Molecular Characterization of Neuroblastoma Tumors

A Basis for Personalized Medicine

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Cover image:

Single nucleotide polymorphism arrays, used for risk-group stratification of neuroblastoma patients. Raw intensity data from a SNP-array, featuring a photograph of the GeneChip® 500k mapping set of arrays (Courtesy of Affymetrix), as well as the resulting genomic profile of a neuroblastoma sample, viewed with the CNAG software.

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Abstract

Neuroblastoma is a very heterogeneous tumor, with a clinical course ranging from spontaneous regression to aggressive tumor growth. A proper stratification of the patients into different risk groups is therefore important in order to provide the most suitable treatment for each patient. The primary aim of this thesis was therefore to further characterize the genes and mechanisms important for neuroblastoma development using genome-wide copy number data from single nucleotide polymorphism (SNP)-arrays as a starting point for more detailed studies of interesting regions of the genome. Furthermore, we wanted to investigate the clinical usefulness of SNP-arrays, both directly as a prognostic tool, and indirectly as a starting point for the generation of patient-specific assays.

As to the genes and mechanisms important for neuroblastoma development, we have identified a chromosomal instability phenotype in the 11q deleted subgroup, possibly caused by the DNA-repair gene *H2AFX* located in the commonly deleted region. Furthermore, we have identified and characterized a small subgroup of neuroblastoma with amplification of two regions on 12q, occasionally accompanied by 11q amplification. Gene expression analysis and siRNA knockdown of the genes included in these amplicons indicate that *CDK4* and *CCND1* are possible drivers of this subgroup and we therefore suggest that this group of neuroblastoma is characterized by a cell cycle de-regulation phenotype.

Regarding the clinical usefulness, our results show that SNP-arrays are powerful tools for the stratification of neuroblastoma patients into different treatment groups. Not only is it possible to detect known prognostic markers such as *MYCN* amplification and 11q deletion, but the genome-wide copy number profile in itself is also important, especially for the identification of patients with a favorable prognosis. Moreover, we show that the array-data can be used for detailed mapping of the rearrangement boundaries, which in combination with a multiplex PCR reaction makes it possible to detect tumor-specific fragments that span the junction of the rearranged DNA. These junction PCR assays were also tested for the detection of minimal residual disease, and were found to be sensitive enough to detect very small amounts of tumor DNA in the blood or bone marrow from patients during treatment or follow-up.

To conclude, genome-wide techniques, such as SNP-arrays are useful not only for research purposes but also as a clinical tool. These arrays give valuable information for the risk-group stratification of neuroblastoma patients, and provide a robust foundation for the development of a personalized treatment strategy for patients with neuroblastoma.

List of papers

This thesis is based on the following papers listed in reverse chronological order. They are appended at the end of the thesis and will be referred to in the text by their roman numerals.

I. Carén H, **Kryh H**, Nethander M, Sjöberg RM, Träger C, Nilsson S, Abrahamsson J, Kogner P, Martinsson T. High-risk neuroblastoma tumors with 11q-deletion display a poor prognostic, chromosome instability phenotype with later onset. Proc Natl Acad Sci U S A. 2010 Mar 2;107(9):4323-8.

II. **Kryh H**, Abrahamsson J, Jegerås E, Sjöberg RM, Devenney I, Kogner P, Martinsson T. MYCN amplicon junctions as tumor-specific targets for minimal residual disease detection in neuroblastoma. Int J Oncol. 2011 Nov;39(5):1063-71.

III. **Kryh H**, Carén H, Erichsen J, Sjöberg RM, Abrahamsson J, Kogner P, Martinsson T. Comprehensive SNP array study of frequently used neuroblastoma cell lines; copy neutral loss of heterozygosity is common in the cell lines but uncommon in primary tumors. BMC Genomics. 2011 Sep 7;12:443.

IV. **Kryh H**, Hedborg F, Øra I, Ambros P.F., Gartlgruber M, Kogner P, Martinsson T. Amplification of two regions on chromosome arm 12q defines a clinically distinct subgroup of high-risk neuroblastoma patients. 2012 Manuscript

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Abbreviations

aCGH	Array comparative genome hybridization
BAC	Bacterial artificial chromosome
CGH	Comparative genome hybridization
CN-LOH	Copy neutral loss of heterozygosity
DM	Double minutes
DNA	Deoxyribonucleic acid
EBRT	External beam radiation thearpy
FISH	Fluorescence in situ hybridization
HSR	Homogeneously staining region
IDRF	Image defined risk factors
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group staging system
INSS	International Neuroblastoma Staging System
LOH	Loss of heterozygosity
M-FISH	Multiplex-fluorescence in situ hybridisation
MMBIR	Microhomology-mediated break-induced replication
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
PAC	P1-derived artificial chromosome
PCR	Polymerase chain reaction
QRT-PCR	Quantitative reverse transcription PCR
RNA	Ribonucleic acid
SKY	Spectral karyotyping
SNP	Single nucleotide polymorphism
SRO	Shortest region of overlap
tRNA	Transfer RNA

Preface

Neuroblastoma is a cancer of the early childhood that has long been intriguing both researchers and clinicians. It is a very heterogeneous disease, ranging from rather benign tumors, capable of spontaneous regression, to highly malignant tumors progressing even despite the most intensive therapy. Initially, clinical risk factors, such as age over one year and presence of metastases (in particular to the bone), were used to identify patients with high-risk neuroblastoma. In the 1980s it also became evident that genetic factors, such as the *MYCN* amplification could improve this stratification.

To improve prognosis, treatment has gradually been intensified since the early 90s and modern therapy now includes dose-intensive induction chemotherapy, surgery of the primary tumor, high-dose chemotherapy combined with autologous stem cell rescue, and local irradiation. In the last decade maintenance therapy with retinoic acid has been added. This has led to an increase of the survival rate for high-risk patients, from 20% in the beginning of the 1990s to around 40-50% today.

Despite intense research efforts, we are still struggling to understand the biology and mechanisms underlying this complex disease, and although survival of patients with neuroblastoma has improved over the last decades, the major treatment strategies are still largely based on intensive chemotherapy. The ultimate goal would be to provide a personalized strategy for neuroblastoma treatment, in which a thorough initial characterization of each tumor is followed by a treatment protocol specifically targeting the biological properties of that particular tumor.

My thesis describes our contribution towards this ultimate goal, focusing largely on the use of SNP-arrays for diagnostic and prognostic purposes. SNP-arrays have proved to be useful tools, not only for treatment stratification of neuroblastoma patients, but also for the general understanding of tumor biology in different subtypes of neuroblastoma. In Sweden, SNP-array investigation is now included as a standard procedure in the molecular characterization of neuroblastoma tumors, thus providing an important first step in the direction towards personalized medicine. Hence, although the scenario of personalized medicine in neuroblastoma treatment is still set in the future, we are definitely getting closer!

Although this journey has been at the same time both fascinating and frustrating, it has also been very educational. Most of all I have come to realize that most scientific discoveries are not *Eureka* moments, but small, struggling steps forward, trying desperately to find the next piece of the puzzle!

I hope you will enjoy reading my book!

1 Introduction

1.1 Basic genetics

All living organisms, from bacteria to multi-cellular animals and plants, carry a blueprint in their cells instructing them how they should develop and function. This information is stored in the DNA molecule which is passed on from a cell to its daughter cells in each cell division, and ultimately from parent to offspring in each generation.

For a single-cell organism, the main developmental decision concerns whether or not there is sufficient material available for cell division to take place. However, for a multi-cellular organism, containing cells and organs specialized for different functions, maximal cell growth is no longer the most beneficial outcome. Instead, in order for the organism to function properly, a delicate balance and timing of proliferation and differentiation is essential.

If the DNA of a cell gets damaged, it may lead to a disruption of this regulation and instead allow the cell to grow and divide, also in the absence of growth signals. This uncontrolled proliferation of a single cell in a multi-cellular context could then lead to the development of a tumor. Hence, in order to properly understand the biology of a tumor cell, we need to understand the underlying genetics.

1.1.1 Nucleic acids and the genetic code

The structure of deoxyribonucleic acid (DNA) was determined in 1953 [1-4], as well as its implication for accurate transmission of genetic material [5]. This molecule is arranged as a double-helix, with four different nucleotides, or bases, linked to a sugar-phosphate backbone (Figure 1). The four nucleotides; adenine (A), cytosine (C), guanine (G) and thymidine (T), are similar in structure, but differ in the binding area such that specific hydrogen bond formation, compatible with a β-helix formation of the DNA, is possible only between certain base pairs: A-T, and C-G.

Ribonucleic acid (RNA) has a similar structure, although with ribose instead of deoxyribose in the sugar-phosphate backbone, and with the nucleotide uracil (U) replacing thymidine. In contrast to DNA, RNA, is commonly found in a single stranded form, and is also capable of creating complex secondary structures.

The similarity between DNA and RNA, allows these two types of nucleic acids to hybridize with each other. This hybridization is also central for the replication of DNA prior to cell division, thus ensuring a robust way of transferring the genetic information to the daughter cells. The transcription and translation of genes into their protein counterparts, a process known as the central dogma, also relies on specific hybridization between nucleic acids (Figure 2a) [6].

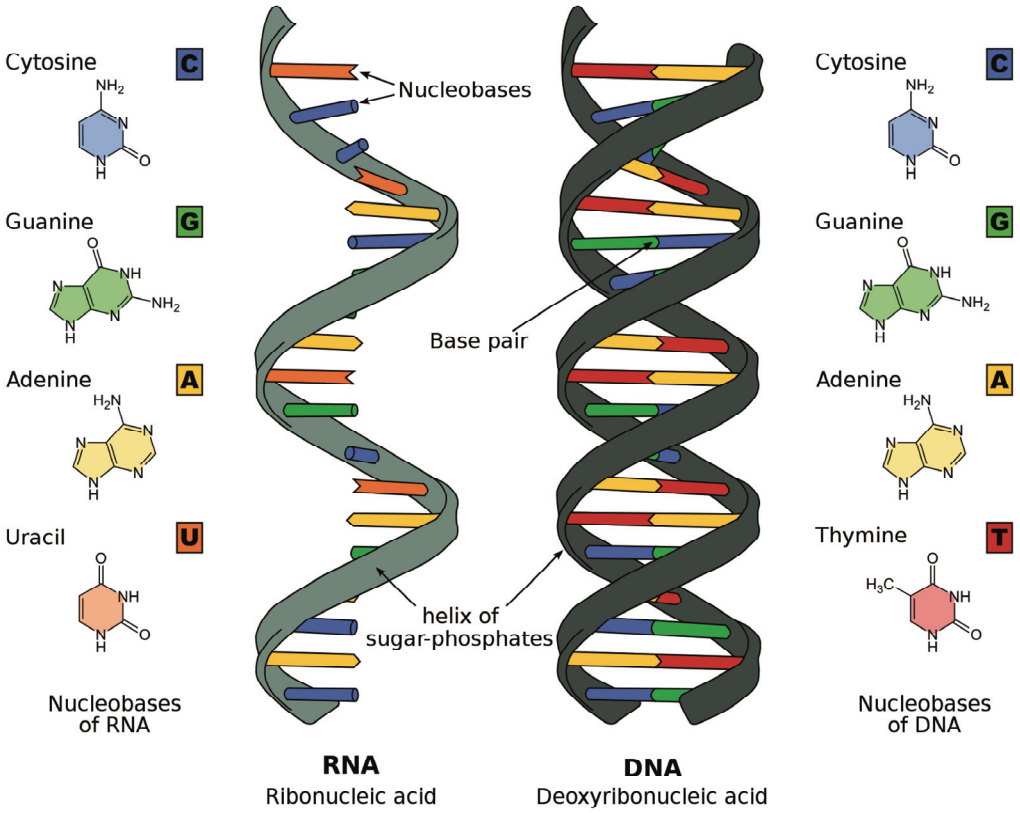

Figure 1 DNA and RNA share a similar structure. Both these nucleic acids consist of a sugar-phosphate backbone and four nucleotides. Furthermore specific base-pairing (A-T/U, and G-C) may occur through hydrogen bond formation between the nucleotides, thus enabling hybridization either between DNA-DNA or DNA-RNA.

In replication, a semi-conservative approach is taken in which the DNA strands separate and a new strand is synthesized onto the existing strand. The resulting DNA molecule is therefore a hybrid between new and old DNA [1, 5]. Transcription is based on the same principle but the synthesis occurs locally for the gene to be expressed. In this case, the newly synthesized strand consists of a messenger RNA (mRNA), which is released from the DNA and transported out of the nucleus to be used as a blueprint for the protein to be produced.

Translation, the process in which the mRNA sequence is transferred into its protein counterpart, is also regulated by specific hybridization. This process takes place in the ribosome, a multiunit RNA-protein complex with a catalytic pocket consisting of ribosomal RNA [7]. The connection between mRNA and protein is made through a set of transfer RNAs (tRNAs), each containing a three nucleotides long recognition sequence. When the nucleotide triplet matches the corresponding triplet, or codon, in the mRNA sequence, the amino acid that is linked to the tRNA is detached and connected to the growing peptide. Each codon corresponds to a specific tRNA, which in turn carries a specific amino acid. However, since there are as many as 64 possible tri-nucleotide combinations but only 20 standard amino acids, some amino acids are represented by more than one codon (Figure 2b) [8].

