# The Neuroblastoma Genome and Epigenome

## - Patient Stratification and Identification of Candidate Genes

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To my wonderful family

## ABSTRACT

**The Neuroblastoma Genome and Epigenome - Patient Stratification and Identification of Candidate Genes**



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Neuroblastoma (NB) is a tumor of the sympathetic nervous system, and the most common extracranial tumor of childhood. The prognosis for high-stage NBs is still poor, with survival rates of about 35%. Side-effects of treatment in these young children can also be severe. It is therefore important to develop better tools for improved patient stratification as well as to identify new targets for therapy.

**Aims:** Using genetic and epigenetic approaches, this thesis aimed to analyze candidate genes with potential involvment in the initation/progression of NB and to identify genes that can be used for improved patient stratification.

**Results:** The six candidate genes located in chromosome region 1p36.22 were down-regulated in tumors from patients with an unfavorable outcome compared with a favorable. DNA methylation was shown not to be involved in the down-regulation of gene transcripts.

In a more comprehensive analysis of 1p36, four genes, *ERRFI1*, *PIK3CD*, *RBP7* and *CASZ1*, were up-regulated by epigenetic treatment. Bisulfite sequencing revealed that DNA methylation most likely was not involved, suggesting for the potential involvement of other epigenetic mechanisms such as histone deacetylation. Missense mutations were identified in *PIK3CD* and *ERRFI1* and the down-regulated mRNA expression of *PIK3CD* and *CASZ1* was detected in high-stage NB. *CASZ1* plays a role in neural development and is therefore an interesting candidate for further study.

In a genome-wide analysis of DNA methylation, a group of methylated genes for which we showed gene expression was affected by epigenetic treatment was selected for further analysis. A selected group, e.g. *SCNN1A, PRKCDBP and KRT19* could be used to distinguish between patients with an unfavorable outcome from those with a favorable one.

Whole-genome copy number analysis of NB tumors identified homozygous deletions in the *CDKN2A* and *RBMS3* genes. Moreover, copy neutral loss of heterozygosity was rare, but could be detected in three chromosomal regions. Tumors with *MYCN* amplification and those with 11q deletion displayed very different genomic profiles. The 11q-deletion group had significantly more chromosomal breaks than the other group, indicative of an 11q localized chromosomal instability gene (CIN). This group also had a significantly higher age at diagnosis. The groups defined by 11q deletion, *MYCN* amplification and 17q gain were the only groups associated with poor patient outcome.

**Conclusions:** Whole-genome profiles add valuable information about genomic aberrations, which are important prognostic factors in NB. Aberrant DNA methylation can be a very early event in tumor development as well as in tumor progression. It is therefore of great importance to learn more about both the genetic and epigenetic profiles of NB. This thesis has added to the current knowledge in these regards and has also identified important genetic aberrations, as well as aberrantly methylated genes. In the future, these aberrations could possibly be used in patient stratification, as biomarkers or as targets for therapy.



**Keywords:** tumor, embryonal, neural crest, neuroblastoma, tumor suppressor gene, DNA methylation, epigenetics, bisulfite sequencing, microarray, 1p36, 11q, MYCN, CASZ1, PIK3CD, PRKCDBP, SCNN1A, TGFBI, DHRS3, KRT19, DUSP23, APITD1, H2AFX

## LIST OF PAPERS

This thesis is based on the following papers, which will be refered to in the text by their Roman numerals.

**I.** **Carén H**, Ejeskär K, Fransson S, Sjöberg R-M, Krona C, Hesson L, Latif F, Martinsson T. A cluster of genes located in 1p36 are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation. *Mol Cancer. 2005 Mar 1;4(1):10*.

**II.** **Carén H**, Fransson S, Ejeskär K, Kogner P, Martinsson T. Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumours. *Br J Cancer. 2007 Nov 19;97(10):1416-24. Epub 2007 Oct 16.*

**III.** **Carén H**, Djos A, Nethander M, Sjöberg R-M, Enström C, Nilsson S, Martinsson T. Identification of epigenetically regulated genes that predict patient outcome in neuroblastoma. *2009, submitted*

**IV.** **Carén H**, Erichsen J, Enerbäck C, Olsson L, Sjöberg R-M, Abrahamsson J, Kogner P, Martinsson T. High-resolution array copy number analyses for detection of deletion, gain, amplification and copy-neutral LOH in primary neuroblastoma tumors; Four cases of homozygous deletions of the *CDKN2A* gene. *BMC Genomics. 2008 Jul 29;9(1):353.*

**V.** **Carén H,** Kryh H, Nethander M, Sjöberg R-M, Nilsson S, Abrahamsson J, Kogner P, Martinsson T. High-risk neuroblastoma without *MYCN* amplification; Characterization of the 11q deletion tumors reveal a poor prognostic chromosome instability phenotype with later onset. *2009, submitted*

## OTHER RELEVANT PUBLICATIONS NOT INCLUDED IN THIS THESIS

**Carén H**, Abel F, Kogner P, Martinsson T. High incidence of DNA mutations and gene amplifications of the *ALK* gene in neuroblastoma tumours. *Biochem J. 2008 Dec 1;416(2):153-9. Epub, 2008 Oct 7.*

**Carén H**, Holmstrand A, Sjöberg R-M, Martinsson T. The two human homologues of the yeast *UFD2* ubiquitination factor, *UBE4A* and *UBE4B*, are located in common neuroblastoma deletion regions and are subject to mutations in tumours. *Eur J Cancer. 2006 Feb;42(3):381-7.*

Krona C, **Carén H**, Sjöberg R-M, Sandstedt B, Laureys G, Kogner P, Martinsson T. Neuroblastoma tumor progression; Loss of *PHOX2B* on 4p13 and 17q Gain are Early Events in Neuroblastoma Tumorigenesis. *Int J Oncol. 2008 Mar;32(3):575-83.*

Krona C, Ejeskär K, **Carén H**, Abel F, Sjöberg R-M, Martinsson T. A novel 1p36.2 located gene, *APITD1*, with tumour suppressive properties and a putative p53 binding domain, shows low expression in neuroblastoma tumours. *Br J Cancer. 2004 Sep 13;91(6):1119-30.*

Thorell K, Bergman A, **Carén H**, Nilsson S, Sjöberg RM, Kogner P, Martinsson T, Abel F. Verification of genes differentially expressed in neuroblastoma tumours: a study of potential tumour suppressor genes. *BMC Med Genomics. 2009 Aug 17;2(1):53.*

Ejeskär K, Krona C, Sjöberg R-M, **Carén H**, Ioannou P. Introduction of *in vitro* transcribed *ENO1* mRNA into neuroblastoma cells induces massive cell death. *BMC Cancer. 2005 Dec 16;5(1):161*

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## ABBREVIATIONS

| | |
|---|---|
| 5-aza-dC | 5-aza-deoxycytidine |
| bp | base pair |
| BSP | bisulfite sequencing |
| cDNA | complementary DNA |
| CNV | copy number variant |
| CpG | cytosine-guanine dinucleotide |
| DM | double minutes |
| DNA | deoxyribonucleic acid |
| DNMT | DNA methyltransferase |
| dsDNA | double-stranded DNA |
| dsRNA | double-stranded RNA |
| FISH | fluorescence in situ hybridization |
| GUSB | ß-glucuronidase |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HSR | homogeneously staining regions |
| INRGSS | neuroblastoma risk group staging system |
| INSS | International neuroblastoma staging system |
| LOH | loss of heterozygosity |
| LOI | loss of imprinting |
| MBD | methyl-CpG binding |
| miRNA | microRNA |
| MLPA | multiplex ligation-dependent probe amplification |
| mRNA | messenger RNA |
| MSP | methylation-specific PCR |
| NB | neuroblastoma |
| PCR | polymerase chain reaction |
| piRNA | PIWI-interacting RNA |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RT-PCR | reverse transcriptase PCR |
| SAM | S-adenosyl methionine |
| siRNA | short interfering RNA |
| SNP | single nucleotide polymorphism |
| SNS | sympathetic nervous system |
| SRO | smallest region of overlap |
| ssDNA | single-stranded DNA |
| tRNA | transfer RNA |
| TSA | trichostatin A |
| TSG | tumor suppressor gene |
| UCSC | University of California, Santa Cruz |

Gene symbols approved by the HUGO Gene Nomenclature Committee (HGNC) are used in the thesis. For full gene names see NCBI Entrez Gene (URL:http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

# INTRODUCTION

## BASIC GENETICS

### DNA and genes

In humans, the genetic information is organized into 23 chromosome pairs consisting of approximately 25,000 genes. The DNA (deoxyribonucleic acid) is composed of a double-stranded polymer composed of four bases; adenine (A), cytosine (C), guanine (G) and thymine (T). Complementary base pairs form between A and T and G and C. The nucleotides are linked together by covalent phosphodiester bonds that join the 5' carbon of one deoxyribose group to the 3' carbon of the next. The two DNA strands that make the double helix run in opposite directions. The structure of the double helix was first published by Watson and Crick in 1953 (Watson & Crick, 1953).

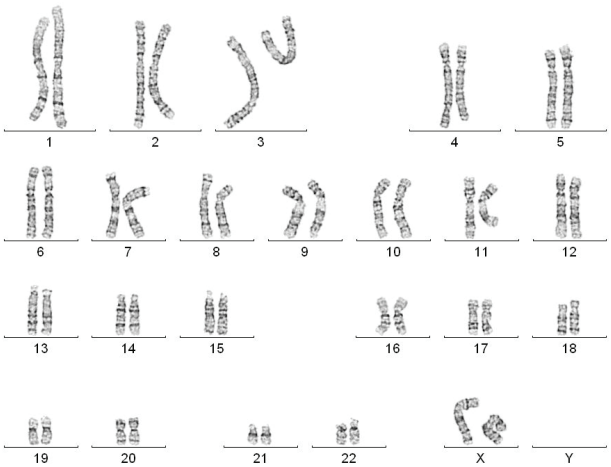


**Figure 1.** A human female karyotype showing 46 chromosomes, 23 chromosome pairs. Karyotype kindly provided by Kirsten Schultz, Department of Clinical Genetics, SU/Sahlgrenska.

The classical view of a gene is that it is composed of exons and introns. The exons code for amino acids that make up the proteins and the introns are non-coding elements that are spliced off during transcription. The promoter region constitutes the regulatory region of the gene and is located in the 5' region. Transcription factors bind to this region and direct the transcription of the gene. Moreover, regions located far from the gene, called enhancers and silencers, affect transcription. The 3' untranslated region of the gene is important for RNA stability and translation. The definition of a gene is no longer entirely straightforward. The dispersed regulation, non-coding RNAs and non-genic conservation (conserved regions outside genes thought to perform functions; Dermitzakis *et al*, 2002) have challenged the concept of the gene. The definition of a gene has therefore been relaxed and, according to the official Guidelines for Human Gene Nomenclature, is currently defined as "a DNA segment that contributes to phenotype/function. In the absence of demonstrated function, a gene may be characterized by sequence, transcription or homology".

## The central dogma of molecular biology

The flow of genetic material from DNA to RNA to polypeptide has been described as the central dogma of molecular biology (Crick, 1958). In the first step, where DNA is replicated, the DNA strands are unwound by a helicase and each strand directs the synthesis of a new complementary strand, resulting in two daughter duplexes. DNA is transcribed into RNA in the nucleus of eukaryotic cells (and in mitochondria and chloroplasts) and the RNA is then translated into polypeptides at the ribosomes (large RNA-protein complexes) in the cytoplasm. Only a small proportion of the DNA in a cell is ever transcribed and only a portion of the RNA is translated into proteins (transfer RNA (tRNA), ribosomal RNA (rRNA) and non-coding RNA are not translated into proteins). Furthermore, primary RNA transcripts are processed into mRNA. During this RNA processing, introns are excised. Sections of the ends of the mRNA are also kept untranslated. Retroviruses, certain primitive viruses and prions may violate the central dogma. Retroviruses transcribe RNA into DNA using the enzyme, reverse transcriptase. Some primitive viruses do not even have DNA and prions can be inherited in the absence of a DNA or RNA template.

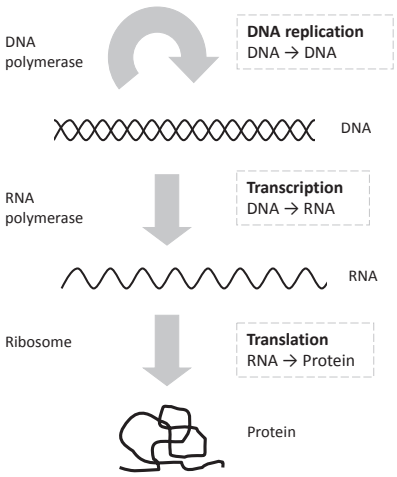


**Figure 2.** The central dogma of molecular biology.

## Genetic variations

A single-nucleotide polymorphism (SNP) is a DNA sequence variation in which one nucleotide differs between individuals. Normally, a SNP has two alleles, although three- and four-allele SNPs do exist, but they are much more unusual. SNPs are located in non-coding regions or in coding regions of genes; however, the location in non-coding DNA is far more common. When located in coding regions, they may affect the amino acid, depending on the position and alleles. A SNP that results in an amino acid variation is called synonymous and one that does not is called non-synonymous. Synonymous can be further divided into missense and nonsense variations. Missense results in a different amino acid and nonsense in a premature stop codon. Any change in DNA is defined as a mutation and a SNP can therefore also be referred to as a mutation. The definition “mutation” has,

however, been used more commonly to describe a DNA alteration that is pathogenic, whereas SNP has been used to define alterations that are not pathogenic. In addition to the basepair substitutions in SNPs, mutations can result from deletions (where one or more bases are lost) or insertions (where one or more bases are inserted). Large-scale aberrations involve the loss or gain of whole chromosomes, called numerical aberrations, the loss or gain of parts of chromosomes, called segmental or structural aberrations, and translocations (where there is a rearrangement of parts between nonhomologous chromosomes).

Much attention has recently been paid to DNA copy number variants (CNVs), defined as stretches of DNA larger than 1 kb that display copy number differences in the normal population (Scherer *et al*, 2007). These variants are likely to play a role in functional diversity and individual CNVs have been shown to be associated with diseases or susceptibility to diseases, reviewed by de Smith *et al* (2008).

## Organization of the genetic material

Each cell contains about 2 meters of DNA, which is compacted and organized by protein structures called histones. The nucleosome consists of a central core of eight histone proteins (two each of H2A, H2B, H3 and H4). Approximately 146 base pairs of negatively charged DNA are wrapped around the positively charged core histones and adjacent nucleosomes are connected by a short stretch of linker DNA. This "string of beads" is coiled into the chromatin fiber. When a cell divides, the chromatin fibers are very tightly folded and can be visualized in the light microscope as chromosomes. Between divisions (during interphase), the chromatin is more extended, a form used when expressing genetic information.

