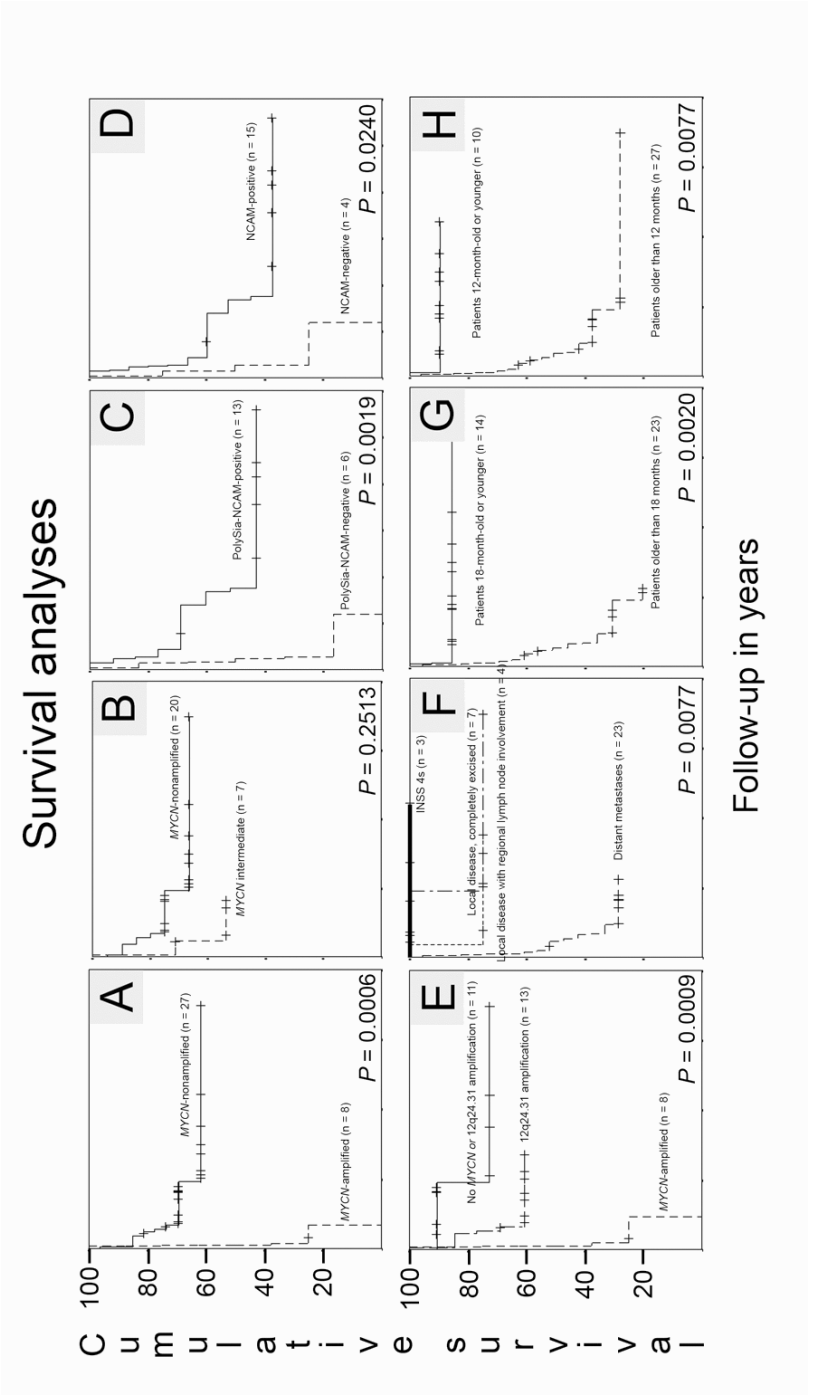

Figure 5. Survival analyses of neuroblastoma patients, which are stratified into various study groups by (A and B) *MYCN* amplification, (C) polySia-NCAM expression, (D) NCAM expression, (E) 12q24.31 and *MYCN* amplification, (F) clinical stage, (G) age dichotomized at 18 months, and (H) age dichotomized at 12 months. The x-axis represents follow-up time in years (40 years in A-B and E-H, 12 years in C-D), and the y-axis represents cumulative survival.

