Aberrant DNA hypermethylation and apoptotic defects in pediatric neuroblastomas
van Noesel, M.M.

Citation for published version (APA):
Buijten & Schipperheijn

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)
Chapter 1

Introduction

Pediatric neuroblastomas: genetic and epigenetic ‘Danse Macabre’

Max M. van Noesel\(^1\) and Rogier Versteeg\(^2\)

Gene 2004; 325: 1-15

\(^1\) Erasmus MC-Sophia Children’s Hospital, department of pediatric oncology-hematology, Rotterdam, The Netherlands

\(^2\) Academic Medical Center, department of human genetics, Amsterdam, The Netherlands
Abstract

Neuroblastomas are the most frequently occurring solid tumors in children under 5 years. Spontaneous regression is more common in neuroblastomas than in any other tumor type, especially in young patients under 12 months. Unfortunately, the full clinical spectrum of neuroblastomas also includes very aggressive tumors, unresponsive to multi-modality treatment and accounting for most of the pediatric cancer mortalities under 5 years of age. It is generally emphasized that more than one biological entity of neuroblastoma exists. Structural genetic defects such as amplification of MYCN, gain of chromosome 17q and LOH of 1p and several other chromosomal regions have proven to be valuable as prognostic factors and will be discussed in relation to their clinical relevance. Recent research is starting to uncover important molecular pathways involved in the pathogenesis of neuroblastomas. The aim of this review is to discuss several important aspects of the biology of the neuroblast, such as the role of overexpressed oncogenes like MYCN and Cyclin D1, the mechanisms leading to decreased apoptosis, like overexpression of BCL-2, Survivin, NM23, epigenetic silencing of Caspase 8, and the role of tumor suppressor genes, like p53, p73, and RASSF1A. In addition, the role of specific proteins overexpressed in neuroblastomas, such as the neurotrophin receptors TrkA, B, and C in relation to spontaneous regression and anti-angiogenesis will be discussed. Finally, we will try to relate these pathways to the embryonal origin of neuroblastomas and discuss possible new avenues in the therapeutic approach of future neuroblastoma patients.
1.0 Clinical behavior of neuroblastomas

Neuroblastomas are neuro-ectodermal tumors of embryonic neural crest-derived cells. The neural crest in normal development gives rise to nerve cells of the sympathetic nervous system. This is formed by the sympathetic side chains and sympathetic ganglia, which run alongside the ventral side of the spine, and the adrenal medulla. The fetal adrenal medulla consists of a mixture of chromaffin cells and clusters of mature ganglion cells. Chromaffin cells are neuro-endocrine in origin and produce the stress hormones norepinephrine and epinephrine. Ganglion cells are interconnecting nerve cells between pre- and postganglionic sympathetic nerve fibers. The adrenal medulla strongly increases the amount of chromaffin cells after birth, in concert with a gradual loss of ganglion cells. In the extra-medullary sympathetic nervous system, the opposite can be observed. Extra-medullary chromaffin cells will rapidly disappear from the ganglia, and the neuronal cells become the predominant cell type. Neuroblastomas most likely originate from both cell types or from a pluripotent precursor cell, since they can contain cells with neuronal and chromaffin cell properties. Also, neuroblastoma tumors arise in both the sympathetic side chains and the adrenal medulla. Neuroblastomas account for 8-10% of all pediatric cancers, and since 80% concerns children under the age of 5 years it is the most prominent solid cancer of this age group. The spectrum of pediatric neuro-ectodermal tumors ranges from undifferentiated, truely malignant neuroblastomas, via ganglioneuroblastomas to well-differentiated, mostly benign ganglioneuromas. Within the group of malignant neuroblastomas, different risk categories can be identified: patients with high, intermediate or low risk tumors. High-risk tumors include disseminated disease or bulky tumors with gross genetic alterations, such as amplification of MYCN (INSS stage 3 and 4). These patients form a group at risk with a 5 years event-free survival of 25-30%, despite multi-modality treatment including myelo-ablative chemotherapy. Patients with intermediate risk disease are characterized by large, unresectable, localized tumors without structural chromosomal defects (INSS stage 2b-3). Their outcome is more favorable (5-year EFS of 60-80%) and can be reached by combining surgery with (neo-) adjuvant chemotherapy. Low risk tumors include children with small tumors (INSS stage 1-2a), which can be managed by surgery alone and will lead to an excellent 5 years EFS of more than 90% (Table 1). Two additional patient categories should be mentioned. The first is formed by infants (under 1 year of age at diagnosis) with a small primary tumor and dissemination of disease. The dissemination is according to a limited and characteristic pattern involving tumor localization in liver, bone marrow and/or skin, but not in bone (stage 4S). These patients have an excellent 5-year EFS of 70-90%, and a high rate of spontaneously regression which allows a ‘wait-and-see’ approach to treatment. The second group is formed by patients uncovered by mass screening. The mass screening programs were initiated in an attempt to early detect patients with unfavorable tumors. However, the screening has led to an increase in the detection of low-stage neuroblastoma patients, but has not resulted in a decrease of unfavorable, high-risk disease in older children. This strongly suggests it concerns a new class of tumors that
Chapter 1

normally would have regressed spontaneously, before the onset of clinical signs. It also suggests that the high-risk tumors, usually in the older child over 1 year, do not evolve from lower-risk tumors, but arise as such.

Apart from the stage of disease, the age of the patient is also an important prognostic factor. For reasons unknown, the prognosis of infants under one year of age at diagnosis is significantly better than for older children with the same clinical stage, particularly for stage 4 disease. Several important questions have been raised by these and other clinical observations. Do neuroblastomas share a common genetic defect at the onset of disease? If so, the different clinical behaviors suggest that low-risk and high-risk tumors divert early after the first oncogenic hit(s) and fare independent courses of tumor progression. Alternatively, low-risk and high-risk neuroblastomas could arise as the result of a different etiology or from different stages of embryonic development. These and other questions will be addressed in view of the (epi-) genetic and molecular alterations in neuroblastomas.

2.0 Genetics of neuroblastoma

The last few decades, several genetic aberrations in different subsets of neuroblastoma tumors and cell lines have been uncovered. The emerging patterns of multiple genetic defects, such as aneuploidy, chromosomal gains and losses, and amplification of chromosomal material seem to mirror the different clinical entities and have led to a better stratification of patients for therapy (Table 1). The most important genetic alterations in neuroblastomas will be briefly discussed in the context of their clinical relevance. For more details on the genetics of neuroblastomas, we refer to excellent and recent reviews by others\textsuperscript{12,13}.