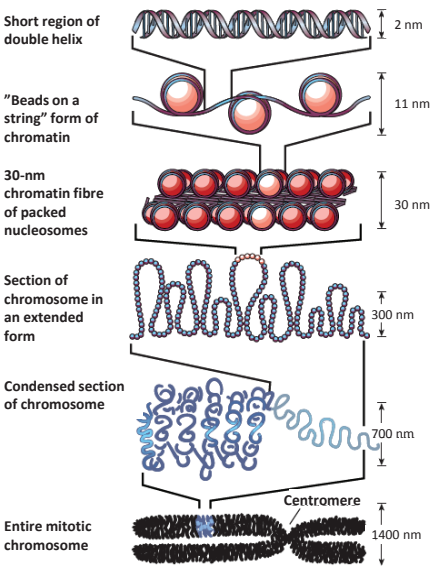


**Figure 3**. The organization of DNA within the chromatin structure. Reprinted with permission from Macmillan Publisher Ltd: Nature (Felsenfeld & Groudine, 2003), © 2003.

## EPIGENETICS

The term “epigenetics” has been used at least since the 1940s, when Conrad Waddington used it to refer to the study of processes by which genotypes give rise to phenotypes (Waddington, 1946). Nowadays, epigenetics is most commonly defined as a mitotic and/or meiotic heritable change in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. DNA methylation and histone modifications are the most studied epigenetic mechanisms that affect gene expression.

### DNA methylation in mammals

The methylation of cytosine in the CpG dinucleotide (where a cytosine is directly followed by a guanine in the DNA sequence) is a common modification of DNA in mammalian genomes. This reaction is catalyzed by the enzymes DNA methyltransferases (DNMTs), which use S-adenosyl methionine (SAM) as the methyl donor.

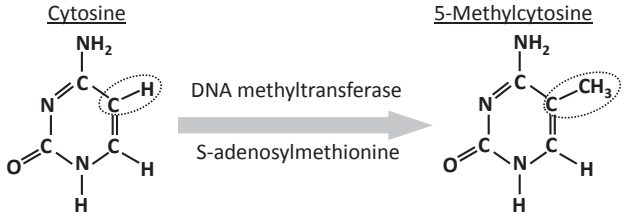


**Figure 4.** Structure of cytosine and 5-methylcytosine. The reaction that converts cytosine into 5-methylcytosine is catalyzed by DNMTs.

Methylated cytosines are more susceptible to deamination into tymines, which have led to an erosion of the number of CpG sites. The majority of CpGs reside within repetitive elements which are methylated. Another place where they are found is in CpG islands associated with promoter regions of genes, normally unmethylated. The DNA hypermethylation of CpG islands is associated with gene silencing and is normally found in imprinted genes and in genes on the inactivated X-chromosome in females. The methylation of promoter CpG islands is also a common mechanism for the inactivation of tumor suppressor genes and has been detected in many different tumor types (Costello & Plass, 2001; Esteller, 2002; Jones & Laird, 1999; Tycko, 2000).

Methyl-CpG-binding (MBD) proteins bind to methylated DNA and recruit repressor complexes which lead to gene silencing. The MBD protein family is composed of MeCP2, MBD1, MBD2, MBD3 and MBD4 (Bird & Wolffe, 1999; Lopez-Serra & Esteller, 2008). In addition, the protein Kaiso can also be involved in this mechanism. Methylated DNA can additionally lead to transcriptional repression by preventing the binding of certain transcription factors that only bind to unmethylated sequences. The DNA methylation patterns are established during embryonic development and are maintained when the cell divides. DNA methylation thus constitutes a form of cellular memory. The DNA methylation

patterns are, however, not fixed. Changes do occur, for example, as physiological responses to environmental exposure, during oncogenic transformation and cellular aging.

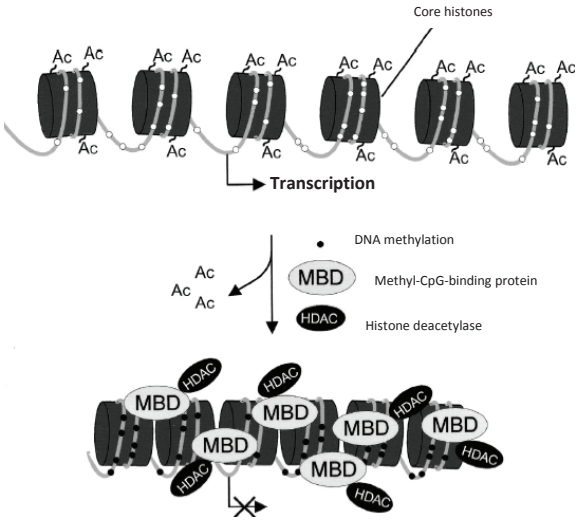


**Figure 5.** Model for methylation-dependent gene silencing. A gene that is actively transcribed is characterized by acetylated histones which cause an open chromatin configuration. When a gene is methylated, the methylated cytosines are recognized by methyl-CpG-binding proteins (MBDs), which in turn recruit histone deacetylases (HDACs) to the site of methylation. This converts the chromatin into a closed structure that is no longer accessible to the transcriptional machinery. Reprinted with permission from Wiley (Worm & Guldberg, 2002).

### Histone modifications

Histones can be modified post-translationally, which alters their interaction with DNA and nuclear proteins. Modifications on the histone tails, the N-terminals that protrude from the nucleosome, include methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation. Modifications such as the acetylation of lysine residues alter the charge and thus change the bulk of the nucleosome. This changes interactions with other nuclear components. Methylation, on the other hand, provides specific binding platforms for chromatin-associated proteins. It has been proposed that the combination of modifications constitute a code, the so-called ”histone code”, which defines the status of the chromatin structure (Jenuwein & Allis, 2001).

### Histone acetyltransferases and histone deacetylases

Histone acetyltransferases (HATs) acetylate lysine residues on the N-terminal of histones, as well as on other proteins (Yang, 2004). Most HATs are present as part of large protein complexes, act as transcriptional coactivators and are generally associated with euchromatin (regions with active transcription).

Histone deacetylases (HDACs) are a family of 18 deacetylating enzymes that remove acetyl groups from lysine residues of histone proteins, as well as on other proteins including transcription factors (Witt *et al*, 2009). HDACs are grouped into four classes among which classes I, II and IV are called "classical" HDACs. This group of HDACs can be inhibited by small molecule compounds called HDAC inhibitors. Class III HDACs are called sirtuins and differ from classical HDACs in their catalytic mechanism and co-factor requirements. HDACs regulate the conformation and activity of chromatin through their deacetylation of the histone proteins H2A, H2B, H3 and H4. The interaction between positively charged histones and negatively charged DNA is thus controlled. HDACs mostly act as part of large multiprotein complexes that function as transcriptional co-repressors. Euchromatic regions with active transcription are associated with low HDAC activity, whereas condensed, transcriptionally inactive heterochromatic regions have high HDAC activity.

**RNA interference**

Small, non-coding RNAs of approximately 20-30 nucleotides are also involved in controlling gene activity. They bind to target RNAs in a sequence-specific manner, as their sequences are complementary to portions of the transcripts they regulate. The main classes of small RNA are short interfering RNAs (siRNA), microRNAs (miRNA) and PIWI-interacting RNAs (piRNAs) (Jinek & Doudna, 2009). Non-coding RNAs such as siRNAs and miRNAs are generated from double-stranded RNA (dsRNA) precursors and their generation depends on the ribonuclease (RNase) Dicer. The siRNAs have a double-stranded structure and the miRNAs a single-stranded. At least 30% of human genes are thought to be regulated by miRNA (Lewis *et al*, 2005). Little is known about piRNAs, but they are generated from single-stranded RNA and have been shown to silence transposons in germ cells.

## CANCER GENETICS AND EPIGENETICS

Cancer is one of the most common causes of death. Cancer is the result of a series of somatic mutations and occasionally also an inherited predisposition. Cancer is not one disease; there are more than a hundred different types of cancer; even within a specific cancer type, the cause and pathology can be very different and a cure is therefore not easy to find. Lifestyle changes can lower the incidence of specific types of cancer and cancer-screening programs, which allow for earlier detection, have improved survival for others.

### Genes involved in cancer

Two major groups of genes, oncogenes and tumor suppressor genes (TSGs), are frequently altered in cancer. Genes whose normal function promotes cell proliferation are called proto-oncogenes. The gain of function mutations in these genes creates forms that are excessively or inappropriately active, called oncogenes. The products translated from TSGs normally inhibit events that lead to tumor formation.

TSGs can be divided into gatekeepers and caretakers (Kinzler & Vogelstein, 1997). Gatekeepers are genes that directly regulate the growth of tumors by inhibiting growth or promoting deaths. There are only one or a few gatekeepers in each cell type and the inactivation of a gatekeeper leads to a very specific tissue distribution of cancer. Both the maternal and the paternal copy of the gene need to be altered for a tumor to develop and the inactivation of the gatekeepers is therefore rate limiting for the initiation of a tumor. The inactivation of a caretaker gene does not promote tumor initiation directly but leads to increased genetic instability which in turn leads to mutations of other genes.

### The two-hit hypothesis

Knudson's two-hit hypothesis from 1971 states that two hits are needed for a TSG to be inactivated, exemplified by retinoblastoma, a tumor in the eye (Knudson, 1971). The first hit can be inherited or somatic; the second hit is always somatic. The two-hit model developed by Knudson has more recently been modified to include the new findings relating to silencing by epigenetic means; the first hit often involves a point mutation or DNA hypermethylation, while the second hit involves a point mutation, DNA hypermethylation or deletion (Jones & Laird, 1999), see Figure 6.

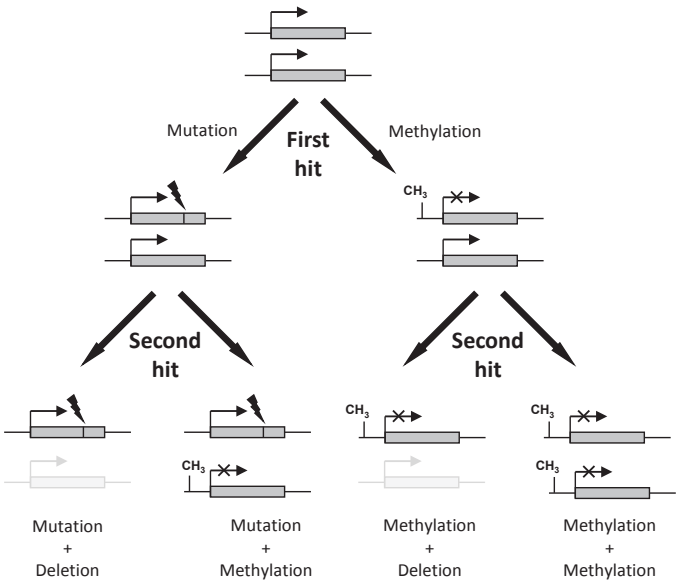


**Figure 6.** Common ways for a TSG to be inactivated. The first hit is often a mutation that affects the function of the gene or DNA hypermethylation which silences the gene. The second hit commonly constitutes the deletion of the second allele or DNA hypermethylation which silences this allele.

## Epigenetic alterations in cancer

Epigenetic alterations in cancer are characterized by genome-wide alterations in DNA methylation and the hypoacetylation of chromatin, as well as gene-specific hypo- and hypermethylation. Genome-wide DNA hypomethylation leads to chromosomal instability and gene-specific oncogene activation, as in the case of R-ras in gastric cancer and cyclin D2 and maspin in pancreatic cancer (Akiyama *et al*, 2003; Nishigaki *et al*, 2005; Oshimo *et al*, 2003). Some genes are aberrantly methylated in specific forms of tumors, while others are commonly affected in many different tumor types. DNA hypermethylation and chromatin hypoacetylation are associated with the silencing of TSGs. Many TSGs have been reported to be silenced by DNA hypermethylation in cancer, including the *RB1* gene in retinoblastoma (Sakai *et al*, 1991), *p16/CDKN2A* in melanoma (Gonzalez-Zulueta *et al*, 1995) and *VHL* in renal-cell carcinoma (Herman *et al*, 1994).

The overproduction of specific histone methyltransferases that catalyze the methylation of lysine 4 or 27 on histone H3 (H3-K4 and H3-K27) is frequently found in neoplasia (Hess, 2004). Moreover, at histone H4, the loss of acetylation at lysine 16 (H4-K16) and the trimethylation of lysine 20 (H4-K20) are commonly seen in cancer (Fraga *et al*, 2005).

miRNA can also be targeted in cancer. The expression profile of miRNA differs between normal tissues and tumors and also between different tumor types (Lu *et al*, 2005). The CpG island hypermethylation of miRNA is responsible for the silencing of a subset of miRNAs (Saito & Jones, 2006).

Loss of imprinting (LOI) refers to the activation of a normally silenced allele or the silencing of the normally active allele of an imprinted gene. Embryos derived from only the maternal or paternal genome frequently form tumors, which underlines the importance of gene expression from the correct parental allele. For example, the LOI of the insulin-like growth factor 2 gene (*IGF2*) accounts for half of all cases of Wilms' tumor in children (Ravenel *et al*, 2001). Other examples of genes with LOI in cancer are *DIRAS3* in breast cancer, *CDKN1C* in pancreatic cancer and *TP73* in gastric cancer (Kang *et al*, 2000; Sato *et al*, 2005; Yu *et al*, 1999).

**Genetic and epigenetic models of cancer**

Cancer has long been thought to arise from a series of genetic alterations in a single cell which is responsible for continued clonal selection and the heterogeneity of the tumor (*the clonal genetic model of cancer*). In this model, epigenetic changes are regarded as alternatives to gene mutations and chromosomal aberrations in disrupting gene expression. The fact that epigenetic changes are found very early in tumorigenesis and even in normal tissues before the tumors occur made Feinberg *et al* (2006) propose *the epigenetic progenitor model*. According to this model, cancer occurs in three steps; (I) an epigenetic disruption of stem/progenitor cells, (II) an initiating mutation in a gatekeeper gene, tumor suppressor gene or an oncogene and (III) genetic and epigenetic plasticity. The first step leads to a polyclonal precursor population of neoplasia-ready cells within a specific organ or system. This step is a key determinant of cancer risk, but also in tumor progression and heterogeneity late in the course of tumor development. The second step involves an initiating mutation in the same population of epigenetically altered progenitor cells, the step that was previously considered to be the first step of a neoplasm. The initiation mutation can be genetic or epigenetic and affects different genes depending on tumor type. The third step leads to increased genetic and epigenetic instability and an enhanced ability to evolve phenotypically.

**Epigenetic therapy**

More than 40 years ago, the cytidine ribose nucleoside analog 5-azacytidine was discovered as a potent agent for cancer treatment (Sorm *et al*, 1964). It was also subsequently shown to be an inhibitor of DNMT. In the cell, 5-azacytidine is modified to deoxyribonucleoside triphosphate and is incorporated into DNA where it is methylated by DNMT. DNMT is unable to dissociate from the methylated base and the methyltransferase activity in the cell thereby rapidly diminishes during replication. 5-aza-2´-deoxycytidine (decitabine) and zebularine are other examples of nucleoside analogs (Zhou *et al*, 2002). 5-azacytidine and 5-aza-2´-deoxycytidine have both been approved by the FDA for the treatment of myelodysplastic syndrome. However, these compounds rapidly degrade in the body. Zebularine is another demethylating agent which is more stable and can be administered orally (Marquez *et al*, 2005). The fact that the nucleoside analogs need to be incorporated into DNA during DNA synthesis limits the activity of the drugs in slowly proliferating cells such as cancer stem cells. Non-nucleoside DNMT inhibitors are therefore under development, also with a second aim of avoiding the toxicity associated with the incorporation of nucleoside analogs into DNA.

