The cell cycle in neuroblastoma: from genomic aberrations to targeted intervention
Molenaar, J.J.

Citation for published version (APA):
Molenaar, J. J. (2009). The cell cycle in neuroblastoma: from genomic aberrations to targeted intervention
Introduction
Introduction

The cancer research field encompasses a broad range of subjects, varying from molecular genetic studies on tumor characteristics to large scale clinical trials with new therapeutic tools. In this thesis we describe part of this process as regards to targeting the cell cycle in neuroblastoma. In the introduction we will try to give an overview of our current knowledge of neuroblastic tumors and we will discuss strategies for the development of new targeted therapies.

1 Neuroblastoma

1.1 Biology of neuroblastoma

Neuroblastic tumors are derived from neuroectodermal cells that originate from the neural crest during fetal development. These cells are normally destined to form the adrenal medulla and sympathetic nervous system(1-4). Failure of these cells to respond to differentiation signals is the first step towards malignant transformation of these neuroblastic cells. Histological markers of the developmental lineage from which tumors cells originate can still be found in the mature tumor(5;6). The histological classification of neuroblastic tumors occurs according to the balance between neural type cells (neuroblasts and ganglion cells) and Schwann type cells. The neuroblastoma are the most aggressive of this family of tumors and in turn may be classified as differentiating, poorly differentiated and the most aggressive undifferentiated. The undifferentiated neuroblastoma are composed almost entirely of neuroblasts which appear as small round blue cells. The ganglioneuroblastoma contain neuroblasts with a more mature appearance that are clustered in small foci surrounded by Schwannian stroma. Ganglioneuroma are predominantly composed of Schwann cells with mature ganglion cells(7;8).

1.2 Genetic aberrations

Various types of genetic aberrations occur in neuroblastoma. First the DNA content of neuroblastoma falls in two categories: near diploid and hyperdiploid. Hyperdiploid tumors are thought to have defects in mitosis, associated with whole chromosome gains and losses. This could explain why these tumors tend to be less aggressive(3;9). On the contrary, more malignant neuroblastoma have defects in genomic stability resulting in chromosomal rearrangements and unbalanced translocations. These tumors maintain their diploid DNA content. Diploid as well as hyperdiploid tumors have gains or losses that frequently involve the same chromosomes. Only in the diploid tumors these events concern chromosome arms or regions while hyperdiploid tumors tend to have gains or losses of whole chromosomes.

Gain most frequently involves chromosome 17. The 17q arm is gained in almost all high grade neuroblastoma. Breakpoints vary, but gain from 17q21 to the telomere suggests a dosage effect of several genes in that region(10). Candidate tumor driving genes are NME1, PPM1D and BIRC5(11-13). The chromosome 2p arm also shows frequent gain.
This region encompasses MYCN and ALK, the two best known oncogenes in neuroblastoma which are discussed below. Apart from these two genes, MEIS1 and NAG are possible tumor drivers in the 2p region. Hyperdiploid tumors also show frequent gain of whole chromosome 7.

Partial loss is most frequently seen for chromosome 1p and 11q. Both are associated with bad prognosis independent of other molecular genetic characteristics, though 1p deletions are strongly correlated with MYCN amplification. For chromosome 1 the Smallest Region of Overlap (SRO) is located at 1p36. Several potential tumor suppressor genes have been identified in this region. Most interesting are CHD5 and a tumor suppressing micro RNA, MIR-34A. On chromosome 11 the SRO is located around 11q23. The tumor suppressor genes here have not yet been identified. Loss of whole chromosome 3 and 4 does occur very frequently in hyperdiploid tumors.