2.1 Ploidy

Aneuploidy (gains and losses of one or more chromosomes of a diploid genome) is a form of genetic instability frequently observed in neuroblastomas. Different patterns of aneuploidy seem associated with the different clinical entities. Near-diploid and near-tetraploid tumors are usually detected in patients over one year of age and associated with structural abnormalities involving allelic loss of chromosome 1p, amplification of the MYCN gene and with aggressive tumors and dismal outcome. Hyperdiploid or near-triploid tumors are usually found in patients under 12 months or in low-risk tumors (Stage 1, 2, 4s) with few or no structural chromosomal abnormalities. Near-pentaploid tumors are rare and found in patients with favorable prognostic factors and excellent prognosis, as in near-triploid tumors\textsuperscript{14,15}.

The mechanism(s) leading to this form of genetic instability in human cancers or neuroblastomas is still unclear. In general, incorrect segregation of chromatids and aneuploidy are considered to result from amplification or hypertrophia of centrosomes\textsuperscript{16}. It has been shown that several common genetic alterations in cancer like p53 gene mutations and CDK2-cyclin E overexpression can induce
Table 1. Characteristics of Neuroblastoma Risk Groups

<table>
<thead>
<tr>
<th>Tumor Characteristics</th>
<th>Low-Risk</th>
<th>Intermediate-Risk</th>
<th>High-Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Typically &lt; 1 yr</td>
<td>Typically &gt; 1 yr</td>
<td>Typically &gt; 1 yr</td>
</tr>
<tr>
<td>Stage</td>
<td>1,2,4s</td>
<td>3,4</td>
<td>3,4</td>
</tr>
<tr>
<td>5-year OS</td>
<td>95%</td>
<td>50%</td>
<td>25%</td>
</tr>
<tr>
<td>Genetic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ploidy</td>
<td>3N</td>
<td>2N/4N</td>
<td>2N/4N</td>
</tr>
<tr>
<td>1p loss</td>
<td>Rare</td>
<td>Rare</td>
<td>Frequent</td>
</tr>
<tr>
<td>11q loss</td>
<td>Rare</td>
<td>Frequent</td>
<td>Rare</td>
</tr>
<tr>
<td>14q loss</td>
<td>Rare</td>
<td>Frequent</td>
<td>Rare</td>
</tr>
<tr>
<td>17q gain</td>
<td>Rare</td>
<td>Frequent</td>
<td>Frequent</td>
</tr>
<tr>
<td>N-myc</td>
<td>Normal</td>
<td>Normal</td>
<td>Amplified</td>
</tr>
<tr>
<td>Molecular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TrkA</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>TrkB</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>TrkC</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>P75NTR</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>CCND1</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>NM23</td>
<td>Low</td>
<td>?</td>
<td>High</td>
</tr>
</tbody>
</table>

OS, Overall Survival; LOH, Loss of Heterozygosity; Trk, Tyrosine Receptor Kinase; CCND1, gene for Cyclin-D1 (adapted from reference Brodeur, 2003).

such aberrations of the centrosomes leading to chromosomal instability in murine cell lines and tumors developed in p53 null mouse models\(^{17,18}\). Also, it has been shown in vitro that overexpression of the \(\text{myc}\) oncogene can induce genomic instability in rodent fibroblast cell line\(^{19}\) and in genetically normal, primary human fibroblasts\(^{20}\). However, the relevance of these general mechanisms for understanding aneuploidy in neuroblastomas is not clear. In general, it seems likely that, so far unidentified genetic lesions underlie the ploidy defects in neuroblastoma.

2.2 Allelic loss at 1p

One of the most prominent regions of Loss of Heterozygosity (LOH) in neuroblastomas is 1p, commonly identified in 30-35% of all tumors. The shortest region of overlap (SRO) of allelic loss in all tumors is within 1p36, but there is no consensus about the polymorphic markers that define the borders of the SRO\(^{21-23}\). Loss of chromosomal material on 1p is frequently associated with an unbalanced translocation with the long arm of chromosome 17, t(1;17). Unfortunately, this has not led to the identification of important genes in the chromosomal breakpoint areas, due to the fact that the breakpoints are highly variable on both chromosomes\(^{24,25}\). Efforts to define the SRO and putative tumor suppressor genes by cytogenetic and molecular analyses of large tumor cohorts and cell lines have identified at least two different SRO’s and one region of homozygous deletion (HD). The region of HD was found in one cell line at chromosome 1p36.2, spanning approximately 500 kb. Expression and sequence analysis of multiple genes within the region of HD has not revealed oncogenic mutations in this region so far\(^{26}\). In tumors with amplification of \(\text{MYCN}\), LOH of 1p usually affects large areas, often reaching to 1p32 or even more proximal. The SRO for
MYCN amplified tumors has been defined to 1p35-1pter\textsuperscript{27,29}. This defect is strongly associated with dismal outcome. In contrast, the SRO for 1p deletions in MYCN single copy tumors was found to be smaller, and defined at 1p36.3\textsuperscript{21,30,31}. The initial finding of preferential maternal allelic loss in this subcategory\textsuperscript{27} was not confirmed in later studies\textsuperscript{32}. Furthermore, tumors with the smaller LOH are often near-triploid and associated with a better outcome. The different regions of deletion associated with different biological entities suggest the existence of more than one tumor suppressor genes at chromosome 1p.

Epigenetic silencing of potential tumor suppressor genes as an alternative mechanism in the absence of genetic mutations has not yet been studied systematically in neuroblastomas and needs further investigation. In our own analysis of the methylation status of 23 putative tumor suppressor genes at 1p36 in 22 neuroblastoma cell lines, we could not detect de novo methylation in the promoter regions of any of the genes\textsuperscript{33}.

Deletion of 1p is considered an independent predictor of unfavorable outcome in neuroblastomas of every stage\textsuperscript{34-36}. It is more commonly found in patients with advanced disease (INSS stage 3, 4) than in low-risk patients. In high-risk patients it is strongly associated with structural abnormalities, in particular amplification of MYCN, di- or tetraploidy, and gain of chromosome 17q. In a multivariate analysis of 89 patients, loss of 1p reduced the 3 years event free survival from 53 ± 10 to 0 percent for stage 3 and 4 tumors and from 100 to 34 ± 15 percent for stages 1, 2, or 4\textsuperscript{34}. Therefore, LOH1p is considered an important predictor of poor outcome for all subsets of neuroblastoma disease.