HDAC inhibitors affect histone acetylation but also facilitate replication-independent DNA demethylation and can therefore be utilized to induce demethylation in post-mitotic non-dividing tissues, such as brain and heart, and in slowly proliferating cells (Cervoni & Szyf, 2001). The HDAC inhibitor SAHA (Vorinostat) has been successfully utilized in clinical trials of patients with cutaneous T

cell lymphoma (Duvic *et al*, 2007). This and other HDAC inhibitors are currently being used in clinical trials for many different cancer types. When using epigenetic therapy, different approaches and strategies may be used in the future (Graham *et al*, 2009). As single agents, they can be used to activate a particular TSG that is fundamental to that specific cancer; as a chemosensitizer to be given prior to chemotherapy in order to make treatment more effective; as maintenance treatment after chemotherapy to prevent relapse; or as prophylaxis for patients running a high risk of developing cancer.

## NEUROBLASTOMA

### Epidemiology

Neuroblastoma is the most common extracranial tumor of childhood. The prevalence is about 1 in 7,000 live births, with 15-20 new diagnosed cases a year in Sweden. The median age at diagnosis is about 18 months, with approximately 40% of cases diagnosed before the age of one and nearly all by the age of ten (Brodeur, 2003). It is an embryonal tumor of the postganglionic sympathetic nervous system (SNS). Most NB tumors are composed of neuroblasts, undifferentiated sympathetic nerve cells arising from the neural crest. Primary tumors are located in areas of the peripheral SNS; about half of all NBs originate from the adrenal medulla and the rest occur in thoracic or abdominal paraspinal sympathetic ganglia or in pelvic ganglia. Metastases often spread to regional lymph nodes, bone and bone marrow. NB displays a high degree of heterogeneity, including a milder or a benign tumor, lethal tumor progression despite intensive therapy and the unusual ability to regress spontaneously, the latter occurring particularly in infants.

### Symptoms and therapy

The symptoms of neuroblastoma can vary widely, depending on the size and location of the original tumor, the extent of spread to other parts of the body and whether or not the tumor cells secrete hormones. An abdominal mass, diarrhea, fever, high blood pressure and pain are some of the symptoms that occur among patients, but there are also patients with no symptoms at all.

The treatment used for neuroblastoma includes surgery, chemotherapy, radiotherapy and biotherapy. In some cases of localized disease, only observation is used to monitor the tumor.

### Germline genetic alterations

A small subset of neuroblastoma cases is inherited in an autosomal dominant manner (Knudson & Strong, 1972; Kushner *et al*, 1986). A family history of NB is found in about 1-2% of cases (Friedman *et al*, 2005). Familial cases are diagnosed at an earlier age compared with sporadic cases and often have several primary tumors. NB can occur with other disorders related to the abnormal development of tissues derived from the neural crest, including Hirschsprung's disease and central congenital hypoventilation syndrome. In this subset of familial cases, mutations in the gene *PHOX2B* have been found (Bourdeaut *et al*, 2005; Krona *et al*, 2008; Mosse *et al*, 2004; Trochet *et al*, 2004). Recently, the anaplastic lymphoma kinase gene (*ALK*) has been identified as a major familial predisposition gene (Janoueix-Lerosey *et al*, 2008; Mosse *et al*, 2008), see below.

### Prognostic factors

The likelihood of cure varies widely, according to age at diagnosis, extent of disease and tumor biology, with the stage of the tumor as the most important prognostic factor. Children less than one year of age generally have a much better prognosis than children diagnosed above this age with equivalent stages (Breslow & McCann, 1971).

NB tumors from children with a favorable outcome are likely to have near-triploid karyotypes with few segmental rearrangements, whereas aggressive tumors often have near-diploid karyotypes and chromosomal rearrangements.

*Expression of neutrophin receptors*

The tyrosine kinase receptors TrkA, B and C play an essential role in normal neural development. In neuroblastoma, the high expression of TrkA is an indicator of favorable outcome, possibly as a result of mediating apoptosis or differentiation (Kogner *et al*, 1993; Nakagawara *et al*, 1992; Suzuki *et al*, 1993). TrkC is also expressed in low-stage neuroblastomas without *MYCN* amplification (Ryden *et al*, 1996; Yamashiro *et al*, 1996). The expression of full-length TrkB, on the other hand, is associated with *MYCN* amplification and advanced disease (Nakagawara *et al*, 1994). Low-stage tumors have no expression of TrkB or express a truncated form.

*Tumor histology*

Most neuroblastomas are undifferentiated tumors, consisting of small, round cells with little or no neural differentiation. The classification schedule devised by Shimada *et al* (1984) relates the histopathological features of a tumor to clinical behavior. The degree of neuroblast differentiation, Schwannian stroma content, nuclear pathology and age at diagnosis are used to classify NB into favorable or unfavorable tumors.

*Risk stratification*

The International Neuroblastoma Staging System (INSS) was developed in 1986 (and revised in 1993) to facilitate the comparison of clinical trials worldwide, see Table 1 (Brodeur *et al*, 1993; Brodeur *et al*, 1988). The INSS uses clinical, radiographic and surgical assessments of children with neuroblastoma.

**Table 1.** International Neuroblastoma Staging System

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

To develop a uniform approach to pretreatment risk stratification, the International Neuroblastoma Risk Group Staging System (INRGSS) has recently been established (Monclair *et al*, 2009). It is based on tumor imaging rather than the extent of surgical resection, Table 2. The International Neuroblastoma Risk Group (INRG) classification system includes INRGSS, age, histology, grade of tumor differentiation, *MYCN* status, presence/absence of 11q deletion and tumor cell ploidy to classify NB tumors.

**Table 2**: INRG consensus pretreatment classification schema.

| INRG stage | INRGSS Description | Age (months) | Histologic category | Grade of tumor differentiation | *MYCN* | 11q-del | Ploidy | Pretreatment risk group |
|---|---|---|---|---|---|---|---|---|
| L1/L2 | | | GN maturing; GNB intermixed | | | | | A Very low |
| L1 | Localized tumor not involving vital structures as defined by the list of image-defined risk factors[1] and confined to one body compartment | | Any, except GN maturing or GNB intermixed | | NA | | | B Very low |
| | | | | | Amp | | | K High |
| L2 | Locoregional tumor with presence of one or more image-defined risk factors | <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 | | |
| | | | | Poorly differentiated or undifferentiated | NA | | | H Intermediate |
| | | | | | Amp | | | N High |
| M | Distant metastatic disease (except stage MS) | <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 | Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow | <18 | | | NA | No | | C Very low |
| | | | | | | Yes | | Q High |
| | | | | | Amp | | | R High |

GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified

[1]For image-defined risk factors see Monclair *et al* (2009)

Adapted from Cohn *et al* (2009) and Monclair *et al* (2009).

**Chromosomal abnormalities**

*1p deletion*
The deletion of parts of chromosome arm 1p, first reported by Brodeur *et al* (1977), is found in 20-35% of all NB (Bauer *et al*, 2001; Carén *et al*, 2008b; Cohn *et al*, 2009; Maris *et al*, 2001; Martinsson *et al*, 1995). This aberration is associated with tumors which also have amplification of the *MYCN* proto-oncogene and is found in approximately 70% of aggressive NBs. The regions of deletion are often large and generally contain the terminal of 1p. Many research groups have attempted to identify the shortest region of overlap (SRO) of deletions in this region. The identified regions are not entirely consistent and several tumor suppressor genes are therefore believed to be located in chromosome 1. The chromatin remodeling family member *CHD5*, located in chromosome region 1p36.31, has been reported to act as a TSG in NB (Fujita *et al*, 2008). The gene is mostly expressed in the nervous system and was shown to have low expression in NB cell lines and tumors with 1p deletion. Functional assays further proved TSG function of this gene in NB. Another gene in chromosome region 1p36, *KIF1B*, was recently reported to function as a haploinsufficiency TSG in NB (Munirajan *et al*, 2008; Schlisio *et al*, 2008).

*Chromosome arm 2p*
The amplification of chromosome region 2p24 is found in 15-30% of NB tumors (Carén *et al*, 2008b; Cohn *et al*, 2009; Schwab *et al*, 1983). The amplified region often contains many genes of which *MYCN* is thought to be the target of the gene amplification. Amplified *MYCN* is localized in double minutes (DMs) or homogeneously staining regions (HSRs). The amplification of *MYCN* is associated with advanced disease stage (Brodeur *et al*, 1984). *MYCN* encodes for a transcription factor that is normally expressed during embryonic development.

Recently, the *ALK* gene has been identified as a major familial predisposition gene targeted by DNA mutations and gene amplifications (Janoueix-Lerosey *et al*, 2008; Mosse *et al*, 2008). *ALK* aberrations are also detected in sporadic cases of neuroblastoma (Carén *et al*, 2008a; Chen *et al*, 2008; George *et al*, 2008; Janoueix-Lerosey *et al*, 2008; Mosse *et al*, 2008). *ALK* is situated in chromosome region 2p23.2, often present in the 2p gain region found in 15-25% of primary NB. Mutation in the tyrosine kinase domain of *ALK* is found in 6-12% of sporadic NB cases and *ALK* gene amplification in 3-5%. The *ALK* gene has been shown to be involved in several chromosomal translocations or inversions contributing to oncogenesis. Fusion proteins involving ALK and other partner proteins have been identified in anaplastic large cell lymphoma, diffuse large B-cell lymphomas and inflammatory myofibroblastic tumors (Gascoyne *et al*, 2003; Morris *et al*, 1994; Pulford *et al*, 2004; Shiota *et al*, 1994). *ALK* is therefore an attractive target for novel therapeutic strategies in NB, since kinase inhibitors are already under development for specific targeted cancer therapy (Li & Morris, 2008).

*11q loss*
The deletion of chromosomal material on the long arm of chromosome 11 is found in 20-35% of NB tumors (Carén *et al*, 2008b; Cohn *et al*, 2009; Guo *et al*, 1999; Srivatsan *et al*, 1993). 11q deletion is mostly found in advanced stage tumors without *MYCN* amplification (Carén *et al*, 2008b; Guo *et al*, 1999). One proposed candidate TSG in this region is *CADM1*, which encodes a cellular adhesion molecule and plays a role in the synaptic formation of neural cells (Michels *et al*, 2008).

*17q gain*
The gain of parts of the long arm of chromosome 17 is the most frequent genetic abnormality in NB tumors, detected in about 50% of tumors (Abel *et al*, 1999; Carén *et al*, 2008b; Caron, 1995; Cohn *et al*, 2009; Gilbert *et al*, 1984). The breakpoint on 17q varies but always involves the terminal of 17q. It is hypothesized that a dosage effect of one or several genes in this region provides a selective advantage (Schleiermacher *et al*, 2004). Proposed candidate genes include *BIRC5*, *NME1* and *PPM1D* (Godfried *et al*, 2002; Islam *et al*, 2000; Saito-Ohara *et al*, 2003). Unbalanced gain of 17q frequently occurs as an unbalanced translocation between chromosome 1 and 17, resulting in 1p deletion and 17q gain (Savelyeva *et al*, 1994).

*Other chromosomal regions targeted in NB*
The deletion of chromosome arm 14q has been detected in about 20% of NB, particularly in advanced stages, and the consensus region has been defined as 14q23-32 (Srivatsan *et al*, 1993; Suzuki *et al*, 1989; Thompson *et al*, 2001). The loss of heterozygosity of chromosome arm 3p has been identified by our group (Ejeskär *et al*, 1998; Hallstensson *et al*, 1997) and is present in approximately 15% of NB. It has subsequently been suggested that the chromosomal region defined as 3p22 contains tumor suppressor genes, since this region was found to be homozygously deleted in a NB cell line (Mosse *et al*, 2005).

*Genome-wide association studies*
Genome-wide association studies (GWAS) have identified chromosome 6p22 as a susceptibility locus for the development of NB. A significant association was found between aggressive NBs and SNPs or variants in this region (Maris et al, 2008). A second locus, at 2q35, was subsequently reported in the *BARD1* gene (Capasso et al, 2009). The BARD1 gene product is essential for the tumor suppressive activity of *BRCA1*. A third susceptibility locus involves chromosome region 1q21.1 and the gene *NBPF23* (Diskin et al, 2009).

**Epigenetic regulation**

*DNA methylation*
Most analyses of DNA methylation in NB have been performed on single genes and several genes in various cellular pathways (apoptosis, cell cycle, differentiation, invasion and metastasis) have been identified as methylated. Recently, genome-wide analyses of NB have also been reported.

Caspase-8 is a key enzyme at the top of the apoptotic cascade. The gene that codes for this protein, *CASP8*, located in chromosome region 2q33, was one of the first genes to be reported as methylated in NB (Teitz *et al*, 2000). Methylation of this gene was strongly correlated with *MYCN* amplification; in 63% of NBs with *MYCN* amplification, *CASP8* was completely methylated, while fewer than 4% of NBs without *MYCN* amplification displayed the same methylation pattern. The overall methylation frequency of *CASP8* has been reported to range between 38% (Michalowski *et al*, 2007) and 56% (Hoebeeck *et al*, 2009). *CASP8* methylation has also been reported to be significantly associated with poor event-free survival (Hoebeeck *et al*, 2009).

The hypermethylation of the promoter region of *RASSF1A* in NB was reported in 2001 (Astuti *et al*, 2001) with a frequency of 55%. The *RASSF1A* gene is a tumor suppressor gene located in chromosome region 3p21.3. This region is subjected to deletions in different tumors, including NB (Ejeskär *et al*, 1998). It has also been shown that the promoter region methylation of *RASSF1A* is

associated with the loss of gene expression in tumor cell lines and that the expression can be restored with the demethylase inhibitor 5-aza-2-deoxycytidine (5-aza-dC) (Agathanggelou *et al*, 2001). In subsequent studies, the frequency of methylation in NB has been reported to be up to 93% (Hoebeeck *et al*, 2009; Michalowski *et al*, 2007). The hypermethylation of *RASSF1A* in pretreatment serum has recently been reported as a prognostic marker in NB (Misawa *et al*, 2009).

Another gene in the 3p21 region that has been reported to be silenced through methylation in NB is *ZMYND10* (also known as *BLU*), which is methylated in 15-41% of NBs (Agathanggelou *et al*, 2003; Hoebeeck *et al*, 2009; Michalowski *et al*, 2007). The methylation of the promoter region in *ZMYND10* is correlated with the down-regulation of the mRNA in NB cell lines, which can be reversed by 5-aza-dC treatment. The exogenous expression of Z*MYND10* in a NB cell line resulted in reduced colony formation efficiency *in vitro*, supporting its role as a tumor suppressor gene in NB (Agathanggelou *et al*, 2003).