Essentially all of the molecular genetic methods used today make use of the specific hydrogen bond formation between nucleotides, either through hybridization between strands of DNA/RNA as in the case of FISH, CGH, and Microarrays, or through the synthesis of DNA, from an existing RNA or DNA template, exemplified by techniques such as PCR, reverse transcription, and sequencing. Some of these methods will be described in more detail in chapter 1.4.

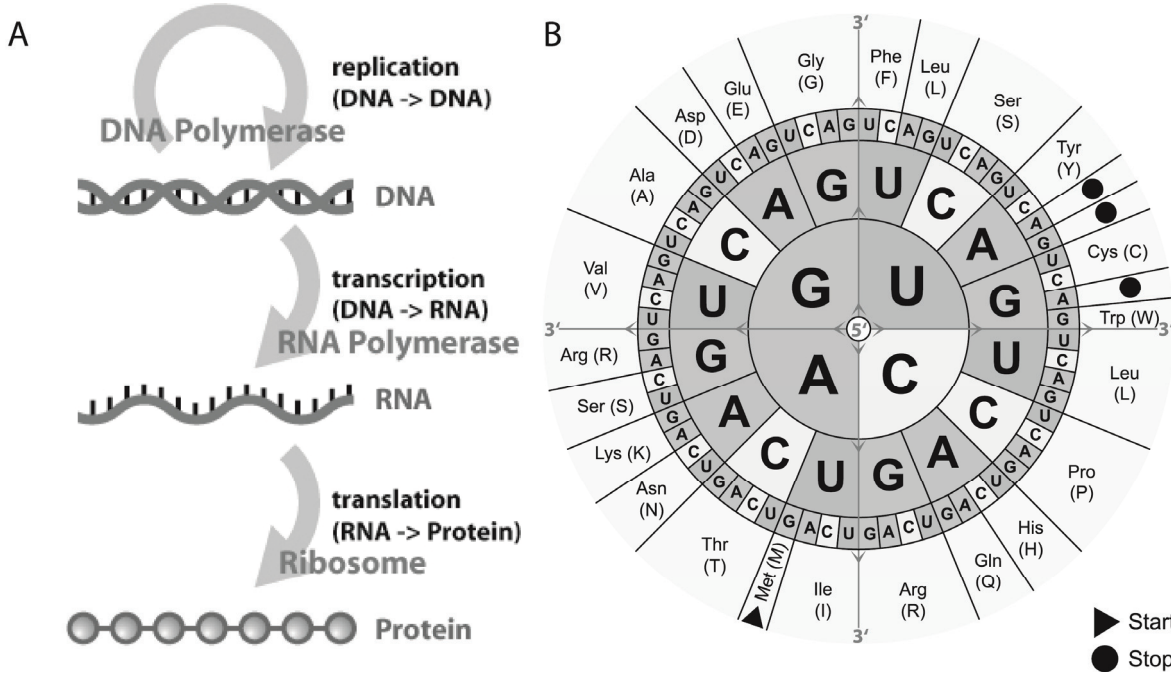

Figure 2 A) The central dogma. The hybridization of DNA and RNA allows for accurate copying of the genetic material either prior to cell division (replication) or for the production of RNA (transcription) and proteins (translation) from specific genes in the DNA.

B) The genetic code. The three nucleotide codons and their corresponding amino acids. Note that many amino acids are represented by more than one codon.

1.1.2 Organization of the genome

The DNA of a human cell contains approximately 3 billion base pairs (bp) [9], organized into 23 pairs of chromosomes [10]. Surprisingly, despite the vast size of our genome, the number of protein coding genes has been estimated to 20,000-25,000, thus taking up only about 1% of the DNA [9]. However, the traditional view of a gene as a protein coding stretch of DNA, containing a promoter that regulates the gene expression, exons that encode the amino acid sequence, and introns that are spliced off during RNA processing, is an over-simplification. It has lately been recognized that regulatory elements can be located also at a large distance from the gene of interest, and that non-coding RNAs are relatively common in our genome [11, 12].

This has led to a more relaxed definition of a gene: "a DNA segment that contributes to phenotype/function. In the absence of demonstrated function, a gene may be characterized by sequence, transcription or homology" [13]. Furthermore, alternative splicing and post-translational modification are common features of our genome, thus enabling multiple versions of a protein to be produced from the same gene. This means that although the number of genes in the human genome may be less than expected, there is a vast complexity in the RNAs and proteins produced.

Gene expression is also regulated by the packing of the DNA. The core packaging unit of the chromosome is the nucleosome. This complex consists of a central core of eight histones with a stretch of DNA wrapped around it in approximately two turns [14]. The nucleosomes are situated at regular intervals along the DNA, with approximately 200 bp allocated to each nucleosome, viewed as 'beads on a string' in the electron microscope. Depending on chemical modifications of the histone-tails, a gene can be more or less easily accessible to regulatory proteins influencing the transcription of the gene. These epigenetic mechanisms play an important role during embryogenesis and development, ensuring that genes are properly regulated depending on time and cell type. Furthermore, defects in these mechanisms may give rise to cancer. Although, the nucleosomes are relatively loosely packaged throughout the majority of the cell cycle, a dramatic condensation of the DNA begins once the cell enters mitosis, causing the DNA to end up in tightly packed chromosomes that are visible even in the light microscope (Figure 3A)[15]

The human chromosomes are generally numbered in order of decreasing size, such that chromosome 1 is the largest, followed by chromosome 2 etc. The exceptions to this rule are the sex chromosomes X and Y, and also chromosome 21, which has actually been found to be smaller than chromosome 22. The short arm of a chromosome is denoted p and the long arm q, and more specific genomic positions have traditionally been referred to in relation to the banding pattern obtained using Trypsin/Giemsa staining of mitotic chromosomes (Figure 3B) [16]. According to this system the chromosomal bands are numbered from the centromere and outwards along the chromosome. Lately however, the convention has changed somewhat and the position of a particular gene is now commonly given in mega bases (Mb), as calculated from the p-terminal of the chromosome.

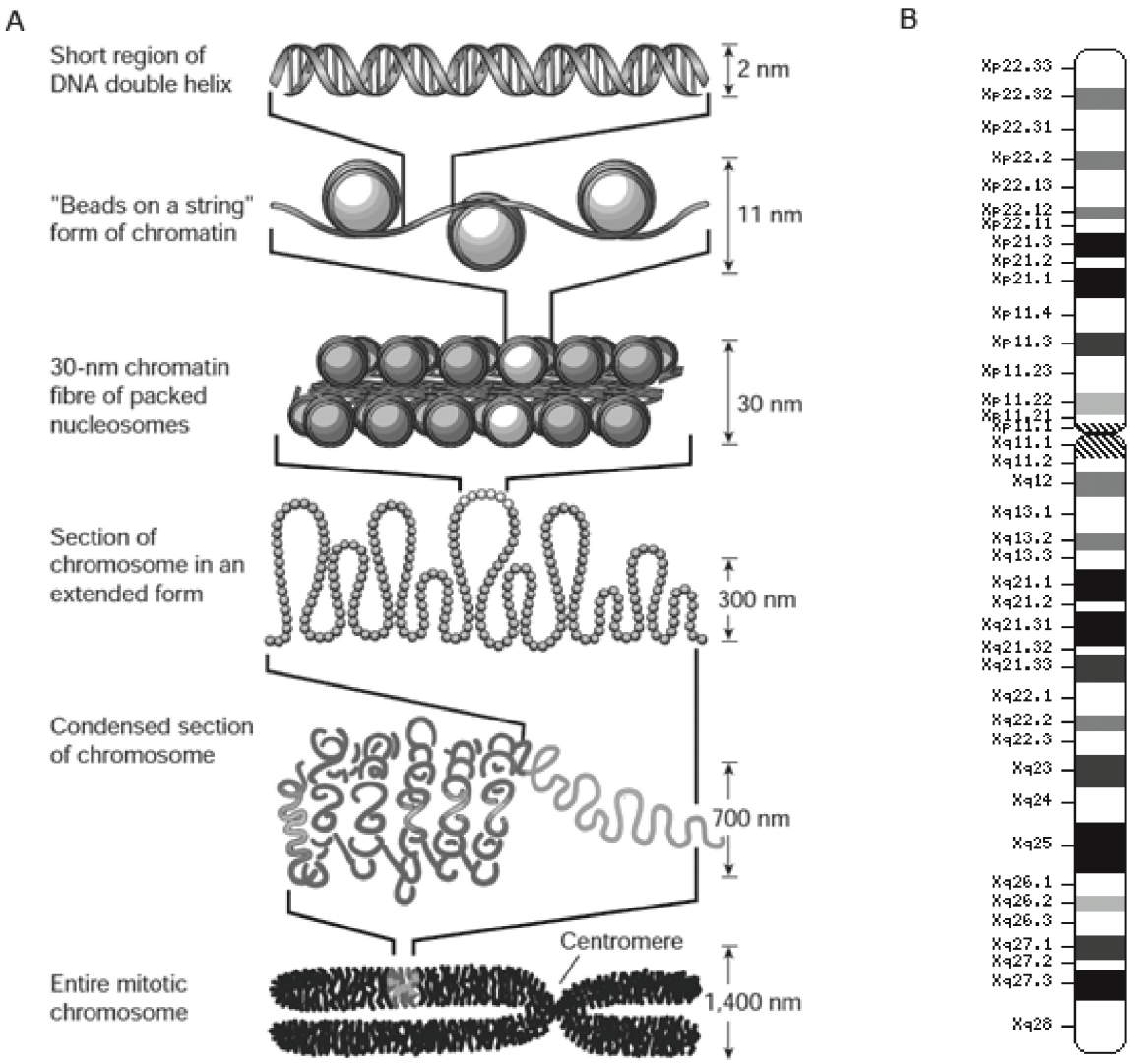

Figure 3 A) Overview of the DNA organization. Nucleosomes are the core packaging units which are densely packed into chromosomes as the cell enters mitosis [15]. Reprinted with permission from Macmillan Publishers Ltd: Nature (Felsenfeld &Groudine, 2003), © 2003

B) Schematic picture of the X chromosome, displaying the banding pattern obtained by Trypsin/Giemsa staining (NCBI).

1.2 Cancer

The term cancer refers to a collection of diseases caused by uncontrolled cell proliferation. In Sweden, approximately 50.000 patients are diagnosed with cancer each year, and the overall 10 year survival is about 60% [17]. There are many different types of cancer, typically named according to the cell type of origin. The most common cancers among adults are prostate cancer (34% of all cancers in men) and breast cancer (29% of all cancers in women) [17, 18]. In Sweden, approximately 300 children are diagnosed with cancer each year, with leukemia and lymphoma as the most common cancer forms (40%) followed by brain cancer (30%) [17]. Neuroblastoma accounts for 6-10% of all childhood cancers, and is the most common cancer among infants [19, 20].

Although surprisingly dissimilar, all types of cancer share a common set of characteristics essential for the development of an aggressive tumor. The classic review "The hallmarks of cancer" by Hanahan and Weinberg [21], lists these as: (i) sustaining proliferative signaling, (ii) evading growth suppressors, (iii) resisting cell death, (iv) enabling replicative immortality, (v) inducing angiogenesis, and (vi) activating invasion and metastasis. Recently, (vii) reprogramming of energy metabolism, and (viii) evading immune destruction, have also been added to this list [22]. These tumor characteristics are acquired through genetic or epigenetic changes in the tumor cells, a sequential process that requires multiple cell divisions. Typically, some of these genetic events affect DNA repair processes or the checkpoint systems controlling them, thus leading to genome instability in the tumor cells. This allows the cancer cells to acquire mutations at a much higher frequency as compared to normal cells.

Generally there are two main types of genes involved in cancer; oncogenes and tumor suppressor genes. Oncogenes are growth promoting genes that normally act to initiate cell division, while, tumor suppressor genes normally have either a growth repressing or DNA protective role, e.g. ensuring that cells do not continue to divide in the presence of DNA damage. In cancer, aberrant inactivation or activation of genes is commonly achieved through genetic alterations such as mutations, deletions, gains, amplifications and translocations. Loss of heterozygosity (LOH) is also a common phenomenon in the cancer genome. This refers to a situation where a previously heterozygous region of DNA is converted into either a homozygous state, containing two identical copies of the DNA, or a hemizygous state where one copy is lost. Additionally, epigenetic dysregulation [23], or altered expression of micro RNAs [24] may cause a shift in the expression profile such that genes that are normally active in a particular cell type are silenced and vice versa.

A tumor suppressor gene generally requires two independent hits in order to be inactivated. Commonly the first hit occurs through mutation or epigenetic silencing, while LOH, either through hemizygous deletion or gene conversion, accounts for the second hit. In hereditary cancers, the first hit usually consists of a mutation that has

been inherited from parent to child; hence only one hit is required for complete inactivation of this gene [25]. Alternatively, for some tumor suppressor genes, loss of only one copy seems to be enough to confer the transformed phenotype; a phenomenon referred to as haploinsufficiency [26].

In contrast, oncogenes are usually affected in a dominant genetic manner either by gene amplification, gene fusion, or activating point mutations [27]. These alterations typically lead either to a higher activity than normal or to activity under the wrong circumstances. Furthermore, the oncogenic activity is typically not dependent on inactivation of the second allele. Hence one mutated copy is usually enough to achieve the transforming properties.

