Diplomarbeit

Elucidation of a novel familial tumor predisposition pathway linked to the chromosome locus 22q12.1 – from bedside to bench

eingereicht von

Alexander Sormann

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Kinder- und Jugendheilkunde

Klinische Abteilung für Pädiatrische Hämato-/Onkologie

unter der Anleitung von

Univ.-Prof. Dr.med univ. Markus Seidel

und

Univ. Doz. Dr. Peter Ambros

Graz am 03.12.2020

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Graz, am 03.12.2020 *Alexander Sormann eh*

Danksagungen

An dieser Stelle möchte ich mich bei all denjenigen bedanken, die mich während der Anfertigung dieser Diplomarbeit motiviert und unterstützt haben.

Zuerst gebührt mein Dank Herrn Prof. Markus Seidel, der meine Diplomarbeit betreut und begutachtet hat. Für die Beratung in Fragen Scientific Writing, die zahlreichen Treffen zur Planung des weiteren Procedere und Zwischenstandsbesprechungen, sowie dem geduldigen Erklären von Sachverhalten bin ich sehr dankbar.

Ein besonderer Dank gilt allen Mitgliedern der betroffenen Familie. Nur durch ihre Zustimmung und außerordentliche Mitarbeit war die Erstellung dieser Forschungsarbeit möglich.

Ebenfalls möchte ich mich bei meiner Familie bedanken, welche mich durch das gesamte Studium begleitet haben. Dich, liebe Mama, möchte ich besonders hervorheben. Durch deine aufopferungsvolle Erziehung hast du mir und Lisa all die Jahre, mit einer Selbstverständlichkeit die Ihresgleichen sucht, gezeigt, dass deine Kinder für dich immer an erster Stelle standen. Ein Wert, den ich stets im Herzen tragen werde.

Abschließend möchte ich mich bei dir, meine liebe Anne bedanken. Dafür, dass du mir Motivation geschenkt hast wenn ich keine hatte, mich zur Ruhe gebracht hast wenn ich rastlos war und dich fürsorglich und jederzeit um mein körperliches und seelisches Wohl, während der Erstellung dieser Arbeit, gekümmert hast.

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Abkürzungen und deren Erklärung

AAA .... *ATPase associated with diverse cellular activities*
AFP .... *Alpha Feto Protein*
AI .... *allelic imbalance*
CaMP .... *cancer mutation prevalence score*
CCC .... *Cancer Centre Vienna*
CCRI .... *Cancer Research Institute*
Ce-M-M-..... *Research Center for Molecular Medicine of the Austrian Academy of Sciences*
CT .... *computer tomography*
DTC .... *disseminated tumor cells*
ERM .... *ezrin-radixin-moesin*
FGFR1 .... *fibroblast growth factor receptor 1*
FNCLCC.................... *French Federation Nationale de Centres de Lutte Contre Le Cancer*
GCT .... *germ cell tumors*
GVHD.. *Graft versus Host Disease*
HR-NBL-1/SIOP .... *high-risk Neuroblastoma of the Société Internationale D´Oncologie Pédiatrique*
IGCCCG .... *International Germ Cell Cancer Collaborative Group*
INRG .... *International Neuroblastoma Risk Group*
INRGSS .... *International Neuroblastoma Risk Group Staging System*
INSS .... *International Neuroblastoma Staging System*
IQ-Motif .... *Isoleucine-Glutamine-Motif*
KFS .... *Klippel-Feil Syndrome*
LDH .... *lactate dehydrogenase*
LOH .... *loss of heterozygosity*
MFB .... *myofibroblasts*
MFS .... *myofibrosarcoma*
miRNA .... *micro RNA*
MRI.. *magnet resonance imaging*
mRNA.. messenger RNA
Myo18A.. Myosin 18 A
Myo18B.. *Myosin 18 B*
NLS.. nuclear localization signal
NMD.. *nonsense mediated decay*
NMM2 .... *non-muscle myosin 2*
NSCLC .... *non-small cell lung cancer*
PB .... *peripheral blood*
PEB .... *cisplatin, etoposid and bleomycin*
SCLC .... *small cell lung cancer*
siRNA .... *small interfering RNA*
SNP .... *single nucleotide polymorphism*
SOP .... *standard operating procedure*
SUMO... *Small Ubiquitin-related Modifier*
TNM .... *Tumor, Node, Metastasis*
UICC... *International Union Against Cancer*
VEGF .... *vascular endothelial growth factor*
WES... *Whole Exome Sequencing*
β-hCG .... *human Chorionic Gonadotropin*

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Zusammenfassung

Ziel der vorliegenden Diplomarbeit war es die Mikrodeletion am langen Arm des Chromosoms 22 an Position 12.1 in Zusammenhang mit dem zugrundeliegenden klinischen Fall einer Neuroblastompatientin zu beleuchten und sowohl die durchgeführten molekular-genetischen Untersuchungen, sowie den derzeitigen Stand der Literatur zu präsentieren. Dazu wurden eine Literaturrecherche in zur Verfügung stehenden online Datenbanken und eine Aufbereitung und Visualisierung von erhobenen Daten vollzogen. Wir konnten die vermutete Rolle des betroffenen Gens als Tumorsuppressor, in Kongruenz mit vorhandener Literatur, untermauern und es war uns möglich den ersten Fall einer Patientin mit Neuroblastom und der genannten Mikrodeletion zu beschreiben. Ebenso unterstützt der Krankheitsverlauf der Patientin die durch Untersuchung von Zellkulturen gewonnene Vermutung eines aggressiven und disseminierenden Tumorzellwachstums bei vorhandener Alteration des Genlocus Myo18B. Daraus resultiert die Notwendigkeit der Fortführung der Erforschung von physiologischen und pathophysiologischen Signalwegen, um für Erkrankte neue Therapieoptionen zu entwickeln und um betroffenen Gesunden eine notwendige Vorsorge zukommen lassen zu können.

Abstract

Aim of this thesis was to elucidate the microdeletion at gene locus 22q12.1 in context with the case of a paediatric neuroblastoma patient and further, to visualize results of molecular-genetic investigations, as well as to present the current state of literature. A review of available research papers was performed using databases such as Pubmed. After evaluation, the redeemed information was visualized together with data from our index patient and her family and data from our genetic investigations. With our findings we further support the role of *Myo18B* functioning as a tumor suppressor and we present the first case of a microdeletion at the culprit locus in a neuroblastoma patient in literature. The aggressive and disseminating course of disease of our index patient also goes hand in hand of the finding of anchorage independent growth of cells in cell cultures reinforcing its metastatic quality when silenced. Upon closer investigation, the necessity emerged to ascertain more about the role *Myo18B* plays in physiological and pathophysiological pathways. This knowledge will allow for targeted therapy options and customized medical screening for affected individuals without disease manifestation.

1 Introduction

Carcinogenesis can be defined as the initiation of a tumor, whereas oncogenesis is rather seen as its maintenance and evolution. Extensive research has shown that cells are more susceptible to carcinogenic factors during the division process than when at rest. Examples, such as bronchial carcinoma in smokers, hyperplastic endometrial processes due to hormonal fluctuation or bone cancer at young age demonstrate more frequent carcinogenic events at stages of increased cellular division and activation.(1) There are multiple combinations of interactions between environmental influences, genes and endogenous signalling that occur in somatic, stem and immune cells, which collectively have the potential to promote cancer. It has become increasingly difficult to decisively determine the mechanisms that cause cells in humans or other species to degenerate into cancer, as the responsible driver mutations need to be identified and separated from non-harmful DNA alterations.

1.1 Congenital tumor predisposition and tumor predisposition syndromes

Genetic changes inherited from our parents, called germ line changes or congenital mutations, are found in every cell of the body of the offspring. If the mutation occurs only in the germ cells (cells becoming sperm or ovum) the genetic condition will be passed on without being present in the parents' somatic cells. Whether or not a mutation is dominant decides the phenotype of heterozygous germ line mutations. A recessive mutation requires both alleles to be affected in order to produce the phenotype in a diploid organism. Germ line mutations are distinct from somatic mutations.

Tumor predisposition syndromes present a genetic situation where cancer arises at an accelerated rate in a variety of different organ systems. Patients with a tumor predisposition syndrome need to be monitored more intensively than the normal population, as they present a more sensitized genetic background. Furthermore, the familial anamnesis is essential, as more relatives might be carriers of a hereditary DNA defect. In **Figure 1** four tumor predisposition syndromes are listed, together with their responsible genes and the associated malignancies that go with each syndrome.