*EMP3* is a myelin-related gene involved in cell proliferation and cell-cell interaction. This gene is located in chromosome region 19q13, a region that is heterozygously deleted in aggressive NBs, especially in local-regional recurrent cases (Mora *et al*, 2001). *EMP3* has been identified as being transcriptionally silenced by methylation in 24% of NB tumors (Alaminos *et al*, 2005). Expression can be restored with demethylating agents and colony formation density and tumor growth in nude mouse xenograft models support the tumor suppressor function in NB.

In NB, the two antiapoptotic decoy receptor genes *TNFRSF10C* and *TNFRSF10D* (also known as *DcR1* and *DcR2*) were found to be methylated in 21% and 25% respectively (van Noesel *et al*, 2002). Methylated samples lacked expression and the expression in cell lines could be restored with 5-aza-dC. The hypermethylation of *DcR2* in serum has recently been reported as an indicator of prognosis and therapeutic efficacy in patients without *MYCN* amplification (Yagyu *et al*, 2008).

Examples of other genes reported as being methylated in NB are *CD44*, 11p13, (Hoebeeck *et al*, 2009; Yan *et al*, 2003), *PTEN*, 10q23, (Hoebeeck *et al*, 2009), *TIMP3*, 22q12, (Michalowski *et al*, 2007), *SFN*, 1p36.11, (Banelli *et al*, 2005), *SEMA3B*, 3p21, (Nair *et al*, 2007) and *THBS1*, 15q15 (Gonzalez-Gomez *et al*, 2003).

The study by Alaminos *et al* (2004) was one of the first to demonstrate that the clustering of NB tumors based on the methylation profile of ten genes could divide NB into clinical risk groups. Since then, genome-wide analysis of DNA methylation has revealed a DNA methylator phenotype in NB with poor prognosis, characterized by the methylation of a set of multiple CpG islands (Abe *et al*, 2005).

*miRNA expression*

The expression profiles of specific miRNAs have been reported to correlate with specific prognostic subgroups of NB (Chen & Stallings, 2007). Most of these miRNAs are down-regulated in *MYCN*-amplified tumors. Specific miRNAs have also been implicated in NB. For example, *miR-34a*, located in the chromosomal region 1p36.23, is expressed at lower levels in unfavorable primary NB tumors and cell lines relative to normal adrenal tissue (Welch *et al*, 2007). The reintroduction of this miRNA into NB cell lines was also shown to cause a dramatic reduction in cell proliferation. *miR-34a* is a target of p53 (Chang *et al*, 2007) and *MYCN* has been reported to be a direct target of *miR-34a* (Wei *et al*, 2008). *miR-17-92* family members have also been implicated in the pathobiology of NB (Schulte *et al*,

2008). In contrast to *miR-34a*, which acts as a tumor suppressor, the *miR-17-92* family members have oncogenic functions.

*The epigenetic machinery*

There are no comprehensive reports on histone modification patterns in neuroblastoma. The expression profile of HDACs has, however, been documented (Oehme *et al*, 2009). The high expression of HDAC8 is associated with high-stage NB, whereas low expression is associated with low-stage NB. HDAC8 expression is also correlated with well-known clinical and molecular risk factors of NB. An inhibitor of HDAC8 has also been shown to induce differentiation of NB cells. As a result, there is hope that selective HDAC inhibitors could be beneficial in the treatment of NB in the future.

## OBJECTIVES

The overall aim of this thesis was to identify genes that are involved in the development and/or progression of neuroblastoma and try to find new targets that can be used in patient stratification.

**Specific aims**

*Paper I*

- To investigate the expression of candidate tumor suppressor genes located in a homozygously deleted region of our reported SRO of deletions at 1p36.22
- To explore DNA methylation as a possible causal mechanism of the down-regulation of gene transcripts

*Paper II*

- To perform an epigenetic screening of 30 genes in chromosome region 1p36 in order to pinpoint candidate tumor suppressor genes
- To analyze the chosen genes in greater detail using bisulfite sequencing, expression analysis of primary NB tumors and mutation screening

*Paper III*

- To identify novel candidate genes epigenetically silenced in neuroblastoma tumors using genome-wide, array-based approaches
- To explore whether the candidate genes can be utilized for patient stratification

*Paper IV*

- To characterize chromosomal aberrations, define breakpoints and SRO of deletions in the neuroblastoma tumor material using a genome-wide approach

*Paper V*

- To analyze chromosomal aberrations defined by high-resolution SNP array, and relate them to clinical features of neuroblastoma
- To explore the features of high-risk neuroblastoma subtypes in detail

# MATERIALS AND METHODS

## TUMORS, CELL LINES AND CONTROL MATERIAL

In this thesis, we have used primary NB tumors from patients from the Scandinavian countries but with the emphasis on Swedish cases. In addition, we have used nine NB cell lines and control tissues from blood lymphocytes and normal adrenal tissue.

The tumor cell content of the samples was histologically assessed in tumor tissue adjacent to that used for DNA or RNA extraction. Genomic DNA was extracted from NB cell lines or from fresh NB tumor tissue or fresh frozen (-70°C) with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany), according to the protocol provided by the supplier, or was phenol extracted using phase lock gel (Eppendorf AG, Hamburg, Germany), according to standard procedure. Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen) or using Totally RNA (Ambion, Austin, TX). Total RNA was reverse transcribed to cDNA using Superscript II (Amersham, Buckinghamshire, UK) and random hexamer primers, all according to the protocol of the supplier. The cDNA samples were quality tested by amplifying the *GUSB* (b-glucuronidase) gene.

*Paper I*

- Tumor DNA and cDNA from 10 NB patients
- DNA and cDNA from seven NB cell lines
- Blood lymphocytes from 8 healthy individuals

*Paper II*

- A total of 66 NB tumors were used:
  - Tumor DNA from 46 NB patients
  - Tumor cDNA from 35 NB patients (corresponding cDNA and DNA from 15 tumors)
- Cultures of nine NB cell lines (IMR-32, SK-N-AS, SK-N-BE(2), SK-N-DZ, SK-N-F1, SK-N-SH, Kelly, NB69 and SH-SY5Y)
- Blood lymphocytes from 120 healthy individuals

*Paper III*

- Cultures of nine NB cell lines
- Tumor DNA from 60 NB patients
- Tumor cDNA from 25 of the same patients
- One adrenal sample
- Blood lymphocytes from ten healthy individuals

*Paper IV*

- Tumor DNA from 92 NB patients
- DNA from four NB cell lines (IMR-32, SK-N-AS, Kelly and NB69)

*Paper V*

- Tumor DNA from 165 NB patients

## METHODS

### Polymerase chain reaction (PCR)

PCR has revolutionized molecular genetics since its introduction in the mid-1980s (Mullis *et al*, 1986). A PCR run consists of a series of cycles comprising three steps; (I) the denaturation of the template, (II) primer annealing and (III) extension/DNA synthesis, see Figure 7. DNA is elongated from the bound primers in the presence of heat-stable DNA polymerase and deoxynucleoside triphosphates (dNTPs). A PCR run can be divided into three phases. During the first phase, the exponential phase, the PCR product is doubled at every cycle (in the event of 100% PCR efficiency). The next phase is the linear phase in which the reaction slows down because the reaction components are consumed and the product starts to degrade. In the last phase, the plateau phase, the reaction has stopped and no more products are made. In traditional PCR, agarose gels are used for the detection of PCR products in the final phase. This is often called end-point detection.

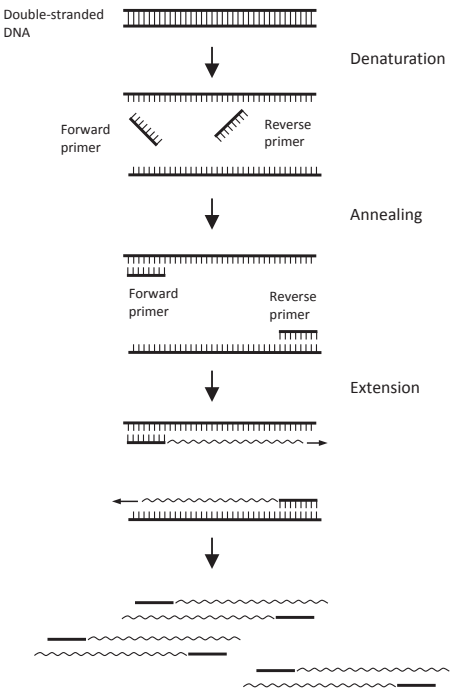


**Figure 7.** Schematic drawing of PCR amplification.

"Hot Start" Taq DNA polymerase for the PCR amplification of DNA has mainly been used in this thesis. These types of Taq polymerase utilize different techniques to keep the polymerase inactivated until the initial heat activation step, which results in more specific, selective PCR amplification.

## Reverse transcriptase PCR (RT-PCR)

RT-PCR is a technique for measuring the amount of mRNA transcripts. The RNA strand is first reverse transcribed into complementary DNA (cDNA), using the enzyme reverse transcriptase, followed by the amplification of the resulting cDNA using polymerase chain reaction with gene-specific primers.

*Real-time RT-PCR*
cDNA is used as the template in the quantitative real-time RT-PCR. In this thesis, TaqMan chemistry (Applied Biosystems) has been used and the PCR reactions have been analyzed using the ABI PRISM® 7900HT Sequence Detection System. Fluorescent probes are used to monitor the amount of PCR product that is produced in each cycle of the PCR and the data are measured during the exponential phase of the PCR reaction. Real-time RT-PCR was used in *Papers I*, *II* and *III*.

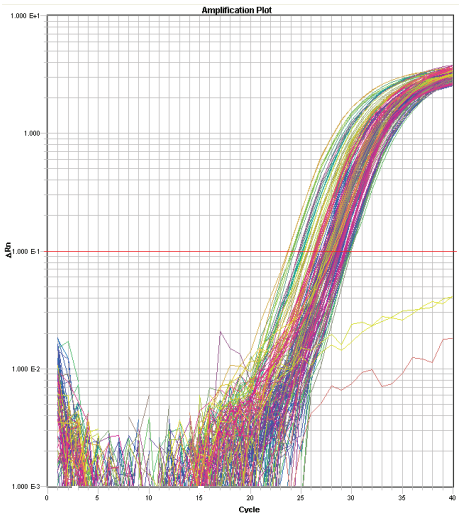


**Figure 8.** Real-time RT-PCR amplification plot from a TaqMan experiment. Fluorescence emission is plotted against the number of PCR cycles. The threshold (red line) is set in the exponential phase of the amplification.

## DNA sequencing/mutation screening

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The most common method of sequencing, originally developed by Sanger *et al* (1977), is called the dideoxy method or Sanger method. In this method, fluorescently labeled nucleotides that lack the -OH at the 3′ carbon atom (dideoxynucleotides; ddNTP) are added to a purified PCR product, together with normal deoxynucleotides, DNA polymerase I and one primer. The ddNTPs are randomly incorporated into the growing DNA strand which stops the chain elongation because of the lack of 3′ -OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the chain termination method. The products from the sequencing reaction are then precipitated and resuspended in a denaturating formamide solution to keep them single standed and separated on an automated sequencer where laser excitation of the terminal fluorescent dyes makes it possible for a

software program to present the bases as peaks in an electropherogram. In this thesis, Big Dye Terminator ddNTP chemistry from Applied Biosystems was used to sequence PCR products from genomic DNA or genomic DNA treated with bisulfite (bisulfite sequencing; see below). In the first paper, PCR products were purified with ExoSAP-IT (USB) and sequencing reactions were precipitated with ethanol and salt, while, in the other papers (*Papers II* and *III*), PCR purification and sequencing precipitation were conducted with AMPure magnetic beads and CleanSeq magnetic beads, both Agencourt (Agencourt Bioscience Corporation), using the Biomek NX pipetting robot (Beckman Coulter).

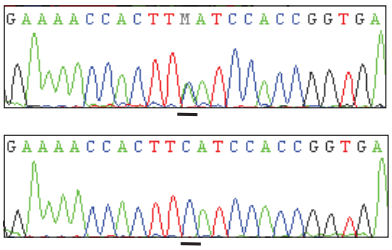


**Figure 9.** Example of DNA sequencing electropherogram. Mutation (F1245C) in the *ALK* gene in a neuroblastoma tumor, upper sequence, and reference DNA, bottom sequence, (Carén *et al*, 2008a).

## Multiplex ligation-dependent probe amplification (MLPA)

MLPA is a multiplex PCR method which is used for the relative quantification of DNA (or RNA) (Schouten *et al*, 2002). It can be used to detect deletions, duplications and copy number variations. The MLPA reaction comprises 5 steps: (1) the denaturation of DNA and the hybridization of MPLA probes, (2) ligation, (3) PCR reaction, (4) Separation of the amplification products by electrophoresis and (5) data analysis. MLPA was used to verify the homozygous deletions found in the *CDKN2A* gene in *Paper IV*.

## Cell culture

*Pharmacological treatments*

The chemicals 5-aza-2-deoxycytidine (5-aza-dC) and Trichostatin A (TSA) can be used to analyze DNA methylation and histone deacetylation respectively. 5-aza-dC is a cytosine analog that is incorporated during DNA synthesis and binds up all the available DNA methyltransferase in the cell, thereby preventing it methylating DNA. This hypomethylation leads to gene activation. TSA targets the HDAC enzyme group that removes acetyl groups from the histone tails and thereby enables gene transcription. Genes that are up-regulated after treatment with 5-aza-dC and TSA may potentially be methylated. Drug treatments with 5-aza-dC and/or TSA have been used in *Paper II*, analyzed by real-time RT-PCR, and in *Paper III*, analyzed by expression microarrays and real-time RT-PCR.

**Bisulfite modification and PCR amplification**

The most common way to analyze DNA methylation is to use bisulfite modified DNA. Methylated cytosines cannot be detected with conventional DNA sequencing, but, using the bisulfite technique (Frommer *et al*, 1992), methylation can be analyzed with sequencing. Bisulfite deaminates unmethylated cytosines into uracil; methylated cytosines are protected from this conversion. The modified template is then amplified by PCR; methylated cytosines as cytosines and unmethylated cytosines converted to uracils are amplified as thymines.

The template can be analyzed in different ways, with primers without any preference for methylated or unmethylated DNA (bisulfite sequencing PCR; BSP), followed by direct sequencing or cloning and sequencing, or with primers designed to amplify methylated templates in one reaction and unmethylated in another, followed by visualization on agarose gel (methylation-specific PCR; MSP). BSP was used in *Papers I-III* and MSP in *Paper III*.

After bisulfite modification, all Cs that are not methylated are converted into uracil, which is amplified as thymine in the PCR reaction. This leads to long T stretches in the DNA and, at sites with low methylation, only three bases are present. Primers for BSP thus contain 3 bases and therefore need to be fairly long to obtain specificity. These T-rich primers are prone to mismatch hybridization and primer dimer formation with the A-rich reverse primer. It is frequently difficult to design these long primers while avoiding CpG sites. Additionally, long stretches of one base (T) in the product can lead to polymerase slippage and thereby poor amplification. Methylated and unmethylated templates have different melting temperatures ($T_m$), which can lead to PCR bias, where the unmethylated strand is more frequently amplified than the methylated one in a sample with mixed methylation. To reduce PCR bias, hot-start Taq is used and a PCR denaturant (such as glycerol or betaine) can be added to the PCR reaction, as has been done in this thesis. In addition, to control for PCR biases, mixed samples of methylated and unmethylated controls can be analyzed along with the unknown samples, which has also been done in this thesis.