### 2.3 Allelic loss at 11q

Genetic aberrations at chromosome 11q in neuroblastoma patients include sporadic constitutional changes such as a balanced translocation involving 11q21 and 11q22, deletion of 11q23, inversion of 11q21-q23 and more frequently allelic loss. Allelic loss of 11q has been reported in 15 to 44 percent of neuroblastomas. The common region of deletion was defined between 11q14-23 in a cohort of 129 neuroblastomas with loss of 11q\textsuperscript{37}. Loss of 11q is inversely correlated with MYCN amplification and associated with a poor prognosis, particularly in patients with MYCN single copy tumors\textsuperscript{38,39}. The data strongly suggest that one or more tumor suppressor gene(s) is located at chromosome 11q and inactivated in the malignant progression of high-risk, MYCN single copy tumors.

### 2.4 Allelic loss at other chromosomes

Allelic loss of various other chromosomal arms and regions have been reported, such as 2q, 3p, 4p, 9p, and 14q. The observed frequencies were highly variable, possibly as a result of different sensitivities between cytogenetic, molecular and Comparative Genomic Hybridization (CGH) studies. Loss of 2q has been defined at 2q33 and is associated with loss of expression of the gene for
Caspase 8 (see section 3.6)\textsuperscript{40}. For 3p, a SRO was defined at chromosomal band 3p25.3-p14.3\textsuperscript{41}, and a candidate tumor suppressor gene, RASSF1A has been identified (see section 3.9). The SRO for 4p has been defined at 4p16, and is possibly also associated with hereditary neuroblastoma\textsuperscript{42, 43}. The SRO at 9p has been established at 9p21\textsuperscript{44}, and for 14q at 14q23-32\textsuperscript{45}. Both 3pLOH and 14qLOH were found to be associated with loss of 11q in localized, MYCN single copy tumors\textsuperscript{38, 45}. The loss of 9p was present in low frequencies in clinically detected tumors, but in high frequency among tumors from mass screening programs\textsuperscript{46}. The SRO includes the tumor suppressor genes CDKN2A (encoding both p16\textsuperscript{INK4A} and p14\textsuperscript{ARF}). However, mutations, homozygous deletions, gross chromosomal abnormalities or promoter hypermethylation with down-regulation of expression of CDKN2A in neuroblastomas have not been found\textsuperscript{47, 48}. In our own experience, we could not detect de novo methylation of p16\textsuperscript{INK4A} in 22 neuroblastoma cell lines\textsuperscript{33}.

2.5 Extra copies of 17q
Gain of a long segment of the q-arm of chromosome 17 is associated with a poor outcome\textsuperscript{49, 50}. Often it results from an unbalanced translocation with chromosome 1p or 11q\textsuperscript{51, 52}. Gain of 17q, in unbalanced translocations or as part of whole chromosome gain is seen in 80% of neuroblastomas\textsuperscript{53-55}. Whole chromosome 17 gain is typically seen in near-triploid tumors with favorable prognosis. Selective gain of the long arm of chromosome 17 is primarily found in advanced disease. The independent predictive power of gain of 17q status was established in tumors with gain of 17q, without MYCN amplification or allelic losses of 1p or 11q\textsuperscript{49, 56}. It appears that unbalanced 17q gain identifies a larger population at risk than any other clinical or cytogenetic factor.

The mechanism(s) involved in the adverse prognosis could be either the fusion of a gene flanking the 17q breakpoint, or a dosage effect of one or more genes at the extra 17q region. A common amplified region has been identified in several studies, ranging from 17q21.3-17qter, corresponding to a distance of approximately 60 cM\textsuperscript{54, 57}. In addition, the breakpoint region is highly variable, which makes it very unlikely that just a single gene at 17q is involved in the tumorigenic progression. It is therefore commonly believed that a dosage effect of one or more genes or class of genes in the unbalanced gain is responsible for the altered phenotype. Several genes have been implicated for a role in tumor progression in the common region of gain, but the most prominent candidates are nm23-H1, nm23-H2 and survivin (see sections 3.10 and 3.11).

2.6 MYCN, the oncogene
Amplification of the proto-oncogene MYCN is the most prototypic genetic aberration in neuroblastomas and is found in 20-25% of all neuroblastomas. MYCN was identified as a gene homologous to c-MYC and overrepresented in neuroblastomas\textsuperscript{58}. Amplified MYCN sequences usually form double minute (dmins) chromosomes or homogeneously staining regions (HSR's), which contain 50-500 copies of the MYCN gene. Overexpression of transfected MYCN in cultured
mammalian cells strongly increases proliferation rates and is able to induce cellular transformation\textsuperscript{59, 60}. Transgenic mice with overexpression of \textit{MYCN} in neural crest-derived tissues frequently develop neuroblastomas\textsuperscript{61}. Reduction of \textit{MYCN} mRNA by the use of antisense \textit{MYCN} can decrease proliferation and/or induce differentiation in cultured human neuroblastoma cell lines\textsuperscript{62, 63}. The \textit{MYCN} product (MYCN) is a nuclear phosphoprotein with an N-terminal transactivation domain and a C-terminal basic Helix-Loop-Helix/Leucine zipper (bHLH-LZ) motif. It functions as a transcriptional activator by forming DNA binding heterodimers with the MAX protein, which is a member of the MAX/MAD family of proteins\textsuperscript{64}. \textit{MYCN} transcriptionally activates many genes, directly and indirectly. Direct, known targets of \textit{MYCN} have long been limited to a handful of genes, like \textit{Prothymosin Alpha}, \textit{Ornithine Decarboxylase} and \textit{ID2}. \textit{MYCN}, like C-MYC, most probably acts directly to promote proliferation, cell growth and protein synthesis. Recently, a comparison by SAGE (Serial Analysis of Gene Expression) analysis of a cell line with ectopic \textit{MYCN} expression indicated that \textit{MYCN} regulates cell growth through genes involved in protein synthesis and ribosome biogenesis\textsuperscript{65}. For numerous other genes the expression levels have been reported to correlate with \textit{MYCN} levels, but the significance of these findings is unclear. \textit{MYCN} amplified tumors follow a very aggressive course and are strongly associated with additional structural abnormalities, especially loss of 1p, 17q gain, and near-diploidy or -tetraploidy. In multivariate analyses, a significant correlation between \textit{MYCN} amplification and poor outcome was established in all patient subsets\textsuperscript{34, 66}. \textit{MYCN} amplification is currently used for the identification of high-risk patients and is implemented in all international clinical trials for the treatment stratification of neuroblastoma patients.

### 3.0 Biological pathways in neuroblastoma

Neuroblastomas are considered to be embryonal tumors, which means that they originate as the result of a developmental defect during the normal differentiation from progenitor cells to mature tissue cells. Although many molecular pathways must be involved in the normal development of the neuro-endocrine cells, our knowledge hereof is scanty and limited. The introduction of molecular tools for high-throughput analysis of expressed genes has accelerated our insight into many biological pathways seemingly important in the pathogenesis of neuroblastomas. We will discuss the most prominent and promising genes, proteins and pathways. Then, we will attempt to correlate them to known genetic alterations and important developmental processes of the neuro-endocrine cells (summarized in Table 2 and Figure 1).