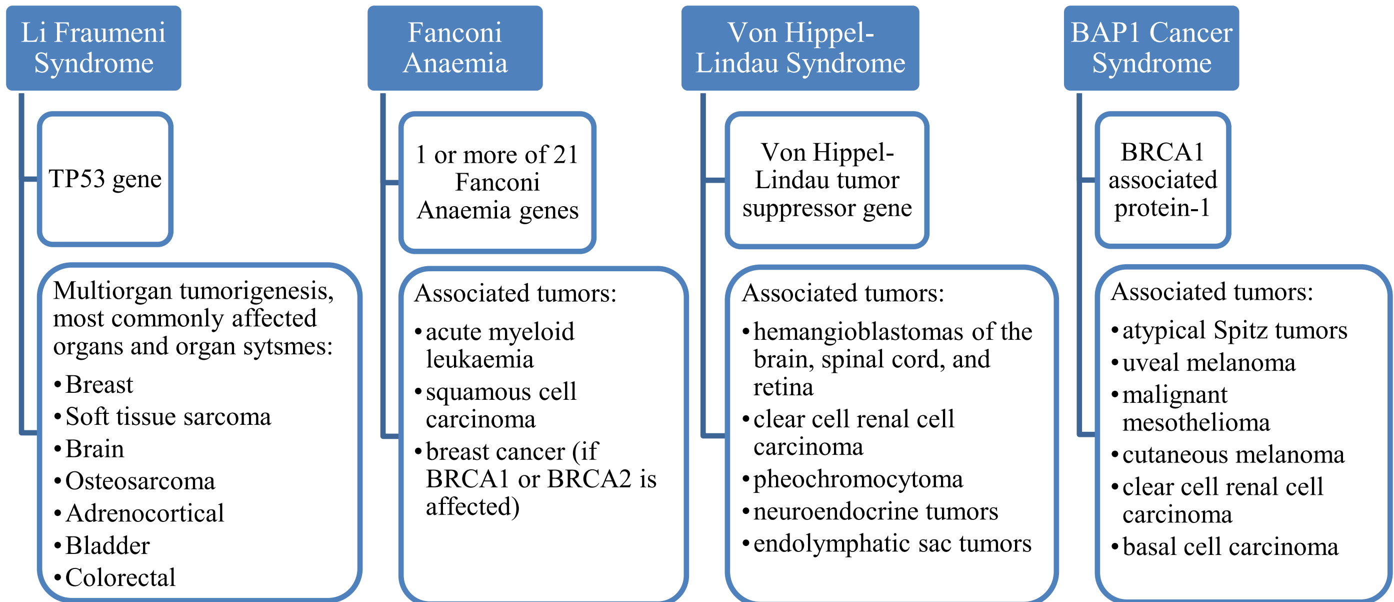


Table 1 - A selection of tumor predisposition syndromes. Shown is the name of the syndrome (blue box), the culprit gene (small white box) and the associated malignancies (large white box).

1.2 *Oncogenes and tumor suppressor genes*

Genetic alterations affecting the underlying nucleotide sequence directly include genetic mutations - ranging from a single base pair to a large segment of a chromosome, genomic instability - increased tendency of genome alteration during cell division, and gene copy number variation. (2) Two critical groups of genes play the main role in the process of a normal cell becoming cancerous: proto-oncogenes and tumor suppressor genes. Proto-oncogenes take action in pathways that promote cellular growth and mitosis. Tumor suppressor genes are considered the counterpart to proto-oncogenes as they are involved in inhibition of cell division, induction of apoptosis, suppression of metastasis and DNA damage repair. (2) Subsequently, loss of tumor suppressor gene function will promote onset and progression of cancer. Tumor suppressor genes and their pathways have been subject of extensive research. Due to this advance in the understanding of how cancer cells work targeted options of therapy emerged. Platinum compounds are highly effective in treating germ-cell derivative cancers, such as metastatic testicular cancer, which is being treated with cisplatin. Another example of targeted therapy is the group of tyrosine kinase inhibitors. Imatinib (Gleevec®; Novartis, Basel, Switzerland) is able to block the BCR-ABL (due to a chromosomal translocation activity-boosted version of tyrosine kinase ABL) fusion protein in the targeted hematopoietic cells and as a consequence suppresses the pathologically triggered increased cell division in the hematopoietic stem cells.

Gene Name	Function	Mutation
C-myc	Cell proliferation	Translocation t(8:14)
Abl	Tyrosine Kinase	SH3 domain, ATP-binding domain mutation
Rb	Cell proliferaion	C-terminal caspase cleavage site mutation; loss of heterozygosity
APC	Mitotic regulation	Truncating mutation; loss of heterozygosity
miRNAs	Function in the negative regulation of gene expression	Variety of regulatory and modulating post-transcriptional tasks
Notch	Signaling pathway for cell–cell communication and gene regulation	Int3 (insertion site)
PI3K	A family of lipid kinases that propagates intracellular signaling cascades	Somatic mutations in *PIK3CA*
p53	Tumor suppressor called the guardian of the genome	DNA-binding domain mutations

Modified Table from Jiao Zhang et al., Future Oncol. 2010 Apr;6(4):587-603.

Table 2 - Some of the best-known pro-oncogenic and anti-oncogenic genes, with their physiological function and known mutation sites or domains.

Genetic changes that occur after conception and are typically restricted to certain tissues (instead of involving all cells of the organism) are called somatic mutations. Such changes can be caused due to exposure to carcinogenic factors. These factors can be of exogenous or endogenous origin. Examples of exogenous agents are ultraviolet light, smoke or other forms of air pollution, ionizing radiation, metals and viruses. Reactive oxygen species or reactive nitrogen species released by macrophages or neutrophils constitute cases of endogenous agents. In case of gene damage in a critical coding sequence, for example for one of the genes listed in **Table 2**, the result can be the initiation or promotion of cancerogenesis.

1.3 Epigenetic factors in cancerogenesis and tumor progression

To produce a phenotype DNA does not always need to be affected directly in its nucleotide sequence, as there are additional epigenetic factors that can undergo modification, which physiologically regulate gene expression. The field of study that investigates the set of

epigenetic modification and its effects on the genetic material of a cell is called epigenomics.

Epigenetic mechanisms are divided into three groups: (3)

- DNA methylation - can lead to gene silencing, chromatine compacting or modifications of histones
- Histone modification - can be altered by processes such as methylation, acetylation, phosphorylation or sumoylation (SUMO - Small Ubiquitin-related Modifier)
- non-coding RNAs - play a role in post translational modifications and other regulatory pathways, this is the so called RNA-interference; include small interfering RNA (siRNA) and micro RNA (miRN)

Depending on the gene or the region of a gene affected, the outcome of epigenetic alterations is plentiful. A methylation process taking place in a promoter region has the potential to down regulate gene expression. If the gene hit by hypermethylation happens to be a tumor suppressor gene DNA repair or protective mitotic regulations might be impaired. Adversely, hypomethylation leads to upregulation of gene transcription and expression, by facilitating entry of the replicative and transcription machinery. In high-risk neuroblastoma (NB) expression of DNA methyltransferases was found to be increased, as well as in cisplatin resistant NB cells. (4)

Histones are a ways of compacting DNA in the nucleus, such a structure (a histone wrapped with DNA) is called a nucleosome. Nucleosomes compose chromatin, which can exist as euchromatin (decondensed and transcriptionally active) or heterochromatin (condensed and transcriptionally inactive). Modification of histones, via acetylation and de-acetylation, has an impact on the accessibility of DNA for transcription factors and can thereby influence gene expression. Histones are objected to different enzymes forming covalent bonds with the N- and C-terminal, such as methylation, acetylation, phosphorylation, and sumoylation. (4)

Micro RNAs are small non-coding strands of RNA that have a regulatory role in gene expression by inducing messenger RNA degradation and thus preventing ribosomal translation. As miRNAs also regulate gene expression for genes coding transcriptional regulation, cell proliferation and apoptosis imbalance can promote carcinogenesis.

1.4 Physiological role of myosins

Myosins are a superfamily of motor proteins in eukaryotic cells. The family tree of these proteins branches into XVIII classes. Class II includes myosins from skeletal muscle fibres and similar filament forming myosins from cardiac muscle, smooth muscle and some non-muscle myosins. They play a role in muscle contraction or in non-muscle cells as stress fibres in the form of contractile bundles, where they support cell to cell adhesion, cell to extra cellular matrix adhesion, cell migration and morphogenesis. (5) These class II myosins are also called conventional myosins. Originally myosins were thought to be only found in muscle cells (hence the name "myo"-(s)+"in"). The other classes (I, III-XVIII) are classified as unconventional myosins. (6) Functions of unconventional myosins include, but are not limited to intracellular transport of vesicles or cell organelles, endocytosis, cell growth, cell differentiation and interaction with kinesin, dynein and actin for the purpose of transport and cell movement or adhesion. Most myosin proteins consist of three distinct parts: the head, the neck and the tail domain. At the head domain the binding location for the binding partner, for example an actin filament, is located. The neck region acts as a lever for transducing force generated by the motor domain, as well as executing regulatory functions. The tail domain can be an anchor point for cargo molecules and/or also other myosin subunits. (7)

1.4.1 Class XVIII Myosins

The class XVIII myosins are characterized by the presence of large N- and C-terminal extensions enclosing a generic motor core. This subgroup is comprised of two myosins Myosin 18 A (Myo18A) and Myosin 18 B (Myo18B), each expressed from different genes. Available data indicates that both myosins lack ATPase-driven motor activity, suggesting that Myo18A and Myo18B function rather as structural myosins than performing dynamic actions. Both of the unconventional myosins can be predominantly found non muscle cells and within sarcomeric structures of striated muscle. (8) Further elucidation of the roles these proteins play showed their presumably essential part for the organization, maturation and regulation of the contractile machinery and the cells itself in non-muscle and muscle cells.