6 DISCUSSION

Due to the well-documented heterogeneous nature of neuroblastoma, several different geno- and phenotypic variants are apparent in a single tumour. Despite heterogeneity, neuroblastoma is probably one of the first tumours for which genetic markers have been used as an adjunctive clinical tool for prognostic definitions. Improvements in survival have been achieved and will probably continue to be achieved with the use of innovative treatment approaches based on a better understanding of the biological pathways responsible for neuroblastoma progression. It has now become clear that a vast number of biological markers have a role in tumourigenesis and progression of neuroblastoma, but the current evidence relating to the published markers is rather conflicting (Riley et al., 2004). Since studies of relatively rare diseases such as neuroblastoma often involve a small number of patients, it is particularly important to evaluate new neuroblastoma markers in combination with previously known significant ones.

The expansion in the number of possible new biological markers is an indirect indication that neuroblastoma is a complex disease with multiple changes taking place within a single tumour. Our studies consist of a small number of patients, thus, at their best, introducing primary knowledge of novel prognostic factors and diagnostic applications for future research. Age, stage and *MYCN* status, which are the most important previously published risk factors (Cohn et al., 2009), were highly significant prognostic factors in our study, too, suggesting that results from our small study group may be representative for a larger population.

6.1 Novel prognostic factors of neuroblastoma

6.1.1 PolySia-NCAM

Our results demonstrate that polySia-NCAM expression in neuroblastoma is a significant factor associating with dissemination status at the time of diagnosis: patients with polySia-NCAM-expressing primary neuroblastomas were shown to have an advanced disease (P = 0.047). Most interestingly, positive polySia-NCAM expression appeared to be a strong prognostic factor for favourable OS (P = 0.0004) in advanced disease, especially in patients with *MYCN*-nonamplified neuroblastoma. Given that polySia-NCAM and NCAM expression did not associate with *MYCN* amplification, which is a common feature in metastatic neuroblastoma, it appears that polySia-NCAM may be an independent prognostic marker for metastatic activity in neuroblastoma.

In survival analyses, polySia-NCAM-expressing neuroblastoma patients with distant metastases (13 patients) survived significantly better (6 out of 13 alive, P = 0.0004) than polySia-NCAM-negative neuroblastoma patients with distant metastases (6 patients, all dead), as stated above. Five out of seven dead

patients in the favourable prognostic group had polySia-NCAM-positive primary neuroblastomas (a favourable factor) with *MYCN* amplification (an unfavourable factor). In other words, *MYCN* amplification seems to counteract the positive prognostic value of polySia-NCAM in the advanced disease group. The majority of the polySia-NCAM-negative neuroblastoma patients (4 out of 6) were diagnosed before 1983, and the disease was widely disseminated at diagnosis among all these patients. All these four patients with polySia-NCAM-negative neuroblastomas (diagnosed before 1983) died within 190 days. Thus, it is possible that the lack of early interventions and the quality of treatment may have worsened the outcome in patients with disseminated polySia-NCAM-negative neuroblastomas, which were diagnosed before 1983. Thus, we cannot exclude the possibility that the difference between recent and former treatment protocols may partly explain our results. Even though not all archival samples were polySia-NCAM-negative, since we detected polySia-NCAM expression in five neuroblastoma samples taken before 1983 (the total number of samples diagnosed before 1983 was 12), we cannot exclude the possibility that long archiving of paraffin-embedded neuroblastoma samples affects the staining results.

Bone marrow is the most important secondary organ for the detection of disseminated tumour cells. The vast majority of bone marrow disseminated primary neuroblastomas harboured polySia-NCAM and NCAM, and the bone marrow metastases also expressed polySia- NCAM and NCAM. Previously, bone marrow metastases in advanced stages of neuroblastoma were shown to be NCAM-positive, but the polysialylation of NCAM molecules was not determined (Tsang et al., 2003). The detection of these disseminated neuroblastic cells in bone marrow was performed easily and specifically by using the polySia-binding fluorescent fusion protein. PolySia together with NCAM may play an important role in the cascade of metastasis formation, where the presence of polySia on cell surfaces of primary tumours possibly permits cells to detach from their neighbours, thereby allowing them to migrate or to undergo changes in shape related to motility and/or structural plasticity. If polySia is involved in the primary formation of metastases, and polySia-expressing cells are found in distant metastases, it is a conceivable that the expression of this carbohydrate in metastatic cells is a more permanent than transient change in phenotype. In addition, polySia-NCAM as well as NCAM expression in untreated primary neuroblastomas is also associated with higher proliferation activity *in vivo*, and not only *in vitro*, as has been presented previously (Hildebrandt et al., 1998). On the contrary, polySia-NCAM-positive tumour cells in bone marrow showed low proliferation activity, which might explain, to some extent, the relative resistance of bone marrow metastases to conventional chemotherapy. The higher proliferation activity of polySia-NCAM-positive primary tumours may, on the other hand, increase metastatic potential, as is commonly believed.