#### 3.1 Expression and function of the neurotrophins and their receptors

Neurotrophins are important soluble factors that regulate growth, development, survival and repair of the nervous system. They use two classes of receptors for their signaling pathways, the Trk tyrosine kinase receptors and the p75 neurotrophin receptor (p75\textsuperscript{NTR}). The receptors TrkA,
### Table 2. Genetic and molecular characteristics of neuroblastomas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Association with Genetic / Molecular defect</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ploidy 3N/5N</td>
<td>Unknown</td>
<td>Good</td>
</tr>
<tr>
<td>Ploidy 2N/4N</td>
<td>Amplified N-myc</td>
<td>Poor</td>
</tr>
<tr>
<td>Gain 17q</td>
<td>t(1;17) or t(11;17) Nm23 overexpression</td>
<td>Poor</td>
</tr>
<tr>
<td></td>
<td>Survivin overexpression Amplified N-myc</td>
<td></td>
</tr>
<tr>
<td>LOH 1p</td>
<td>Amplified N-myc</td>
<td>Poor</td>
</tr>
<tr>
<td>LOH 2q</td>
<td>Loss of CASP8 Amplified N-myc</td>
<td>Poor</td>
</tr>
<tr>
<td>LOH 3p</td>
<td>LOH 11q, 14q N-myc normal</td>
<td>Intermediate</td>
</tr>
<tr>
<td>LOH 4p, 9p</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>LOH 11q</td>
<td>LOH 3p, 14q N-myc normal</td>
<td>Intermediate</td>
</tr>
<tr>
<td>LOH 14q</td>
<td>LOH 3p, 11q N-myc normal</td>
<td>Intermediate</td>
</tr>
<tr>
<td>TrkB overexpression</td>
<td>Ha-Ras overexpression</td>
<td>Good</td>
</tr>
<tr>
<td>TrkA overexpression</td>
<td>Ploidy 2N/4N Amplified N-myc</td>
<td>Poor</td>
</tr>
<tr>
<td>TrkC overexpression</td>
<td>Unknown</td>
<td>Good</td>
</tr>
<tr>
<td>P75NTR overexpression</td>
<td>Unknown</td>
<td>Good</td>
</tr>
<tr>
<td>CCND1 overexpression</td>
<td>Amplification CCND1</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

LOH, Loss of Heterozygosity; Trk, Tyrosine Receptor Kinase; CCND1, gene for Cyclin-D1.

TrkB and TrkC can bind the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), respectively, while neurotrophin-4/5 (NT-4/5) binds to TrkB and weakly to TrkA. p75NTR is a low-affinity receptor and member of the Tumor Necrosis Factor (TNF) receptor super family, and can bind all four neurotrophins.67,69.

### 3.2 TrkA expression in neuroblastomas

High expression of the high-affinity receptor TrkA is found in mature sympathetic ganglia as well as in tumors with favorable prognosis. High TrkA expression is associated with younger age, lower stage and absence of MYCN amplification. Vice versa, low TrkA expression is associated with a poor prognosis and MYCN amplification. In the absence of NGF, TrkA expression will lead to apoptosis, while binding of NGF increases cell survival and differentiation of neuroblasts. Schwann cells, which can be part of the stromal environment of the neuroblasts, are a known source of NGF production. Increase of stromal components in neuroblastoma tumors is also associated with a better prognosis and cellular differentiation. Conversely, the absence of NGF may lead to increased apoptosis in ganglia and TrkA expressing tumors. Therefore, absence of NGF in the microenvironment of tumors may play a role in the regression of neuroblastomas seen in individual patients, especially infants.

Another possible connection of TrkA to regression of neuroblastoma tumors is formed by the oncogene HRAS. Increased HRAS expression is a favorable predictor of outcome in neuroblastomas and strongly correlated to high expression of TrkA. This was shown for clinically detected neuroblastomas and neuroblastomas detected by mass screening. Furthermore, increased expression of HRAS protein within tumor samples was preferentially found in areas of cellular degeneration. Interestingly, the areas of high HRAS staining did not stain for active caspase 3 or...
Figure 1. Apoptotic Defects in Neuroblastoma. Defects in the intrinsic and extrinsic routes to apoptosis are prominent in high-risk neuroblastomas.

1. Methylation and down-regulation of the CASP8 gene blocks the extrinsic route (see section 3.6).
2. Increased expression of BCL2 and/or BCLX_L prevents formation of BAX and release of pro-apoptotic mitochondrial factors, such as Cytochrome c and SMAC/DIABLO (see section 3.7).
3. MYCN is a pro-apoptotic factor that increases BAX and Cytochrome c release (see section 3.8).
4. TP53 in neuroblastoma cells is mostly locked in the cytoplasm, possibly by the Parc protein. This prevents normal, pro-apoptotic TP53 activity (see section 3.12). The role of the related TP73 in neuroblastomas is unclear. However, both TP53 and TP73 may be inhibited by overexpression of anti-apoptotic DeltaNp73.
5. NM23 is a strong candidate oncogene on chromosome 17q, a region of frequent chromosomal gain in neuroblastomas. Nm23H2 may prevent doxorubicin induced apoptosis (see section 3.10).
6. Increased expression of Survivin, an IAP protein inhibits Caspase 9 activation in the apoptosome (see section 3.11).

fragmented 3' DNA ends (TUNEL assay). In neuroblastoma cell lines, overexpression of wild-type HRAS, caused the cells to undergo cell death, whereas overexpression of an inactive HRAS mutant did not cause cell death. Interestingly, the process of cell death appeared to be independent of caspase activation. This suggests that expression of the HRAS, in concert with high TrkA expression can induce caspase-independent cell death in neuroblastoma cell lines and may play a role in the intriguing process of spontaneous regression of neuroblastomas. A role for TrkA as an inhibitor of angiogenesis was suggested in SY5Y cells transfected with TrkA and TrkB. In comparison to the parental SY5Y cells, the mRNA and protein levels of angiogenic factors were significantly reduced, whereas this was not demonstrated in the TrkB transfectants. In xenograft models, tumorigenicity was reduced in the TrkA transfected xenografts, and was associated with decreased vascularization. Lastly, genetic alterations have not been demonstrated for either of the Trk
receptors. Their role in the origin of neuroblastomas therefore remains speculative. They may reflect the level of differentiation at the time of developmental arrest of the neuroblast, and seem to have an important role in the phenotype of the arrested cell.