1.4.2 Myo18A

In humans, the Myo18A gene is located on chromosome 17q11.2. It was first discovered in 2000 where upregulation in cells with increased hematopoietic supportive ability was found. (9) A common feature for a myosin head domain is to hydrolyse adenosine triphosphate (ATP) in order to transmute the released energy into physical force, utilizing a conformational change of the myosin head changing its orientation. (7) However, Guzik-Lendrum et al. showed in myosin 18 of Drosophilia, that neither actin-activated ATPase activity, nor ATP binding was present at the motor domain. Furthermore, neither the binding affinity nor the stochiometrically calculated reactants were affected by ATP. (10) The study suggests that the Myo18A motor may function as an actin tethering protein. This was further investigated for human Myo18A, which revealed a similar ATP-independent binding to actin. (11) Physiologically this mechanism plays a role in applying a tensile force to the trans-Golgi membrane supporting trafficking of vesicles, as well as giving the Golgi its characteristic appearance in fluorescence and electron microscopy.(12) Myo18A was identified as a protein labelled as SP-R210, the Surfactant Protein A Receptor, which is highly expressed in myeloid cells, bone marrow, spleen, lymph nodes and lung. (13) This very distinct function as a receptor suggests, that Myo18A might play a modulation role in hematopoietic and immune cells. On a cellular level, the myosin is involved in focal adhesions, actin stress fibres and lamellar actomyosin bundles. A potential role in cancer development has been shown by Langer et al. in 2010. An exon array analysis of non-small cell lung cancer (NSCLC) revealed alternative splicing of Myo18A, thus postulating a cancer associated feature of the myosin. (14) Furthermore, a fusion of the genes Myo18B and the gene for the fibroblast growth factor receptor 1 (FGFR1), due to the chromosomal translocation t(8;17) found in a patient with atypical myelodysplastic-myeloproliferative disease, has been shown to inhibit correct segregation during mitosis of the fusion genes, leading to thrombocytopenia with decreased number and size of megakaryocytes accompanied by increased counts of monocytes, eosinophils and basophils. (15) Myo18A has been found to modulate non-muscle myosin 2 (NMM2) filament formations and organisation, by co-polymerisation with its coiled-coil domain. (16) In a metastatic prostate cancer cell line (PC-3) the overexpression of Myo18A leads to reduction of NMM2 stress fibres impairing cell shaping, cell adhesion and migration, thus contributing to formation of metastasis. (16) Overexpression has yet to be found outside of PC-3 cells. So far, the gene or its expressed protein is not associated with any other cancers, besides NSCLC and metastatic prostate cancer. (14)

1.4.3 Myo18B

1.4.3.1 Physiological roles

In 2001 a novel myosin similar to the human PDZ-myosin (Myo18B) was identified on chromosome 22, at locus 22q12.1. (17) Due to its structural similarity to the other class XVIII Myosin Myo18A, it was put in the same class and named Myo18B. It is characterized by two distinct regions, one at the amino (5') and one at the carboxyl (3') terminus that show no perceivable similarity with any other myosin class. Unlike Myo18A, which is found in most human cells and tissues Myo18B's expression is highly tissue specific. Localization experiments showed, that the protein is exclusively cytoplasmatic in undifferentiated myofibroblasts, yet in differentiated muscle cell the protein can also be traced in the nuclei. (17) The highest levels of expression can be measured in cardiac and skeletal striated muscle. Additionaly low levels of Myo18B messenger RNA (mRNA) can also be found in the testis, bone marrow, placenta, pancreas, prostate, lungs, mammary glands, thymus and brain.

In **Figure 1**, a schematic protein organization and domain sequence are depicted. The C- and N-term extensions show no similarity to any known protein domain with the exception of a nuclear localization signal (NLS), an amino acid sequence, which tags a protein for import into the cell nucleus by nuclear transport, situated in the C-terminus. (18) This validates the findings of the protein in the nuclei of differentiated muscle cells. The motor domain, also called the myosin head, harbours one section that is essential for ATP-dependent force generation. Surprisingly due to amino acid substitutions in this essential region it is suspected, that Myo18B lacks motor ATPase activity. (8) The underlying function of the motor domain remains to be decrypted. Following the motor domain in 3' direction a single Isoleucine-Glutamine-Motif (IQ-Motif) follows. This region may also be called the neck region of the myosin. IQ domains in other peptides have proven to be capable of binding calmodullin, which can stimulate changes in the actin cytoskeleton mediated by proteins such as myosins. (19) The characteristic myosin structure continues with its coiled coil tail domain. A coiled-coil is an alpha-helical secondary structure, which consists of 2-7 alpha helices that are twisted together like the strands of a rope yet to form another helical structure. Within this region an ezrin-radixin-moesin (ERM) and an ATPase associated with diverse cellular activities (AAA) domain have been located. (18) ERM sequences have the ability of crosslinking actin filaments with plasma membranes.

(19) AAA motifs are involved in a variety of cellular functions such as protein degradation, membrane fusion, DNA replication, assembly and disassembly of protein complexes and intracellular transport as a chaperone like ATPase. (20) To better understand the proteins functions, interactions and underlying mechanisms and its mentioned domains and motifs further investigation is needed.

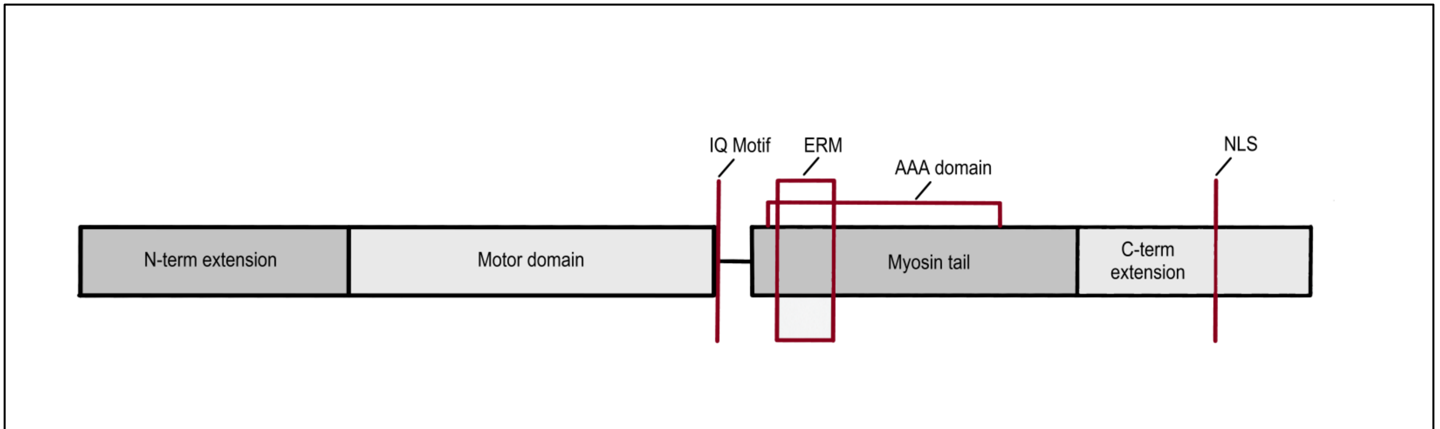


Figure 1 - Schematic representation of the protein structure of human Myo18B illustrating the ~285 kDa and 2567-amino acid long peptide. Sections from left to right: N-terminal extension, generic motor domain, neck region containing one myosin light chain binding IQ motif, followed by the myosin tail containing a coiled-coil region (here not shown), an ERM and AAA domain ending into the C-terminal extension, which contains a putative NLS.