Previously, NCAM expression has been detected in 100% of neuroblastoma samples (Figarella-Branger et al., 1990; Molenaar et al., 1991; Phimister et al., 1991; Wachowiak et al., 2008; Winter et al., 2008), whereas according to our results polySia-NCAM and NCAM expression are found only in 54% and 58% of neuroblastoma samples, respectively. Whether this difference is due to the fact that the archival paraffin-embedded neuroblastoma samples in our series lose some immunohistochemical properties during years remains to be studied. One explanation for the detected difference could be that if heterogeneous neuroblastoma does not express polySia-NCAM and NCAM in all focuses, which is probable, our hotspot analysis of most proliferative (malignant) spots will have led to different results. We found 11 NCAM-negative neuroblastomas out of 31 samples, and 10 of these NCAM-negative neuroblastomas were also *MYCN*-nonamplified tumours. Very interestingly, *NCAM* is located at locus 11q23, which is frequently deleted in *MYCN*-nonamplified neuroblastomas (Guo et al., 1999; Plantaz et al., 2001; Attiyeh et al., 2005). This LOH at 11q23 manifests in 34% of neuroblastomas (Attiyeh et al., 2005), and features high-risk neuroblastoma (Guo et al., 1999), despite the lack of *MYCN* amplification in the same tumours.

Therefore, it is highly conceivable that a loss of functional NCAM happens in at least some of LOH 11q23 neuroblastomas, and explains, indeed, the previously reported prognostic value of LOH 11q23. In particular, the unbalanced 11q23 LOH, where some genetic material is missing due to deletions (possibly *NCAM* deletions), strongly associates with unfavourable OS (Attiyeh et al., 2005). Our results suggest that the polySia-NCAM and NCAM-negative phenotypes form a prognostically significant (P = 0.0004 and P = 0.0088, respectively) finding especially in advanced disease, where no subgroup-specific prognostic risk factors have been reported earlier. Since NCAM is the carrier of polySia, our results may at least partially associate with *NCAM*-deletion caused by unbalanced 11q23 LOH. Unfortunately, there are no previous studies addressing the prognostic value of 11q23 LOH in the advanced disease subgroup of neuroblastoma, or examining NCAM expression and 11q23 LOH in the same tumours, or more specifically in the same tumour spots.

In summary, patients with polySia-NCAM-negative a.) local neuroblastoma with involvement of regional lymph nodes (equal to INSS stage 2B-3 tumours), or b.) neuroblastomas with distant metastases (equal to INSS stage 4 tumours) have an unfavourable prognosis despite *MYCN* amplification status, whereas patients with polySia-NCAM-positive neuroblastomas (similar to INSS stage 2B-4 tumours) have a more favourable prognosis, especially when the *MYCN* copy number is normal. From a technical point of view, the fusion protein, which we have developed and produced (Jokilammi et al., 2004), seems to suit the clinical detection of primary as well as disseminated polySia-NCAM-expressing neuroblastoma cells excellently. PolySia-NCAM is considered to be a neural stem cell marker, and widely spread polySia-NCAM neuroblastoma

cells (of neural crest origin) may be neural stem cell-like cells obviously harbouring other than proliferative and mitotic stem cell characteristics, such as differentiation, which improves prognosis. In addition to the small patient cohort, the main criticism of the results concerns the possible confounding effect of treatment differences between study subjects.