### 3.3 p75<sub>NTR</sub> expression in neuroblastomas

The role of p75<sub>NTR</sub> in neuroblastomas is still unclear. In general, activation of p75<sub>NTR</sub> leads to increased apoptosis in neuroblasts. Whether this is dependent upon stimulation with neurotrophins is still controversial. Different cell systems have created contradictory results about the p75<sub>NTR</sub>-induced apoptosis. Some found it to be correlated only to the expression level of the receptor, while others showed that apoptosis was activated in a ligand-dependent manner<sup>78-80</sup>. High expression of p75<sub>NTR</sub> is more pronounced in lower-risk neuroblastomas, whereas MYCN amplification is strongly associated with low expression of p75<sub>NTR</sub><sup>81</sup>.

### 3.4 TrkB and TrkC expression in neuroblastomas

In contrast to TrkA and p75NTR, high expression of TrkB is preferentially found in high-risk tumors, particularly in those with amplification of MYCN<sup>82</sup>. In the presence of BDNF, TrkB signaling promotes cell survival and induces modest neurite outgrowth and this may represent an autocrine or paracrine loop leading to survival<sup>83</sup>. This paracrine loop seems to enhance both angiogenesis and drug resistance<sup>78, 84, 85</sup>. There is evidence that TrkB and TrkC are expressed in the earlier development of the ganglion cells of the sympathetic nervous system, which will switch to mainly TrkA expression in the mature ganlion cell<sup>86</sup>. The data suggest that the maturation arrest of cells with high expression of TrkB takes place at an earlier stage of neuroblast development than those with high TrkA expression. TrkC expression is, like TrkA predominantly found in lower stage, MYCN single copy tumors.

### 3.5 Retinoic acid

Retinoic acid (RA) is involved in neuronal differentiation and apoptotic pathways. Different naturally occurring retinoids (All-trans-RA or ATRA and 9-cis-RA) mediate their effects by interaction with two types of receptors, the RA receptors (RARs) and the Retinoic X receptors (RXRs), both non-steroid nuclear hormone receptor family members. It has been shown that the induction of apoptosis by synthetic RA such as 13-cis-RA is dependent upon the co-stimulation of both RAR α and RAR γ<sup>84, 87</sup>. Differentiation is induced by RAs through the activation of many genes, although the key pathways may not be known yet. In neuroblastomas with expression of BDNF, RA increases transcription of TrkB, leading to cell survival and neurite extension<sup>88</sup>. RA also increases expression of glial-derived neurotrophic factor (GDNF) and its receptor RET, and this combination also enhances neuritogenesis<sup>84, 89</sup>. The ability of RAs to induce apoptosis or differentiation in vitro has led to clinical use. The synthetic
compound 13-cis-RA was found to be most active and improves the event-free survival of patients with minimal residual disease or complete remission after treatment. Currently, it is included in all major clinical studies. A recently introduced synthetic retinoid, N-(4-hydroxyphenyl) retinamide (fenretinide) does not lead to differentiation of tumor cells, but solely induces apoptosis. Fenretinide is currently undergoing clinical trials in neuroblastoma patients.

3.6 Caspase 8 and the extrinsic route to apoptosis

Alterations of the Caspase 8 gene, CASP8, have been shown in neuroblastomas, medulloblastomas, rhabdomyosarcoma, retinoblastoma and neuroendocrine lung cancer. Caspase 8 is a cysteine protease and a member of the Caspase family of proteolytic enzymes, activated in programmed cell death. Caspase 8 can be activated by most members of the TNF-Receptor Super Family (TNFRSF members, e.g. Fas, Death Receptor 3, DR4, DR5, TNFR2, p75TNFR) through the intermediate FADD (FAS-associating protein with death domain). The intracellular death domain (DD) of the TNFRSF members, FADD and pro-caspase 8 assemble to form a death-inducing signaling complex (DISC). Activated Caspase 8 will lead to activation of pro-Caspase 3 and initiation of the final pathway to apoptosis. CASP8 is located at human chromosome band 2q33, a region associated with LOH in neuroblastomas and several other tumor types (see section 2.4). Genetic analysis of the region showed a homozygous deletion of 20-35 kb in one neuroblastoma cell line and one tumor, encompassing the immediate CASP8 region, but not involving flanking genes like CASP10. Further analysis showed loss of expression of CASP8 mRNA and Caspase 8 protein in 13/21 neuroblastoma cell lines. The overall loss of CASP8 expression in neuroblastomas is estimated at 25-35%, predominantly in high-risk tumors, and seems strongly associated with the presence of amplified MYCN. The lack of expression was associated with hypermethylation of a 5' flanking sequence, and re-expression could be induced by treatment of the tumor cells with the demethylating agent 5-aza-2'deoxycytidine (5-AZA). There was a strong association of hypermethylation of this particular area and down-regulation of CASP8. Interestingly, the area subjected to de novo methylation is not a classical CpG island, as it has a C + G content of less than 60% (49%) and a CpG:GpC ratio below 0.6 (0.25). Furthermore, the proposed regulatory region maps at the boundary between exon 3 and intron 3. Also, promoter studies did not identify this region as a gene promoter, whereas a cloned DNA fragment at the 5' terminal region of exon 1 has promoter activity in neuroblastoma cell lines, but only in cells that express the CASP8 gene. No differences in methylation were detected in this promoter between cells with or without expression of the CASP8 gene. Furthermore, in cell lines that do not express CASP8, 5-AZA treatment of the cells was able to activate CASP8 promoter constructs, suggesting that demethylation of a trans-acting factor or gene controls the activity of the CASP8 promoter.

The biological relevance of loss of Caspase 8 follows from its central position in the extrinsic apoptotic route. CASP8 acts as a tumor suppressor gene, and inactivation will result in cell survival.
Indeed, tumor cells with loss of CASP8 do not respond to TNF-receptor mediated triggers like TRAIL (TNF Receptor Apoptosis Inducing Ligand) or FasL (Fas ligand)\textsuperscript{101}. In tumors with hypermethylated CASP8, treatment with 5-AZA will restore the responsiveness to TRAIL or FasL and induce apoptosis\textsuperscript{102}. Therefore, the epigenetic and genetic down-regulation of CASP8 is the first evidence of alterations in apoptosis in neuroblastomas.