1.3 Neuroblastoma

The term neuroblastoma was first introduced in 1910 by the pathologist J. H. Wright, describing a set of childhood tumors with features of neuronal origin [28]. Neuroblastoma is believed to stem from immature cells of neural crest origin, and the primary tumors are typically found in the adrenal medulla or along the paraspinal sympathetic ganglia. It is a clinically heterogeneous disease, with some tumors showing spontaneous regression while others are highly aggressive, often leading to progression despite intensive therapy. The most common metastatic sites are bone, bone marrow and lymph nodes, although a separate metastatic pattern, confined to the liver and skin, is often seen in infants [20].

Neuroblastoma mainly affects small children, with a median age at diagnosis of only 18 months, and with 75% of the patients diagnosed before the age of four [20]. In Sweden, 15-20 children are diagnosed with neuroblastoma each year [19, 29], and the yearly incidence rate of neuroblastoma for 1983-2007 was 10.3 cases per million children [30].

The symptoms of neuroblastoma are usually diffuse and depend largely on the location of the primary tumor as well as the presence and location of metastases. Patients with a local disease sometimes present with severe abdominal pain, while in other cases, the patient is free of local symptoms and the tumor is incidentally discovered [31]. In contrast, patients with metastatic neuroblastoma are typically quite ill at diagnosis, presenting with unspecific symptoms such as fever, pallor and anorexia. Metastatic specific symptoms, such as bone pain, limping and sometimes even marrow failure, related to bone or bone marrow metastases are also common [31].

The diagnosis of neuroblastoma requires either histopathological characterization of tumor tissue or the combined finding of tumor cells in the bone marrow, and an increased level of catecholamines in urine or serum [32, 33].

1.3.1 Familial neuroblastoma

About 1-2% of neuroblastoma patients have a family history of the disease [31]. However, since advanced neuroblastoma commonly results in death before the patient has reached reproductive age, the true frequency of hereditary mutations is hard to estimate. In 2008, the *ALK* gene, located to 2p23.2, was found to be mutated in 8 out of 14 families with well documented familial neuroblastoma (either more than three affected individuals, or two affected individuals of first-degree relation) [34]. The *ALK* gene encodes a receptor tyrosine kinase that is expressed at high levels during the development of the nervous system [35]. Furthermore, it is commonly activated through gene-fusion in anaplastic large cell lymphomas and inflammatory myofibroblastic tumors [36].

The most common *ALK* mutation in familial neuroblastoma results in a change from arginine to glutamine at amino acid 1275 (R1275Q), but other mutations, such as T1087I, T1151M, G1128A, and R1192P have also been reported [34, 37-39]. Several of these mutations show incomplete penetrance, leaving many carriers of the mutation free of disease [34, 37]. Mutations in the *ALK* gene have also been found in sporadic neuroblastoma (see below).

Familial neuroblastoma is also associated with some neural-crest related developmental disorders, such as central congenital hypoventilation syndrome and Hirschprung´s disease, causing a lack of ganglia in the colon [40]. In most of these cases, mutations are found in *PHOX2B*, a gene associated with the development of the peripheral nervous system [41, 42]. However, this gene is rarely found to be mutated in sporadic tumors [43, 44].

1.3.2 Somatic genetic alterations in neuroblastoma

A majority of neuroblastoma tumors arise spontaneously through genetic and epigenetic changes in somatic cells (i.e. non-germ line cells). Sporadic neuroblastoma is usually associated with a multitude of large scale genetic aberrations, such as gain or amplification of genetic material or hemizygous deletions. These DNA copy number changes are generally divided into numerical aberrations, affecting whole chromosomes, and segmental changes, affecting only a part of the chromosome. However, except for the *MYCN* amplification (see chapter 1.3.2.3), the driving genes of these regions are largely unknown.

1.3.2.1 Ploidy

The overall copy number or ploidy of neuroblastoma tumors has been found to be of prognostic importance, at least in infants, and is currently included as a prognostic marker in the treatment stratification of neuroblastoma [45]. Favorable tumors of lower stages are generally associated with a hyperdiploid or near-triploid karyotype while more aggressive neuroblastomas are near-diploid or near-tetraploid [46, 47]. This seemingly odd distribution probably reflects different modes of acquisition of the genetic aberrations, such that the diploid or tetraploid cases are affected with segmental

aberrations resulting from a chromosomal instability phenotype, while the near-triploid cases have a mitotic dysfunction causing loss and gain of whole chromosomes [20, 48].

Lately, genome-wide copy number analyses have found that the favorable neuroblastomas with a near-triploid DNA content are associated with a '*numerical only*' profile consisting solely of whole chromosome gains and losses [49-52]. Hence, it seems that it is the presence or absence of segmental aberrations that is predictive for the prognosis, rather than the actual ploidy [50, 53-55].

1.3.2.2 1p deletion

Hemizygous deletion of a part of the short arm of chromosome 1 (1p loss) occurs in 25-30% of neuroblastoma tumors [45, 51, 56], and has been found to be associated with metastatic disease and a poor prognosis [57, 58]. The deletion is quite large, and typically extends all the way to the telomere [57, 59]. Moreover, homozygous deletions are rare and have this far only been encountered in a neuroblastoma cell line [60, 61]. Nevertheless, many groups have engaged in the search for a tumor suppressor gene believed to be located in this region.

The shortest region of overlap (SRO) is generally located to 1p36.2-3, although there is no ultimate consensus between research groups as to the exact boundaries [59, 62-64]. The SRO also differs depending on the *MYCN* status of the tumors, with the non-*MYCN* amplified tumors having a smaller and more distal SRO (0-10Mb), compared to the *MYCN* amplified tumors (17-32Mb) [59, 65]. Additionally, interstitial deletion at 1p32-34 has been reported in a few tumors [57, 66]. It therefore seems likely that there is more than one putative tumor suppressor gene on 1p.

1.3.2.3 Chromosome arm 2p

Neuroblastoma cells frequently have cytogenetic signs of gene amplification, either as double minutes (DMs), i.e. small extra-chromosomal markers, or as homogeneously staining regions (HSRs) inserted into a chromosome. In 1983, these abnormal structures were shown to contain the *MYCN* gene, normally located at 2p24 [67]. This amplification, sometimes containing more than 100 copies of the *MYCN* gene, is present in 15-30% of neuroblastoma tumors and is known to be a marker of poor prognosis [45, 51, 56]. Although some neighboring genes, e.g. *NBAS* and *DDX,* are sometimes co-amplified, *MYCN* is the only consistently included gene, and is believed to be the primary target of the amplification [68].

The *ALK* gene (2p23.2), found to be mutated in a majority of familial cases, is also affected in sporadic neuroblastoma. Somatic *ALK* mutations occur in 4-11% of neuroblastoma tumors, with the most common alterations being F1174L and R1275Q [34, 37, 39, 69]. Both these mutations cause changes in the tyrosine kinase domain of the ALK protein, leading to auto-phosphorylation and activation of this receptor tyrosine kinase [38].

Furthermore, amplification of this gene has been found in 2-4% of primary neuroblastoma tumors, and a low copy gain at 2p, has been reported in 17-50% of neuroblastoma tumors [34, 37, 39, 69]. Although the 2p gain region is large, it is intriguing to note that both *MYCN* and *ALK* are typically included.

1.3.2.4 11q deletion

Hemizygous deletion of a part of chromosome arm 11q has been identified as a risk factor of importance for the identification of high-risk neuroblastomas that lack *MYCN* amplification [49, 58, 70, 71], and it has now been included in the treatment stratification of neuroblastoma [45]. Deletions of the 11q arm occur in 15-20% of neuroblastoma tumors [45, 51, 56], usually starting somewhere between 69-84.5 Mega bases (Mb) (11q13.3-14.1) and extending all the way to the telomere [49].

1.3.2.5 17q gain

The most common somatic change in neuroblastoma is 17q gain, present in 40-50 % of all neuroblastomas [45, 51, 56, 72]. This aberration often appears in aggressive neuroblastoma tumors and is correlated with 1p deletion, *MYCN* amplification and 11q deletion [72]. The breakpoints are quite variable, although most of the gains start around 30-50Mb and extend to the telomere [65, 73-75]. The 17q gain is believed to arise as a result of an unbalanced translocation, with chromosome 1 and 11 as the most common translocation partners [72, 76-78].

1.3.3 Expression profiling in neuroblastoma

Already in 1993, expression of the neurotrophic tyrosine kinase receptor *NTRK1* (also known as *TRKA*) was found to be linked to the outcome of neuroblastoma patients. High mRNA expression of this receptor tyrosine kinase was found in favorable neuroblastoma tumors, while low or absent expression correlated with a poor prognosis [79-81]. Shortly thereafter, another neurotrophin receptor, *NTRK2* (also known as *TRKB*), was identified. This receptor has two isoforms that are differentially expressed in neuroblastoma tumors. The full length version is preferentially expressed in high stage *MYCN* amplified neuroblastoma tumors [82]. Furthermore, it is often co-expressed with its ligand brain-derived neurotrophic factor (*BDNF*), thus indicating the presence of an autocrine or paracrine loop [83]. In contrast, the truncated version, lacking the tyrosine kinase domain, is usually found in maturing neuroblastoma tumors, while *MYCN* non-amplified tumors generally do not express *NTRK2* [82].

The general mRNA expression profile in neuroblastoma has been extensively studied, resulting in several gene expression profiles indicative of the prognosis for neuroblastoma patients [84-91]. Many of these profiles include an extensive set of genes and are hence impractical to evaluate in a clinical setting. Recently however, Garcia et al. [92] proposed a three gene signature capable of separating the low- and high-risk neuroblastomas. These genes can be assayed with a quantitative PCR assay, making clinical use feasible.

1.3.4 Risk group stratification of neuroblastoma patients

Although staging systems for neuroblastoma have been in use since the 1970s [93], there was no consensus as to which system to use until the International Neuroblastoma Staging System (INSS, Table 1) was proposed in 1986 [94, 95]. This staging system takes into account the age at diagnosis, the spread of disease, and the resectability of the tumor, grading the tumors from stage 1 to 4, with stage 4 being the most aggressive tumors. There is also a special stage called 4s, defined as children < 1 year of age with a localized primary tumor and metastases limited to the liver, skin and/or bone marrow (<10% tumor cells in bone marrow). These children have a very good prognosis despite the metastatic appearance, and their tumors may even regress spontaneously [95].

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 nonadherent lymph nodes negative for tumor microscopically.
2B	Localized tumor with or without incomplete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
3	Unresectable unilateral tumor infiltrating across the midline*, 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† (limited to infants < 1 year of age).

Table 1 The international neuroblastoma staging system (INSS) [95].

Multifocal primary tumors (e.g. bilateral adrenal primary tumors) should be staged according to the greatest extent of disease, as defined above, and followed by a subscript letter M (e.g. 3M).

*The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.

†Marrow involvement in stage 4S should be minimal, i.e. < 10% of total nucleated cells identified as malignant on bone marrow biopsy or on marrow aspirate. More extensive marrow involvement would be considered to be stage 4. The MIBG scan (if performed) should be negative in the marrow.

Together with other factors of prognostic importance, such as *MYCN* amplification, DNA ploidy, and histopathology [96], the INSS has been widely used for the last 20 years for the treatment stratification of neuroblastoma tumors. However, since the INSS relies on surgical resection to determine the stage of the low and intermediate risk groups (stage 1-3), it is not suitable as a pre-treatment staging system. As a consequence, in 2009, the International Neuroblastoma Risk Group Staging System (INRGSS) [32, 45] was developed in order to facilitate the generation of homogenous pre-treatment cohorts in risk-based clinical trials performed around the world.

The INRGSS (Table 2), in contrast to INSS, employs a set of 20 image-defined risk factors (IDRFs, Table 3) to distinguish between the low and intermediate stage tumors [32]. This staging system grades the tumors as L1, L2, M and MS. L1 is a local tumor in the absence of IDRFs, while L2 is a local tumor exhibiting at least one of the IDRFs. Stage M, similar to stage 4 of the INSS system, represents tumors with distant metastatic spread, and MS, although with an age-cutoff extended to < 18months, represents the group previously known as INSS stage 4S.

To assign the patients into risk groups, the INRG task force has also established a risk classification schema that, in addition to the INRG stage, includes age, histology, *MYCN* status, 11q status and tumor cell ploidy (Table 4) [45].