In *Paper III*, tailed PCR primers were used. This produces a universal sequencing primer-binding site (Han *et al*, 2006). After the first round of PCR amplification, the longer primer-binding site provides for higher $T_m$ and thereby specificity. The GC-rich tag also incorporates dCTP and dGTP into the PCR products and thereby produces a more evenly distributed nucleotide stoichiometry in the sequencing reaction.

**Bisulfite sequencing**

BSP is the "golden standard" for methylation analysis. It enables the analysis of a large number of CpG sites per amplicon, in contrast to MSP, for example. Another advantage of BSP is that it also provides information on possible incomplete bisulfite conversion. MSP is used to analyze a couple of CpG sites in a large number of samples. It can also be used when BSP primers cannot be designed because of an overly high frequency of CpG sites in the region of interest. Sequencing PCR products from bisulfite-modified products is more complicated than the standard sequencing of genomic DNA. To obtain optimal results, BigDye Terminator v1.1 and a two-temperature thermal cycling protocol were used in *Paper III*. BigDye Terminator v1.1 was used, because it provides better signal resolution at the beginning of the sequence, while the two-temperature thermal cycling profile (instead of the normal three-temperature profile) was used as it maintains the level of signal strength. Another

problem that we encountered when working on *Paper I* was that the older basecallers from Applied Biosystems "over-normalized" the missing base in samples with low or no methylation. A great deal of time was therefore spent on raw data analysis in *Paper I*. This problem was, however, subsequently reduced (but not eliminated) by the introduction of the KB™ basecaller, used in *Papers II* and *III*.

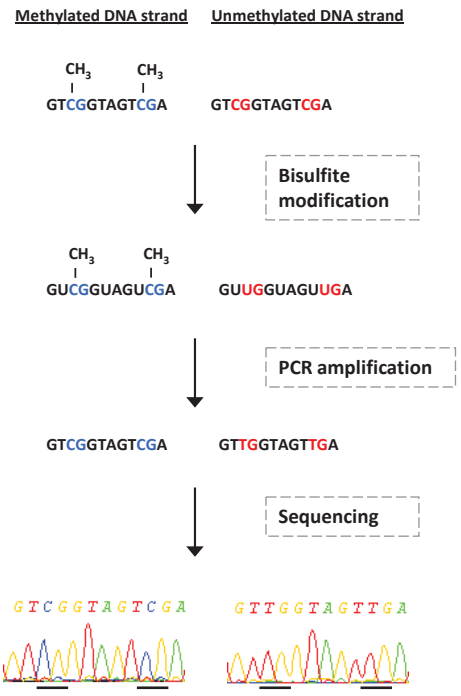


**Figure 10.** Bisulfite modification of DNA followed by PCR amplification and sequencing. Unmethylated cytosines are converted into uracils by the bisulfite treatment, while methylated cytosines are protected. In the PCR reaction, cytosines are amplified as cytosines and uracils as thymines, which can be seen in the sequence chromatogram after the sequencing step. Methylated CpGs are shown in blue and unmethylated CpGs in red. The CpG positions are underlined in the chromatogram.

## Microarrays

*SNP arrays*

SNP arrays were used in *Papers IV* and *V*. They provide both copy number and allele-specific information and have the capacity to detect duplications, amplifications, homozygous and hemizygous deletions and copy-neutral LOH (genomic regions that have a normal gene copy number, even though both gene copies originate from the same parental chromosome, i.e. uniparental disomy). The Mapping 500K Array Set from Affymetrix is comprised of two arrays, each capable of genotyping on average 250,000 SNPs. In this thesis, the array that uses the Nsp I restriction enzyme (approx. 262,000 SNPs) has been used, with an average distance between SNPs of 11 kb.

Total genomic DNA is digested with the restriction enzyme and is ligated to adapters. After ligation, the template is subjected to PCR amplification. A generic primer that recognizes the adapter sequence is used to amplify adapter-ligated DNA fragments. The amplified DNA is then fragmented with DNAse I, labeled with deoxynucleotidyl transferase, and hybridized to the array.

In this thesis, CNAG (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays) software was used to analyze the data (Nannya *et al*, 2005; Yamamoto *et al*, 2007).

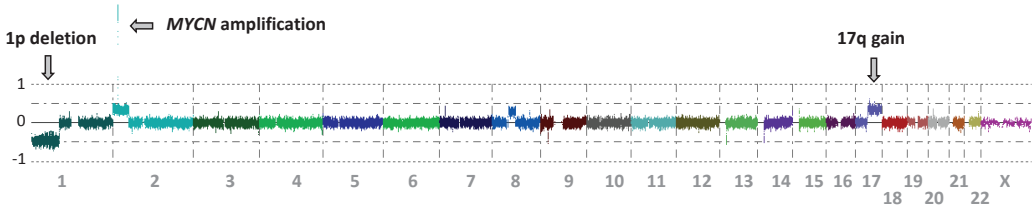


**Figure 11.** Results of a neuroblastoma tumor analyzed with the Affymetrix SNP array, visualized with CNAG software (Carén *et al*, 2008b). Classical features of NB are shown, 1p deletion, *MYCN* amplification and a gain of a part of chromosome arm 17q; indicated by arrows. In this tumor, a 2p gain and a gain of a part of chromosome 8 can also be seen.

*RNA arrays*

RNA expression arrays were used in *Paper III*. RNA extracted from cell treatment experiments was analyzed with the Human-6 v2 Expression BeadChip from Illumina which targets > 48,000 probes for six samples on the same beadchip. Total RNA is reverse transcribed and the first-strand cDNA is used to make the second strand. The purified second-strand cDNA, along with biotin UTPs, is then *in vitro* transcribed into biotinylated cRNA. Purified, labeled cRNA is hybridized to the beadchip before being washed and stained with streptavidin-Cy3. The beadchip is then dried and scanned on the Illumina BeadArray Reader confocal scanner. Expression data generated by BeadStudio software (Illumina) were exported and analyzed using IlluminaGUI (Schultze & Eggle, 2007).

*DNA methylation arrays*

In *Paper III*, the HumanMethylation27 BeadChip from Illumina was used. With this chip, some 28,000 CpG sites from 14,000 genes can be analyzed in twelve samples simultaneously. The probes on the chip are derived from the NCBI database and the array is supplemented with more than 1,000 cancer-related genes and more than 150 well-established cancer genes known to show differential methylation patterns. The BeadChip content also targets the promoter regions of 110 miRNA genes. After bisulfite treatment of the DNA samples, the cytosines in the CpG sites are genotyped as C/T polymorphisms. The fluorescence signals are measured from the BeadChips and the fluorescence data are then analyzed using BeadStudio. This software assigns a score called a “beta value” to each CpG site, which corresponds to the ratio between the fluorescence signal from the methylated allele (C) and the sum of the fluorescent signals of the methylated (C) and unmethylated (T) alleles (Bibikova *et al*, 2006).

## Statistical methods

*Student's two-sided t-test*
Student's two-sided t-test is used to compare two normally distributed populations. This test was used in all papers. In *Papers I*, *II* and *III,* it was used to compare the difference in gene expression between two groups of NB; tumors with favorable and unfavorable biology. For this purpose, the relative gene expression values were first log transformed to make them normally distributed.

*Bonferroni correction*
Each test that is conducted has a chance of being positive, even though there is no real difference (false positive result). When conducting a large number of tests on the same data, the chance of obtaining false positive results is increased. To deal with this, Bonferroni correction can be used. With this method, the p-value for a test is multiplied by the total number of tests that have been conducted. Bonferroni correction was used to adjust for multiple testing in *Paper II*.

*Pearson Product Moment Correlation (Pearson's correlation)*
Pearson's correlation reflects the degree of linear relationship between two variables and is the most common method for measuring correlation. It reflects the degree of linear relationship between two variables and ranges from +1 to -1. A correlation of +1 means that there is a perfect positive linear relationship between variables. This test was used in *Paper III* to analyze the correlation between DNA methylation and gene expression.

*Fisher's exact test*
Fisher's exact test is a test of statistical significance which is used in the analysis of 2x2 association tables where sample sizes are small. This test was used in *Paper IV* to investigate the association of *MYCN* amplification and 1p deletion and *MYCN* amplification and features of 1p deletion.

*Kaplan-Meier survival analysis*
In *Papers III* and *V*, Kaplan-Meier survival analysis was used to measure the percentage of patients living for a certain length of time after diagnosis, divided into different subgroups of NB.

*Regression*
Regression analysis is used to study the relationship between two variables. In linear regression, the dependent variable is a linear function of the independent/predicting variable. Linear regression requires the dependent variables to be quantitative. In the event of binary data, such as the presence or absence of a given condition, logistic regression is used. This method predicts the probability of being affected divided by the probability of not being affected and gives the result as an odds ratio (OR). Logistic regression was used in *Paper III*.

## RESULTS AND DISCUSSION

### Epigenetic (and genetic) analysis of NB tumors and cell lines

***Paper I***

As the distal part of chromosome 1p shows LOH in a large percentage of advanced NB tumors, it has been suggested that the region contains one or more tumor suppressor genes. Our group, as well as others, has attempted to identify the critical region(s) by comparing deletions found in the tumors. We had previously defined the shortest region of overlap (SRO) of deletions in a tumor panel to about 25 cM located between markers D1S80 and D1S244 (Martinsson *et al*, 1997; Martinsson *et al*, 1995). By adding germ cell tumors, an approximately 5 cM combined SRO of deletions was defined by markers D1S508 and D1S244 (Ejeskär *et al*, 2001). As an overlapping homozygous 500 kb deletion of 1p36.2–3 was found in an NB cell line (Ohira *et al*, 2000), this region has been analyzed in more detail. We had previously analyzed the genes in this region for sequence mutations and only a few mutations were found in rare tumors (Abel *et al*, 2002; Abel *et al*, 2004; Ejeskär *et al*, 2000; Krona *et al*, 2004; Krona *et al*, 2003). We therefore hypothesized that mRNA translated from these genes could be down-regulated due to the DNA methylation of the respective promoter CpG islands. See Figure 12 for the genomic organization of the gene region.

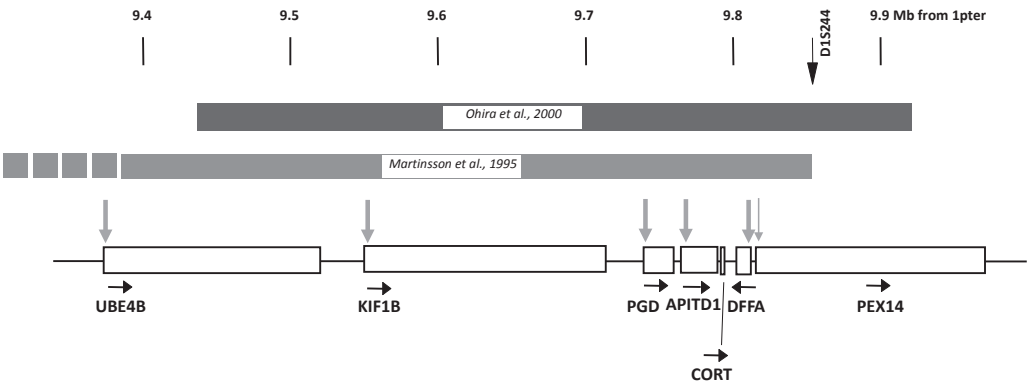


**Figure 12.** Genomic organization of the analyzed gene region. Grey vertical arrows indicate the presence of CpG islands. Figure adapted from Krona *et al* (2004).

Of the seven genes located in this region, *CORT* was without a CpG island and thus was excluded from the analysis. All the other six gene transcripts were down-regulated in high-stage NB tumors as compared to low-stage tumors. Promoter hypermethylation, apart from the CpG island of *APITD1,* was not detected in the genes. However, the methylation in the *APITD1* gene does not appear to be tumor specific, since methylation was also detected in healthy blood controls. The lower expression of these genes in tumors with unfavorable biology compared with those with favorable biology has subsequently been confirmed in other studies (Abel *et al*, 2004; Fransson *et al*, 2007; Oberthuer *et al*, 2007). Following the publication of *Paper I, KIF1B* has been reported as a haploinsufficient tumor

suppressor gene in NB by two groups (Munirajan *et al*, 2008; Schlisio *et al*, 2008). In one of these studies, the promoter region was confirmed as being unmethylated in NB. In summary, the six genes *UBE4B*, *KIF1B*, *PGD*, *APITD1*, *DFFA* and *PEX14* were shown to be down-regulated in high-stage NB tumors, a feature that could not be explained by DNA methylation but instead by a mechanism that is still waiting to be discovered.

***Paper II***

In the following study, we wanted to expand our investigation of the 1p region, more specifically to our combined NB/germ cell SRO of deletions. This region, extending from markers D1S508 and D1S244, bp 7.765.595–11.019.814 (UCSC version May 2004; URL: http://genome.ucsc.edu), was therefore analyzed. Since it is very time consuming to design, optimize and validate bisulfite sequencing assays, we used an alternative strategy in this paper. We treated NB cell lines with the demethylating agent 5-aza-dC or TSA, a histone deacetylase inhibitor, to explore epigenetic mechanisms in the regulation of 30 genes in the 1p36 chromosomal region. The concentrations of drugs and the time of treatment were first optimized by analyzing the expression of a panel of known methylated genes in NB. A dose and time at which they were re-expressed by treatment was chosen. Moreover, a panel of reference genes for the normalization of real-time RT-PCR experiments was tested in order to choose reference genes that were consistently expressed and not affected by the treatments. Genes identified as candidates for epigenetic regulation were studied further using expression analysis and mutation screening of primary tumors. A small number of the analyzed genes showed indications of epigenetic inactivation among which two contained mutations in NB tumors. Bisulfite sequencing was subsequently used to validate methylation status of the identified genes.

The known functions of the four identified genes are interesting in relation to NB. *ERRFI1*, also known as *MIG-6* or *RALT*, is a negative regulator of epidermal growth factor (EGF) signaling which can be induced by stress, growth factors and the protein Ras. *ERRFI1* is described as a tumor suppressor gene and the loss of its activity contributes to the initiation of lung carcinogenesis, as well as other tumor types (Zhang *et al*, 2007; Zhang & Vande Woude, 2007). In our study, we found that *ERRFI1* was up-regulated by TSA treatment but not by 5-aza-dC treatment. Since the gene does not harbor a CpG island, we thus concluded that this gene is most likely not regulated by DNA methylation. The up-regulation after TSA treatment indicated that histone deacetylation could be involved in the regulation, or that the gene is affected by an indirect mechanism or turned on by a stress response during treatment. We did not detect any difference in the expression of this gene in tumors with favorable biology compared with those with unfavorable biology. Resequencing identified two tumors with two different mutations, one of which resulted in an amino-acid change in an evolutionary conserved gene region. The mutations were not detected in any of more than 100 healthy controls.