Apart from these large chromosomal aberrations, small regions of amplification do occur. The MYCN oncogene at chromosome 2p is amplified in 20-30% of neuroblastoma. Amplification strongly correlates with a bad prognosis. Myc oncogene family members, MYC (c-Myc), MYCN and MYCL, are transcription factors, which activate, together with their dimerization partner Max, gene expression of a number of genes in an E-box dependent manner. MYCN and MYC probably have very similar molecular functions. The Myc Oncogene family members are involved in cell growth through protein synthesis, transcriptional regulation of ribosomal RNA processing, cell adhesion and tumor invasion. Recently MYCN has shown to be involved in the origin of replication complex as a co-factor in the complex as well as through direct transcriptional up-regulation of the MCM family of genes. Also amplifications of the Cyclin D1 oncogene at 11q13 are found in neuroblastoma. These amplifications have a much lower frequency of about 2-5%. 11q13 Amplifications are not correlated with prognosis or tumor subtypes. The function of Cyclin D1 will be discussed further in this chapter.

Finally single nucleotide mutations are identified for a number of genes in neuroblastoma. Recently the ALK gene was found to be mutated in 6-10% of the neuroblastoma. Mutations are located in the kinase domain of the ALK gene and are thought to have an activating function. PHOX2B is also found to be sporadically mutated. The functional consequences are still being studied. The established tumor suppressor P53 is not found to be mutated in primary neuroblastoma but mutations do occur in tumors that relapse after treatment.

1.3 Clinical presentation

Clinically neuroblastoma can arise anywhere throughout the sympathetic nervous system. The adrenal gland is the most common primary site followed by abdominal, thoracic, cervical and pelvic sympathetic ganglia. Neuroblastoma may metastasize to the lymph nodes, bone marrow, bone, liver and skin. Bone metastasis tend to appear in the orbit and therefore periorbital ecchimoses is a classical sign of disseminated neu-
neuroblastoma. Around 40% of the tumors present with localized disease that ranges from small intra-adrenal mass found by ultrasonography, to very large and locally invasive tumors. Paraspinal located tumors tend to invade the neuronal foramina and can cause compression of the spinal cord. These cases are oncological emergencies and require acute treatment. About half of the patients present with evidence of metastatic disease, ranging from loco regional spread in lymph nodes to distant metastasis. Children with metastasis mostly have extensive tumor burden and are frequently very ill. About 5% of the patients present with a striking phenotype of 4S disease. Infants present with small tumors that metastasize to skin, liver and bone marrow. 4S Tumors tend to disappear spontaneously without treatment(3;4;23).

1.4 Diagnosis and staging
Neuroblastoma diagnosis requires either a positive histological analysis or evidence of neuroblastoma cells in the bone marrow with positive catecholamines in the urine. Tumors are staged according to the International Neuroblastoma Staging System (INSS) which was revised in 1993 (33). Stage 1, 2 and 3 represent loco regional tumors with or without positive lymph nodes. Stage 4 tumors are those with distant metastasis. The specific subgroup of stage 4S tumors is reserved for patients less than 1 year of age with dissemination limited to skin liver and/or bone marrow.

1.5 Risk stratification
Risk classification of neuroblastoma is not uniform in different collaborative groups. However, all risk classification system use age, INSS stage, and MYCN copy number as risk factors. Histopathological grading is used in different variants, in Europe all groups include neuroblastoma and ganglioneuroblastoma in the treatment protocols for malignant neuroblastoma. Ganglioneuroma are classified as a benign disease and not included.

The Dutch Childhood Oncology Group (DCOG) Neuroblastoma Disease Committee has launched a new treatment protocol in 2007 for which the stratification is shown.
in figure 1. Recently several high throughput methods have shown additional value in risk stratification. Specifically the analysis of the overall genomic pattern adds critical prognostic information to conventional markers and will be included in future treatment stratification (9).

1.6 Current treatment and prognosis

The DCOG treatment regimen is based on the German Pediatric Oncology Group treatment strategy. The schedule for high risk patients is shown in figure 2. The medium risk protocol contains parts of the high risk treatment. In low risk patients a wait and see strategy is followed.