Stage	Description
L1	Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment
L2	Locoregional tumor with presence of one or more image-defined risk factors
M	Distant metastatic disease (except stage MS)
MS	Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow

Table 2 The International Neuroblastoma Risk Group Staging System (INRGSS) [32]

Image-Defined Risk Factors
Ipsilateral tumor extension within two body compartments
Neck-chest, chest-abdomen, abdomen-pelvis
Neck
Tumor encasing carotid and/or vertebral artery and/or internal jugular vein
Tumor extending to base of skull
Tumor compressing the trachea
Cervico-thoracic junction
Tumor encasing brachial plexus roots
Tumor encasing subclavian vessels and/or vertebral and/or carotid artery
Tumor compressing the trachea
Thorax
Tumor encasing the aorta and/or major branches
Tumor compressing the trachea and/or principal bronchi
Lower mediastinal tumor, infiltrating the costo-vertebral junction between T9 and T12
Thoraco-abdominal
Tumor encasing the aorta and/or vena cava
Abdomen/pelvis
Tumor infiltrating the porta hepatis and/or the hepatoduodenal ligament
Tumor encasing branches of the superior mesenteric artery at the mesenteric root
Tumor encasing the origin of the coeliac axis, and/or of the superior mesenteric artery
Tumor invading one or both renal pedicles
Tumor encasing the aorta and/or vena cava
Tumor encasing the iliac vessels
Pelvic tumor crossing the sciatic notch
Intraspinal tumor extension whatever the location provided that:
More than one third of the spinal canal in the axial plane is invaded and/or the perimedullary leptomeningeal spaces are not visible and/or the spinal cord signal is abnormal
Infiltration of adjacent organs/structures
Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block, and mesentery
Conditions to be recorded, but not considered IDRFs
Multifocal primary tumors
Pleural effusion, with or without malignant cells
Ascites, with or without malignant cells

Table 3 Image-Defined Risk Factors in Neuroblastic Tumors [32]. Abbreviation: IDRFs, image-defined risk factors.

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

Table 4 International Neuroblastoma Risk Group (INRG) Consensus Pre-treatment Classification schema [45]. Pre-treatment risk group H has two entries. 12 months = 365 days; 18 months = 547 days; blank field = "any"; diploid (DNA index ≤ 1.0); hyperdiploid (DNA index > 1.0 and includes near-triploid and near-tetraploid tumors); EFS, event-free survival; very low risk (5-year EFS > 85%); low risk (5-year EFS > 75% to ≤ 85%); intermediate risk (5-year EFS ≥ 50% to ≤ 75%); high risk (5-year EFS < 50%). GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; M, distant metastatic disease (except stage MS); MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age (for staging details see Cohn et al. [45] and Monclair et al. [32])

1.3.5 Treatment options

Neuroblastoma therapy is stratified according to the risk grouping described above. Due to the extreme heterogeneity in the clinical behavior of this disease, a challenging task is not only to find a cure for the high-risk patients, but also to avoid over-treatment for the patients with a favorable prognosis.

Surgical removal of the primary tumor remains an important treatment element for neuroblastoma tumors. For localized tumors, surgery is the treatment of choice, while for metastatic tumors, its use has been debated [97, 98]. However, as local relapse is common in patients with metastatic disease, surgical removal of the primary tumor is recommended in most high-risk treatment protocols [33].

Chemotherapy is mainly used in the treatment of the intermediate and high-risk subtypes of neuroblastoma that requires a systemic approach due to metastases and/or advanced localized disease. The aim of chemotherapy is to reduce tumor size in order to facilitate surgical removal and to eliminate metastatic disease [33, 99]. There are many chemotherapeutic agents used in neuroblastoma treatment today, such as alkylating drugs (cyclophosphamide, busulphan, melphalan), vinca-alkaloids (vincristine), antracyclines (doxorubicin), and platinum analogues (cis-platinum, carboplatinum) [33]. Further options include agents such as topotecan, irinotecan, and temozolomide that are currently being tested in phase II clinical trials [100, 101]. Initial therapy (induction) consists of intensive therapy with several agents, such as the COJEC protocol (cisplatin (C), vincristine (O), carboplatin (J), etoposide (E), and cyclophosphamide (C)) that is used in the SIOP high-risk neuroblastoma study. Most high-risk protocols also include consolidation with high-dose chemotherapy, combined with autologous stem cell rescue, in order to eliminate residual disease after induction therapy and surgery [33, 99].

Although neuroblastoma tumors are generally sensitive to radiation therapy, it is associated with severe late effects. External beam radiotherapy (EBRT), applied to the primary site of the tumor, is therefore avoided in most primary treatment protocols for low-and intermediate stage patients. However, in high-risk patients, EBRT against the primary tumor site is used during the consolidation phase, and patients with refractory or relapsed localized tumors may also benefit from radiation [33].

Although many patients with advanced stage tumors respond quite well to initial treatment, they often experience relapse, which is the result of minimal residual disease. Since retinoic acid has long been known to induce differentiation in neuroblastoma cells [102, 103], a common way to deal with minimal residual disease in neuroblastoma involves different forms of retinoic compounds. Today, most high-risk treatment protocols include a maintenance phase following high-dose chemotherapy, where 13-cis-retinoic acid is given in order to induce differentiation or apoptosis in any remaining tumor cells [33, 104, 105].

1.3.5.1 Current treatment strategies

Many of the tumors with INSS stage 4S regress spontaneously and patients without severe symptoms and unfavorable prognostic markers usually do not require therapy. They are closely monitored and treatment is initiated only in case of disease progression [33].

Similarly, localized neuroblastoma tumors are primarily treated with surgery, if feasible with respect to surgical risk factors. In some instances an induction treatment with chemotherapy is needed in order to shrink the tumor prior to resection. However, a clinical trial in which patients with INSS stage 1 and 2, without unfavorable prognostic markers, were randomized either to surgery or a '*wait-and-see*' strategy, showed that 47% of the tumors regressed spontaneously [106]. This indicates that a thorough molecular characterization of each tumor can identify which patients need intensive therapy, and which patients may be spared from unnecessary treatments. It is therefore possible that, this strategy will be applied to a wider range of patients in the near future.

For patients with intermediate risk, the preferred treatment is surgery combined with moderate doses of chemotherapy. However, due to the excellent prognosis of this patient group it has been discussed whether the amount of chemotherapy could be further reduced. In contrast, high-risk neuroblastoma is usually treated with a highly intensive therapy, combining multiple chemotherapeutic agents, surgery, radiation therapy, autologous stem cell rescue and treatment with cis-retinoic acid [33, 99].

1.3.5.2 Novel therapies

At present, only about half of the patients with high-risk neuroblastoma are completely cured from their disease. Furthermore, there is a substantial amount of toxicity and late side effects associated with the current treatment strategies. This calls for the development of novel therapeutic drugs that target specific properties of the tumor. However, in order to find targets suitable for therapeutic intervention, thorough molecular characterization is needed.

Although this scenario of a personalized treatment strategy is still set largely in the future, the inhibitor Crizotinib (PF02341066), targeting the receptor tyrosine kinase *ALK*, is now being tested in a clinical trial for the treatment of neuroblastoma patients with mutated or amplified *ALK* (clinical trial: NCT00939770). However, recent reports have indicated that the F1174L mutation, commonly occurring in neuroblastoma, confers at least partial resistance to Crizotinib [107, 108]. Hence, higher doses of this drug, or the use of a structurally different inhibitor, such as TAE-684, will probably be needed to treat these patients [107-109]

There are also several large scale efforts dedicated to the search for new therapeutic agents for neuroblastoma treatment. One such effort is the Pediatric Preclinical Testing Program (PPTP), which uses mouse models of pediatric cancers, to screen for drugs effective against these diseases. This screening showed that the inhibitor MLN8237,

targeting the Aurora A kinase (AURKA), had a broad activity against neuroblastoma tumors [110]. This kinase plays a central role for the segregation of chromosomes at mitosis, and overexpression of this gene results in centromere duplication and aneuploidy [111]. Furthermore, it has been shown to prevent degradation of the MYCN protein [112], and overexpression of *AURKA* is associated with high-risk neuroblastoma and *MYCN* amplification [113]. The inhibitor MLN8237 is now in phase I/II trials for the treatment of neuroblastoma (clinical trials: NCT00739427 and NCT01154816).

1.4 Molecular genetic methods in neuroblastoma

Ever since the prognostic importance of 1p deletion and *MYCN* amplification was established some 30 years ago [57, 114], attempts have been made to stratify patients into suitable treatment groups based on genetic testing. Generally, there are two main types of aberrations to be assayed for: small scale alterations such as point mutations and insertions/deletions in specific genes, and large scale aberrations such as translocations, or gains and losses of large chromosomal regions. The latter type, with the exception of balanced translocations, also cause a change in the DNA copy number at the affected region, which can be detected using a variety of molecular methods.

For the purpose of treatment stratification in neuroblastoma, assays against small scale alterations have been of limited use, mainly due to the few genes known to be of prognostic importance. In contrast, detection of large scale copy number changes in DNA has successfully been used for treatment stratification. Both isolated genomic changes, as well as the copy number profile for the entire genome have been shown to be informative for the prognosis of the patients [51-55, 115, 116].

The methods available for DNA copy number detection have changed over the years, from the Trypsin/Giemsa staining of metaphase chromosomes in the 1970s, through fluorescence in situ hybridization (FISH) and comparative genome hybridization (CGH), to the many types of multilocus analysis techniques, e.g. multiplex ligation-dependent probe amplification (MLPA) [117], or the high density microarrays used today [118]. Generally the methods have increased either in precision, enabling the discovery of smaller genetic aberrations, or in generality, covering the entire genome at a higher and higher resolution.

1.4.1 Fluorescence in situ hybridization (FISH)

With the introduction of the FISH technique in the late 1970s [119, 120], genetic analysis was no longer limited to the dense chromosomes found in metaphase, but also the more dispersed DNA of cells in interphase, could be analyzed. This also made the direct analysis of tumor biopsies feasible, since cell culturing in order to achieve cells synchronized in mitosis was no longer required. This was a major breakthrough, especially for solid tumors such as neuroblastoma, for which obtaining metaphase spreads of the required quality have been difficult.

The FISH technique is based on a fluorescently labeled DNA probe that is hybridized to the DNA of the cells to be studied [118]. The probe is complementary to the gene/region of interest, and the hybridization occurs directly in the nuclei of cells attached to a microscope slide. The result is then obtained by viewing the slide in a fluorescence microscope, recording the number of fluorescent signals per cell.

FISH is commonly performed as a two fluorophore experiment, using one probe for the region of interest while the second, differently labeled probe is targeting a region believed to reflect the overall copy number of the tumor, i.e. a control probe. It is also possible to do FISH in a highly multiplex setting, with probes covering entire chromosomes. In 1996, two techniques for 24-color chromosome painting were published; multiplex-FISH (M-FISH) [121] and spectral karyotyping (SKY) [122]. These highly similar techniques have been widely used in the field of cancer biology, especially for the detection of translocations. Although these techniques have been used on interphase nuclei to determine the spatial localization of chromosomes [118], using them for karyotyping purposes yet again requires the cells to be in metaphase.

The resolution of FISH, has also improved over the years from approximately 5Mb, using metaphase spreads, to 50kb-2Mb in interphase cells, and down to 5kb-500kb using fibre-FISH [118]. Although far better than the ~10 Mb resolution obtained with techniques such as Trypsin/Giemsa staining, traditional FISH requires a priori knowledge about the target(s) of interest. Alternatively, using the chromosome painting techniques, provides a genome-wide analysis, but at the cost of a lower resolution (~10Mb). It should also be noted that regular M-FISH/SKY is designed to detect inter-chromosomal rearrangements, such as balanced or unbalanced translocations, where two separate chromosomes are connected to each other, whereas rearrangements occurring within a chromosome, such as inversions, small deletions or tandem-duplications will go unnoticed.

In neuroblastoma, interphase FISH has commonly been used for the detection of single alterations such as *MYCN* amplification, and 1p deletion [123, 124]. Although microarray techniques are increasingly being used for the analysis of these aberrations, *MYCN* analysis by FISH is still valuable as a first analysis due to its rapid processing time.

1.4.2 Comparative genome hybridization (CGH)

At the end of the 1990s, comparative genome hybridization (CGH) entered the field of DNA copy number detection [125]. This method makes use of a normal metaphase spread as a backbone for hybridization. However, the DNA to be analyzed could be retrieved from any phase of the cell cycle, making this method feasible also for the direct analysis of tumor tissue. Whole genome DNA from both a test- and a reference sample are then labeled with two different fluorescent dyes and allowed to competitively hybridize to the metaphase backbone. The ratio of fluorescence intensity

for the two dyes is then calculated in order to detect the copy number alterations of the test sample [118].

The output is given as a relative change in copy number between test and reference sample, without any structural information about the gains and losses. Therefore, rearrangements such as balanced translocations that do not cause a change in copy number will not be detected [118]. The resolution of CGH depends largely on the copy number of the target sequence. Although high grade amplifications as small as 2Mb have been detected using CGH, the resolution for low copy gains and losses is rather around 10-20Mb [126]. However, the main advantage of CGH is that it does not require any *a priori* knowledge about the target, making it suitable as a discovery tool.

In neuroblastoma genetics, CGH has been used extensively for the genome-wide detection of copy number changes. It has been an important method, either for the discovery of specific gains such as the 17q gain [72, 127], or for genomic profiling in the context of treatment stratification [52, 115, 128]. Lately however, CGH has been replaced with array based methods due to its low resolution.

1.4.3 Micro-arrays

The term micro-array refers to a large collection of DNA probes arranged in an orderly manner, either on a solid surface, such as a silica chip, or on a set of microscopic beads. Based on their intended usage, micro-arrays can be divided into two main categories. Mapping arrays detect levels of DNA copy number, either genome-wide or for a particular region, while expression arrays measure the relative level of RNA for a defined set of genes. The general principles for these different types of arrays however are similar. As is shown in Figure 4, micro-arrays depend on the hybridization of a labeled sample of RNA/DNA to DNA probes attached to the surface of a chip or beads. A laser then excites the fluorescent molecules and a scanner is used to record the fluorescence intensity for each spot on the array [118].