1.4.3.2 Potential pathophysiological functions

To further elucidate the functions and possible consequences of malfunctioning proteins, several studies have been performed to pinpoint the intracellular location of highest Myo18B peptide expression and to find potential binding sights. The relevance in embryonic-cardiac development of Myo18B was investigated by mating Myo18B heterozygous mice. No Myo18B deficient offspring was born, implying that the embryos had died during embryogenesis. Following this discovery homozygous knockout embryos were observed and showed severe internal haemorrhage in the cardiac and ventral body wall regions, as well as dilated pericardial cavities. These malformations consequently cause embryonic death between E10.5-E11.5 (Carnegie Mouse Stage). Ultrastructural analysis showed disordered alignment and unbalanced distribution of thick and thin filaments of the cardiac myofibril structure.(21) In 2007 HOMER2 was identified as a binding partner of Myo18B. By binding to HOMER2 the induced suppression of

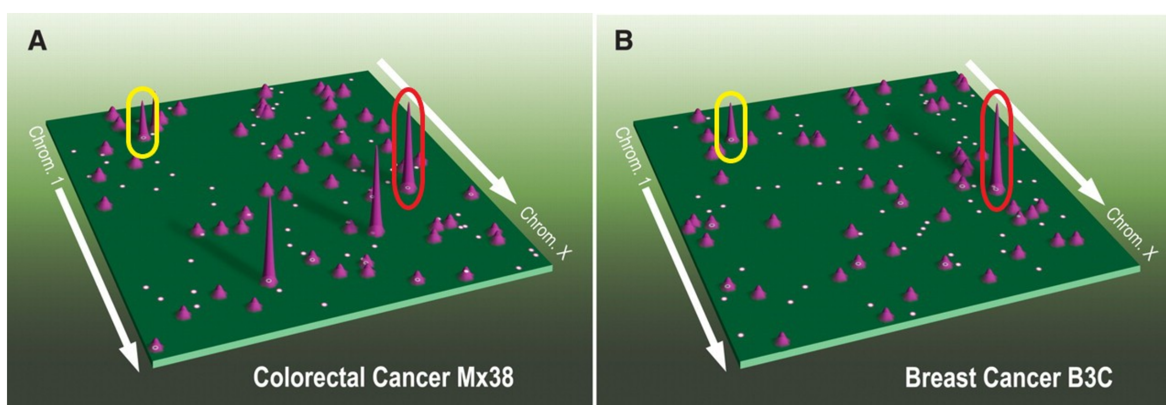
anchorage-independent growth, which is one of the hallmark properties acquired by fibroblasts and epithelial cells before they can become tumorigenic, was observed in a NSCLC cell line. (22).

1.4.3.3 Aberrant Myo18B expression identified in tumor suppressor / oncogene screens

It is widely accepted, that the process of cells becoming carcinogenic consists of multiple steps rather than one specific event. The observable phenotypic changes of cancer cells reflect the genetic alterations of modified DNA. The agglomeration of researched proto-oncogenes and tumor suppressor genes are involved in a multitude of intracellular processes such as cell growth, intracellular trafficking, cell and cell organelle alignment, cell differentiation, cell death, apoptosis and response and repair of damaged or faulty DNA. Yokota et al. looked for “hot spots” of chromosomal loss of heterozygosity (LOH) in lung cancer cells to identify novel tumor suppressor genes, which are involved in formation and genesis of lung cancer in humans. (23) Myo18B was frequently deleted, mutated and hypermethylated in lung cancers and in lung cancer cell lines. Gene expression was reduced in 88% (30/34) of NSCLC and in 47% (8/17) of small cell lung cancer (SCLC) cell lines. In primary lung cancer somatic mutations were detected in 13% (6/46) and CpG promoter island methylation was found in 35% (14/40) concluding to about 50% of gene deletion, mutation and promoter methylation. (23) The treatment with 5-aza-2’-deoxycytidine of 14 cell lines resulted in 11 (79%) cell lines with restored gene expression. In the remaining 3 cell lines Myo18B expression was reintroduced by treating the cells with trichostatin A, a histone deacetylase inhibitor, suggesting that histone deacetylation contributes to silencing Myo18B in lung cancer cells.(24)

Two studies investigated the influence of Myo18B expression in ovarian cancer. (25,26) Methylome analysis showed that high Myo18B methylation trends towards better response to treatment, but no association with overall survival was found. (25) This postulates a potential role as a novel chemoresponse marker. The overall expression of the gene was reduced in close to 70% of ovarian cancers tested, yet some cancer cells were able to express considerate levels of wild type Myo18B despite of allelic imbalance (AI). (26) This suggests that in order to lose function of its tumor suppressing features LOH is required.

Due to the detected AI found in ovarian cancer of 50-70% (26) investigation of other types of cancers regarding the culprit locus on chromosome 22 has been established. In colorectal cancers, 20-40% showed AI in the same chromosomal region. (27) Nine of eleven cell lines showed reduced Myo18B expression assessed by real-time quantitative PCR. In all nine cell lines increased expression was measured after treatment with 5-aza-2'-deoxycytidine and/or trichostatin A. (27) This understanding further strengthens the assumption that one of the underlying mechanisms of Myo18B silencing is histone deacetylation and/or promoter hypermethylation. Blecker et al. investigated, in a multicentre study, candidate cancer genes in glioblastoma, melanoma and pancreatic carcinoma. (28) An earlier study identified 280 candidate cancer genes distributed between colorectal and breast cancers. (29) Mutational data was raised to identify candidate cancer genes - genes that are most likely to be drivers for carcinogenesis and tumor progression, but are not identified as such yet. The genes were then ranked according to their cancer mutation prevalence score score (CaMP), a metric used to rank genes by the number and nature of the mutations observed. This way can be distinguished whether a mutation rate is higher than the anticipated passenger mutation rate and thereby, according to Wood et al., it is more likely to be a driver mutation rather than a passenger mutation.



Modified Figure from Wood et al, Science .2007 Nov 16;318(5853):1108-13.

Figure 2 - On the green plane, 60 somatic mutations each of colorectal cancer (A) and breast cancer (B) are mapped, beginning with the short arm of chromosome 1 in the top left corner. Ascending chromosomal positions follow in the direction of the white arrow. The chromosomes are laid out continuously on the plane, meaning when the front edge is

reached the chromosome will continue from the back edge one row over to the right. Two well-known genes shared by both tumors are highlighted here: the oncogene PIK3CA (yellow, on chromosome 3) and the tumor suppressor gene TP53 (red, on chromosome 17). Myo18B was also found mutated twice in both tumors; however, this gene is not depicted here on the gene landscape. (Data from table S4 A and table S4 B found in the "Supporting Online Material" from Wood et al, Science .2007 Nov 16;318(5853):1108-13.) (29)

The majority of candidate cancer genes fall into the "hill" category. Four of these genes were also found to be mutated in melanoma and pancreatic cancer, one of the being *Myo18B*. (28) However, no alteration of Myo18B was found in glioblastoma tumor samples. The validation of mutated Myo18B in lung cancer, colorectal cancer, ovarian cancer, pancreatic cancer and melanoma together with its known capability of suppressing anchorage independent growth as well as the its role in intracellular trafficking reinforces its putative role as a tumor suppressor.

Further, an association between Myo18B and Klippel-Feil Syndrome (KFS) or other Klippel-Feil anomaly syndromes has been established.(30–34) KFS is rare anomaly characterized by congenital fusion of the cervical vertebrae. Alazami et al presented in 2015 a paper about two not-related patients from consanguineous parents. Exome sequencing of both patients revealed a homozygous nonsense mutation and as a result loss of Myo18B RNA expression in peripheral blood samples, assuming due to nonsense mediated decay (NMD).(31) Another patient with KFS was also diagnosed with large-cell anaplastic medulloblastoma (at the age of 3 years). (33) Exome analysis displayed a compound heterozygous state of Myo18B, surprisingly transcriptomic analysis showed that tested tumor tissue had higher levels of expressed Myo18B, compared to other paediatric tumors of the central nervous system.(33)

1.5 Relevant malignancies of the patient's pedigree

Hereafter, a selection of two tumor classes, germ cell tumors and soft tissue sarcomas, with a special focus on myofibrosarcoma is presented. In addition, the index patient's malignancy, the NB, will be introduced as well. The goal here is to give an overview about each neoplasia with its epidemiological situation in Austria, diagnosis, genetics, treatment options and prognostic factors.

1.5.1 Neuroblastoma

1.5.1.1 Epidemiology

The NB is the second most common form of malignant cancer during childhood and the most frequent extra-cranial solid tumor in infants and children. (35) According to Statistik Austria there are 126 reported cases of NB in the age group 0-19 years in the years 2008 to 2017. The age-standardised incidence in the age group 0-19 is 7.3 new diagnoses per 1.000.000 citizens per year. (36) Boys are diagnosed 1.2 times more often than girls are. The age group showing the highest incidence are infants from age 0 to 1with 7.7 new diagnosis per 100.000 citizens per year. (35)

1.5.1.2 Histology

The NB is a tumor, that has its origin in the primordial neural crest. (37) Histology of tumor tissue ranges from immature ganglia cell precursors, so called neuroblasts, to areas mostly consisting of neuropil, sections with a high densitiy of dendrites and axons with few cell bodies, to Schwann cells, that occour in more differentieted parts of the tumor. (36,37) Occasional Homer-Wright-rosettes, surrounding the neuropil, can be observed in histological dissection.

1.5.1.3 Tumor development

Depending on diverse genetic factors, the NB can develop in different ways. Progression and metastasis, differentiation forced through chemotherapy or spontaneous regression have been observed.