An important regulator of Caspase 8 and the DISC is FLIP (Flice Inhibitory Protein), a caspase 8-related protein. The FLIP gene is a structural homologue of CASP8 and co-localizes at chromosomal band 2q33. Two isoforms of the protein, a long FLIP\textsubscript{L} and a short FLIP\textsubscript{S} have been identified. FLIP\textsubscript{L} is very similar to caspase 8, but is defective in its protease domain, rendering the protein unable to initiate the apoptotic cascade. Initial reports were inconclusive whether FLIP proteins are stimulatory or inhibitory on caspase 8 and apoptosis. However, experiments in which FLIP was stably overexpressed in cell cultures or in mice deficient in FLIP support an antiapoptotic function for FLIP\textsuperscript{103}. FLIP\textsubscript{L} and FLIP\textsubscript{S} seem comparable in their ability to inhibit apoptosis, but their functional differences still need further study. Furthermore, high FLIP expression was reported in melanomas and EBV-induced Burkitt’s lymphomas\textsuperscript{103}. In neuroblastoma cell lines, we found a strong association between silencing of CASP8 and FLIP. The majority of these cell lines were hypermethylated for Casp8 and FLIP\textsuperscript{104}.

### 3.7 Intrinsic route to apoptosis

Apoptosis can also be mediated through the intrinsic, Caspase 9 dependent route. Activation of Caspase 9 results from release of Cytochrome c (Cyt c) from the mitochondria, which associates with APAF-1 (Apoptotic Protease-Activating Factor-1) and pro-caspase 9 to form the apoptosome. Within this protein complex, caspase 9 activation is achieved by an autocatalytic cis-acting processing event of the pro-caspase 9\textsuperscript{105}. Caspase 9 will, similar to activated Caspase 8 activate the downstream caspase effector cascade, which involves Caspase 3, among others. The autolytic process will eventually lead to the demise of the cell.

Necessary for the release of cyt c is the initiation of the mitochondrial outer membrane permeabilization (MOMP). BCL2 and BCL-X\textsubscript{L} suppress apoptosis by blocking MOMP, whereas pro-apoptotic BAX (BCL2-associated X protein) can shift the balance and induce MOMP\textsuperscript{106}. This will induce conformational changes in the pore proteins like VDAC (Voltage Dependent Anion Channel), and the release of Cyt c. Also SMAC/DIABLO (Second Mitochondria-Derived activator of Caspase/ Direct IAP-Binding Protein with Low pi) is released, which acts as an inhibitor of the IAP’s (Inhibitor of apoptosis). IAP’s, like Survivin (see section 3.11) are negative regulators of the apoptosome. Therefore, release of SMAC/DIABLO will also activate apoptosis.

In neuroblastomas, increased levels of BCL2 and BCL-X\textsubscript{L} have been observed, and correlate with decreased apoptosis\textsuperscript{107, 108}. Overexpression of BCL2 blocked TRAIL-induced apoptosis in neuroblastoma cell lines\textsuperscript{109}. Also, increased BCL2 expression was correlated with poor prognostic
factors like MYCN amplification and unfavorable histology. Finally, overexpression of both BCL2 and BCL-X_L was able to block apoptosis induced by various chemotherapeutic agents, like cisplatin, doxorubicin, etoposide, and betulinic acid. Therefore it was suggested that the observed expression of these proteins in tumors may contribute to the drug resistance, characteristic of high-risk neuroblastomas. It should be stressed however, that structural alterations of the BCL2 and BCL-X_L genes, or any of the other genes involved in the intrinsic apoptotic pathway have never been shown.

3.8 MYCN and apoptosis

Many observations have shown that MYCN and its family member c-MYC can induce apoptosis. The apoptotic properties of c-MYC have been studied in more detail than for MYCN, and several data to support a role for MYCN in apoptosis are based on studies with c-MYC. However, all the available data suggest that MYCN and c-MYC have similar functions, but in different cell types. In fact, it was even shown that MYCN can functionally replace c-MYC in murine development.

Initially, it was shown that apoptosis could be induced in fibroblasts with ectopic expression of c-MYC if cultured in the absence of sufficient survival factors. A widely supported interpretation of these and similar observations about the apoptotic potential of oncogenes is that the induction of the cell cycle after expression of oncogenes also sensitizes cells to apoptosis. Apoptosis however, will be suppressed as long as appropriate survival factors are available for proliferation and growth. This suggests a coupling between the cell cycle and apoptosis, and implies that cells with oncogenic mutations can only outgrow their paracrine environment in the presence of sufficient growth factors, or if apoptosis is inhibited. Examples of inhibition of apoptosis in combination with up-regulation of the cell cycle are ample in carcinogenesis. In fact, in the majority of all cancer types, a combined loss of TP53 or ARF and activation of oncogenes like c-MYC or RAS can be found. In particular for c-MYC it was shown that in normal, mature cells activation of c-MYC induces uniform cell proliferation, accompanied by overwhelming apoptosis that rapidly erodes cell mass. However, upon induced co-expression of BCL-X_L, c-MYC triggered rapid and uniform progression into invasive tumors. Subsequent c-MYC deactivation induced rapid regression associated with vascular degeneration and cell apoptosis. It seems that in neuroblastomas a similar interplay between cell growth and apoptosis exists involving MYCN. It has been convincingly shown that exogenous overexpression of MYCN sensitizes neuroblastoma cell lines to many apoptotic triggers, such as γ-IFN, TRAIL, FasL, but also to exogenous stimuli like doxorubicin. However, neuroblastomas characteristically overexpress MYCN, and do not apoptosis spontaneously or after stimulation. In fact, they appear to be highly resistant to induction by TRAIL. This is considered to result from overexpression of BCL2 or BCL-X_L in neuroblastomas. Increased levels of BCL2 can counteract the MYCN induced activation of BAX, which would lead to release of Cyt c into the cytosol. Overexpression of BCL2 or BCL-X_L has been observed in high-risk neuroblastomas with amplified MYCN.
Introduction: La Danse Macabre

evidence of the clinical relevance and influence of BCL2 on apoptosis comes from experiments in which the influence of BCL2 overexpression is bypassed by overexpression of pro-apoptotic SMAC/DIABLO. This strongly increased the TRAIL sensitivity of glioblastoma cells and neuroblastomas, suggesting that ineffective or defective apoptosis is a basic defect in these tumors.

The role of MYCN in the extrinsic apoptotic route is less clear. Induction of apoptosis by MYCN or c-MYC through this route has never been shown. Nevertheless, down-regulation of CASP8 (see section 3.6) in neuroblastomas seems closely connected to overexpression of MYCN. However, a direct influence of MYCN on CASP8 has not been shown. Overexpression of MYCN does not induce CASP8 hypermethylation or down-regulation. Interestingly, epigenetic down-regulation of CASP8 has mainly been observed in neuro-endocrine tumors with MYC overexpression, such as neuroblastoma, medulloblastoma and neuro-endocrine lung tumors. It is possibly that the Caspase 8 dependent pathway is an essential pathway in neuro-endocrine development and that blocking of this route is important in the pathogenesis of aggressive neuro-endocrine tumors.