6.1.2 12q24.31 amplicon

A high-resolution aCGH identified the genomic regions involved in genetic alterations in two neuroblastoma cell lines (NGP and IMR-32). FISH in a neuroblastoma TMA format was adopted to assess the clinical significance of the identified genomic alteration event at 12q24.31. Given that neuroblastomas display intratumoural heterogeneity of biological variables including the *MYCN* copy number (Ambros et al., 2001), an overall estimation of the prognostic impact of copy number changes and genetic alterations in one randomly selected spot of neuroblastomas could be erroneous. Moreover, as the sample under evaluation might contain only a small subpopulation of genetically changed tumour cells, and non-cancer cells may diminish the actual copy number, low-level copy number increases of prognostically significant genes are difficult to detect. Therefore, we utilized a hotspot tumour TMA, where the hotspots represented the most proliferative spots of the neuroblastoma samples, and thus most probably associate with prognostically significant chromosomal alterations. An *in silico* approach was used to explore existing gene expression data on genes at 12q24.31 region.

Hotspot FISH analysis revealed a high frequency (42%) of low-level gain of 12q24.31 in the neuroblastoma samples. Patients with the gain had an intermediate prognosis in comparison to *MYCN*-amplified patients (poor prognosis) and patients having neither of the two alterations (best prognosis) (P = 0.0016). Although the NGP cell line contained both *MYCN* and 12q24 amplifications, the FISH analysis revealed only one neuroblastoma with both genetic modifications. To further examine the detected 12q24.31 amplicon and the possible new oncogenes in this region, *in silico* data mining was used to explore existing gene expression data from 445 healthy and 598 malignant tissue samples of nerve cell origin. With this approach, we identified upregulation of *DIABLO* among the 24 analysed genes in the 12q24.31 amplicon region.

DIABLO is a proapoptogenic mitochondrial protein which is released in response to apoptotic stimuli into the cell cytosol. Therefore, it is conceivable that the role of DIABLO overexpression is to sensitize cancer cells to apoptotic death (Kashkar et al., 2003; Kashkar et al., 2006). However, previous contradictory results suggest that there is both a direct correlation, such as in cervical cancer and gastric cancer (Espinosa et al., 2004; Shibata et al., 2007), and inverse correlation, such as in renal cell carcinoma, lung cancer and hepatocellular carcinoma (Sekimura et al., 2004; Mizutani et al., 2005; Bao et

al., 2006), between DIABLO expression and cancer progression. In this sense, the carcinogenetic role of DIABLO is ambiguous, and needs to be examined more thoroughly, in neuroblastoma, too.

In summary, in addition to the previously identified oncogenes in neuroblastoma, our analyses led to the identification of the high frequency of gain of 12q24.31, which associated with intermediate prognosis in neuroblastoma patients. This low-level alteration may serve as a novel additional biomarker to assess neuroblastoma progression and prognosis.

6.2 Novel methods and strategies in molecular neuroblastoma research

Rapidly developing molecular techniques have revealed many potentially important genetic aberrations of neuroblastomas in recent years. Despite the technical progress, there is still no uniform method to assess the only prognostically significant example of oncogene activation, *MYCN* amplification. The classical technique for analysing *MYCN* amplification has been Southern blot hybridization. In order to assess the *MYCN* amplification status with Southern blot, it is necessary to extract DNA from a sufficient amount of fresh tumour sample. To analyse archival tumour samples, FISH has been used (Shapiro et al., 1993). The poor availability of fluorescence microscopes in clinical diagnostics in many countries has been one of the major obstacles for the widespread use of the FISH technique, and FISH has remained a complementary method in studies of *MYCN* amplification.

6.2.1 Hotspot-CISH technique in the analysis of *MYCN*

We have formulated a new *MYCN* copy number assessing strategy that utilizes the CISH method and the hotspot selection of neuroblastomas. Currently, an overall estimation of various tumour progression-associated parameters in one randomly selected spot may lead to faulty reasoning. In addition, results presented as an average of a molecular analysis of a whole tumour sample do not represent the small proportion of highly malignant cells, and therefore the results may be misleading. For example, in Southern blot hybridization analysis of the *MYCN* copy number, the sample under evaluation may contain only a small subpopulation of *MYCN*-amplified cells, and therefore non-cancer cells may diminish the actual copy number. Together with a high border value this may result in a statistical bias. In accordance with the idea that malignant changes in protein as well as in genomic level associate with accelerated proliferation, we measured the proliferation index of the whole neuroblastoma sample with the MIB-1 antibody, and selected the focus (Ø 2 mm) containing the highest fraction of proliferative cells for the TMA block and for further analysis of various molecular markers. The hotspot analysis of *MYCN* amplification revealed that the selection of a single focus appears to be reliable