70% of patients at time of diagnosis show signs of remote metastasis, most commonly located in bone, bone marrow, liver and skin. (38) Locoregional infiltration of lymphatic pathways is seen in 30% of patients, without remote metastasis, at the time of diagnosis.
The interruption of the mitotic pathway of tumor cells in NB and thereby the reduction of tumor mass is the primary goal of chemotherapy. Chemotherapy induced cell differentiation has been investigated in the NB90 trial. (36) The underlying mechanisms of differentiation induction are not fully understood yet, the influence of present Schwann cells precursor cells in relation to tumor progression and differentiation is being discussed. (39)
Another developmental pathway for the NB is spontaneous regression. The case of cell revertance, the loss of malignant phenotype and properties of the cells, is most commonly observed in patients below the age of 12 months at time of diagnosis, even if a specific pattern of metastasis is present. These young patients are likely to undergo spontaneous regression with just supporting care and occasional minimal therapy.(40) Studies looking for potential value in mass screening programs for NB in infants were performed in Japan (41), North America (42) and Europe (43). The studies showed a substantial increase in prevalence, but the overall mortality remained unchanged. (42) Mechanisms and factors that are being discussed to play a role in the occurrence of spontaneous regression are neurotrophic receptors, telomerase and telomere interaction, immunological and epigenetic regulators. (44)

1.5.1.4 Genetics

A familial predisposition can be found within 1-2% of NB patients. Cases with familial NB are on average younger at time of diagnosis and about 20% show multifocal primary tumors. Germline mutations that are associated with familial predisposition are ALK gene mutation, PHOX2B gene mutation and deletion at the 1p36 or 11q14-23 locus, as well as with commonly known cancer predisposition syndromes such as Li-Fraumeni Syndrome or Wiedemann-Beckwith Syndrome. (38)
In 1971, Knudson introduced the two hit model via his statistical study of retinoblastoma patients. He postulated that the dominantly inherited retinoblastoma is caused by one part germline mutation, the 1st hit, and that the disease is ultimately triggered due to a somatic mutation, caused by environmental influences emerging during lifetime, which he called the 2nd hit. (45) The very same principle is thought to play a role in the formation of the NB.

Genetic factors that are thought to play a role in non-familial NB formation and that have been taken into account in the risk assessment by the International Neuroblastoma Risk Group (INRG). (45) Among many others, the most important players are the amplification of the proto-oncogene MYCN, 11q aberration and ploidy of the tumor cells. (46) Furthermore, deletion of one or more putative tumor suppressor genes located in the region of 1p36 is associated with an increased risk for disease relapse; the same association goes for MYCN amplification. (47) Some other chromosomal alterations with clinical and biological significance, such as loss of heterozygosity and DNA copy number change, are located on the chromosome arms 3p, 11q and 17q. (48)

1.5.1.5 Staging

The International Neuroblastoma Staging System (INSS) is a surgical staging system. Due to different national surgical approaches and guidelines regarding tumor surgery and the ongoing better understanding of NB, its development, genetics and biology the Children's Oncology Group updated and added to the INSS. (38) The staging system, that is now most commonly used for pretreatment risk stratification is the International Neuroblastoma Risk Group Staging System (INRGSS), introduced by the INRG Task Force, based on clinical criteria and image-defined risk factors. (49) It includes the INSS as well as risk group and treatment assignments, which offer a more complete pretreatment overview for treating physicians.

Table 3 shows the INRG Pretreatment Classification Schema. (50) Depending on INRG stage, age, histological category, grade of tumor differentiation, MYCN amplification status, 11q aberration status and tumor cell ploidy the patients are assigned to one of 16 risk groups. Different combinations of the risk criteria listed above conclude in very low risk, low risk, intermediate risk or high risk groups. To further distinguish between different risk criteria combinations, which would result in the same risk group, letters from A to R are assigned, resulting in 16 individual pretreatment risk groups.

INRG Stage	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	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)
			Amplified			K (high)
L2						
Age <18 mo	Any, except GN maturing or GNB intermixed		NA	No		D (low)
				Yes		G (intermediate)
Age ≥18 mo	GNB nodular neuroblastoma	Differentiating	NA	No		E (low)
				Yes		H (intermediate)
		Poorly differentiated or undifferentiated	NA			H (intermediate)
			Amplified			N (high)
M						
Age <18 mo			NA		Hyperdiploid	F (low)
Age <12 mo			NA		Diploid	I (intermediate)
Age 12 to <18 mo			NA		Diploid	J (intermediate)
Age <18 mo			Amplified			O (high)
Age ≥18 mo						P (high)
MS						
Age <18 mo			NA	No		C (very low)
				Yes		Q (high)
			Amplified			R (high)

Table 3 - INRG Pretreatment Classification Schema.

GN = ganglioneuroma; GNB = ganglioneuroblastoma; NA = not amplified

1.5.1.6 Prognosis

The prognosis of NB patients depends on factors already mentioned in the INRG Pretreatment Classification Schema such as age at diagnosis, tumor histology, genomic and biological tumor features and the INRG stage. An additional factor that is of utmost importance is the tumors response to treatment. Cases with poor response to initial treatment often show progressive and/or recurrent disease.

1.5.1.7 Therapy

The decision of which therapy applies to which patient is made by taking the assigned risk group into account that was determined at the time of diagnosis. In Table 4 an overview of the treatment options for low risk, intermediate risk, high risk and stage 4S/MS is given. (38)

COG Risk-Group Assignment	Treatment Options
Low-Risk Neuroblastoma	Surgery followed by observation.
	Chemotherapy with or without surgery (for symptomatic disease or unresectable progressive disease after surgery).
	Observation without biopsy (for perinatal neuroblastoma with small adrenal tumors).
	Radiation therapy (only for emergency therapy).
Intermediate-Risk Neuroblastoma	Chemotherapy with or without surgery.
	Surgery and observation (in infants).
	Radiation therapy (if needed).
High-Risk Neuroblastoma	A regimen of chemotherapy, surgery, tandem cycles of myeloablative therapy and SCT, radiation therapy, and dinutuximab, with interleukin-2/GM-CSF and isotretinoin.
Stage 4S/MS Neuroblastoma	Observation with supportive care (for asymptomatic patients with favorable tumor biology).
	Chemotherapy (for symptomatic patients, very young infants, or those with unfavorable biology).
	Radiation therapy (rarely for patients with symptoms related to hepatomegaly from metastatic disease).

Table 4 - Treatment Options for Neuroblastoma

COG = Children's Oncology Group; GM-CSF = granulocyte-macrophage colony-stimulating factor; SCT = stem cell transplant.

In cases of recurrent NB differentiations are made between locoregional and metastatic recurrence, as well as the originally determined pretreatment risk group. Re-surgery, intensified chemotherapy, radiation therapy, immunotherapy and other novel therapeutic approaches are used to treat recurrent NB.

1.5.2 Germ cell tumors

The majority of germ cell tumors (GCT) in patients occur between birth and young adulthood. GCTs are most commonly located along the midline ranging from the hypophysis to the sacrococcygeal area; these tumors are thought of as defects that arise due to errors in cell migration during embryonic development. Primary tumors that pathophysiologically are not connected to malfunctioned embryonic cell migration can develop in the ovaries or testis, as germ cells naturally occur in the gonads. In the following, the focus will be on different GCTs that develop in the testis.

1.5.2.1 Epidemiology

According to Statistik Austria, there were 438 reported cases of testicular cancer in 2017 in Austria, histologically 90% were classified as GCT. (51) The cumulative risk to develop testicular cancer until the age of 75 has increased from 0.3 in the year 1983 to 0.7 in the year 2017. (51) A German study investigated in 2004 a large population-based paediatric cohort of GCTs to evaluate the parameters age, sex, site of the tumor, histology, and potential correlations between these parameters. (52) The age distribution showed two peaks, the first one during infancy, the second after onset of puberty. A histological differentiation displayed that almost all tumors at birth were teratomas, while yolk sac tumors were the predominant tumor histology during childhood. The term germinoma is often used to refer to an intracranial GCT of the same or very similar histology subtype as the dysgerminoma (in the ovaries) or the seminoma (in the testis). During the adolescent years gonadal germinomatous tumors become the most represented type of GCT. In the age group 25-45, testicular cancer is the most common form of malign neoplasia. Histologically two thirds of these cancers are pure seminoma, followed by malign teratoma (17%). (53) Epidemiological risk factors for the development of testicular tumors are cryptorchidism, hypospadias, decreased spermatogenesis, infertility, familial history of testicular tumors among first-grade relatives and the presence of a contralateral tumor or

testicular intraepithelial neoplasia. Klinefelter's syndrome is considered a predisposing factor in the development of GCTs arising from the testis and mediastinum. (54)

1.5.2.2 Classification

A Classification Based on the WHO classification of tumors of the testis and paratesticular tissue is shown below in Table 5. (55) Only GCTs were taken into account for creating this table.