3.9 Hypermethylation of RASSF1A

Allelic loss at the short arm of chromosome 3 is a common event in many different cancers, including pediatric neuroblastomas. The SRO in neuroblastomas has been defined at 3p25.3-p14.3 (see section 2.4). RASSF1A (Ras-association domain family 1) is within the SRO in chromosomal band 3p21.3 and frequently inactivated by hypermethylation in neuroblastoma cell lines and tumors, medulloblastoma, rhabdomyosarcoma, retinoblastoma, phaeochromocytoma, lung, breast, kidney cancer, head and neck squamous cell carcinomas, testicular germ cell tumors and several others. Hypermethylation of RASSF1A in neuroblastomas was reported in 40-55% of tumors and 86% of cell lines. A relationship with concomitant hypermethylation of CASP8 was suggested since methylation of CASP8 was detected in 56% of tumors with RASSF1A methylation and only in 17% in tumors without RASSF1A methylation (p=0.003). A role as a tumor suppressor gene of RASSF1A was suggested since re-expression of RASSF1A in human cancer cell lines reduces the cellular growth. The function of RASSF1A seems to negatively effect the oncogenic HRAS GTPase. However, HRAS overexpression in neuroblastomas is a favorable event, possibly related to spontaneous tumor regression (see section 3.2). This contrasts with the oncogenic potential of HRAS and challenges any interpretation about the influence of RASSF1A on Ras function.

3.10 Nm23-H1 and nm23-H2

NM23H1 is a highly interesting candidate gene for an important role in neuroblastoma pathogenesis, associated with gain of chromosome 17q. NM23H1 maps at 17q22 just within the common amplified region of 17q. NM23H1 was originally described as a suppressor of metastasis with low or absent expression in mainly breast cancer and melanoma. In several other malignancies, NM23H1
acts as an oncogene and increased serum levels of NM23H1 correlated with poor prognosis and increased aggressiveness. This is well documented for non-Hodgkin's lymphomas and this correlation is likely to exist for various hematological malignancies.\textsuperscript{125} NM32-H1 overexpression in neuroblastomas is also strongly related to increased aggressiveness. Interestingly, NM23H1 is part of a gene family, of which its close relative NM23H2 co-localizes at 17q22. NM23H1 and NM23H2 are both up regulated by MYCN and c-MYC in neuroblastoma. Importantly, this implies that amplification of MYCN in tumors with a gain of 17q will lead to a synergistic accumulation of the NM23H1 and H2 mRNA levels. Functionally, it seems that NM23H2, but not H1 has an anti-apoptotic effect. Down regulation of NM23H2 expression by RNAi enhanced apoptosis after addition of doxorubicin in neuroblastoma cell lines, but did not influence apoptosis induced by TRAIL.\textsuperscript{126} These preliminary data suggest that NM23H2 is involved in chemotherapy resistance in neuroblastomas with 17q gain. Taken together, the NM23 gene family members at 17q seem important candidate genes in the 17q-gain related pathogenesis of neuroblastomas.

3.11 Survivin
Recently, the 17q located Survivin gene, which codes for the anti-apoptotic regulatory protein Survivin has also been implicated in neuroblastoma. Survivin maps at chromosome 17q25, within the minimal region of chromosomal gain. The Survivin protein is a so-called IAP (Inhibitor of apoptosis), a negative regulator of Caspase 9 in the intrinsic apoptotic route. In two studies of neuroblastoma samples of 72 and 34 patients respectively, increased expression of Survivin correlated significantly with unfavorable histology, additional adverse clinical factors (age, stage) and outcome.\textsuperscript{127} Moreover, exogenous overexpression of Survivin was able to block apoptosis induced by RA in a RA sensitive cell lines.\textsuperscript{128} Increased expression of Survivin has been observed in embryonic and fetal organs and the majority of malignancies, but is undetectable in most terminally differentiated normal tissues.\textsuperscript{129} The role of Survivin in cancer formation or progression and more specific in neuroblastoma needs to be established.

3.12 p53, p73 and DeltaNp73
The nuclear phosphoprotein TP53 plays a central role in the pathogenesis of human cancers. TP53 is a checkpoint protein for the cell cycle and monitors DNA damage. Activation of TP53 will lead to cell cycle arrest or apoptosis. Defects in the p53 gene play a role in more than 50% of all human neoplasias. It usually concerns LOH of one allele, in combination with a (missense) point mutation. In neuroblastomas, p53 is rarely mutated.\textsuperscript{130} However, it has been shown in a cohort of 46 neuroblastomas that the TP53 protein is functionally inactivated through sequestration in the cytoplasm in undifferentiated neuroblastomas.\textsuperscript{131} Recently, a cytoplasmic protein Parc was identified, responsible for cytoplasmic sequestration of ectopic TP53. RNAi-mediated reduction of endogenous Parc mRNA significantly sensitized neuroblastoma cells with cytoplasmic TP53 sequestration to a DNA damage
response. Therefore, it seems that the subcellular localization of TP53 is regulated by Par132.

The p73 gene is a structural homologue of p53, located at chromosome 1p36.3133 and TP73 induces apoptosis similar to TP53. Therefore, TP73 seemed an excellent candidate tumor suppressor gene for neuroblastomas. However, despite the fact that p73 maps within the SRO of 1p deletion in neuroblastomas, mutations have not been found so far. Only in malignant lymphomas, inactivation of p73 has been observed as a result of bi-allelic hypermethylation134. Recently, a truncated anti-apoptotic isoform, DeltaNp73, which antagonizes both TP53 and the full-length TP73 protein, has gained attention. So far, expression of this variant in neuroblastoma patients significantly correlated with age at diagnosis, urinary excretion of catecholamine-derivatives, and reduced survival. The role of this protein needs further investigation in larger cohorts to establish its role in the pathogenesis of neuroblastomas135.

3.13 Cyclin D1

Genetic aberrations and overexpression of Cyclin D1 (CCND1) have been identified for several human neoplasms, such as mantle cell lymphoma, head and neck squamous cell carcinoma, lung cancer and breast cancer136. D-type cyclins play an essential role in cell cycle progression, as they control Cyclin Dependent Kinases (CDKs). Activation of CDK4 and CDK6 by CCND1 induces phosphorylation of the retinoblastoma protein Rb, release of E2F transcription factors and progression of the cell cycle from G1 to S. Recently, very high expression of CCND1 RNA and protein levels was found in approximately two thirds of cell lines and tumors. In addition, amplification of the CCND1 gene was found in one neuroblastoma cell line and 4 neuroblastoma tumors137. There was no obvious relation to prognostic factors, such as amplification of MYCN, age, stage or any other factor. It suggests that CCND1 is a frequently overexpressed oncogene in neuroblastomas. This is of particular interest since the downstream kinases CDK4/6 are potential targets for future kinase inhibitors138, 139.