procedure since the proportion of *MYCN* amplified tumours (22.9%) is in accordance with the common view of the frequency of *MYCN* amplification. Furthermore, detected *MYCN* amplification leads to statistically diminished survival, as in numerous previous studies. In addition, the results of CISH and Southern blot hybridization analysis of *MYCN* amplification did not differ from each other. Therefore, it appears that the *MYCN* amplification status can be reliably examined with a bright light microscope using the CISH technique focusing on the hotspot, instead of the laborious and error-prone fluorescent microscopic evaluations of a whole heterogeneous tumour specimen.

Even though ploidy and chromosome two copy number may distort the unadjusted CISH analysis of *MYCN* copy numbers, our results also provide evidence that the chosen cut-off value of 6 dichotomizes *MYCN*-amplified neuroblastomas at least as sensitively as Southern blot hybridization, in which the amplification was defined as ≥ 10 copies. Therefore, setting a cut-off value in CISH analysis further facilitates and eases the CISH procedure, as only one probe is required to be hybridized and evaluated microscopically. However, the cut-off value of 6 excludes an intermediate increase of the *MYCN* copy number (between 3 and 6 copies per nucleus), which may reflect the initiation of *MYCN* amplification. In the present study, we could easily detect the intermediate increase of *MYCN* copy number with CISH. These tumours did not differ prognostically from unamplified tumours in OS. Previously, an intermediate increase in *MYCN* copy number in a single focus (Ø 2 mm) of neuroblastomas did not seem to lead to diminished survival (Valent et al., 2002). It should be noted that ploidy and the chromosome two copy number may distort the unadjusted CISH analysis of intermediate *MYCN* copy numbers, too. Therefore, in order to estimate the role of low-level *MYCN* copy numbers in survival prognostics, further studies of the unadjusted CISH analysis are needed. On the other hand, tumour aneuploidy (three or more copies of chromosome two per cell) does not seem to associate with a real amplification of *MYCN* (Shapiro et al., 1993), which suggests that the unadjusted analysis with a cut-off value of 6 may be appropriate at least when intermitotic cells compose the majority of the tumour sample.

6.2.2 Hotspot PI

The mitotic activity in hotspots, assessed with Ki-67 antigen detection, seems to correlate with other prognostic variables, such as *MYCN* amplification, histological subtype and INCP prognosis. As *MYCN*-amplified tumours had higher PIs (PI 38.39 ± 18.90 versus 24.63 ± 16.64, P = 0.047), it is tempting to speculate that genetic aberrations are associated with higher proliferation. In a previous study of 87 neuroblastomas, it was also shown that Southern blot hybridization-detected *MYCN*-amplified neuroblastomas (21.8%) were associated with disease progression and higher PIs (PI 36.4 ± 24.4 versus 18.7 ± 16.9 in tumours without *MYCN* amplification, *P* = 0.0034) (Rudolph et al., 1997). Larger studies are needed to determine possible subgroups of PIs,

which would contribute to the prognostic histopathological analysis by substituting for MKI. Whether the PI of hotspots acts as an independent prognostic factor remains to be elucidated.

6.2.3 Array-based profiling of neuroblastoma genetics

In general, our findings suggest that the integration of array-based profiling at DNA, RNA and tissue levels is a powerful modern strategy to identify new genetic markers for neuroblastoma. The presented strategy highlights the significance of the *in silico* data mining opportunities of existing data sets in providing further evidence of the putative genes involved in tumourigenesis. In practice, this relatively systematic and rapid methodology is applicable to a wide range of cancer cell lines.

6.2.4 Flow cytometry in detecting MRD

Flow cytometry provides information on multiple cellular parameters including cell size and cell surface proteins in a multicolour assay. Large numbers of cells can be screened with flow cytometry, which is a rapid, relatively simple and cost-effective technique. However, flow cytometry assays also have limitations. Indeed, it is virtually impossible to detect one neuroblastoma cell out of 10^6 (sensitivity far less than 0.0001%), which is more or less the sensitivity of RT-PCR. However, there is no evidence that such an extreme sensitivity level is clinically relevant. It is known that if the number of neuroblastoma cells in bone marrow exceeds 100 tumour cells per 10^5 bone marrow cells (0.1%) within 12 weeks after start of cytotoxic treatments, the prognosis is unfavourable (Seeger et al., 2000). This indicates that the sensitivity of flow cytometry assays must be better than 0.1% to study MRD in neuroblastoma patients. Despite all the technical progress in MRD analysis, the clinical significance of these observations has remained unclear. This is partly due to the fact that published reports disagree on a.) the tumour cell count when bone marrow infiltrating tumour cells appear to have prognostic significance, and b.) the cell surface markers and cell size limits which should be used in analyses.