Non-invasive germ cell neoplasia

- Germ cell neoplasia in situ (GCNIS; synonyms: carcinoma in situ testis, intratubular germ cell neoplasia unclassified)
- Gonadoblastoma

Germ cell tumors derived from GCNIS

- Seminoma
- Non-Seminoma
  - Embryonal carcinoma
  - Teratoma (post-pubertal)
  - Yolk sac tumor (post-pubertal)
  - Choriocarcinoma and other trophoblastic tumors

Germ cell tumors unrelated to GNCIS

- Childhood tumors
  - Teratoma (pre-pubertal)
  - Yolk sac tumor (pre-pubertal)
- Spermatocytic tumor

Table 5 - WHO Classification of Germ Cell Tumor Pathology

1.5.2.3 Diagnosis

In clinical examination patients often present with a unilateral mass in the testis, accompanied by redness, pain or tenderness to the touch frequently found via self-examination. In up to 27% of the cases of testicular cancer, pain is the first symptom, other symptoms than can occur are gynecomastia due to secretion of hormones of the tumor tissue, swelling of lymph nodes or lower back pain due to tumor cell dissemination. (56) About 1-2% of patients present with bilateral affected gonads upon diagnosis. As infectious epididymitis or orchitis are far more common than tumors and symptoms such as swelling and pain are similar ultrasonography should be used to determine whether the mass is intra- or extratesticular and to display volume and anatomical location of the testicular lesion. (56) An examination of the contralateral testis is also advised.

Listed below is the protocol for the diagnosis and staging of testicular cancer by the European Association of Urology. (56)

- Discuss sperm banking with all men prior to starting treatment for testicular cancer
- Perform bilateral testicular ultrasound in all patients with suspicion of testicular cancer.
- Perform physical examination including supraclavicular, cervical, axillary and inguinal lymph nodes, breast and testicles.
- Measure serum tumor markers both before and after orchiectomy taking into account half-life kinetics.
- Perform orchiectomy and pathological examination of the testis to confirm the diagnosis and to define the local extension. In a life-threatening situation due to extensive metastasis, commence chemotherapy prior to orchidectomy.
- Perform contrast enhanced computerised tomography scan (chest, abdomen and pelvis) in patients with a diagnosis of testicular cancer. If iodine allergy or other limiting factors perform abdominal and pelvic magnetic resonance imaging.
- Perform magnetic resonance imaging of the brain (or brain computer tomography if not available) in patients with multiple lung metastases, or high beta subunit of human Chorionic Gonadotropin (β-hCG) values, or those in the poor-prognosis International Germ Cell Cancer Collaborative Group (IGCCCG) risk group.
- Encourage patients with testicular cancer to perform self-examination and to inform first-degree male relatives of the need for self-examination.
- Discuss testis-sparing surgery with frozen section examination in patients with a high-likelihood of having a benign testicular tumor which are suitable for enucleation.
- Offer biopsy of the contralateral testis to patients with testicular cancer and at high-risk for contralateral germ cell neoplasia in situ.

1.5.2.4 Tumor markers

The measurement of tumor markers is necessary for diagnosis and staging purposes in patients with GCTs. Just as important is the reassessment post-orchidectomy to interpret the marker dynamics. At time of diagnosis Alpha Feto Protein (AFP), β-hCG and lactate dehydrogenase (LDH) should be assessed. AFP, which is produced in cells of yolk sac tumors and β-hCG, which is expressed by trophoblasts, can be used to determine the type of tumor by comparing the marker levels to specific patterns of marker elevation. (57) Furthermore, the quantity of the markers mentioned aids in guiding therapeutic

management and are as well implemented in the prognostic-based staging system for metastatic germ cell cancer by the IGCCCG. (58) Elevated tumor markers post-orchidectomy should normalise within several weeks. Given the half-life for AFP is five to seven days and the half-life for β-hCG is one to three days, a slower than expected decrease of levels in the blood of any of these markers could be an indicator for metastatic disease.

1.5.2.5 Prognosis

The widely spread Tumor, Node, Metastasis (TNM) classification is used to specify the extent of disease and should be assessed at time of diagnosis. 13 different prognostic groups ranging from stage 0 to stage IIIc have been defined by the International Union Against Cancer (UICC). Testicular cancer in general is considered a well treatable form of neoplasia with a favourable 5 year survival rate of 97%. (53) Klepp et al. showed in the SWENOTECA study that in a cohort of 370 patients with non-seminoma upon diagnosis 63% of the patients were categorized as early clinical stage. (59) The early clinical stage was defined as cases with no clinical or radiological sign of distant metastasis. The early diagnosis for patients with pure seminoma has been shown to be higher at around 75-80%. In the IGCCCG's prognostic-based staging system for metastatic testicular cancer good, intermediate and poor prognosis groups are categorized, by using histology, location of the primary tumor, location of metastasis and pre-chemotherapy serum tumor marker levels as prognostic factors. (58)

1.5.2.6 Therapy

Treatment for patients with GCTs is highly variable and depends on factors such as tumor histology and pathology, signs of metastasis, tumor marker assessment, staging and the assigned IGCCCG's prognostic-group. For early primary tumors located in the testis orchiectomy can be the only treatment necessary. In patients with unilateral seminoma measuring a diameter smaller than 4cm and without confirmed infiltration of the rete testis there is only a small chance of occult metastasis (<5%). (60) In patients with a tumor greater than 4cm in diameter adjuvant care in form of radio therapy of the paraaortic region or chemotherapy with carboplatin can be considered and need to be discussed with patients individually. In a cohort of 353 non-seminoma 8.5% clinical stage I patients showed infiltration of the retroperitoneal lymph nodes, showing a higher risk for occult metastasis

in early stages, compared to pure seminoma patients. (61) High risk patients adapted therapy with triple chemotherapy consisting of cisplatin, etoposid and bleomycin (PEB) should be considered. Continously elevated tumor markers or rising markers post orchiectomy without any suspect findings in computer tomography (CT) or magnet resonance imaging (MRI) should be regularly checked up on until a lesion is located and therapy can be initiated, or until the markers start to drop according to their half life. For cases in later stages a selection of high dose chemotherapy, lymph node resection or radiation therapy is available for the treating physician to discuss with the patient individually.

1.5.3 Soft tissue sarcoma

There is a wide spectrum of soft tissues oh which soft tissue sarcomas can originate. It is not easy to give a definition that includes all soft tissues of the human body. The Merriam-Webster medical dictionary defines soft tissue by what it is not: "*body tissue that is not hardened or calcified*". (62) In the WHO's latest update on the classification of tumors of soft tissue and bone the following chapters are mentioned in the soft tissue sarcoma section of the lexicon: (63)

- Fiborblastic/myofibroblastic Tumors
- So-called fibrohistiocytic tumors
- Smooth-muscle tumors
- Skeletal-muscle tumors
- Nerve sheath tumors
- Tumors of uncertain differentiation
- Undifferentiated / unclassified sarcomas

1.5.3.1 A glance at Myofibrosarcoma

Myofibroblasts (MFB) are cells that have acquired a phenotype intermediate between fibroblasts and smooth muscle cells. Their origin and path of differentiation is heterogeneous and scattered throughout different tissues of the body. A fibroblast can differentiate into a proto-MFB, hepatic stellate cells can be activated and transform into MFB, smooth muscle cells have the ability to de-differentiate and become a MFB, epithelial or endothelial cells are able to undergo epithelial-mesenchymal transitions and become a MFB and fibrocytes released by the bone marrow might be able to contribute to

the MFB count. (64) The main role by this cell is the synthesis of extracellular matrix and the build up of tension in tissues, both of which play an essential role in tissue repair during wound healing, but can also impair organ integrity and function when excessive stimulation or delayed apoptosis are present. The malfunction of MFB regulation can result in hypertrophic scars, Dupuytren's disease, heart and kidney fibrosis, scleroderma and pathological deformation of the liver and lung. Some reactive and soft-tissue lesions have their principal cell in the MFB. There is a variety of low- and high-grade myofibrosarcoma (MFS) and other tumors with myofibroblastic differentiation.