3.14 Is neuroblastoma an embryonal tumor?

It is widely assumed that neuroblastomas are embryonal tumors. This means that they are considered to originate from a developmental defect, which prevents normal cellular differentiation and locks cells in a state of increased growth. How true is this? Certainly, neuroblastomas are tumors of early childhood and are almost non-existent after the age of 10 years. In murine neural crest cells, induction of neuroblastomas by forced overexpression of MYCN is limited to the first months of life. These data suggest that neuroblastomas arise only during the normal development of the sympathetic nervous system. Paradoxically, the younger patient enjoys a better prognosis than the older child does (> 1 year at diagnosis). This is unexpected, since an early onset of disease would imply earlier defects during development and the formation of more undifferentiated, aggressive tumors.
Except for the fact that certain developmental genes have a prominent expression in specific subsets of neuroblastomas\(^{140, 141}\) (see also sections 3.2-4), there is still little support for an embryonal origin of neuroblastomas at the molecular level. Firstly, there are no known alterations in developmental control genes. It could be argued that \( MYCN \) is an important developmental control gene, but \( MYCN \) and its family member \( c-MYC \) are also implicated in many adult tumor types. Secondly, the additional (epi-) genetic alterations present in neuroblastomas are related to an increase of the cell cycle (overexpression of oncogenes as \( MYCN \) and \( CCND1 \)) and loss of apoptosis (\( CASP8 \) hypermethylation, overexpression of \( BCL-2, NM23H2, Survivin \)). This bears resemblance to the common themes in adult type cancers. Almost all adult types of cancer can be characterized by increased proliferation, in combination with apoptotic defects. These observations direct us to the understanding that embryonal tumors are dependent upon similar mechanisms for cancer formation as epithelial carcinomas. 

In summary, the embryonal origin of pediatric malignancies may be reflected by the strong expression of intact genes of neuro-endocrine development, which contribute to the phenotype of the cell. However, no genetic alterations in developmental control genes have ever been shown. The overexpression of oncogenes and loss of apoptotic potential in neuroblastomas are reminiscent of the mechanisms well known in the pathogenesis of adult type cancer formation.

### 3.15 Future prospects

The molecular properties and defects of the neuroblastoma cells predict multiple possible ways to specifically target the malignant cell\(^{142}\). Targeted therapy can be directed against defective genes or proteins, which contribute to the malignant phenotype of the cell. However, the physiological properties of the cell can also be used as a specific target for therapeutic modulators. The adrenergic properties of the neuroblast have inspired the development of targeted radiotherapy with \(^{131}\)labeled MetalodoBenzylGuanidine (MIBG), a compound structurally related to nor-adrenaline. It has proven to be an effective agent with minimal toxicity, even in patients with decreased chemosensitivity\(^{143, 144}\). The neuronal antigen disialoganglioside (GD2) is highly expressed on neuroblastoma cells. Monoclonal antibodies against GD2 have shown anti-tumor activity in multiple phase II studies and are currently being tested in the European HR-NBL-1/ESIOP phase III trial. RAR receptors can induce apoptosis and/or differentiation upon stimulation with RA. This has led to the use of 13-cis-Retinoic acid and more recently the development of fenretinide, which more specifically induces apoptosis (see section 3.5).

Approaches to target molecular defects of the neuroblastoma cell are aimed at reversing the aggressive phenotype. Most of these approaches are still in development, and often involve the restoration or stimulation of apoptosis\(^{145}\). Several molecular defects could potentially be targets for intervention in neuroblastomas.

a. In xenograft and ex vivo tumors it was shown that apoptotic defects induced by overexpression
of BCL2 and/or BCL-XL can be corrected by using a SMAC/DIABLO peptide\(^{119}\).

b. Hypermethylation of CASP8 is widespread in high-risk neuroblastomas. Several demethylating agents and deacetylation inhibitor compounds are currently being tested for clinical use and could potentially restore the CASP8 function\(^{146}\).

c. Inhibition of genes by RNAi is a promising novel strategy that has proven useful in vitro and waits clinical testing\(^{147}\). Several genes are potential targets in neuroblastomas, such as BCL2 and/or BCL-XL and the overexpressed Parc gene that locks TP53 in the cytoplasm. Also overexpressed oncogenes like MYCN, and CCND1 could be excellent targets.

d. Tyrosine kinase inhibitors have become the most promising new drugs against cancer since imatinab (Gleevec) has proven effective in hematological malignancies and others. The overexpression of the TrkB receptor kinases in high-risk neuroblastomas potentially enables targeted inhibition. Specific kinase inhibitors have proven effective in several human-derived neuroblastoma xenografts\(^{148}\). The widely observed overexpression of CCND1 in neuroblastomas suggests that the down-stream kinases CDK4/6 are potential targets for future kinase inhibitors, which may be useful in a wide range of patients (see section 3.13).

3.16 Concluding remarks
Neuroblastomas are a complex and diverse group of tumors of early childhood. Their clinical behavior is reflected by the multiple genetic alterations found in different clinical subgroups. This has enabled us to better identify patients at risk. However, this has not led to novel therapeutic strategies or better survival. While the treatment of the low-risk and intermediate-risk patients has mostly been satisfactory, this is not so for patients with high-risk, aggressive disease. Particularly in high-risk neuroblastomas we are starting to uncover molecular markers and essential biological pathways, which will help us to unveil the secret of the neuroblast. More importantly, it will direct our future efforts towards new tumor-specific therapeutic approaches aimed at overcoming or compensating for the molecular alterations in the tumor cell. Hopefully this will be beneficial to the prognosis of neuroblastoma patients.
References


Tumor-specific down-regulation of the TRAIL decoy receptors DcR1 and DcR2 is associated with dense promoter hypermethylation

Max M. van Noesel1,2,3,4, Saskia van Bezouw1, Gajja S. Salomons1, P. A. Voûte2, Rob Pieters4, Steve B. Baylin3, James G. Herman3, Rogier Versteeg1

Cancer Research 2002; 62: 2157-2161

Acknowledgement
This work was supported by the Dutch Cancer Society (MMvN), and the Stichting Kindergeneeskundig Kankeronderzoek (SKK).

1 Dept. of Human Genetics, Academic Medical Center, Amsterdam, The Netherlands
2 Dept. of Pediatric Oncology Emma Kinderziekenhuis/AMC, Amsterdam, The Netherlands
3 The Johns Hopkins Oncology Center, Tumor Biology Laboratory, Baltimore, USA
4 Dept. of Pediatric Oncology-Hematology, Erasmus MC-Sophia Children’s Hospital, Rotterdam, The Netherlands