*RBP7* is a cellular retinoid-binding protein. Retinol is important in embryonic development. *RBP7* is epigenetically silenced by DNA methylation in the promoter region in a high frequency of nasopharyngeal carcinomas, as well as in some cancer cell lines (colon, prostatic and ovarian cancer) (Kwong *et al,* 2005). In our study, *RBP7* was up-regulated after treatment with either 5-aza-dC or TSA. Bisulfite sequencing did not reveal methylation around the transcription start site. The observed up-regulation of this gene by epigenetic treatment in NB is probably due to unspecific drug effects or an indirect mechanism (other genes demethylated by treatment that in turn enhance the transcription of *RBP7*). Illumina DNA methylation arrays later confirmed that the region around the transcription

start site was unmethylated (Carén, unpublished data and *Paper III*). However, a CpG site located 317bp upstream of the TSS was found to be methylated at different frequencies in NB tumors and cell lines. It has recently been reported that methylation in CpG island shores (regions located outside CpG islands with a lower GC content) can have a strong correlation with gene expression (Irizarry *et al*, 2009). This might also be the case for *RBP7* in NB. The methylation frequency at this site was, however, not inversely correlated with gene expression, as would be expected (Carén, unpublished data and *Paper III*). Since Kwong *et al* found that the region surrounding exon 1 was methylated and the mRNA down-regulated in nasopharyngeal carcinomas (Kwong *et al*, 2005), it is likely that the detected methylated site outside the promoter CpG island of *RBP7* by the DNA methylation arrays does not affect the transcription of the gene.

*CASZ1* is a putative homolog to the zinc finger transcription factor Castor, required for CNS neuronal development in Drosophila melanogaster, where it is involved in neuronal cell lineage specification (Mellerick *et al,* 1992; Edenfeld *et al,* 2002). The expression of *CASZ1* is increased when cells of neural origin are induced to differentiate (Liu *et al*, 2006). Four fragments were used to analyze the methylation status of the *CASZ1* gene; one in CpG island 1 surrounding the transcription start site, two in CpG island 2 where the translation begins and one in CpG island 3 (see Figure 13). The fragment in CpG island 1 was unmethylated, as was the second fragment in CpG island 2. The first fragment in CpG island 2 was, however, heavily methylated in the NB cell lines. The primary tumors, as well as blood from healthy donors, were also partially methylated at this fragment, suggesting that the methylation of this region is not restricted to NB.

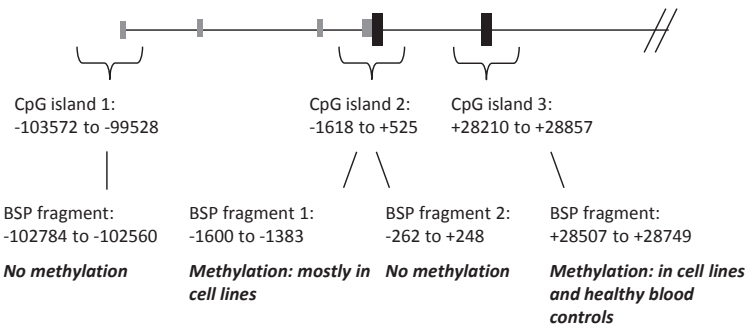


**Figure 13.** Genomic organization of the *CASZ1* gene and results of bisulfite sequencing, figure from Carén *et al* (2007). Grey boxes indicate untranslated exons, while black boxes represent translated exons. No methylation specific to NB could be identified.

Methylation data from the Illumina methylation array confirmed that the region surrounding the TSS was unmethylated (Carén, unpublished data and *Paper III*). The *CASZ1* gene showed lower gene expression in tumors with unfavorable biology compared with those with favorable biology. The *CASZ1* gene has recently been reported to be a tDMR gene, i.e. a gene that is differentially methylated in different tissues (Igarashi *et al*, 2008; Irizarry *et al*, 2009). *CASZ1* is almost completely methylated in testes but has a varying methylation pattern in other tissue types. One could speculate that genes with different methylation patterns in different tissues are strictly regulated by DNA methylation and that this type of genes could therefore easily be aberrantly methylated by DNA

hypermethylation in tumors. Tight regulation of the *CASZ1* gene could be important, in that the gene needes to be expressed at correct times and in the right tissues during development. However, if the gene is aberrantly methylated and not expressed correctly during differentiation of the neural crest cells, this could perhaps contribute to the development of NB. In contrast, if the gene is methylated in blood lymphocytes, for example, this might not have any consequences. The fact that the gene is down-regulated in high-stage NB and the important function this gene appears to perform during development, make *CASZ1* an interesting candidate for further study in the development of neuroblastoma.

*PIK3CD*, encoding the protein p110d, is a catalytic subunit in class IA phosphoinositide 3-kinase (PI3K). Phosphoinositide 3-kinases play an important part in regulating signaling involved in cell cycle progression, cell growth, survival and migration. The PI3 kinases are generally considered to function as oncogenes. In our study, the partial methylation of *PIK3CD* was found around the transcription start site only in the NB cell lines. On the Illumina methylation array, two CpG sites are present for *PIK3CD*, both located in CpG island 1. The first site, located 162bp downstream of the TSS (between the two fragments analyzed in the paper), was unmethylated. The second fragment is located 405 bp upstream of the TSS, close to but upstream of BSP fragment 1 analyzed in the paper. This site was methylated in cell lines and primary tumors and also in the adrenal sample analyzed with the array. The methylation frequencies of this site were not correlating with the expression of the gene (Carén *et al*, 2007 and unpublished data). As for *CASZ1*, the *PIK3CD* gene has subsequently been reported to be differentially methylated in different tissues (Irizarry *et al*, 2009).

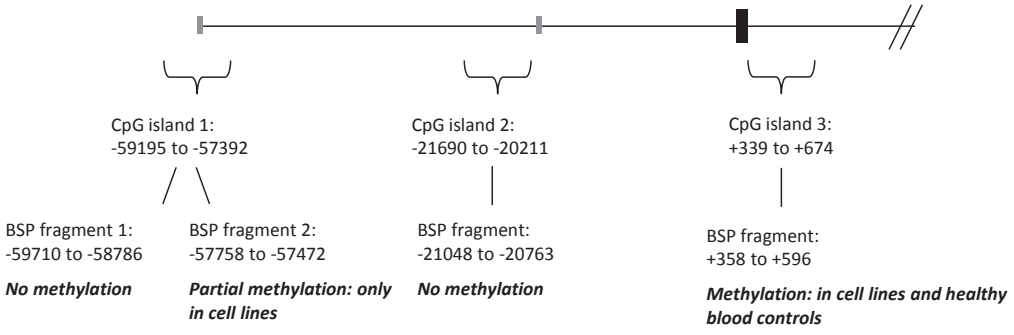


**Figure 14.** Organization and results from methylation analysis of *PIK3CD*, figure from Carén *et al* (2007). Three CpG islands were detected in the *PIK3CD* gene. The CpG islands are located in the proximity of the first three exons; two in the untranslated region and one in the translated region. No methylation specific to NB could be identified.

RNA expression analysis revealed less *PIK3CD* transcripts in aggressive NB, compared with more favorable NB tumors, also significant after Bonferroni correction. This is consistent with a previously published expression profiling study (Janoueix-Lerosey *et al*, 2004). We have recently confirmed that this difference is also valid at the protein level (Fransson *et al, manuscript in preparation*). The down-regulation appears, however, not to be correlated to DNA methylation of the analyzed regions.

The resequencing of *PIK3CD* revealed two patients with missense mutations; a total of five different amino acids were targeted in the two patients. The changes were *de novo* mutations, not present in constitutional DNA from the tumor, or hemizygous for one of the alleles found in the constitutional DNA. The mutations were all located in evolutionary conserved gene regions and could not be detected in any of 200 alleles in healthy controls. The tumors with mutations were found to have low gene expression, indicating that the mutations could affect the expression of the gene.

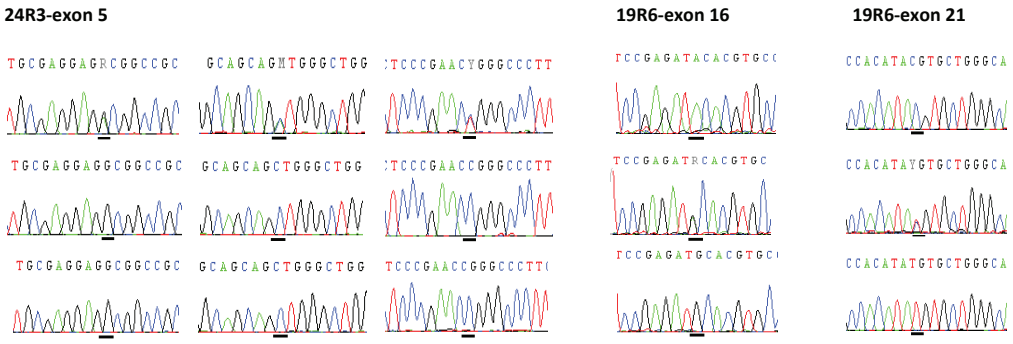


**Figure 15.** Mutations in the *PIK3CD* gene. Bars under each chromatogram indicate the position of the mutation. Lower panels: Healthy control individuals. Central panels: Normal tissue from the respective patient. Upper panel from the left: mutations 448G>A, 469C>A and 562C>T in patient 24R3 giving rise to amino-acid changes from Ala to Thr, Leu to Met and Arg to Trp respectively. In patient 19R6 (in the centre), the 1965G>A mutation results in an amino-acid change from Met to Ile in exon 16 and, in the same patient, in exon 21 (to the right), 2661T>C mutation. Figure from Carén *et al* (2007).

The fact that aggressive NB tumors have a lower expression of *PIK3CD* than low-stage tumors does not indicate that *PIK3CD* would act as an oncogene in NB. Nevertheless, it is possible to speculate that this oncogenic pathway is active in the low-stage tumors but not in aggressive NBs, which can have other pathways leading to the activation of tumorigenesis. Since the genomic structure and genetic profiles of these subgroups of NB are very different, this could be the case. Alternatively, the *PIK3CD* gene could perform tumor suppressive functions in NB, which are lost in the high-stage tumors. Further studies of the *PIK3CD* gene in relation to NB, as well as other tumor types, are ongoing at our laboratory.

***Paper III***

In *Paper III,* we used genome-wide approaches to identify genes silenced due to epigenetic regulation in NB. Four neuroblastoma cell lines were treated with the demethylating agent 5-Aza-dC separately and in conjunction with the histone deacetylase inhibitor TSA. Expression was analyzed using whole-genome expression arrays (Illumina Human-6 v2 Expression BeadChip) to identify genes that were activated by treatment. These data were then combined with data from whole-genome DNA methylation arrays (Illumina Human Methylation27 DNA analysis BeadChip) to identify candidate genes silenced in neuroblastoma due to DNA methylation. The whole-genome DNA methylation

arrays provide a quantitative measurement of the methylation frequency at specific CpG sites. The validity of the array analyses were verified with bisulfite pyrosequencing (Carén, unpublished data). The validity of the cell treatment approach was demonstrated, as imprinted genes were identified, together with genes previously known to be methylated in NB, such as *ZMYND10*, *SFN* and *THBS1*. Genes with important functions in relation to tumorigenesis and early development or a chromosomal location in regions which show LOH in NB were selected (see Table 3 and text below).

**Table 3.** Genes analyzed in the study.

| Symbol | Chr | Methylated in other cancers | Comment |
|---|---|---|---|
| *DHRS3* | 1p36 | Melanoma cell lines | Putative TSG, crucial for the development of neural crest cells, located in NB SRO of deletions |
| *DUSP23* | 1q23 | | Role in cell cycle control and early development |
| *TGFBI* | 5q31 | Leukemia, renal cell, lung, esophageal cancer | TSG, tumor suppressing function in NB |
| *COL1A2* | 7q22 | Medulloblastoma, colorectal and breast cancer | TSG |
| *PRKCDBP* | 11p15 | Breast, lung and gastric cancer | Putative TSG |
| *SCNN1A* | 12p13 | Breast cancer | |
| *POU2F2* | 19q13 | | Transcription factor, regulator of neuronal differentiation |
| *KRT19* | 17q21 | Renal cell carcinoma | |
| *LRCH4* | 7q22 | | |

*DHRS3* is located in chromosome region 1p36.22, a region that is often deleted in NB, as well as in other tumor types. The gene, also called *retSDR1*, encodes a widely expressed enzyme that is involved in retinol metabolism (Cerignoli *et al*, 2002; Haeseleer *et al*, 1998). It has been reported that DHRS3 is induced by retinoic acid in NB and the authors speculated that *DHRS3* could act as a haploinsufficiency gene. Chromosomal deletion of this region could compromise the ability to form the storage form of retinol, leading to the impaired growth-inhibitory pathway which could contribute to cancer initiation and/or progression. *DHRS3* has been reported to be methylated in melanoma cell lines (Furuta *et al*, 2006).

*DUSP23*, in chromosome region 1q23, belongs to the dual-specificity protein phosphatase family of protein tyrosine phosphatases. This family plays an important role in mitogenic signaling and in controlling the cell cycle (Tonks & Neel, 2001). *DUSP23* has been shown to be expressed in most fetal tissues, implying a possible role in early development (Wu *et al*, 2004).

*TGFBI* (*BIGH3*/keratoepithelin), located in chromosome region 5q31, is ubiquitously expressed in human tissues, but the down-regulation of the gene has been reported in a variety of tumor cell lines and primary tumors (Shao *et al*, 2006). The protein is induced by transforming growth factor-beta and binds to type I, II and IV collagens. It has recently also been found that the loss of TGFBI promotes cell proliferation and predisposes mice to spontaneous tumor development (Zhang *et al*, 2009). In NB, *TGFBI* reduces proliferation and invasion *in vitro* and *in vivo* (Becker *et al*, 2006). *TGFBI* expression is inversely correlated with *MYCN* expression in NB (Becker *et al*, 2008).

*COL1A2*, located in chromosome region 7q22.1 is one part of collagen type I, which is the major fibrillar component of the stroma in most solid malignancies (Sengupta *et al*, 2003). COL1A2 can be produced by the tumor or the stromal fibroblast cells. Tumor cells that produce collagen type I have low tumorigenic potential (Dahlman *et al*, 2002). *COL1A2* has been reported to be methylated and silenced in multiple cell lines and primary tumors (Sengupta *et al*, 2003), including the pediatric brain tumor medulloblastoma, where it can discriminate specific disease subgroups (Anderton *et al*, 2008).

*PRKCDBP*, also known as *hSRBC*, is a putative tumor suppressor gene located in chromosome region 11p15. Previous studies have revealed that the epigenetic or mutational inactivation of *PRKCDBP* is involved in breast, lung and gastric cancer (Lee *et al*, 2008; Martinez *et al*, 2009; Xu *et al*, 2001; Zochbauer-Muller *et al*, 2005).

*SCNN1A*, located in chromosome region 12p13, is an ion transport gene. This gene has recently been reported to be one of six genes that contribute to a hypermethylator phenotype that is seen in a subset of breast cancer cell lines and primary tumors (Roll *et al*, 2008).