![Figure 2: Overview of neuroblastoma high risk treatment protocol (S=surgery, N5/6=chemotherapy cycles, MIBG=MIBG treatment, MEGA+ASCT= myeloablative high-dose-chemotherapy with autologous stem cell transplantation, 13-cis-RA=13-cis-retinoic acid)](image)

The DCOG found the GPOG treatment unsatisfactory for high-risk neuroblastoma patients and therefore they chose to add 2x radioactive MIBG treatment upfront to the high risk protocol. MIBG is a neurotransmitter-like substance that can be radioactive labeled with 131I for treatment or 123I for diagnostics. The radiopharmacon shows uptake in 95% of the neuroblastoma. After initial radiation 3 cycles N5 chemotherapy (cisplatinum, etoposide, vindesine) and 3 cycles N6 chemotherapy (vincristine, dacarbazine, ifosfamide, doxorubicin) are given. Resection is performed if substantial tumor remains after induction therapy. This resection should be attempted after the 4th or 6th chemotherapy cycle. After the primary induction therapy and surgery a myeloablative high-dose-chemotherapy with melphalan, etoposide and carboplatin is given, followed by autologous stem cell reinfusion. Finally retinoic acid is given in 6 cycles followed by a short break and 3 more cycles. The prognosis for patients with high risk tumors is very poor as shown in the Kaplan Meier curve. (fig 3.) The numbers however were taken from the 98 neuroblastoma and ganglioneuroma panel used for high throughput profiling in our lab. These patients received various treatment regimens. The prognosis is better for patients that are treated with the current protocol, with a 5 year overall survival around 30% for high risk patients (34;35).

2 Targeted therapy

The poor prognosis of neuroblastoma despite extensive treatment using the complete spectrum of current therapeutic options implies that a new approach is needed. In the last two decades knowledge on the pathogenesis of cancer has increased exponen-
15

Introduction

Initially. It is now clear that cancer is a multistep process which leads to a common set of properties in cancer cells. These include the hallmarks of cancer: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis(36). The most powerful strategies for intervention will be described in this chapter, followed by a description of the stepwise process of targeted drug development. We will finish with a discussion on the new therapeutic options in neuroblastoma and the cell cycle as potential therapeutic target.

2.1 New treatment strategies

Despite the fact that many properties are altered through genetic and epigenetic changes, the primary driving oncogenic aberrations in a tumor are thought to be few. These can be gain of oncogenes or loss of tumor suppressors. The mechanism of oncogene addiction describes the phenomenon in which tumor cells can only survive with the continued activity of a certain driving oncogene(37). The subset of oncogenes whose inhibition can lead to cell death or differentiation is of great clinical importance. The best know example is the BCR-ABL fusion gene which can be specifically targeted by the kinase inhibitor Imatinib. Other genes that are successfully targeted in subsets of tumors are EGFR (Gefitinib/Erlotinib) and HER2 (Trastuzumab). For the well known oncogenes MYC and RAS the intervention has proven more difficult. Also the reverse mechanism to use tumor suppressors as therapeutic target has lagged behind. It is often difficult to use a small molecule to restore or mimic the function of a protein that is absent and gene transfer to restore tumor suppressor function is not yet possible in a clinical setting.

As we know now, tumors also depend on activity of many other signal transduction routes that are not directly oncogenic themselves. For many aberrant pathways the activity is required for tumor cell survival but normal cells do not depend on this to the same degree. This dependence opens the opportunity for synthetic lethal interventions, meaning that inhibition or stimulation of a certain gene will only be lethal in cells with activation of a certain oncogene or inactivation of a certain tumor suppressor. So, targeting a gene or pathway that is synthetic lethal to a cancer relevant mutation should kill only cancer cells and spare normal cells(38). Though theoretically very prom-

![Figure 3: Kaplan Meier curve showing survival of patients with high, medium and low risk neuroblastoma. The number between brackets indicates the number of patients in the group. Data were derived from the 98 neuroblastoma tumors panel that is used in the R2 web application.](image-url)
ising, the number of true synthetic lethal combinations that are described is still very small. High throughput screening methods are now used to identify more potential targets. In chapter 6 we describe the discovery of a synthetic lethal relation between MYCN and CDK2.