We created an artificial MRD bone marrow samples by mixing fresh bone marrow cells and *in vitro* cultured polySia-NCAM-positive SH-SY5Y neuroblastic cells in different ratios. These cell suspensions were labelled with the polySia-binding fluorescent fusion protein, which was able to detect polySia-NCAM-positive tumour cells from normal bone marrow cells in flow cytometry. We noticed that normal bone marrow samples contained a few polySia-NCAM-positive cells, which were significantly smaller in size than polySia-NCAM-positive tumour cells. Our preliminary results (not shown) suggested that the sensitivity of polySia-NCAM-based detection of tumour cells in flow cytometry is about 1%, which is probably not sufficient for clinical use. Moreover, since normal bone marrow stem cells may express polySia-NCAM,

more detailed flow cytometry studies are needed to determine the clinical applicability of polySia-NCAM-based MRD detection. However, given that polySia-NCAM is expressed in most of the bone marrow disseminated neuroblastomas, it appears reasonable to develop the polySia-NCAM-based flow cytometry methodology.

6.3 Important negative results

As more and more potential drugs are to be tested in phase I clinical trials, it is extremely important to have a firm biological rationale and evidence of efficacy in preclinical models to help to avoid unnecessary trials with possible adverse effects on neuroblastoma patients. Similarly, the highly expanded search for new prognostic molecular markers produces a high number of markers which do not have biological relevance in neuroblastoma, but are still considered to be possible new molecular markers or even treatment targets.

6.3.1 *c-kit* mutations

Assessing different type of tumours suitable for imatinib treatment has been one of the vogue topics for clinical cancer researchers in recent years. According to a previous report, imatinib inhibits the growth of c-kit positive neuroblastoma cells *in vitro*, and decreases the size of tumours in gastrocnemius neuroblastoma xenograft model mice treated orally with imatinib every 12 hours for 14 days (Beppu et al., 2004). GIST, one of the main target tumours of imatinib, shows a variety of gain-of-function mutations across the *c-kit* gene. These mutations predict the responsiveness of GISTs to imatinib treatment (Heinrich et al., 2003). Our series of neuroblastomas is the first that has been examined for GIST-type *c-kit* gene mutations in exons 9, 11, 13 and 17. Based on our results, GIST-type *c-kit* mutations are rare events in neuroblastomas. Whether other than GIST-type *c-kit* mutations exist in neuroblastomas remains to be elucidated.

A previously published study demonstrates imatinib-driven growth inhibition in a c-kit negative neuroblastoma cell line, too (Vitali et al., 2003). Therefore, imatinib may suppress the growth of neuroblastoma through c-kit independent pathways. The expression of the c-kit receptor only in a low fraction of neuroblastomas and the paucity of GIST-type *c-kit* mutations suggest that the therapeutic activity of imatinib through c-kit kinase suppression would benefit at most only a small subset of patients suffering from neuroblastomas. The molecular subclassification of neuroblastomas on the basis of c-kit expression appears to be insignificant in identifying tumours bearing *c-kit* mutations. It is conceivable that *c-kit* mutations do not fundamentally contribute to the pathogenesis of neuroblastomas. Whether *c-kit* has any significant importance in the growth of neuroblastomas remains to be investigated.

6.3.2 Id2 and nestin expression

In a previous study, Id2 mRNA expression levels did not correlate statistically with OS, though there was a tendency among patients with lower mRNA levels to have a more favourable prognosis (P = 0.0643) (Vandesompele et al., 2003). In contrast, Lasorella et al. found a high correlation between *MYCN* and Id2 expression in immunohistochemical analysis of primary neuroblastomas. They also showed that the expression of Id2 strongly predicts poor outcome (Lasorella et al., 2002). In our study, the expression of the indirect proliferation stimulant, Id2, did not associate with higher PIs or *MYCN* expression in hotspot analysis. It may be concluded that Id2 expression does not seem to serve as an independent prognostic marker in neuroblastomas.

