## Network: Systems Biology of Embryonal Tumors – Neuroblastoma as Model

# Project: Molecular Pathways of Spontaneous Neuroblastoma Regression

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## Introduction

Neuroblastoma is a malignant embryonal tumor in young children consisting of undifferentiated neuroectodermal cells derived from the neural crest. Clinical courses are extremely variable and range from rapid progression despite intensive therapy to spontaneous regression in approximately 10% of the patients, which in most instances occurs in children below the age of 1 year. Understanding the molecular and cellular events that trigger spontaneous regression is a major challenge in neuroblastoma research and could provide a rational basis for developing new therapeutic approaches. During the past years, several non-random genetic alterations at multiple genetic loci have been described, which are likely to contribute to neuroblastoma development and progression. Although several of these individual alterations have been associated with particular patient outcome, only the genomic status of the *MYCN* gene – amplified or non-amplified- represents a sufficiently robust molecular parameter to be used world-wide as a therapy stratification tool[1]. However, a substantial number of advanced stage neuroblastomas with poor outcome lack amplified *MYCN*. Accordingly, additional markers are urgently needed which specifically identify patients with normal genomic *MYCN* status and high risk to experience a tumor progression.

To understand the divergent biological behavior of neuroblastomas and to translate this knowledge for patients benefit, there is need to address the complexity of cellular signaling coordinates in neuroblastoma tumor progression, in spontaneous tumor regression and in therapy resistance. The German Research Association for Neuroblastoma Targeted Therapies (GRANT) within the NGFN2 has established a well-managed network with strong links between all participating groups. High-throughput data from the participating groups and from different technical platforms have been combined in the iChip database (see project: Bioinformatics/Data Management) and serves as the basis for neuroblastoma-oriented clinical and basic research. Our group has focused on the elucidation of molecular mechanisms involved in neuroblastoma tumor initiation and progression by a combination of genomic and gene expression surveys and functional approaches .

## Results

### Neuroblastoma phenotype-specific genetic programs

As a point of departure, we have used microarray technology to identify genetic determinants of progressing *versus* regressing neuroblastomas. We have generated gene expression profiles from 53 neuroblastoma tumors using cDNA microarrays consisting of 42,578 clones. By using different mathematical methods to analyze the relationship among these samples, we demonstrated that the global patterns of gene expression largely reflect the clinical phenotype of the tumors independent of currently used risk factors including amplified *MYCN[2]*. These results strongly suggest that shared genetic programs are activated in aggressive neuroblastomas irrespective of the genomic status of *MYCN*. In order to identify biological processes and signaling pathways that may contribute to increasing aggressive phenotype of the tumors, we used two independent approaches: (1) a principle component analysis (PCA)-based approach to data analysis, which allows one to associate gene expression profiles with gene ontology (GO) annotations[3], (2) a combined analysis of gene expression data from tumor samples and *in vitro* cell culture models, which allow the targeted activation of *MYCN* or *E2F1,* two major determinants of neuroblastoma development.

We identified a specific subset of cell cycle and/or chromosome segregation genes that are deregulated in aggressive neuroblastomas including all tumors with amplified *MYCN*. *In vitro,* targeted *MYCN* expression in neuroblastoma cells similarly activates this particular genetic program. By functional analyses, we could demonstrated that *MYCN* does not activate this genetic program directly but indirectly *via* disrupting specific components of the Rb pathway (Rb-Skp2). These components are similarly disrupted in aggressive neuroblastomas lacking deregulated *MYCN*, obviously through as yet unknown *MYCN* independent mechanisms. Targeted inactivation of the Rb-Skp2 pathway *in vitro* is associated with uncoupling of cell cycle progression from mitotic control, a frequently observed feature in cancer cells leading to increased genomic instability and unrestrained proliferation. In contrast to aggressive neuroblastomas, regressing neuroblastomas fail to activate this specific genetic program indicating that components controlling Rb-Skp2 are still functional in these tumors[4].

### Design of a neurobastoma-specific oligo-nucleotide microarray

Current classification systems based on clinical, histomorphological, and genomic data assessing the individual risk of each neuroblastoma patient are still error prone. It is reasonable to suspect that individual neuroblastoma phenotypes emerge from the expression repertoire of particular genetic programs. To develop a robust risk stratification tool based on gene expression data, all key genetic determinants dictating neuroblastoma phenotypes should be adequately represented on such a diagnostic tool. Because general mass sequencing approaches of human cDNAs have not incorporated to significant extent cells of neuroectodermal origin, or neuroblastoma tumors, it is likely that key genetic determinants are underrepresented in currently established expression databases on which standard expression arrays are based. In a collaborative effort within the German Research Association for Neuroblastoma Targeted Therapies (GRANT), we have gathered gene expression data from a large set of NB tumors using different high-throughput gene expression analysis tools: Standard expression arrays, customized arrays based on subtractive cDNA libraries and SAGE libraries were used to define a comprehensive list of genes, which largely reflects the expression repertoire of individual neuroblastoma phenotypes. In addition, transcripts mapping to frequently altered chromosomal regions were also included. Based on this unique compilation of neuroblastoma phenotype-specific transcripts, we design a customized oligonucleotide microarray consisting of 11.000 oligos (60-mers). As appropriate probes for approximately 2000 transcripts were not available, we newly designed 60-mer probes specific for these transcripts.