Both strategies (i.e. targeted inhibition of oncogenes or synthetic lethal combinations) yield potential tumor specific treatment strategies. Still the high mutation rate and genetic plasticity of tumors will lead to tumor resistant subclones. One of the most striking examples is the therapy resistance to imatinib caused by BCR–ABL gene mutation in Chronic Myeloid Leukemia (39). Therefore it is vital to search for optimal combination strategies using targeted compounds and achieve additional or even synergistic therapeutic effects.

2.2 Identification of targets

These new treatment strategies will not select treatment based on tumor type but rather on tumors characteristics defined by genetic mutations or aberrations in signal transduction routes. This requires detailed knowledge of the genetic and epigenetic status of the tumor to be treated. In the last decade this became possible through various large scale profiling techniques. Firstly the identification of gained oncogenes and deleted tumor suppressor genes can be done genome wide through array Comparative Genomic Hybridization (CGH). This method is based on the hybridization of fluorescently labeled tumor DNA with one label and normal DNA with a different label, to DNA micro arrays containing probes mapping across the whole human genome (40). Recently the use of Single Nucleotide Polymorphism (SNP) arrays for this purpose has become possible as well. This technique uses the existence of over a million SNPs scattered across the human genome. Loss or gain of allelic variants can be used to score genetic aberrations. Single nucleotide mutations or small deletions can be detected through DNA sequencing for genes known to be involved in a certain cancer. More high-throughput methods that use large scale sequencing techniques are now being developed.

In addition to the genetic aberrations, it is essential to analyze the activity of signal transduction routes involved in cancer. Single marker gene scoring by immunohistochemical staining can give an indication for some routes. More complete insight can be obtained through mRNA profiling of tumor material(41). Expression aberrations of driving genes in signaling pathways can indicate whether activation of these routes is driving a certain tumor. Moreover, through manipulation experiments and correlative analysis, mRNA gene signatures have been generated that indicate the activity of pathways or the probability for drug sensitivity. These signatures can be related to mRNA profiles of tumor samples to predict the activity of signal transduction routes (42;43).

Integrated analysis of array CGH, SNP array and Affymetrix expression data can be used to pinpoint tumor driving aberrations. To that purpose Jan Koster and colleagues at our department of human genetics have designed the bioinformatic platform R2. Affymetrix mRNA profiles of 143 neuroblastoma and 25 neuroblastoma cell lines, array
CGH of 110 neuroblastoma tumors and SNP array data of 50 tumors and 25 cell lines have been generated in our lab and are available through the R2 website. Also mRNA profiles of many manipulation experiments have been incorporated in the application. Moreover we can compare our data with public available profiles of over 15000 tumor samples of 50 different tumor types. R2 facilitates an integrated analysis and allows identification of important regulatory pathways. This platform has been used extensively in chapter 5.

### 2.3 Validation of targets

The next step in targeted drug development is the validation of a gene or pathway as viable drug target. Cell line systems are used to test this. In our lab we use a panel of 25 well characterized neuroblastoma cell lines. Currently we are also isolating tumor initiating cells from primary tumors to include in this panel(44). In these models the expression levels of potential drug targets is manipulated. Various systems are used for over-expression or silencing of tumor suppressors and oncogenes. Silencing is mainly performed using the RNA interference technique. Classically this is done by transfecting 21 base pair double strand RNA molecules targeting the gene of interest (45). More recently the transfection of plasmids that express short hairpin RNA molecules using lentiviral systems has become the state of art. The SIGMA TRC library is used in our lab. In addition we use a doxycycline inducible system (46) if knock down of a certain gene is studied in more detail. Over-expression analysis is studied using stable inducible plasmid systems. If inhibition or over-expression of a certain target gene leads to a significant phenotype like apoptosis or differentiation, further analysis is performed using targeted compounds. The final step is in vivo proof of principle using the inhibiting compound in xenograft or transgenic models.