Nestin is a neural stem cell marker that is suggested to be a mediator of *MYCN*-associated tumour aggressiveness in neuroblastoma (Thomas et al., 2004). These *in vitro* findings also suggested that nestin is present in the nucleus of *MYCN*-amplified neuroblastic cells (Thomas et al., 2004). According to our findings on tumour samples, nestin expression does not correlate with *MYCN* amplification, INPC, INSS, PIs, histopathological prognosis, or outcome. Furthermore, the nestin expression pattern was shown to be cytoplasmic in immunohistochemical analysis. Thus, nestin appears to have a less important role in neuroblastomas than might have been expected.

6.4 Limitations of the study

The major limitation of the study is the small sample size. In addition to the limited statistical power, comparison of subgroups of patients and individual prognostic markers is compromised due to the same reason. In practice, the reported results of possible new prognostic markers are not based on high-level evidence, since the small sample size together with the use of measures that lack reliability and established validity hinder the conclusive evaluation of the results. We should also take into account the confounding effect of heterogeneous neuroblastoma treatments, which most probably varied significantly from patient to patient, since the patients were diagnosed between years 1967 and 2001. Moreover, the archival tumour samples may have undergone changes that have led to conformational changes in expressed proteins. Therefore, our results are inconclusive and should be interpreted cautiously.

Despite the small number of patients, which is partly due to the low population-based incidence of neuroblastomas in Finland, some of our results were statistically highly significant as well as biologically relevant. We believe that the reported new markers and methods are applicable in neuroblastoma research, and may benefit clinical diagnostics and decision making after they have been confirmed in larger studies. Therefore, to overcome the

methodological compromises, large clinical trials with patients receiving standardised neuroblastoma treatments are required.

6.5 Future challenges and ideas

The rarity of neuroblastomas at population level as well as the multiplicity and complexity of reported neuroblastoma markers underline the need for collaboration and co-operation between neuroblastoma research groups. In general, large multicentre collaborative studies with standardizing methodology and reporting are needed to address the questions of interest, such as which of the markers provides the best prognostic value, or whether monitoring and screening of neuroblastoma patients using biomarkers is cost-effective. In addition to the new methodology and strategies in biomarker screenings, we have provided a new basic knowledge of the highly interesting potential biomarkers, polySia-NCAM and the 12q24.31 amplicon, in neuroblastoma risk stratification. A future challenge will be to translate the current information into clinical benefit. The first and most important future step is to confirm our findings in large cohorts of neuroblastoma patients. Some of the possible future ideas and modifications are discussed below in more detail.

Prevention of neuroblastoma cell invasion into bone marrow and targeted treatment of bone marrow disseminated cells could be powerful therapeutic approaches, especially for patients who have a high-risk disease without clear bone marrow dissemination at diagnosis. Several antibodies against a neuroblastoma surface antigen, disialoganglioside GD2, have been used as therapeutic agents (Frost et al., 1997; Cheung et al., 1998; Yu et al., 1998), especially in INSS stage 4 neuroblastoma. This therapy, however, has painful side effects because of the antibody cross-reactivity with peripheral pain fibres. In addition, treatment of neuroblastomas with an anti-GD2 antibody is ineffective against progressive disease or soft-tissue masses (Kushner et al., 2001a). Therefore, the development of more specific and less toxic immunotherapies in the form of polySia-NCAM-binding cytotoxic fusion protein might overcome the problems of antibody treatments. Whether polySia-NCAM-targeted therapeutic approaches alone would efficiently diminish the dissemination of tumour cells, or even eradicate, for instance, bone marrow metastases, is questionable. However, given that most of bone marrow disseminated neuroblastomas express polySia-NCAM, polySia-NCAM-targeted treatments for disseminated disease might be worth testing. As outlined above, clinical development of anti-NCAM immunotherapies have already been conducted (Tassone et al., 2004; Smith, 2005), and anti-NCAM treatments of small cell lung cancer, NCAM-positive solid tumours and multiple myeloma are in clinical studies (http://www.immunogen.com/wt/page/IMGN901b).