Micro-arrays can be further divided into subtypes depending on: 1) their mode of manufacturing (probes can either be spotted onto the surface or synthesized directly on the surface), 2) their mode of comparison (one-channel vs. two-channel), and 3) the type and size of probes used (BACs, oligonucleotides, SNP-specific, methylation specific etc.).

1.4.3.1 Array comparative genome hybridization (aCGH)

The first arrays to be used for mapping purposes consisted of a library of long stretches of DNA, such as bacterial artificial chromosomes (BACs) or P1 derived artificial chromosomes (PACs), spotted onto a glass slide or silica membrane [129, 130]. These arrays were typically run as a two channel experiment, comparing two samples, generally tumor DNA and its matched control, on the same array. As the name implies, the procedure is similar to that of traditional CGH. The samples are labeled with two different fluorescent dyes, typically Cy3 (green) and Cy5 (red), and

then allowed to competitively hybridize to a single array. The fluorescence intensity for each dye is then recorded for each spot on the array, and the resulting ratio is used to determine the copy number changes of the test sample [118].

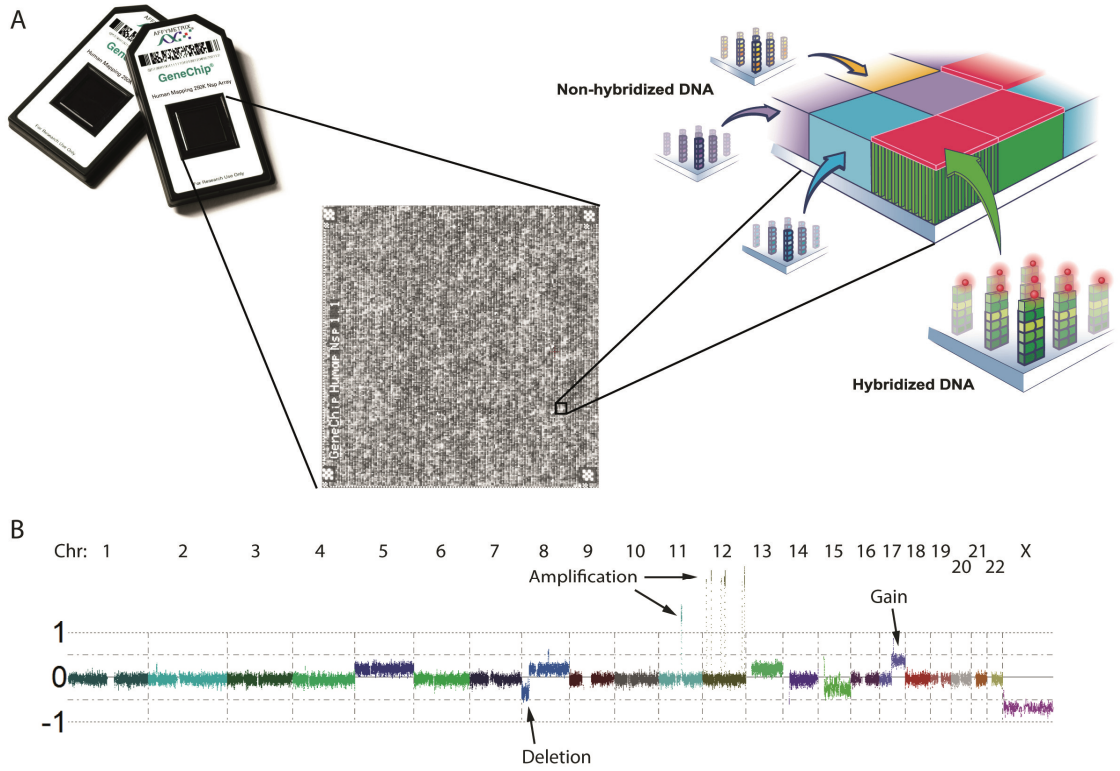

Figure 4 A) The microarray-principle, as exemplified by the Genechip® 250k NSP SNP-array (Affymetrix). The array contains assays against approximately 250.000 SNPs, distributed in small rectangular features on the array. Fluorescently labeled DNA is hybridized to the array, and the fluorescent intensity for each feature is then detected. The photograph of array cartridges and the schematic representation of the array features are reproduced with courtesy of Affymetrix

B) SNP array output for copy number estimation. The overall copy number for each probe is shown, on a logarithmic scale, color coded for each chromosome. The appearances of gains, deletions and amplifications, are indicated with arrows.

The resolution of aCGH depends on the number of probes spotted to the array, as well as the size and genomic location of the individual probes. Generally the trend has been to move from the large BAC or PAC clones (~100kb) to much smaller oligonucleotides (25-60bp) in order to improve the resolution [131]. Oligonuclotide arrays are now commercially available through companies such as Agilent Technologies and Roche Nimblegen. These companies provide a variety of arrays suitable for copy number detection, targeting the human genome with a median probe spacing of about 2.1kb. It is also possible to order custom made tiling arrays for smaller regions of the genome. These arrays typically have a probe spacing of

approximately 100bp, thus making even small micro deletions visible [132, 133]. Array CGH has now replaced conventional CGH as the method of choice for genome wide copy number detection in neuroblastoma [51, 53, 55, 116, 133-138].

1.4.3.2 SNP-arrays

Single nucleotide polymorphisms (SNPs), the most common source of genetic variation in the human genome, refers to genomic positions where two or more bases are found in the population. With an estimated frequency of about 10 million SNPs, evenly dispersed across the human genome, they represent a suitable target for large scale association studies aiming to find the causing genes for the disease in question [139]. The assembly of a large number of SNP assays to an array was first described in 2003 [140]. Although this technique was first used for the purpose of high throughput genotyping [140-142], it was soon recognized that these arrays could also be used for copy number analysis [143, 144].

SNP arrays use short oligonucleotides (~25nt) that, in contrast to the aCGH method, are located such that a particular SNP is covered [118]. Separate probes are synthesized to match each of the possible alleles, thus enabling genotyping of the SNP by comparing the fluorescent intensity between the two sets of probes. In addition to genotyping, the fluorescent intensity for each probe also enables the copy number to be inferred from the array [143, 144]. This works in much the same way as with the aCGH described above, although for SNP-arrays a separate intensity measurement is present for each of the alleles, thus enabling allele specific copy number to be analyzed in addition to the total copy number. This feature makes it possible to detect both loss of heterozygosity (LOH) and copy number changes simultaneously [118, 145].

In contrast to aCGH, SNP-arrays are performed as single channel experiments, with only a single sample hybridized to each array. The fluorescent intensities are then compared *in silico*, rather than on the actual array, either to a set of reference samples from healthy individuals or to the matched control sample from the same patient. The resulting copy number plot, similar to that of an aCGH experiment, then shows the change in copy number relative to the controls used. The most common producers of SNP-arrays today are Affymetrix (silica chips) and Illumina (bead arrays), both providing a multitude of available array-formats, ranging from 10.000 to more than a million SNPs per analysis. In neuroblastoma, SNP-arrays have been used both for large-scale genome wide association studies [146, 147], as well as for detection of LOH and copy number changes [65, 75, 148, 149].

Generally, the difference between aCGH and SNP-arrays are starting to decrease. Array-CGH providers such as Agilent have recently started to include SNP-probes on their arrays, while SNP-array providers such as Affymetrix now include a large set of non-SNP probes to their arrays in order to improve the resolution.

2 Objectives

The comprehensive aim of this thesis was to provide a better understanding of the genes and mechanisms underlying neuroblastoma genesis and progression, with the ultimate goal of achieving a basis for personalized diagnostics and treatment in neuroblastoma.

Specific aims:

Paper I

- To evaluate the use of SNP arrays as a diagnostic and prognostic tool in neuroblastoma
- To refine the prognostic subgrouping of neuroblastoma tumors
- To identify genes of importance for the genome instability phenotype observed in the 11q deleted neuroblastoma tumors

Paper II

- To test whether SNP-arrays are useful as a starting point for the generation of tumor- and patient-specific PCR assays that can be used for the detection of minimal residual disease in neuroblastoma
- To investigate possible mechanisms underlying the *MYCN* amplification

Paper III

- To analyze the frequency and distribution of copy neutral loss of heterozygosity in neuroblastoma tumors and cell lines
- To investigate possible mechanisms underlying copy neutral loss of heterozygosity

Paper IV

- To refine the prognostic subgrouping of neuroblastoma tumors through characterization of a new subtype presenting with two high grade amplifications at chromosome arm 12q
- To identify the driving genes of these amplicons with the aim of finding suitable therapeutic targets

3 Material

This thesis is based on the Swedish neuroblastoma material, consisting of approximately 230 neuroblastoma patients. This essentially represents all the patients of the Swedish Childhood Cancer Registry that were diagnosed with neuroblastoma between 1988 and 2011, for which there were tumor samples available for analysis.

Additionally a few neuroblastoma patients from other countries were also included, as well as a set of twelve neuroblastoma cell lines: IMR32, Kelly, NB69, SH-SY5Y, SK-N-AS, SK-N-BE(2), SK-N-DZ, SK-N-FI, SK-N-SH (ECACC, HPA Culture Collections, Salisbury, UK), SH-EP (ATCC, Manassas, VA), LS, and NGP (kindly provided by Prof. Manfred Schwab).

For most of the cases, pretreatment samples of the tumor, either in the form of biopsy or surgically removed tumor, were used for extraction of DNA and RNA. Additionally, for some of the cases, matched blood and bone marrow samples were collected throughout the treatment period.

Informed consent was retrieved from the parents of the patients, and ethical permission was granted by the local ethics committee (Karolinska Institutet and Karolinska University Hospital, registration number 03-736 and 2009/1369).

4 Results and discussion

4.1 Clinical implications of SNP-arrays in neuroblastoma

The clinical heterogeneity of neuroblastoma has long been intriguing researchers and clinicians. Even before the introduction of chemotherapy, it was noted that some patients with neuroblastoma had benign tumors that regressed also in the absence of treatment, while others had a very aggressive disease ultimately leading to progression and death [33]. Furthermore, most of the therapies currently used against cancer are associated with extensive side effects, some of which may affect the patients for the rest of their lives. In the treatment of small children, whose bodies are still growing, this is especially troublesome. The challenge in the field of neuroblastoma is therefore not only to cure the children with high-risk disease, but also to spare the children with a relatively benign tumor from unnecessary treatment.

This ultimately requires a good and reliable treatment stratification that is capable of separating these types of neuroblastoma tumors, in order to be able to provide the best treatment for each patient. In neuroblastoma, genetic changes such as gains and losses of particular regions have been found to follow this pattern of heterogeneity, hence providing a target for treatment stratification. However, although treatment stratification, using genetic factors, has been applied to neuroblastoma for the last 30 years, the diagnostic methods used have either been of poor resolution or focused on a single aberration, such as the *MYCN* gene.

Researchers around the world have now analyzed the genome-wide copy number profiles of neuroblastoma tumors in search for prognostic profiles that are informative for treatment stratification [51-55, 115, 116]. Similarly, we have used high density SNP-arrays (GeneChip® 250k NSP, Affymetrix) to investigate copy number changes on a genome wide basis in the Swedish neuroblastoma material of approximately 230 tumors [49, 65]. In **Paper I**, 165 of these tumors were grouped according to their genomic profile, revealing six subgroups of neuroblastoma: '*numerical only*', '*MYCN amplified*', '*11q deleted*', '*17q gained*', '*other segmental*', and '*flat profile*' (Figure 5). With exception of the '*flat profile*' group, which is probably the result of a too high contamination of normal cells, these subgroups were also analyzed for their impact on the overall survival of the patients.