1.5.3.2 Epidemiology

C. Wimber et al. have investigated soft tissue sarcoma in Austria in a population-based study for a time from 1984 to 2004. During this period, 5333 cases were registered. The most common histological type found in patients were sarcoma not otherwise specified (36%), leiomyosarcoma (24%), liposarcoma (12%), malignant fibrous histiozytoma (9%) and fibrosarcoma (5%). (65) The analysis of the age distribution showed a peak incidence around the age of 60 years, yet another smaller peak during infancy and the early years of childhood has been revealed. [**Figure 3** - Age distribution of soft tissue sarcomas incidence for the years 1984-2004 in Austria] The age-adjusted incidence of soft tissue sarcoma in Austria has been calculated to be 2.4 per 100.000 per year. (65)

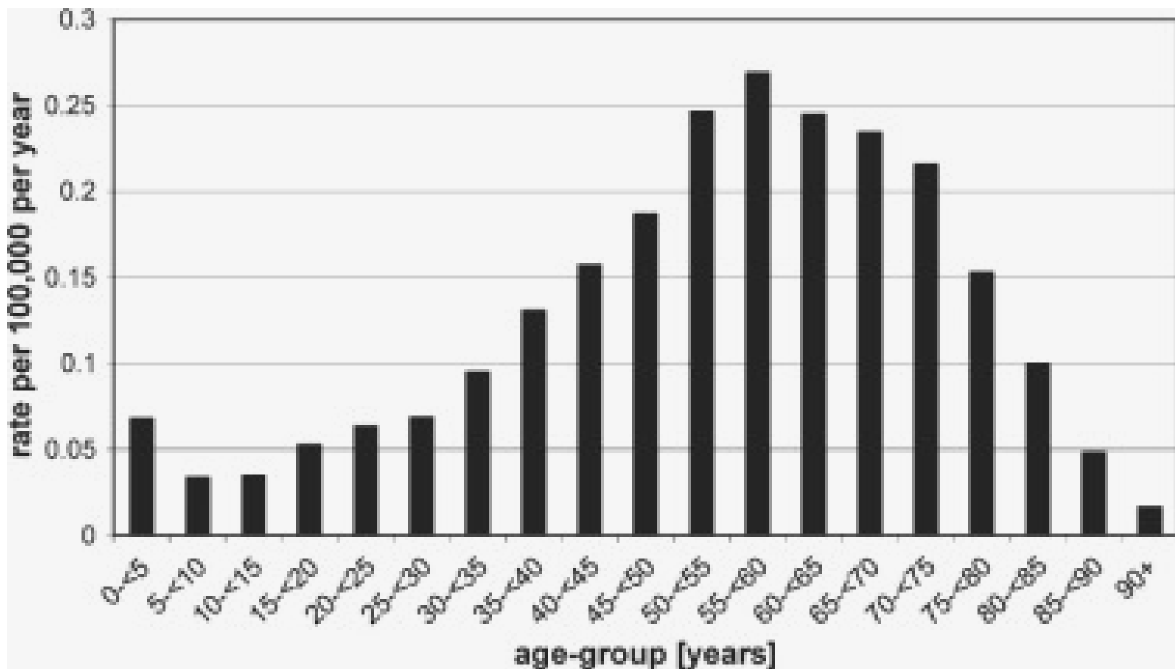


Figure 3 - Age distribution of soft tissue sarcomas incidence for the years 1984 - 2004 in Austria.

1.5.3.3 Diagnostics

According to the standard operating procedure (SOP) of the Comprehensive Cancer Centre Vienna (CCC) primary diagnostic tools are palpation and high-resolution ultrasonography of the suspect are supported by x-ray imaging. (66) In case of lesion diameter greater than 5cm, concomitant pain, deep localization of the suspect mass beneath the muscle fascia, no or disorganized Doppler-Flow in ultrasonography, heterogeneous tissue composition, growth, deformation or destruction of neighbouring anatomical structures or any other suspect diagnostic findings MRI is advised. After completion of non-invasive diagnostics and yet unclear grade of malignancy of the mass a needle biopsy can be performed, taking into account the size and location of the tumor in order not to impair future needed total excision. To evaluate reclaimed material immuno-histochemical stains using antibody-antigen interaction can be used. In context with chromosomal alterations and histomorphological features the immune-histochemical findings are to be seen as seminal, regarding finding the correct diagnosis.

1.5.3.4 Classification

Soft tissue sarcomas include a wide variety of different neoplasia. In 2013 the WHO published the fourth edition of “WHO Classification of Soft Tissue Tumors” a wide variety

of soft tissue entities is described in the mentioned publication: adipocytic tumors, fibroblastic/myofibroblastic tumors, so-called fibrohistiocytic tumors, smooth muscle tumors, pericytic (perivascular) tumors, skeletal muscle tumors, vascular tumors, gastrointestinal stromal tumors, nerve sheath tumors, chondro-osseous tumors, tumors of uncertain differentiation and undifferentiated/unclassified sarcomas. (63) A guide for classification of tumors regarding their malignancy is provided by the book and currently widely used in clinical practice. The lesions are divided into three groups: benign, intermediate and malign, the intermediate group is divided again tumors that show locally aggressive growth, yet with lower risk of metastasis (fasciitis group) and tumors that have a marginally higher chance to become metastatic. (See Table 6 below).

<table>
<tr><th colspan="2">Mannerism</th><th rowspan="2">Therapeutic group</th><th rowspan="2">Characteristics</th><th rowspan="2">Examples</th></tr>
<tr><th>Clinical</th><th>Biological</th></tr>
<tr><td colspan="2" rowspan="2">Benign</td><td>Ia</td><td>Very rarely relapse, no metastasis</td><td>Benign fibrous Histiocytoma</td></tr>
<tr><td>Ib</td><td>Relapse possible, no metastasis</td><td>Lipoma, aponeurotic Fibroma</td></tr>
<tr><td rowspan="2">Intermediate</td><td>Locally aggressive</td><td>IIa</td><td>Numerous relapses, no metastasis</td><td>Fibromatosis</td></tr>
<tr><td>Rarely metastasis</td><td>IIb</td><td>Numerous relapses, metastasis possible</td><td>Infantile Fibrosarcoma, well-differentiated Liposarcoma</td></tr>
<tr><td colspan="2" rowspan="2">Malign</td><td>III</td><td>Numerous relapses, metastasis possible</td><td>Myxofibrosarco-ma</td></tr>
<tr><td>IV</td><td>Assumed “systemic disease”</td><td>Alveolar Rhabdomyosar-coma</td></tr>
</table>

Table 6 - Soft tissue tumor classification according to malignancy

Worldwide mainly two morphological grading systems are in clinical practice. One system, primarily used in the Anglo-American language area, by the National Cancer Institute (67), the other one, which is the predominant one in Austria, first developed by the French Federation Nationale de Centres de Lutte Contre Le Cancer (FNCLCC). (68) The tumor differentiation, rate of mitosis and rate of necrosis are the base parameters to

evaluate the score for the FNCLCC grading-system. The histo-pathological grading follows the well-established TNM-scheme. Problematic cases occur when the two morphological grading systems show only limited accordance with biological features of the individual tumor. Due to these possible incongruities, some tumors are falsely labelled as not assessable. (66)

1.5.3.5 Therapy

The treatment of MFS and most other soft tissue sarcoma involves an interdisciplinary team of physicians including surgeons, radiologists, nuclear medicine specialists and oncologists. Due to the rare nature of these tumor entities, each individual case is usually discussed in tumor boards comprising some or all of the specialists mentioned above. (69)
In patients without metastatic disease, the primary goal is to achieve a R0-resection of the lesion. The scar left from the biopsy and the channel of the needle should always be removed as well as the complete fascial compartment containing the tumor including the neighbouring fascia tissue.
Radiation therapy can be used pre-, post- and intraoperative. Tumors that are primarily classified as inoperable may change their status after applied neoadjuvant radiotherapy. Intraoperative radiotherapy facilitates the exposure of tissue on the very spot of the resected lesion with local and very controllable radiation. Adjuvant radiation treatment is included in protocols for intermediate and high-risk malignant soft tissue tumors. Only in patients with low risk lesions or other successful R0-resection, it is an option to abstain from adjuvant radiotherapy.
As surgery and radiotherapy are the main treatment options for non-metastatic disease, medical treatment is more commonly used in advanced disease. The front line therapy for progressed soft tissue sarcoma consists of anthracyclines and ifosfamide. (70)

2 Aim, Hypothesis and Methods

2.1 Aims

The goal of this work is the assessment of the feasibility and potential informative value of further molecular investigation regarding the detected microdeletion at gene locus 22q12, detected in an index patient with neuroblastoma and other family members with or without malignancies in a tumor-prone Austrian family. In addition, the current factual situation of published literature concerning the mentioned putative tumor suppressor will be summarised and critically looked at. A plan for additional methods of investigation to better understand the pathogenesis and underlying pathways of this mutation and its related consequences will be addressed. Ultimately, the fusion of knowledge from published work and collected data from the index patient and the family will be appraised and the necessity for preventive medical checkups for mutation carriers and also not affected family members will be evaluated, similar to already known tumor predisposition syndromes.

2.2 Hypothesis

We postulate that the deletion at gene locus 22q12.1 functions as predisposing factor for tumors of diverse etiology within the family of the index patient.

2.3 Methods

This work consists of two main parts, the medical record review together with integration of reverse translation laboratory data of the index patient and biological relatives, and the literature review of the present state of scientific knowledge of the putative tumor suppressor. Informed consent forms for analysis of tumor and peripheral blood samples were obtained during lifetime or at time of treatment from the participants. Permission for minors to be included was given by their legal guardian. The conduction of this study was approved by the Ethics Committee of the Medical University of Graz; EK Number 1259/2019.