### 2.4 New therapies in neuroblastoma

Many new therapeutic strategies are developed in neuroblastoma but, only very few are specifically targeting tumor driving genes or use synthetic lethal relations. New aspecific cytotoxic agents in clinical trial are topoisomerase inhibitors (irinotecan, XK469), tubulin binding agents (ABT751, TPI-287), DNA damaging agents (Trabectedin) and an interesting compound that is used in sleeping sickness (Nifurtimox). The last compound was coincidently found to inhibit neuroblastoma growth and is now in phase 2 clinical trial. Many new compounds target signal transduction routes known to be involved in oncogenesis. Several are also clinically tested in neuroblastoma, including VEGF inhibitors (ZD6474, Cediranib, Sunitinib, Bevacizumab), an mTOR inhibitor (Temsirolimus), a proteasome inhibitor (Bortezomib), a COX2 inhibitor (Celecoxib), an EGFR inhibitor (Gefitinib) and a synthetic retinoid (Fenretinide). Antibodies to optimize the immune response targeting neuroblastoma cancer cells are 3F8 and Ipilimumab. Both are in advanced clinical trials. More information on these trials can be found at http://clinicaltrials.gov.

Targeted therapies, specific for genes with a role in neuroblastoma tumors, aim at ALK,
AURKA and NTRK1/2 (Trk A and B). Since the identification of ALK as potential tumor driving gene in neuroblastoma, two targeted compounds were extensively tested in vitro (TAE684 from Nerviano and PF-02341066 from Pfizer). In vivo testing of TAE684 showed poor pharmacokinetics and new derivatives of the compound are now being tested in vitro. The Pfizer compound is in phase I clinical trial in adults and a clinical trial in neuroblastoma patients is planned but has not been registered at the NCI yet (47). AURKA has been identified as potential therapeutic target. The gene was found to be sporadically amplified in neuroblastoma and showed an extensive over-expression. Moreover a synthetic lethal-like relation with MYCN was found (48). A clinical phase I/II trial in neuroblastoma patients with the AURKA inhibiting small molecule MLN8237 is ongoing. Finally the tyrosine kinases NTRK1 and 2 are strongly over-expressed in neuroblastoma and the NTRK inhibitor CEP701 is in clinical trial in neuroblastoma(49).

2.5 Cell cycle as therapeutic target

In chapter 2 to 6 we will describe the identification of cell cycle aberrations in neuroblastoma tumors and the validation of therapeutic targets in the cell cycle. In Chapter 2 we show that the Cyclin D1 gene is extensively over-expressed in neuroblastoma and we identify a low frequency of genomic amplifications of the Cyclin D1 gene. Chapter 3 discusses the mechanism of over-expression of Cyclin D1 in non amplified tumors and we identify the GATA3 transcription factor as a direct transcriptional regulator of Cyclin D1. In Chapter 4 we use RNA interference to show that neuroblastoma functionally depend on over-expression of G(1)-regulating genes to maintain their undifferentiated phenotype. In Chapter 5 we use an integrated analysis of array CGH, SNP arrays and Affymetrix mRNA expression to show a surprisingly high frequency of genetic aberrations in G1 regulating genes, which leads to over-expression of E2F target genes. Finally in Chapter 6 we validate CDK2 as viable drug target in neuroblastoma and we identify a synthetic lethal relation between the MYCN oncogene and CDK2. In Chapter 7 we give a short summary and we discuss future directions.
Reference List


(19) van Noesel MM, Versteeg R. Pediatric neuroblastomas: genetic and epigenetic 'danse macabre'. Gene
The Cell Cycle in Neuroblastoma