*POU2F2* (*OCT2*) is a transcription factor that is widely expressed in the central nervous system during rat embryonic development (He *et al*, 1989). It has also been reported to serve as a bifunctional regulator of neuronal differentiation, with one isoform blocking neuronal differentiation and another inducing it (Theodorou *et al*, 2009).

*KRT19* (*CK19*) is an intermediate filament protein involved in the structural integrity of epithelial cells. The cytokeratins, to which *KRT19* belongs, perform important functions in cell migration, invasion and metastasis (Hendrix *et al*, 1996). *KRT19* shows frequent methylation in renal cell carcinoma (RCC) cell lines and primary RCC, without methylation in normal renal tissue (Morris *et al*, 2008). *KRT19* has also been reported as being down-regulated in squamous cell carcinoma (SCC) of the head and neck and the overexpression of the gene was shown to reduce SCC invasiveness by diminishing migratory capability. In contrast, *KRT19* is widely used as a biomarker for the detection of disseminated tumor cells in blood and bone marrow (Stathopoulos *et al*, 2005).

*LRCH4*, located in 7q22, contains leucine-rich repeats at the amino terminus, known to be involved in ligand binding (Kobe & Deisenhofer, 1995). Not much is known about the functions of this gene.

The nine genes that were selected were analyzed with bisulfite sequencing and expression analysis of primary tumors. Eight of the nine selected genes were also methylated in the promoter CpG islands (analyzed by bisulfite sequencing) and, as a result, the large number of positive negatives normally identified by 5-Aza-dC/TSA treatments was minimized by the additional information from the methylation arrays (positive negatives are genes identified as being potentially methylated that turn out to be unmethylated using other methods; these genes are activated indirectly and not due to the demethylation of the respective gene). Most of the eight identified genes have not previously been reported as being methylated in NB and some of the genes also distinguish between biological subsets of neuroblastoma tumors (*SCNN1A*, *PRKCDBP* and *KRT19)*. This naturally needs to be verified in a larger set of tumor material. The identification of biomarkers will likely be important in the risk stratification of patients with neuroblastoma in the future and, for this purpose, the methylation status of genes could be a suitable marker.

Interestingly, preliminary data from the Illumina DNA methylation arrays show that the hierarchial clustering of the genome-wide methylation pattern distinguishes NB tumors into biologically distinct

subgroups based on five-year overall survival, INRG stage, and chromosomal profiles. Moreover, the methylation frequency of a limited set of genes appears to be able to define the groups just as well. The identification of genes that are methylated selectively in the respective groups is ongoing. It will be interesting to explore whether genes in different pathways or cellular processes are selectively targeted by DNA methylation in the respective groups.

### Whole-genome analysis of chromosomal aberrations in NB tumors and cell lines

***Paper IV***

In the fourth paper, we performed a comprehensive genome-wide analysis of DNA copy number in 92 NB tumors using 50 K and/or 250 K gene chip arrays from Affymetrix. The most common genetic abnormality was a gain in chromosome region 17q, detected in 45% of tumors (see Table 4 for a summary).

**Table 4**. Summary of findings in the study.

| Chromosomal rearrangement | Frequency of NB | SRO |
|---|---|---|
| 1p deletion | 30% (n=28) | for MNA 17.2-37.0; for MNA-neg 0-10.4 |
| 2p gain/amplification | | |
| *MYCN* amplification | 26% (n=24) | |
| 2p gain (without MNA) | 16% (n=15) | |
| 2p gain and MNA | 9% (n=8) | |
| 3p deletion | | |
| heterozygous deletion | 15% (n=14) | 0-5.5; 46.9-51.0 |
| homozygous deletion | 1% (n=1) | 29.6-30.0 (*RBMS3*) |
| 9p deletion | | |
| heterozygous deletion | 4% (n=4) | |
| homozygous deletion | 4% (n=4) | 21.9 (*CDKN2A*) |
| 11q deletion | 22% (n=20) | 84.5-qter |
| 12q amplification | 2% (n=2) | 56.1-56.7; 67.3-68.3; 68.7-69.0 |
| 17q gain | 45% (n=41) | 54.5-qter |
| Copy-neutral LOH | 3% (n=3) | 9p, 5q, entire 11 |
| Only numerical rearrangements | 20% (n=18) | |
| No aberrations | 17% (n=16) | |

MNA, *MYCN* amplification; SRO, shortest region of overlap; neg, negative

Interestingly, homozygous deletions in chromosome region 9p21, in the *CDKN2A* gene, were detected in four NB tumors. In addition, four tumors with heterozygous deletion and one tumor with a copy number neutral LOH (CN-LOH) were identified. This region is frequently deleted in a wide range of malignancies (Rocco & Sidransky, 2001). *CDKN2A* is a tumor suppressor gene that encodes the transcripts $p16^{INK4a}$ and $p14^{ARF}$ in alternative reading frames. $p16^{INK4a}$ inhibits cdk4 and cdk6, two cell cycle activators, which inactivates the tumor suppressor protein pRB. $p14^{ARF}$ stabilizes p53 through the inactivation of mdm2, which otherwise degrades p53 (Sharpless, 2005). Heterozygous deletions of *CDKN2A* in NB have previously been described (Iolascon *et al*, 1998). In that analysis of a limited number of tumors (n=24), mutation screening did not detect any mutations and partial methylation was detected in 35% of tumors. In spite of this, DNA methylation did not correlate to gene expression.

The amplification of chromosome region 12q14 was detected in two NB tumors. Since the publication of this paper, we have detected chromosome 12q amplifications in another two NB tumors. Candidate oncogenes in this region include *MDM2*, *CDK4* and *TSPAN31* (also known as *SAS*; Sarcoma Amplified Sequence).

*MDM2*, *PPM1D,* located in the gain region on chromosome arm 17q, and *CDKN2A* have all been implicated in TP53 inactivation. *TP53* is an important tumor suppressor gene that is inactivated by mutations in approximately half of all human tumors, but it is believed to be eradicated in most tumors (Woods & Vousden, 2001). *TP53* mutations are rare in neuroblastoma tumors (Hosoi *et al*, 1994; Imamura *et al*, 1993; Komuro *et al*, 1993; Vogan *et al*, 1993). However, mechanisms other than *TP53* mutations could prevent p53 activation. The silencing of *CDKN2A* by methylation or deletion, or the amplification of *MDM2* are mechanisms that are responsible for inactivating p53 in human tumors (Auerkari, 2006; Esteller *et al*, 2000; Ho *et al*, 2001; Oliner *et al*, 1992). *PPM1D* has also been reported as a candidate proto-oncogene that may be involved in tumorigenesis through the inactivation of p53 (Woods & Vousden, 2001). In our study, we detected homozygous and heterozygous deletions of *CDKN2A* and the amplification of the *MDM2* region and gain of the *PPM1D* region, which shows that these genes can be involved in the initiation/progression of neuroblastoma through the inactivation of p53.

A homozygous deletion was also detected in one tumor in the gene *RBMS3*, in a patient that had died from the disease (Figure 16). In addition, 14 tumors displayed the heterozygous deletion of chromosome region 3p, ten of which included the *RBMS3* gene. All these tumors were stage 4 NBs or patients who had died from the disease, indicating that the deletion of this region is an adverse prognostic factor. *RBMS3* has two RNA-binding motifs, which are closely related to the structure of the c-myc gene single-strand binding proteins. These proteins are believed to regulate DNA replication, transcription, apoptosis and cell cycle progression by interacting with the c-myc protein (Penkov *et al*, 2000). The deletion of chromosome region 3p is frequently observed in many cancer types. The *RBMS3* gene has previously been described as undergoing copy number decrease in oral squamous cell carcinomas (Garnis *et al*, 2003). In esophageal squamous cell carcinoma, *RBMS3* displays LOH in about 60% of cases and the down-regulation of the mRNA in more than 70% (Qin *et al*, 2008). The *RBMS3* gene can be found in "The Database of Genomic Variants" (URL: http://projects.tcag.ca/variation/; Iafrate *et al*, 2004). This database contains more than 8,000 CNV loci. The tumor with the homozygous deletion of *RBMS3* has one small deletion covering the *RBMS3* gene on one allele which could potentially be a CNV, in this case also present in constitutional DNA from the patient. The other allele has a larger deletion which is likely to have become lost in the tumor. Unfortunately, we did not have access to constitutional DNA from this patient and could consequently not test this hypothesis. It could also be the case that the allele with the smaller deletion is also lost selectively in the tumor. The fact that the deleted region in the tumor is somewhat larger than the reported CNV is indicative of this. We wanted to explore whether *RBMS3* was inactivated by other means in NB. To do this, we screened *RBMS3* for inactivating mutations in 22 NB tumors, 4 of which had 3p deletions; no missense mutations were detected (Carén, unpublished data). *RBMS3* does not harbor a CpG island and data from the Illumina methylation array indicate that there is low methylation around the transcription start site for this gene; as a result, DNA methylation does not appear to be involved in the regulation of this gene (Carén, unpublished data). A meta-analysis by Warnat *et al* combined gene expression data from two studies in order to investigate differences in gene expression in advanced-stage (stage 3 or 4) NB tumors without *MYCN* amplification that had contrasting outcomes (alive or dead) five years after the initial diagnosis

(Warnat *et al*, 2007). In this analysis, *RBMS3* was identified as one of 72 genes that were differentially expressed between these two groups; *RBMS3* was down-regulated in the group with adverse outcome, indicating that this gene can play a role in NB development/progression. Four of the NB tumors with the heterozygous deletion of 3p in our study did not include the *RBMS3* gene, indicating that other genes in the 3p region could be involved. Candidate tumor suppressor genes in SRO 2 (see Figure 16) include *RASSF1A*, *ZMYND10* and *SEMA3B*. Nevertheless, no consistent SRO that harbored all the 3p deletions was identified, indicating that several genes could be involved in NB tumorigenesis.

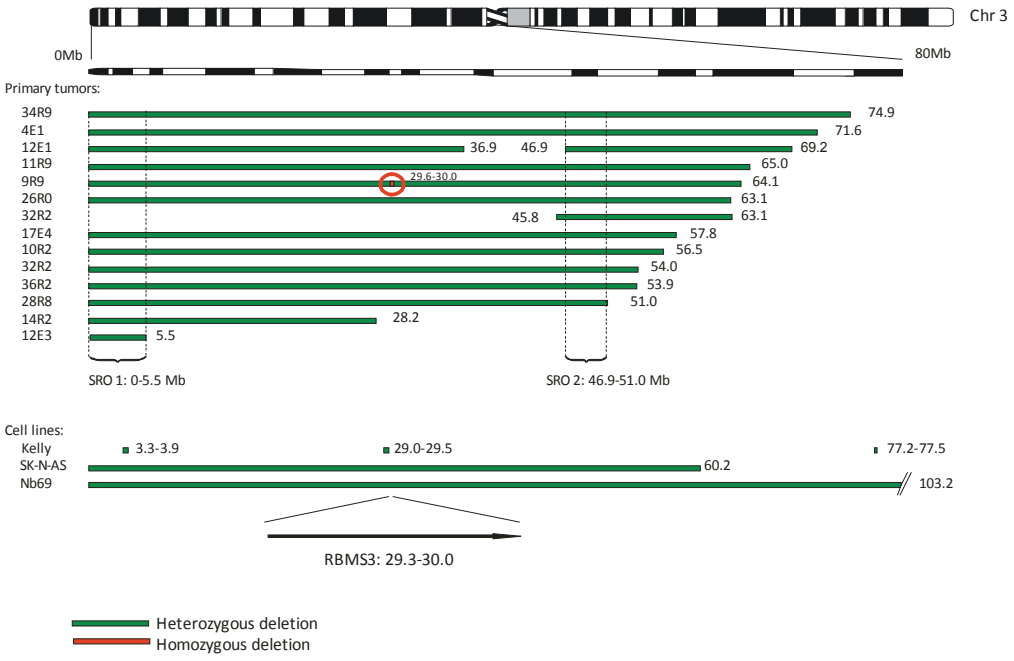


**Figure 16.** Chromosomal aberrations detected in chromosome 3. A homozygous deletion in the *RBMS3* gene at position 29.6-30.0 Mb was detected. Two regions of SRO of deletions were detected; SRO 1 at position 0-5.5 Mb and SRO 2 at 46.9-51.0 Mb. Figure from Carén *et al* (2008b).

One advantage of SNP arrays compared with BAC arrays is that the identification of CN-LOH is possible. CN-LOH was found to be rare but could be detected in three tumors in our study; one in chromosome region 9p21-9p24 including the *CDKN2A* gene, one with a partial loss of chromosome arm 5q and one with the entire chromosome 11.

To summarize, the study identified 59 tumors with structural/segmental abnormalities (64%) and only numerical changes in 18 of the tumors (20%). No rearrangements could be identified in 15 tumors (16%), probably reflecting the presence of too much normal cells in these samples.

***Paper V***

In this study, we wanted to conduct a comprehensive array copy number analysis of all the available Swedish NB tumors in order to draw conclusions from a large set of material, without any biases. We also wanted to compare genomic profile data with clinical data for this large set of material. In our first analysis (*Paper IV*, Carén *et al*, 2008b) we noted specific features of the tumors with 11q deletion and therefore wanted to investigate this in more detail. We analyzed 165 neuroblastoma tumors of all stages using genome-wide, high-density SNP microarrays from Affymetrix (the same 250 K gene chip arrays used in *Paper IV*). We divided the tumors into five subgroups; (i) NBs with *MYCN* amplification (but not 11q deletion), (ii) NBs with 11q deletion (but not *MYCN* amplification), (iii) NBs with 17q gain (without *MYCN* amplification and 11q deletion), (iv) NBs with segmental aberrations not including *MYCN* amplification, 11q deletion or 17q gain and (v) NBs with only numerical changes (whole chromosome gains and/or losses). One additional group constituted tumors with a flat profile – no aberrations at all detected.

The overall survival at eight years after diagnosis was very similar in the *MYCN*-amplified and the 11q-deleted groups, approximately 35%, while the median age at diagnosis differed; 21 months for the *MYCN*-amplified group and 42 months for the 11q-deletion group. The favorable group defined by only numerical aberrations had an overall survival of about 90% and a median age at diagnosis of only three months, while the 17q gain group had a survival rate slightly over 60% and a median age at diagnosis of 21 months (Figure 17). The groups characterized by 11q deletion, *MYCN* amplification or 17q gain respectively were all associated with a poor outcome, whereas the other segmental group was not.