Using this neuroblastoma-specific oligonucleotide microarray, we analyzed retrospectively 160 neuroblastoma tumors[5]. The Prediction Analysis for Microarray (PAM) was applied to gene-expression profiles of a first set of patients with contrasting clinical courses (died of disease despite treatment, n=23 *versus* long-term survivors without cytotoxic treatment, n=54). The resulting 144-gene predictor was

evaluated by classifying a second set of 83 patients and comparing to risk stratification by current classification system used in Germany, USA, and Japan. We could demonstrate that classification errors made by the currently used classification systems would have been corrected by a gene expression based classification system. Improvement of prediction accuracy was observed in all neuroblastoma risk groups. Thus, neuroblastoma patients may largely benefit from a gene expression based classification system and therapeutic intensity ranging from a wait-and-see approach to multimodality therapy could be better tailored to the individual risk of the patient. To further evaluate our gene expression based risk stratification tool, all newly diagnosed patients enrolled in the German Neuroblastoma trial 2004 are prospectively analyzed. To our knowledge, this is the first time that a gene expression based classification system is incorporated into a nation-wide clinical cancer trial.

### Inferring a model of tumor progression from genomic data

The knowledge of key genomic events that are causal to cancer development and progression is invaluable not only for our understanding of cancer biology but may have a direct clinical impact. The task of deciphering a model of tumor progression by requiring that it explains (or at least does not contradict) known clinical and molecular evidence can be very demanding, particularly for cancers with complex patterns of clinical and molecular evidence. We developed a process of model inference and show how a progression model for neuroblastoma can be inferred from genomic data[6]. The main idea of our method is to translate the model of clonal cancer evolution to mathematical testable rules of inheritance. For this study seventy-eight NB samples from stages 1, 4S and 4 with and without *MYCN* amplification were analyzed with array based comparative genomic hybridization (CGH)[7]. We find that the pattern of recurrent genomic alterations is strongly stage dependent and identify traces of inheritance within the genomic data. We infer a model of tumor progression of neuroblastomas, which is in agreement with clinical behavior observed for neuroblastomas and is compatible with existing empirical models of neuroblastoma progression (Fig.1). In order to identify candidate genes within the frequently altered regions in neuroblastomas, which may contribute to neuroblastoma tumor development, we combined genomic and gene expression surveys from neuroblastoma patients. Two candidate genes emerged from these analyses, one, *CAMTA1* within the commonly deleted region on chromosome 1p, and another, *WSB1* on 17q, frequently gained in neuroblastomas, which over-expression seem to have tumor growth suppressive effects in low-risk neuroblastomas [8, 9].

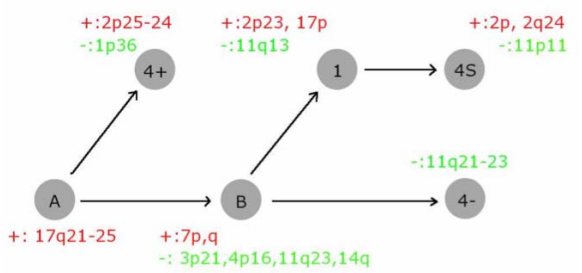


***Fig 1:*** *Graphical representation of the inferred tumor progression model for neuroblastoma. Stage 1, 4s (special), 4- (without amplified MYCN), and 4+ (with amplified MYCN) and two intermediate stages A and B are depicted as circles. Arrows indicate accumulation of mutations.*

## Outlook

In perspective, our combination of functional and quantitative genomic/gene expression approaches is efficiently producing a set of informative molecular markers as coordinates of different neuroblastoma phenotypes. These markers will be subjected to functional characterization to define their individual contribution to neuroblastoma initiation and progression. In future projects, we aim at to identifying new compounds specifically targeting the cellular signaling coordinates disrupted in aggressive neuroblastomas.

*Lit:* ***1.*** *Schwab, M., et al., Neuroblastoma: biology and molecular and chromosomal pathology. Lancet Oncol, 2003. 4(8): p. 472-80.* ***2****. Wei, J.S., et al., Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma. Cancer Res, 2004. 64(19): p. 6883-91.* ***3.*** *Krasnoselsky, A.L., et al., Altered expression of cell cycle genes distinguishes aggressive neuroblastoma. Oncogene, 2005. 24(9): p. 1533-41.* ***4.*** *Westermann, F., et al., Alterations in the Rb-Skp2 pathway are key to progressive neuroblastoma disease. submitted to Cancer Cell.* ***5.*** *Oberthuer, A., et al., Gene-expression based classification of neuroblastoma patients is superior to current risk stratification. submitted to NEJM.* ***6.*** *Chen, Q.R., et al., cDNA array-CGH profiling identifies genomic alterations specific to stage and MYCN-amplification in neuroblastoma. BMC Genomics, 2004. 5(1): p. 70.* ***7.*** *Bilke, S., et al., Inferring a tumor progression model for neuroblastoma from genomic data. J Clin Oncol, 2005: p. 1-9.* ***8.*** *Henrich, K.-O., et al., Low expression of HsCAMTA1 is associated with poor outcome in human neuroblastoma. submitted to Clinical Cancer Res.* ***9.*** *Chen, Q.R., et al., Increased WSB1 copy number leads to its overexpression which correlates with increased survival in neuroblastoma. submitted to Clinical Cancer Res.*