Since the level of polySia-NCAM expression may vary from low to very high levels at different time points of tumourigenesis, polySia-NCAM detection methods should be improved; more sensitive methods are required, especially

for flow cytometry. Because polySia-NCAM-expressing cells were detected in a single cell level both in bone marrow smears and paraffin-embedded bone marrow samples, the sensitivity of flow cytometry could probably be improved through methodological modifications. Highly sensitive polySia-NCAM detection from blood samples could possibly be used in controlling early changes in disease course, and also for diagnostic screenings and follow-up evaluations.

As mentioned earlier, NCAM expression in unbalanced 11q23 LOH tumour spots and the prognostic value of unbalanced 11q23 LOH in the advanced disease subgroup of neuroblastoma have not been studied. In future, it might be worth studying the above-mentioned aspects thoroughly, especially since the *NCAM* gene is located at locus 11q23. Understanding the fact that malignant chromosomal aberrations with survival benefit locate most probably in highly proliferating tumour focuses, our hotspot analysis might be a more reliable approach to study unbalanced 11q23 LOH than analyses of whole tumour samples.

The role of the frequent 12q24.31 low-level gain should also be studied in a more homogenous and large patient group. If the prognostic value of 12q24.31 gain proves to be significant, genes in the amplicon must be studied in greater detail. The suggested ‘driver’ gene in the amplicon, *DIABLO*, should be further studied using *in vitro* experiments, for example genetically manipulated neuroblastoma cell lines.

7 SUMMARY AND CONCLUSIONS

Our specific aims for the study were to construct a simple and reliable method for the assessment of *MYCN* amplification status, to uncover novel prognostic markers of neuroblastoma to further refine treatment decisions, to identify gene mutations for new cytotoxic treatments, and to study sites of genetic aberrations in order to provide insights into mechanisms of malignant transformation and progression. In short, our study covers all the specific aims of the project in detail, and constitutes a collection of results that are of high interest and warrant future research. Despite the limitations of the study, mainly the small and heterogeneous study cohort, the value of the qualitative research design can be considered to be relatively high, especially since the number of published papers consisting of small neuroblastoma patient series, such as ours, has become sparse in recent years.

In summary, the hotspot approach outlined here describes relatively strict criteria for the selection of tumour spots of heterogeneous neuroblastomas for clinical and research practice. The clinical impact of *MYCN* amplification as an independent prognostic factor has been shown once again. The implementation of the fast and simple CISH method in the evaluation of the *MYCN* amplification status seems to greatly improve the previous methodology. We believe that the presented hotspot and CISH methodology may serve as a basis for a uniform *MYCN* assessment protocol which allows the comparison of the results of clinical trials and biological studies performed by different groups in different countries. Therefore, re-evaluation of the *MYCN* amplification status of hundreds of neuroblastomas in tumour banks is also feasible because of the practicability, reliability and signal accuracy of the strategy.

A subpopulation of cells in untreated primary neuroblastomas contains adhesive characteristics, namely polySia-NCAM and NCAM phenotypes, which associate with proliferation activity and clinical stage. Moreover, polySia-NCAM and NCAM appear to be pathogenetically relevant markers predicting outcome, especially in advanced stage neuroblastoma, which to date has remained a therapeutic and prognostic challenge. The specific and sensitive detection of polySia-NCAM-expressing primary and metastatic tumour cells may allow the development of new immunodiagnostic and immunotherapeutic approaches. By using advanced molecular techniques, we showed that neuroblastomas do not possess *c-kit* gene mutations, which are the main prerequisite for effective imatinib response in GIST-tumours. Our results do not support the previous findings suggesting that the *c-kit* gene plays an important role in the tumourigenesis of neuroblastomas. On the contrary, our results suggest that clinical approaches subclassifying neuroblastomas on the basis of c-kit expression appear to be insignificant in identifying imatinib-sensitive neuroblastomas. Finally, our strategy in discovering potential tumour genes

buried in the new genomic amplicon, 12q24.31, which appears to be associated with varying tumour behaviour and patient outcome, is an example of the powerful utilization of bioinformatics and biotechnology.

The array-based methods provided could already be used in diagnostic settings. Although we have made some progress in identifying neuroblastoma-specific molecular targets for diagnostic applications and novel therapeutics, much work needs to be done for thorough standardisation and validation of the reported findings. Regardless, our results warrant additional investigation.

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