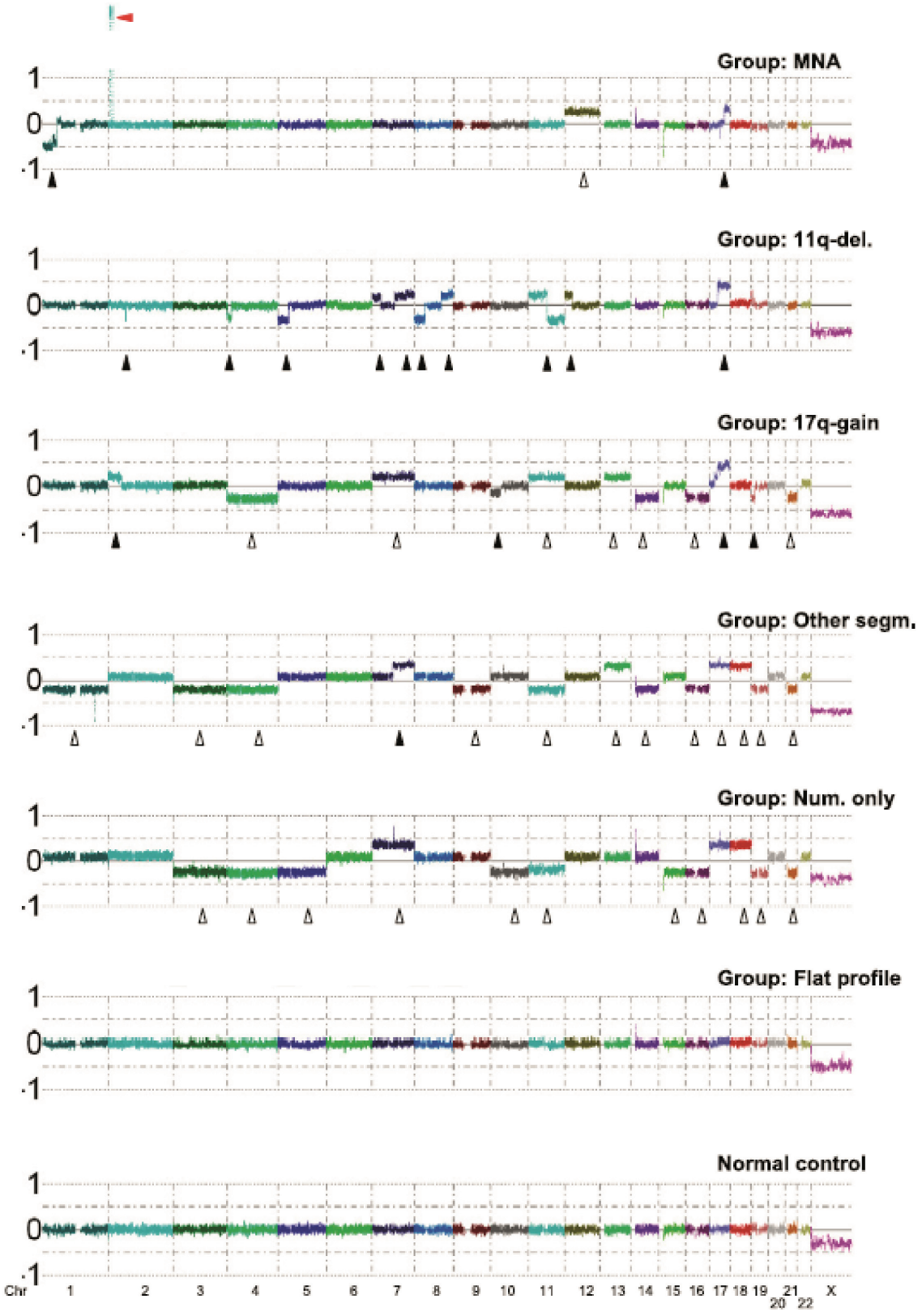

Figure 5 Representative genomic profiles for the six subgroups of neuroblastoma, described in paper I. Profiles are presented on a logarithmic scale as the change in copy number compared to a set of diploid reference samples. Everything above zero represents a gain of genetic material, and everything below zero represents a deletion. All of these profiles show tumors from male patients normalized against a set of female reference samples, hence explaining the low signal of the X chromosome. Red arrow: amplification, black arrows: segmental aberrations, open arrows: numerical changes.

Based on genomic profiles, three main subtypes of neuroblastoma tumors have previously been suggested [51, 52, 116]. These subtypes correlate well with our three largest groups. Type 1 consists of tumors with whole chromosome gains and losses, which corresponds to the '*numerical only*' group in our paper. This group was found to have a very good prognosis (~90% survival), in our material. Type 2A contains tumors with 11q deletion and 17q gain, which match our '*11q deleted*' group. This group was found to have a poor survival (~35%) in our material. Finally, type 2B, characterized by *MYCN* amplification, 17q gain and 1p deletion, corresponds to our '*MYCN amplified*' group. This group was found to have a similar survival as the type 2A group.

Although the observed survival rates in our material were slightly lower than the previously reported survival rates for these groups; 94-100% for type 1, 40-60% for type 2A and 40-50% for type 2B [51, 52, 116], our results show that this classification robustly identifies the main subtypes also in the Swedish neuroblastoma material.

However, we also found that 20% of the neuroblastoma tumors presented with segmental aberrations, but in the absence of *MYCN* amplification and 11q deletion, hence not fitting any of the main groups. These tumors were stratified according to their 17q status, such that the tumors with 17q gain constitute a separate group from the tumors with other segmental changes. This resulted in an intermediate prognosis (~60% overall survival) of the '*17q-gain'* group, and an excellent survival of the '*other segmental'* group (~100% overall survival).

Several research groups have previously pointed to the prognostic importance of the global presence or absence of segmental alterations in neuroblastoma tumors, suggesting that the presence of any segmental aberration confers a worse prognosis compared to a profile with only numerical aberrations [53-55]. The finding that the patients of our '*other segmental*' group, had a 100% survival was therefore a bit surprising. However, as the main discrepancies between classification schemes concern the tumors that do not fit any of the three well characterized subgroups, there are several possible reasons for this discrepancy. In many publications, the tumors corresponding to our '*17q gain*' group have been included in the '*other segmental*' group. Since, the 17q gain was found to be associated with a worse prognosis in our material, exclusion of these tumors might explain the unexpectedly good survival seen in the group with other segmental aberrations. Furthermore, all except one of the '*other segmental'* cases in our material contain only a single segmental aberration in the context of a predominantly numerical profile. Hence, these tumors would have been included in the '*numerical only*' group, had a less stringent cut-off been used.

Our results point to the need for a further refinement of the risk group stratification, and also suggest that the presence or absence of 17q gain is of importance for the stratification of the patients whose tumors do not readily fit any of the three main genomic profiles.

Another important clinical aspect is the follow up of patients after treatment. In the treatment of leukemia and lymphoma it is current clinical practice to use tumor-specific junctions as targets for PCR based detection of tumor cells circulating in the blood or bone marrow [150]. This enables a close follow up of the patients, both during treatment, evaluating the response to therapy, and during follow-up, as an early detection of relapse. These malignancies often contain fusion genes with a recurrent junction, i.e. the position of the rearrangement is the same in many tumors. Hence construction of a PCR assay that targets this junction, and that can be used for tumor detection in a multitude of tumor samples is fairly easy [151, 152].

In several solid tumors, including neuroblastoma, the situation is not that simple. Although the *MYCN* amplicon is recurrent in the sense that it always includes the *MYCN* gene, the endpoints vary over several Mb. The same is true for other genomic aberrations commonly seen in neuroblastoma, such as 1p loss, 11q loss, and 17q gain. However, with a patient-specific approach, it is still possible to design this type of assay. In **Paper II**, we show that the endpoints of the *MYCN* amplicon can be determined with sufficient precision for each patient using SNP arrays, and that combining this with a multiplex PCR, enables the generation of a tumor-specific PCR fragment that can then be used for detection of tumor DNA in blood or bone marrow (Figure 6). This is also supported by the results of Weber et al. [153], although they used a customized tiling array for the mapping of the endpoints.

The INRG task force currently recommends the use of immunocytochemistry with antibodies directed against GD2, or QRT-PCR against tyrosine hydroxylase (TH) mRNA, for the detection of minimal residual disease in neuroblastoma [154]. Although these methods have approximately the same sensitivity (1 in 10^6 cells) as our PCR based assay, they rely on the differential expression of these markers between tumor and normal cells. While both GD2 and TH have been shown to be ubiquitously expressed in neuroblastoma cells, with absent or very low expression in blood or bone marrow, the expression patterns might not be consistent throughout the course of the tumor. Unless absolutely required for tumor cell survival, expression of a particular marker may be down-regulated during tumor evolution, hence resulting in false negative results [155]. Furthermore, detection using RNA put great demands on the handling of samples prior to analysis, as RNA is easily degraded [154]. In our assay, DNA is used instead of RNA, and the target is a tumor-specific junction, not existing at all in normal cells, thus providing a robust method for detection of minimal residual disease in neuroblastoma. Furthermore, since this junction is connected to the amplification of *MYCN*, which is believed to be driving this subtype of neuroblastoma, it is not likely to be altered during tumor evolution.

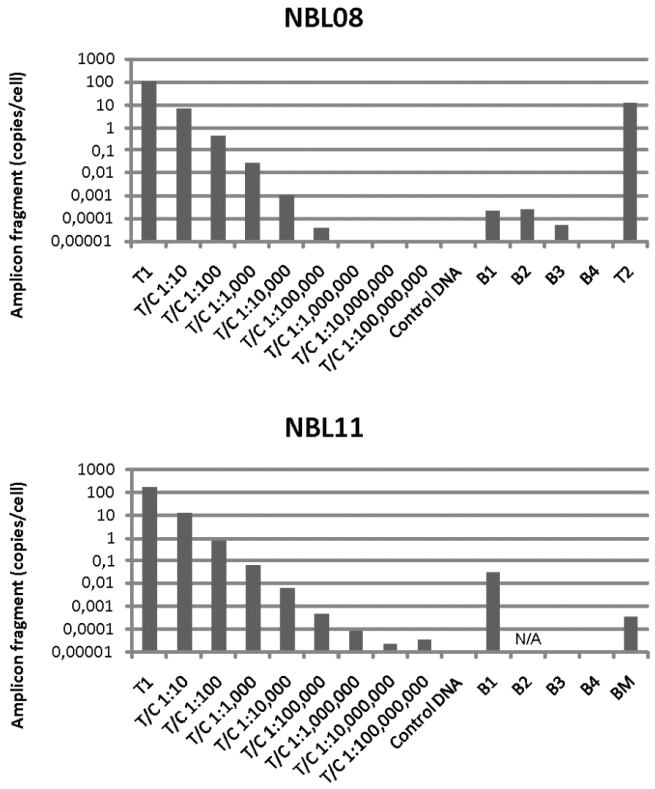

Figure 6 Detection of minimal residual disease using quantitative PCR assays directed against a tumor-specific fragment spanning the *MYCN* amplicon junction. Patient-specific assays were designed for two neuroblastoma patients (NBL08, NBL11) for whom several sequential blood and bone marrow samples were taken during the treatment. These assays were capable of detecting tumor DNA down to a dilution of 1:100,000-1:1,000,000 of tumor DNA diluted with control DNA. Furthermore, the presence of tumor DNA could also be shown in several of the blood and bone marrow samples from the patients. T1: initial tumor biopsy, T2: surgically removed tumor, B1-B4, sequential blood samples taken throughout the treatment of the patients, BM: bone marrow (collected at the same time as B2). Reprinted with permission from Spandidos publications: Int. J. Oncol. (Paper II: Kryh et al., 2011), © 2011

The patient-specific nature of these junctions requires a thorough examination of the endpoints for each patient before the tumor-specific assay can be designed. Although we decided to use SNP-arrays for this purpose, another alternative is to use massively parallel sequencing, a method having the capacity to directly identify the junction sequence [156, 157]. However, at present, the timeframe and computational capacity needed for this analysis is not compatible with standard clinical use. In contrast, SNP-arrays are now routinely used for treatment stratification of neuroblastoma in Sweden. Hence, for a majority of neuroblastoma cases, the SNP-array data will be readily available, thus enabling a quick generation of a tumor-specific assay.

Our results show that a tumor-specific assay can be developed and tested within a few weeks after initial diagnosis, and that these assays are capable of detecting and monitoring tumor DNA in blood or bone marrow of the patient.

4.2 Understanding the mechanisms of neuroblastoma genetics

Ever since the link between DNA amplification and cancer was discovered in the early 1980s [158], researchers have been aiming to understand the mechanisms of this genomic rearrangement. From re-replication models to breakage-fusion bridge cycles, many mechanisms have been proposed over the years [159]. In **Paper II**, we have investigated the detailed structure of the *MYCN* amplicon in four primary neuroblastoma tumors. Two of these tumors carried the amplification as double minutes (DMs), while one had a homogeneously staining region (HSRs), as shown by FISH analysis. Hence, among these tumors, both cytogenetic versions of *MYCN* amplification were represented.

Sequencing the tumor-specific junctions further revealed that the amplicon units were arranged in a head-to-tail tandem conformation, hence confirming previously reported findings [160, 161]. Moreover, the cytogenetic appearance of the amplification did not seem to influence the structure of the junction, as amplicons both in the form of DMs and HSRs shared a similar structure. This is in line with the model by Carroll et al. [162], suggesting that extra chromosomal structures such as episomes or DMs can be re-inserted into a chromosomal location as an HSR.

Furthermore, for each of the four tumors, the sequence at the site of the junction showed only a limited overlap of a few nucleotides (Figure 7), suggesting either non-homologous end joining (NHEJ) or microhomology mediated break induced replication (MMBIR) as possible mechanisms for this rearrangement. The MMBIR mechanism propose that a single double-stranded-break of DNA, resulting from a stalled or broken replication fork, may initiate a rearrangement through a template switch to another open replication fork [163]. This process requires only a few bases of micro homology, and may occur under circumstances when homologous repair is impaired, such as the RAD51 depletion seen in hypoxia [163, 164].

Another aberration sometimes occurring in cancer is copy neutral loss of heterozygosity (CN-LOH) or acquired uniparental disomy (aUPD). This refers to a loss of heterozygosity in the absence of a copy number change, causing the cell to have two identical copies of a certain DNA fragment. In **Paper III**, we investigated the frequency of this aberration in neuroblastoma and showed that although it was uncommon in primary neuroblastoma tumors, it was frequently found in neuroblastoma cell lines. This difference might be an effect of cell line immortalization, reflecting the adaptation of the cell to a life in a dish. Alternatively, CN-LOH might be more common among relapsed tumors, from which most cell lines are derived.