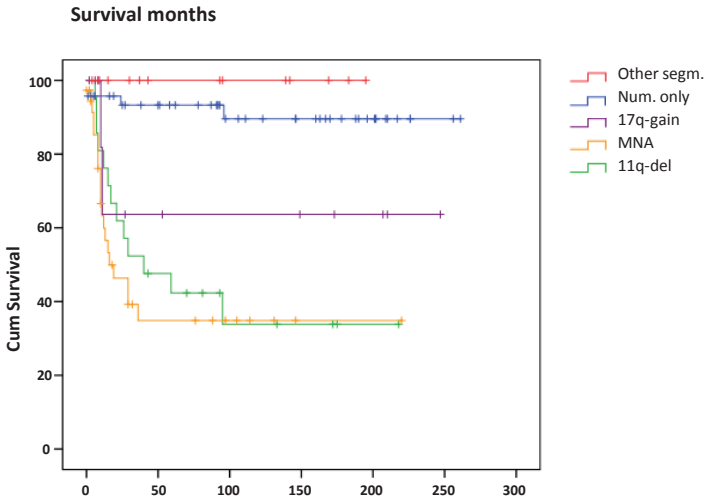


**Figure 17.** Kaplan-Meier analysis of survival probability in the five subgroups of NB.

There was a significant difference in the number of segmental aberrations in the groups; the *MYCN*-amplified group and the 17q-gain group had a median of four aberrations, while the 11q-deleted

group had a median of 12. The lowest number of breaks was found in the other segmental group, with a median of one aberration. The chromosomal breaks associated with the segmental aberrations were more focused on specific regions in the *MYCN*-amplified group, while the 11q-deleted group displayed a more "shotgun-like" distribution. The high frequency of segmental aberrations and chromosomal breaks in the 11q-deletion group of tumors is suggestive of a CIN (chromosomal instability) phenotype gene located in 11q. One such gene is the *H2AFX* (H2A histone family, member X) gene located in 11q23.3 (pos. 118.4 Mb from pter, which is within the shortest region of overlap of the 11q deletions). Further analysis of this gene in relation to NB is ongoing at our laboratory. If the hypothesis of a CIN phenotype gene that is targeted in the 11q-deleted tumors is correct, the treatment of these tumors may gain from the more radical surgical removal of all tumor cells, since remaining tumor cells have a high capacity to mutate.

**Table 5.** Summary of features of the *MYCN* amplification (MNA) phenotype and the 11q phenotype.

| **MNA group** | **11q-deletion group** |
|---|---|
| Amplification of *MYCN* | No amplification of *MYCN** |
| No 11q del* | 11q del |
| Presence of segmental aberrations but only a few (median=4) | Many segmental aberrations (median=12) |
| Chromosomal breaks on a few other chromosomes, in addition to chromsome 2 (location of *MYCN*) | Chromosomal breaks on several chromosomes, in addition to chromsome 11 |
| Break distribution "more focused" | Break distribution "more shotgun like" |
| 1p deletion region more proximal on 1p (consensus in 17-32Mb) | 1p deletion region more distal on 1p (consensus in 0-10.4 Mb) |
| Poor prognosis, 8-year survival approx. 35% | Poor prognosis, 8-year survival approx. 35% |
| Median age at diagnosis 21 months | Median age at diagnosis 42 months |

*Only rare cases of both MNA and 11q deletion in the same tumor

Thirty-one of the 165 analyzed tumors had a flat profile. This probably reflects the contribution of non-relevant cell material in the sample that conceals the true aberrations in the tumor rather than the tumors having no aberrations. The problem of flat profiles was mostly seen in the tumors that had been diagnosed and resected during the 1980s and 1990s. This probably reflects the fact that these samples were not as pure as more recent samples.

We propose from our studies that the two high-risk neuroblastoma groups defined by 11q deletion and *MYCN* amplification should perhaps not have the same type of high-risk treatment due to their very different genetic profiles. Further, we feel that whole-genome analysis is essential in the clinical diagnoses, risk assessments and further treatment choices of neuroblastoma tumors. SNP analyses of all NB tumors in Sweden have now been introduced at the clinic.

## CONCLUSIONS

In this thesis, we have analyzed the epigenetic regulation, as well as genome-wide chromosomal aberrations in NB tumors. A short summary of the findings now follows:

- The genes *UBE4B*, *KIF1B*, *PGD*, *APITD1*, *DFFA* and *PEX14* located in the frequently deleted chromosomal region 1p36.22 in NB were down-regulated in NB tumors with unfavorable biology compared with those with favorable biology. This down-regulation was, however, found not to be due to DNA hypermethylation of their respective CpG islands.

- In a comprehensive analysis of the larger candidate region on 1p36, two patients with missense mutations in the *PIK3CD* gene were identified. The mRNA expression of the *PIK3CD* gene was down-regulated in unfavorable compared to favorable NB tumors. Bisulfite sequencing indicated that DNA methylation was not the mechanism for the observed down-regulation; instead, histone deacetylation was suggested to be involved, since treatment with the histone deacetylase inhibitor TSA led to increased gene transcription. The genes *ERRFI1*, *RBP7* and *CASZ1* were also up-regulated by epigenetic treatment, among which *CASZ1* was found to be down-regulated in NB tumors with unfavorable biology compared with those with favorable biology. Again, our data suggested histone deacetylation as the main candidate epigenetic mechanism responsible for the down-regulation of mRNA expression of this gene. Our data thus suggested the *CASZ1* and *PIK3CD* genes as the best 1p localized genes for further study.

- In a whole-genome methylation analysis combined with a whole-genome expression analysis of NB cell lines treated with epigenetic drugs, we identified eight genes (*KRT19*, *PRKCDBP*, *SCNN1A*, *POU2F2*, *TGFBI*, *COL1A2*, *DHRS3*, *DUSP23*) that are methylated in neuroblastoma, most of them not previously reported as such. Moreover, we showed that the methylation frequencies of a number of these genes could distinguish between biological subsets of neuroblastoma tumors. DNA methylation frequencies of these genes could be used as potential biomarkers to aid in patient stratification in the future.

- In a whole-genome array copy number analysis, we detected homozygous deletions in two chromosomal regions. Homozygous deletion of *CDKN2A*, located in chromosome region 9p21 was found in four NB tumors. In addition, four additional tumors with heterozygous deletion and one tumor with a CN-LOH in this region were identified. One homozygous deletion was also detected in the chromosome region 3p24.1, inside the *RBMS3* gene. Taken together, data from this study provides new interesting candidates for further exploration.

- We demonstrated that there are large chromosomal and clinical differences between the two NB high-risk groups, tumors with *MYCN* amplification and 11q deletion. We found significantly different frequencies of segmental aberrations in these two groups; the *MYCN*-amplified group had a median of four aberrations, while the 11q-deleted group displayed a median of 12 aberrations. The overall survival at eight years after diagnosis was very similar in the two groups, approximately 35%, while the median age at diagnosis differed (21 months for the *MYCN*-amplified and 42 months for the 11q-deleted tumors). The observed high frequency of segmental aberrations and chromosomal breaks in the 11q-deleted group of tumors is suggestive of a CIN (chromosomal instability) phenotype gene located in this

chromosomal segment. This study provides valuable information on distinct and different genetic profiles of the two high-risk groups of tumors that potentially can be used as a basis for the choice of therapy in the future. Our analysis also underlines the importance of using dense genome-wide microarray analyses with aCGH or SNP arrays in the clinical management of these patients to fully evaluate their tumor genomic profiles and thereby to improve diagnosis, prognosis, and terapeutic approches.

## FUTURE PROSPECTS

In NB, mRNA expression analysis has been shown to result in very inconsistent results between different labs. In contrast, DNA methylation is a very stable marker that is suitable for use as a biomarker. We demonstrate that combining DNA methylation arrays and RNA arrays from cell treatment studies using epigenetic drugs is an effective approach for detecting aberrantly methylated genes. We also identified genes that differ in their methylation frequencies between patients with a good and a poor prognosis. These studies need to be performed on more extensive material in order to identify the set of genes that best distinguish between different biological subtypes of NB. Ideally, more suitable control tissues such as normal fetal adrenal medulla could also be included in the analysis in order to identify not just good biomarkers but also genes that really are abnormally methylated compared with the appropriate control tissue to provide more insight into the oncogenic process. Epigenetic drugs can potentially be included in the treatment of NB in the future, as single agents or more likely in combination with existing therapy. In order for this to happen, intensified research is needed to learn more about epigenetic regulation in NB.

Combinatorial analyses of data sets from DNA methylation arrays, RNA expression arrays and SNP arrays on NB are ongoing at our lab. This has a strong potential to enhance our understanding of the biological processes involved in NB, as wells as to identify new targets for patient stratification and therapy,

Even though the prognosis for children with neuroblastoma has improved substantially in recent decades for both low-risk and high-risk neuroblastoma, the prognosis for high-stage NB is still poor (Träger, 2009). More needs to be learnt about the high-risk groups defined by *MYCN* amplification, 11q deletion and 17q gain. Specifically the group defined by 11q deletion is the group that we seem to know least how to cure since the aggressive *MYCN*-amplified tumours have shown to respond to multimodal therapy with intensified chemotherapy (Träger, 2009; Per Kogner, personal communication). It is therefore important to explore all the features of this “11q-deletion phenotype” from different aspects, both genetic and epigenetic, in order to learn more about the pathways that are targeted. It is also essential to learn more about why relapses of NB are common. If there are tumor-initiating cells with cancer stem cell properties in NB, as has been reported, cells of this type are perhaps what needs to be focused on, as they could potentially be the reason why high-risk NB is so difficult to cure and why relapses occur at a high frequency. More knowledge about the genetic and epigenetic features of these kinds of cell could potentially open up new targets for therapy and could improve the survival from NB in the future.

## SAMMANFATTNING PÅ SVENSKA

Neuroblastom är en cancerform som nästan uteslutande drabbar små barn. Sjukdomen uppkommer från stamceller i det perifera sympatiska nervsystemet och primärtumören kan påträffas inom hela det sympatiska nervsystemets utbredningsområde. Sjukdomsfrekvensen ligger på ungefär 1 fall per 100 000 barn under 15 års ålder, vilket motsvarar 15-20 fall om året i Sverige. Neuroblastom är en mycket heterogen cancerform, det kliniska spektrumet sträcker sig från mycket aggressiva, svårbehandlade tumörer till gynnsamma tumörer som botas med mild behandling. Det finns till och med en form som kan tillbakabildas spontant helt utan behandling.

Det första syftet med avhandlingen var att studera epigenetiska förändringar i neuroblastom-tumörer för att därigenom identifiera tumörsupressorgener som är felaktigt avstängda och för att identifiera markörer för att förbättra patient stratifiering. Till skillnad från genetiska förändringar är epigenetiska förändringar reversibla. Det finns därför förhoppningar om att använda läkemedel som riktar sig mot dessa mekanismer som behandling mot cancer. Detta görs redan idag för specifika cancerformer och det kan i framtiden även bli aktuellt för behandling av neuroblastom, möjligtvis i kombination med dagens behandling. Identifiering av DNA metylering kan även användas för att detektera cancer, då dessa förändringar är mkt tidiga förändringar. DNA metylering som biomarkör kan även användas för att skilja olika typer av tumörer och för att förutsäga risk och val av behandling. Detta är särskilt viktigt i fråga om behandling av barn med neuroblastom. Det är viktigt att kunna identifiera de barn som kräver den aggressivaste behandlingen för att få den bästa möjligheten att överleva, men även att identifiera den grupp som klarar sig med mindre aggressiv behandling, för att minska risken för allvarliga biverkningar, vilka kan vara påtagliga för denna unga patientgrupp.

I *arbete I* studerades gener i kromosomområdet 1p36.22, ett område som ofta fattas från en av kromosomerna i neuroblastomtumörer. Uttrycket av generna fanns vara lägre i ogynnsamma tumörer jämfört med gynnsamma men DNA metylering förklarade inte det sänkta genuttrycket.

I *arbete II* studerades ett större kromosomalt område innehållande 30 gener i kromosomregionen 1p36 med avseende på DNA metylering och histondeacetylering. De mest intressanta genkandidaterna från denna studie utgörs av *CASZ1* och *PIK3CD* som har ett lägre genuttryck i ogynnsamma tumörer jämfört med gynnsamma. I *PIK3CD* hittades dessutom mutationer på DNA nivå. Dessa två gener är intressanta att studera vidare rörande deras tänkbara inblandning i neuroblastomutveckling.

I *arbete III* gjordes en analys av DNA metylering över hela genomet. Detta gjordes genom att kombinera analys av DNA metylering med hjälp av kommersiella helgenoms-metyleringsarrayer och en behandlingsstudie där neuroblastom-cellinjer behandlades med epigenetiska droger/läkemedel, och genuttrycket efter behandling jämfördes med uttrycket före behandling med helgenoms-expressionsarrayer. Av generna som identifierades som metylerade på DNA nivå och vars uttryck också påverkades av epigenetiska droger valdes ett antal kandidatgener ut som verifierades med andra metoder. Metyleringsfrekvensen av några av generna visade sig även kunna skilja gynnsamma från ogynnsamma tumörer och förutsäga överlevnad (generna *SCNN1A*, *PRKCDBP* och *KRT19*). Det är troligt att metyleringsstatus för dessa gener eller andra gener identifierade i större patientmaterial i framtiden kan användas som biomarkörer för neuroblastom.

Det andra syftet med avhandlingen (*arbete IV* och *V*) var att göra helgenomsstudier av kromosomala förändringar i ett stort antal neuroblastomtumörer för att bättre kunna karakterisera och identifiera olika riskgrupper. Denna analysmetod används numera kliniskt för att diagnostisera neuroblastom och för att underlätta behandlingsval.

I *arbete IV* studerades 92 neuroblastomtumörer och kromosomala avvikelser i detta material identifierades. Homozygota deletioner (då material från båda kromosomer i ett kromosompar är förlorat) är mycket ovanliga i neuroblastom, men i detta material identifierades homozygota deletioner i tumörsuppressorgenen *CDKN2A* i fyra tumörer. Hos fem tumörer hittades även heterozygota deletioner (material från en kromosom förlorat) eller kopie-neutrala förluster (kromosom-material nedärvt från en förälder är förlorat medan materialet från den andra föräldern är duplicerat). En homozygot deletion påträffades även i genen *RBMS3*, en gen som också ingick i heterozygota deletioner i tio tumörer.

I det följande arbetet (*arbete V*) ville vi utöka materialet till att omfatta hela det svenska neuroblastom-materialet. Vi hade tidigare noterat intressanta fynd hos de tumörer med deletioner omfattande delar av den långa armen av kromosom 11. I *arbete V* visar vi att patienter med 11q deletion i tumören har en relativt högre sjukdomsdebutålder och en dålig prognos. Dessa tumörer har också signifikant fler kromosombrott än andra neuroblastomtumörer vilket kan tyda på att en gen som har betydelse för kromosomstablitet kan vara påverkad i denna grupp av neuroblastom. Tumörer med 11q deletion eller amplifiering av proto-onkogenen *MYCN* är de neuroblastomtumörer som är svårast att bota. På grund av de mycket olika genetiska profilerna som vi identifierat för dessa typer av neuroblastom föreslår vi att det kan krävas olika behandlingsstrategier för att uppnå bästa behandlingsresultat i respektive grupp.

Även om behandlingen av neuroblastom har blivit bättre under de senaste årtiondena är det fortfarande svårt att bota högstadie-neuroblastom. Den här avhandlingen har bidragit till att öka förståelsen kring neuroblastom, men mycket forskning återstår innan vi till fullo kan förklara varför neuroblastom uppkommer och hur vi ska agera för att bota så många patienter som möjligt, men förhoppningsvis kommer vi att komma dit i framtiden.

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