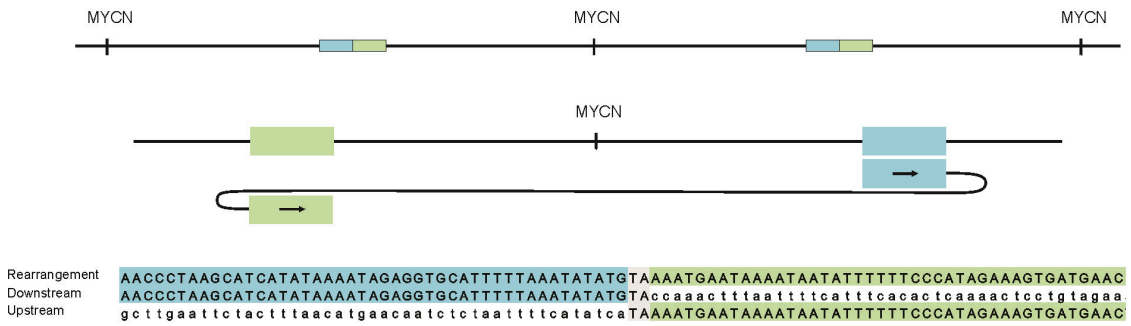

Figure 7 Sequence across the *MYCN* amplicon junction for a representative neuroblastoma sample. Note the limited overlap seen at the actual junction. Blue: sequence originally located distal to the *MYCN* locus, Green: sequence originally located proximal to the *MYCN* locus. Reprinted with permission from Spandidos publications: Int. J. Oncol. (Paper II: Kryh et al., 2011), © 2011

The size and position of the CN-LOH also differed. Three major types of CN-LOH were identified; small interstitial regions; larger telomeric regions extending to the end of the chromosome; and whole chromosome LOH (Figure 8). While the cell lines displayed a strong preference for the telomeric version (74%), with only 21% and 5% of the CN-LOH being of the whole chromosome and interstitial versions respectively, the primary tumors were more diverse with 50% interstitial, 35% whole chromosome, and 14% telomeric events.

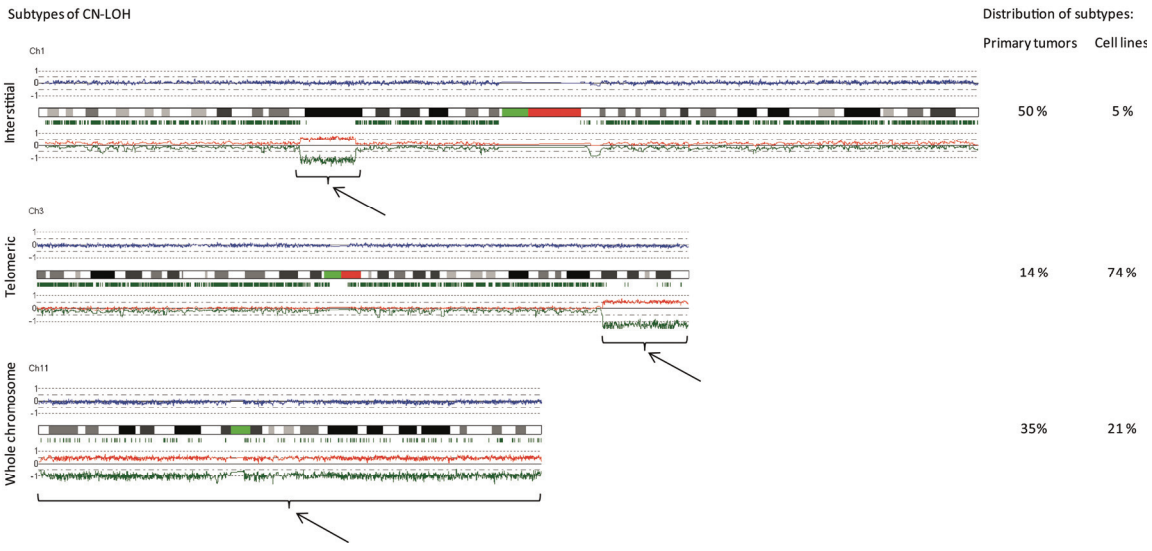

Figure 8 Copy neutral loss of heterozygosity (CN-LOH) in neuroblastoma tumors and cell lines. Three different versions of CN-LOH are shown (indicated with arrows) as well as their relative distribution in the primary tumors and cell lines. Average copy number is shown in blue, allele specific copy numbers are shown in red/green, with red being the allele with the highest intensity.

From a mechanistic point of view this probably reflects different modes of acquisition. Segmental regions of LOH, such as the interstitial and telomeric events, may originate from a double stranded break repair error followed by reduplication of the affected area, or through mitotic recombination between low copy repeats. In contrast, whole chromosome LOH, like other whole chromosome copy number changes, is most likely caused by a mitotic dysfunction such as nondisjunction, causing two sister chromatids to end up in the same daughter cell, or anaphase lag, causing loss of one allele followed by duplication of the remaining allele [165, 166]. Alternatively, a multipolar cell division may give a similar result [48, 167]. In our material, most of the whole chromosome CN-LOH was found in tumors with a numerical only profile, hence supporting the idea of a common mechanism for all whole chromosome aberrations in neuroblastoma.

These results highlight the complexity of neuroblastoma genetics and also show that SNP arrays can reliably identify various versions of allelic imbalances that are undetectable using methods such as array CGH.

4.3 Finding the genes and pathways of neuroblastoma development

Although, DNA copy number profiling has been found to be of prognostic importance for neuroblastoma, the genetic aberrations are usually extensive, and which genes that are responsible for driving the tumor progression remains elusive. Additionally, each tumor usually presents with a multitude of aberrations, making it difficult to determine which aberrations that are drivers and which that are simply passengers resulting from a more general chromosomal instability phenotype.

In **Paper I** we found that the neuroblastoma tumors with 11q deletion have an increased number of chromosomal breaks, hence suggesting a chromosomal instability phenotype in this subgroup of neuroblastoma. Although chromosomal instability is not listed as one of the hallmarks of cancer [21], it allows for tumor cells to acquire further genetic aberrations at an increased pace, and might help the cells acquire cytotoxic resistance.

We hypothesized that one or more genes involved in DNA repair, located in the 11q region would be the cause of this phenotype. This region contains several interesting genes involved in DNA repair mechanisms, such as *MRE11A* (11q21), *ATM* (11q22.3), and *H2AFX* (11q23.3). Among these genes, *H2AFX* was the only one consistently included in the 11q deletion. Furthermore, *H2AFX* was found to have a decreased mRNA expression in the tumors with 11q deletion compared to tumors with an intact chromosome 11. Hence, haploinsufficiency of this gene might be the cause of this defect in DNA repair. However, in most of the tumors, the deletion also included *ATM* and *MRE11A*, suggesting that the simultaneous inactivation of these genes may result in a synergistic effect.

A link between deletion of 11q and DNA repair deficiency has also been found also in other tumors, such as breast cancer [168] and head and neck squamous cell carcinomas [169]. Furthermore, addition of a complete chromosome 11 by micro cell fusion to various cancer cell lines with DNA repair deficiency, have been shown to restore the DNA repair to normal levels, and also to reduce the transforming capacity of these cells [170].

We have also observed amplification or gain of 11q13 in a small subset of neuroblastoma tumors. This region contains the *CCND1* gene that is important for transition through the G1 checkpoint of the cell cycle. Although seemingly contradictive, the starting point of the 11q deletion is almost always located distal to this region. Hence, it seems that although deletion of 11q is generally beneficial for the tumor, loss of the *CCND1* region is not.

Although the introduction of the highly intensive treatment protocol for treatment of high-risk neuroblastoma patients in the 1990s has led to an increased survival for patients with *MYCN* amplified tumors [29], it does not seem to be as successful for the treatment of 11q deleted tumors (Per Kogner, personal communication). This implies that new treatments are needed for this subtype of high-risk neuroblastoma.

Another subtype that might need a modified treatment is the '*12 amp group'* analyzed in **Paper IV**. This small subset of seven neuroblastoma tumors and two cell lines (LS and NGP) share amplification of two separate regions on chromosome 12; at 12q14.1 and 12q15. Furthermore, two of the tumors as well as the cell line LS also presented with amplification of 11q13. Similar amplicons on chromosome 12 [171-176], and 11 [177-179] have previously been found in other tumors, although they have only infrequently been reported for neuroblastoma [51, 116, 180]. The tumors of this subgroup have a peculiar clinical phenotype with a majority of the primary tumors mimicking Wilms tumor. Additionally, three of the tumors were found to have tumors extending into the vena cava, and for two of the patients in this study, as well as for the patient from whom the cell line NGP was derived, the presence of lung metastases was documented, an otherwise uncommon feature of neuroblastoma tumors.

The observed pattern of amplification led us to hypothesize that one or more oncogenes, driving the tumor progression of this subgroup, are located in these regions. Furthermore, a synergistic relationship between these oncogenes, such as an autocrine loop, would be expected from this genomic pattern. In order to investigate which of the genes that drive the tumor progression, we first performed a gene expression analysis for the genes located in the amplified regions. This analysis showed that a majority of these genes responded to the amplification with an increase in gene expression. Additionally, siRNA knockdown revealed that *CDK4* and *CCND1* located in the 12q14 and 11q13 amplicons respectively, both had an impact on cell proliferation and are possible drivers of this subgroup.

CDK4 and CCND1 are important regulators of the cell cycle and their interaction at the start of the cell cycle leads to phosphorylation of RB and progression through the G1 checkpoint [181] (Figure 9). Hence, our findings suggest that cell cycle de-regulation is an important theme in this subtype of neuroblastoma. Furthermore, expression of the *CCND1* gene may also be regulated through the transcription factor GATA3, hence showing high gene expression also in the absence of DNA amplification [182]. Moreover, we have previously detected genetic aberrations of other cell cycle regulating genes in our material, such as the loss of 9p21, containing the CDK inhibitors *CDKN2A* and *CDKN2B* or gain of 7q21 containing *CDK6* [49, 65]. Similar findings have also been reported by Molenaar et al. [183].

Cell cycle de-regulation therefore seems to be a more general theme in neuroblastoma, suggesting that therapeutic drugs targeting these processes might be effective in the treatment of this disease. One interesting option, directly targeting CDK4 and CDK6, is the inhibitor PD-0332991, currently in phase I/II clinical trials for the treatment of a variety of cancers [184, 185].

Alternatively, for tumors with a defective G1 checkpoint, targeting the G2/M checkpoint might be more effective. The simultaneous disruption of both these checkpoints will allow for the cell to proceed through mitosis despite extensive DNA damage [186]. The same holds true for tumors deficient in double-stranded break repair [187]. Hence, in both these cases, the delicate balance of a chromosomal instability phenotype that allows for tumor evolution, but on the same time provides enough genomic integrity for the tumor clone to be able to divide, will ultimately be shifted into a mitotic catastrophe and apoptosis of the tumor cells [186].

Interestingly, an siRNA based screening of the human protein kinome, recently identified inhibition of *CHEK1* to be effective against a panel of neuroblastoma cell lines [188]. CHEK1, in line with the theory described above, is important for the regulation of the G2/M checkpoint, and several inhibitors against this kinase, are now included in clinical trials for other types of cancer [186]. It would therefore be interesting to see whether these inhibitors, possible in combination with chemotherapeutic agents, are efficient also for the treatment of high-risk neuroblastoma patients.

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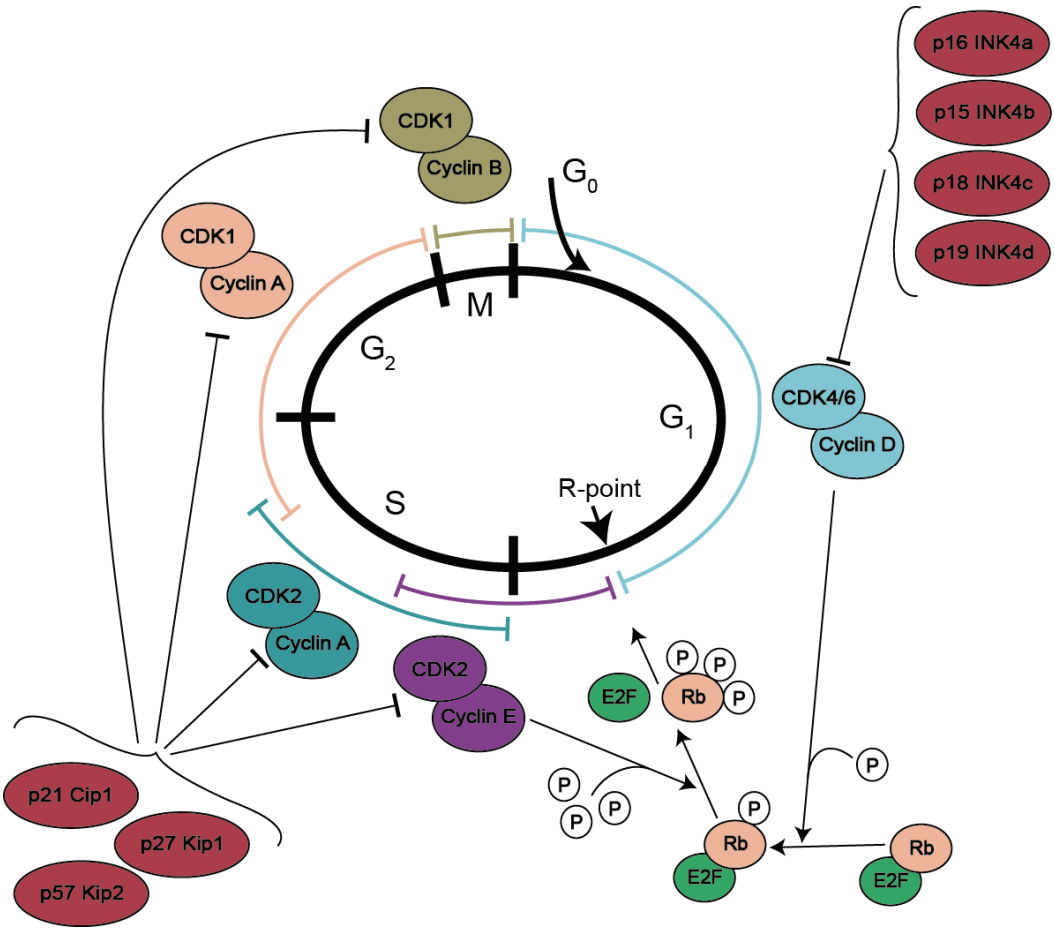

Figure 9 Schematic picture showing the main components of the cell cycle. Cyclin dependent kinases (CDKs) and their respective Cyclins are color-coded to match their time of activation throughout the cell cycle

5 Concluding remarks and future perspectives

Cancer treatment in general has improved greatly over the last decades, showing an increase in the 5 year overall survival, from around 40% in the 1970s to approximately 70% in 2007 [17]. Also for neuroblastoma, there has been an improvement in overall survival, especially for the children with high-risk disease [29, 30]. However, in most cases, the treatment is still very toxic, targeting essentially all fast growing cells in the body. This approach commonly results in long term side effects that are particularly problematic for children whose bodies are still growing.

The ultimate goal for neuroblastoma therapy would be to reach a state of personalized medicine, in which molecular characterization of the tumor provides a basis for the selection of the most suitable therapy for each patient. In reality however, this is not that easy. Not only do you need to have informative and reliable diagnostic methods that are capable of distinguishing between the relevant subgroups, you also need to have a good understanding of the biological mechanisms behind the disease, and last but not least, tumor-specific strategies to deal with the disease once you have characterized it.

5.1 Personalized diagnostics and follow-up

In this thesis, we have shown that high density SNP arrays offer a robust and reliable way of assessing DNA copy number alterations in neuroblastoma tumors, and that the resulting output is indicative for the prognosis of the patient. This has proven to be very useful, not only to find the patients that are in need of intensive treatment, but also to confidently be able to identify those patients that may be spared from unnecessary treatment. Although the classification of neuroblastoma into three main subgroups (*MYCN amplified, 11q deleted, numerical only*) is consistent between studies, there is still some debate on how to classify the remaining cases. According to our classification, the 17q gain identifies a worse prognosis, compared to other segmental aberrations and might identify some of the cases for which no *MYCN* amplification or 11q deletion is present, but that are still in need of intensive therapy. Conversely, we found that the patients with a profile consisting mainly of whole chromosome changes but with a few segmental aberrations had a very favorable prognosis.

The current risk group stratification scheme includes ploidy, *MYCN* amplification and 11q deletion, as the sole genetic factors [45]. Our data suggest that inclusion of additional factors such as 17q gain might improve the stratification of the ~20% of patients whose tumors do not match any of the current genetic profiles. However, due to the relatively small number of tumors in our material, these results need to be validated in a larger set of tumors.

Furthermore, we have shown that array data is a suitable starting point for the generation of tumor-specific PCR assays that can be used for detection of minimal

residual disease. Since the array data is often available from the diagnostic test, this approach is particularly convenient. However, the need for multiple primers to be designed for each patient has made this approach a bit cumbersome. Since the number of primers needed decreases with an increasing resolution of the array, using an even denser array may facilitate this procedure. Alternatively, massively parallel sequencing can also be used for the identification of tumor-specific junctions [156, 157]. This technique, also referred to as next-generation sequencing, has recently entered the field of neuroblastoma genetics [189, 190].

Although we are now capable of sequencing the entire human genome in a single experiment, it is still rather expensive, and the massive amount of data requires extensive use of bioinformatics. More commonly, a certain region is selected, such as all coding exones (exome), all mRNA transcripts (transcriptome) or a specific genomic region of interest. Depending on the selected region, this method is also suitable for the identification of tumor-specific junctions, providing a direct sequence across the junction. Additionally, since the number of sequence reads for a particular region reflects the number of input sequences, massively parallel sequencing can also be used for the purpose of copy number detection [191]. It is therefore possible that this technique will be able to replace many of the currently used molecular methods in a clinical setting for neuroblastoma in the future. However, the time-frame, and amount of bioinformatics associated with this technique are currently hampering its use in a clinical setting.

5.2 Biological characterization and possible therapies

Neuroblastoma tumors, although similar in appearance, differ in their genetic composition, as well as their tumorigenic behavior. It is therefore likely that there are several different lines of neuroblastoma tumors that achieve their transforming properties through more or less distinct pathways. Furthermore, what treatment is the most effective will most likely depend on what subtype of neuroblastoma it is.

In this thesis, we have characterized a few subgroups of neuroblastoma tumors in search for the driving genes that might be useful as therapeutic targets for these groups. The patients with 11q-deleted tumors were generally found to be older at diagnosis and to have tumors displaying a chromosome instability phenotype with a high frequency of chromosomal breaks. This chromosomal instability phenotype is probably caused by one or more of the genes, involved in DNA repair, that are located to this chromosome arm. Our results show that the *H2AFX* gene is consistently included in the deleted region, and that this gene showed a low mRNA expression in the 11q deleted tumors. Hence, the patients of the 11q deleted subgroup might benefit from a modified treatment, possibly involving a combination of *CHEK1* inhibitors and double-stranded break inducing chemotherapeutic agents.

Similarly, the '*12amp'* group, showing a peculiar clinical presentation with primary tumors mimicking Wilms tumor, and with a high frequency of lung metastases, constitute another odd group of neuroblastoma. This group has high grade amplification on chromosome 12 and sometimes also on chromosome 11. We have shown that these amplifications results in a higher mRNA expression of several genes, including *CCND1* and *CDK4,* that are important for regulation of the cell cycle. Furthermore, we have shown that inhibition of these genes cause a decrease in cell proliferation for cell lines with this pattern of amplification. It is therefore likely that drugs targeting the cell cycle will have an effect against this type of neuroblastoma.

In conclusion, although a truly personalized treatment for patients with neuroblastoma is still waiting in the future, we are getting closer. In Sweden, high density SNP arrays are now used as a standard option in the molecular characterization of each newly diagnosed neuroblastoma tumor, thus providing a basis for the selection of the best possible treatment for each patient. Furthermore, several therapeutic drugs, targeting specific pathways of the tumor are currently in clinical trials for other types of cancer and may possibly be included in the treatment of neuroblastoma in the near future.

6 Svensk sammanfattning

I Sverige diagnosticeras 15-20 barn varje år med neuroblastom, en typ av barncancer som uppstår i det perifera nervsystemet. Neuroblastom är en väldigt heterogen typ av cancer; där vissa tumörer kan tillbakabildas spontant även utan behandling, medan andra tumörer är väldigt aggressiva och medför en dålig prognos trots intensiv behandling. Då dagens cancerbehandlingar är associerade med en mängd långsiktiga biverkningar, är det därför av största vikt att kunna särskilja dessa grupper av patienter för att kunna ge en så bra vård som möjligt.

Redan på 80 talet fann man att genetisk karaktärisering av tumören gav värdefull information som kunde användas för att bättre bedöma vilka patienter som behöver en extra intensiv behandling. Även om man tidigare, med hjälp av ålder på patienten, samt förekomst av metastaser, till viss del kunnat skilja ut de patienter som behövt en extra intensiv behandling, så var detta ett stort framsteg. Behandlingen för neuroblastom har sedan dess förbättrats avsevärt, och en modern behandlingsstrategi för hög-risk neuroblastom består av en kombination av intensiv cytostatikabehandling, kirurgi, stamcellstransplantation, strålning, samt behandling med A-vitamin. Detta har lett till en ökad överlevnad för patienter med hög-risk neuroblastom, från ~20% i början av 90 talet till 40-50% idag.

Det ultimata scenariot för behandling av neuroblastom vore en patientspecifik behandling, där man först gör en noggrann analys av genetiska förändringar i tumören, för att sedan utefter det lägga upp en personlig behandling, med läkemedel som slår mot egenskaper specifika för just denna tumör. På så vis skulle många av biverkningarna kunna lindras eller undvikas helt, då kroppens normala celler inte skulle drabbas lika hårt av behandlingen. För att nå dit behöver vi dock dels utveckla känsliga detektionsmetoder för att kunna kartlägga de genetiska förändringarna i tumören, dels få en större förståelse för de biologiska mekanismer som tumören är beroende av, samt sist men inte minst, utveckla nya specifika läkemedel som slår mot dessa mekanismer.

Denna avhandling fokuserar på de första två av dessa förutsättningar, och baseras till stor del på användandet av SNP arrayer för att diagnosticera genetiska förändringar i neuroblastom tumörer. Dessa arrayer, ungefär i storleken av ett frimärke, innehåller hundratusentals små bitar av DNA som matchar olika positioner längs alla kromosomer i det mänskliga genomet. Genom att låta tumör-DNA, infärgat med en fluorescerande markör, hybridisera till arrayen, kan man på så sätt se skillnader i antal kopior, såsom deletioner (en eller två kopior saknas), gain (ett fåtal extra kopior) och amplifiering (>10 kopior), för olika delar av genomet.

Vi har visat att dessa arrayer är användbara för riskgrupps-klassificering av patienter med neuroblastom, vilket har lett till att SNP-arrayer numera används som ett naturligt steg i analysen av neuroblastom i Sverige. Förutom specifika avvikelser, som t.ex. *MYCN* amplifiering, används även den totala kopietals-profilen för klassificeringen.

Till exempel har det visat sig att patienter vars tumörer har enbart numeriska förändringar, dvs. deletion eller gain av hela kromosomer, har en mycket god prognos.

Vi har även visat att SNP-arrayer kan användas för att lokalisera specifika brottspunkter, och på så vis fungera som en grund för utvecklandet av patientspecifika tester. Genetiska avvikelser i tumörer, exempelvis amplifieringar, resulterar ofta i att två DNA segment som normalt är lokaliserade långt ifrån varandra, kopplas ihop. Den skarv som då bildas, är per definition tumör-specifik och kan användas för att detektera tumörceller som spridit sig till blod eller benmärg. I neuroblastom, till skillnad från t.ex. leukemier, är dessa kopplingar även patient-specifika, vilket gör att ett nytt test måste designas för varje patient. Vi har dock visat att man, genom att kombinera arraydatan med PCR och sekvensering, kan ta reda på exakt hur skarven ser ut för varje enskild patient och därefter designa ett PCR-baserat test som kan användas för att kunna följa hur väl patienten svarar på behandling.

Vi har även analyserat olika kromosomregioner i mer detalj för att försöka ta reda på vilka gener eller signalvägar som är av betydelse för uppkomsten av olika typer av neuroblastom. Vi har bland annat sett att tumörer med deletion av den långa armen på kromosom 11 har fler kromosombrott jämfört med övriga typer av neuroblastom, vilket tyder på att en eller flera gener som är viktiga för reparation av dubbelsträngade DNA-brott har blivit påverkade. I denna region på kromosom 11 finns ett flertal gener som är inblandade i DNA-reparation, bland annat *H2AFX*, som även visade sig ha en minskad mRNA-aktivitet i dessa tumörer.

Tumörer med amplifierade områden på kromosom 12 och ibland även kromosom 11, utgör en annan typ av neuroblastom, med speciella egenskaper. Dessa patienter har primärtumörer som är lokaliserade till njuren och därför ofta misstas för att vara en Wilms tumör istället för neuroblastom. Det är dessutom vanligt med lungmetastaser i denna grupp av patienter, vilket annars är väldigt ovanligt för neuroblastom. Vi har visat att de amplifierade områdena på kromosom 11 och 12 innehåller generna *CCND1* och *CDK4* som är viktiga för regleringen av cellcykeln, dvs. de processer som styr huruvida cellen ska dela sig eller inte. Dessa gener var även mer aktiva i tumörer med amplifiering jämfört med tumörer med två normala kopior av dessa regioner, och dessutom minskas cellernas tillväxthastighet om dessa gener slås ut, vilket tyder på att de kan vara lämpliga mål för riktade behandlingar.

Sammanfattningsvis så visar denna avhandling på att SNP arrayer är användbara, dels för diagnostik och riskgrupps-klassificering av neuroblastom, men även för att få en ökad kunskap om vilka gener och mekanismer som är viktiga i utvecklandet av denna sjukdom. Array-metodiken utgör således en bra grund för utvecklandet av en patientspecifik behandlingsstrategi.

7 Acknowledgements

Writing a doctoral thesis is certainly not easy! There has been many times when everything seemed hopeless, but also many joyful moments that has made everything worthwhile. I would therefore like to express my gratitude to all of you who have helped making this thesis possible.

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