2.3.1 Retrospective chart review

The patients whose charts had been reviewed were biologically related to the index patient and including the index patient herself. Ten individuals consented to having a peripheral

blood sample tested for a potential mutation at the gene locus 22q12.1. Primary focus rested on haemato-oncological conditions, disease associated clinical and genetic investigations and whether or not a genetic deletion at locus 22q12 was found. Data from routine and research labs from all patients included was integrated. Initiation and planning of genetic analysis was carried out by the Children's Cancer Research Institute (CCRI, Sankt Anna Kinderspital), subdivision of Tumor Biology for Solid Tumors, under direction of Peter F. Ambors. He contributed greatly to this project by performing the single nucleotide polymorphism(SNP)-arrays and RNA sequencing. Further, the Research Center for Molecular Medicine of the Austrian Academy of Sciences (Ce-M-M-) was involved in this project with its subdivision of Kaan Boztug. This laboratory conducted the whole exome sequencing (WES) approach and data filtering pipeline for this project.

2.3.2 Literature review

To obtain a better insight into recent developments of Myo18B research a literature review was conducted. This part was essential to later on properly plan for further research and to develop a clinically-orientated prophylaxis guideline for family members carrying the putative tumor predisposition trait. The electronic databases of MEDLINE including PubMed, Cochrane Library, Science-direct and Google Scholar, from September 2002 till May, 2020 were searched. The search was carried out by using MeSH terms and Boolean operators suitable for PubMed. The following keywords were used: "Myo18B" OR "Myosin XVIII B" OR "Myosin 18B" AND "Cancer" OR "Myopathy" limiting to publications in English. Using these terms, references were identified. In addition to data base search, further articles were located using the Snowball method, meaning repeatedly referenced works in the already found articles were sought out as well, due to their evident relevance. Sources used from the internet were written by professionals in their fields and published on reliable sites, or on professional organizational or government sites.

3 Results

3.1 Pedigree

With assistance of the mother of the index patient a family history and pedigree were created. CytoScan HD Suite arrays indentified multiple family members with a heterozygous microdeletion of Myo18B. The pattern of affected family members (members with a confirmed genomic deletion) clearly shows a germline transmission traceable from generation II to generation IV of the pedigree.

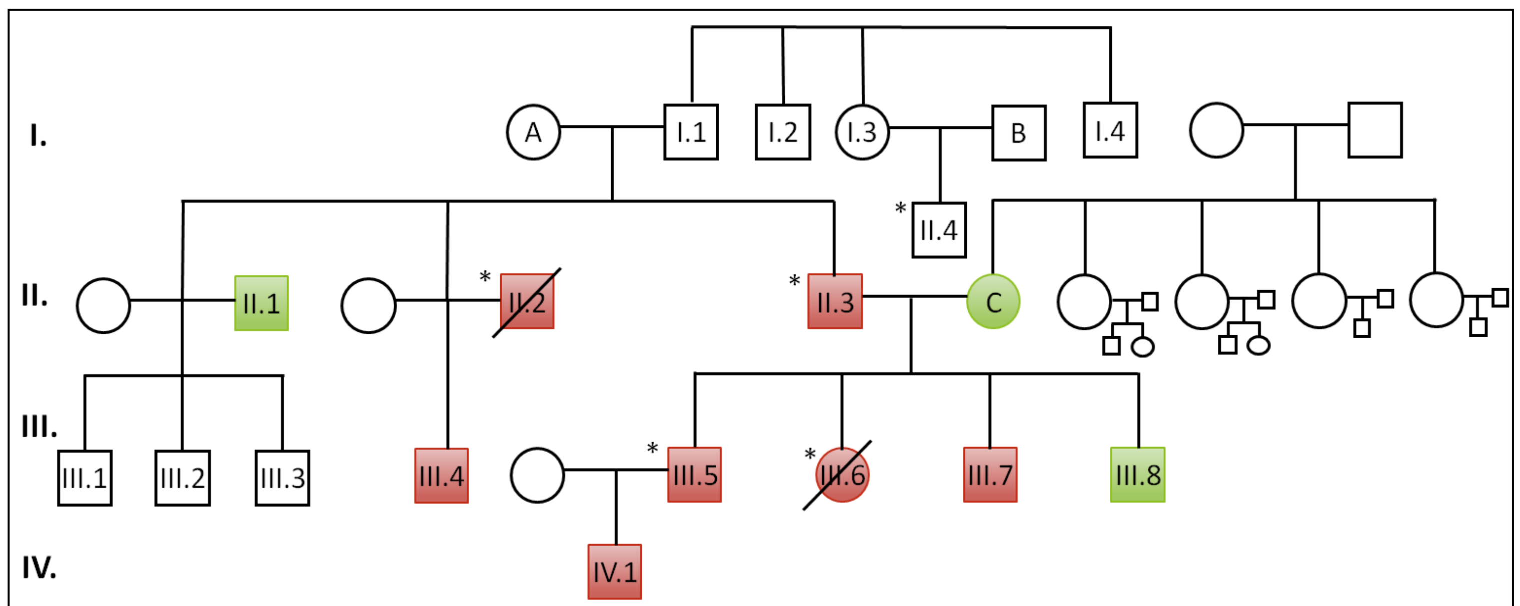


Figure 4 - Pedigree of Austrian family with germline Myo18B mutation. Asterisk, tumor diagnosis in individual; red panel, tested and confirmed heterozygous deletion of Myo18B; green panel, tested and excluded genomic deletion; white panel, not genetically tested.

Out of the ten tested individuals seven (II.2, II.3, III.4, III.5, III.6, III.7 and IV.1) showed a deletion at the long arm of chromosome 22 at the locus 12.1. Four (II.2, II.3, III.5, III.6) of the seven mutation carrying individuals were diagnosed and treated for malignancies. The index patient (III.6) was diagnosed in June 2006 with high-risk stage IV NB, which in the course of relapsing disease resulted in the beginning of the investigation. The other individuals were diagnosed with MFS (II.2), scrotal cancer (II.3) and metastasising scrotal germ cell tumor (III.5). II.4 was not genetically tested for a microdeletion of Myo18B, but through third party anamnesis of family members a case of scrotal cancer was recorded.

3.2 Index patient report

A 11-year-old female patient was transferred in late June of 2006 from the general paediatric out-patient department to the paediatric department for haemato-oncology. During the last 4 months, episodes of sharp recurring abdominal pain occurred every 4th-5th week, accompanied by fever of 39.7°C, which lasted for several days each episode. The punctum maximum of the abdominal pain was located by the patient in the left flank and upper left region of the abdomen. Application of Laevolac resulted in regular dejection. The young girl reported reduced appetite and weight loss of 2kg during the last couple of weeks. A resident paediatrician had performed an abdominal ultrasound examination two months earlier, which resulted in virtually no clinical abnormalities. Two days before admission, the patient was taken to the emergency room due to abdominal pain and feverish body temperature. The capillary blood count showed an elevated CRP level of 40 mg/l and a left shift with 81% neutrophil count. The 6 days earlier prescribed antibiotic therapy with Tricef (cephalosporin) was changed to Augmentin (amoxicillin with clavulanic acid). On the day of admission, abdominal ultrasonography was performed at the pediatric department and a 5x5 cm mass was found adjacent to the left kidney in the region of the suprarenal gland. This finding justified admission to the department of paediatric haemato-oncology with the suspected diagnosis of abdominal neuroblastoma (NB).

3.3 Diagnostic procedures

According to the INSS criteria for NB diagnosis a certain diagnosis can only be given via histological examination of tumor tissue acquired by biopsy of the suspect mass, or cytologically by identified infiltration of the bone marrow and simultaneously elevated catecholamine-metabolite levels in the patient's urine or blood. (30)
No palpable mass was found during manual examination of the abdomen, borders of liver and spleen could not be clearly identified. The initial analysis of catecholamine metabolites in the urine showed elevated vanillylmandelic acid, homovanillin mandelic acid and dopamine. Furthermore, neuron-specific enolase measured at 44.4 µg/l (norm <12). As these findings supported the tentative diagnosis of NB an extensive abdominal ultrasonography and a CT-scan of the thorax and abdomen were performed the same day.
The results of the imaging (CT-scan, MRI, MIBG-scintigraphy) revealed a thoraco-abdominal mass, originating from the left adrenal gland, stretching upwards to the distal

oesophagus and even further along the aorta, almost reaching the bifurcation. On the level of the diaphragm the tumor crossed the midline and protruded towards the porta hepatis, the left lobe of the liver cannot be clearly demarcated from the mass. A compression of the vena renalis sinistra was present at the confluence with the vena cava inferior. Furthermore, am 4mm nodule of high density was found on diaphragm level on the left, no intrapulmonary nodules were located. According to the radiologist these findings were highly compatible with NB. In addition, axillary lymph nodes on the left, an area at the left kidney base and in the soft tissue of the right thigh MIBG enhancement were found.

As the next step a bone marrow puncture and biopsy was performed. The material sent to St. Anna Kinderspital in Vienna was analyzed immuno-histologically and molecular-cytogenetically. The first one revealed GD2-positive cells and a wide range of criteria positive cells. The latter one showed no MYC-N amplification, but a 17q gain was verified. Collectively these findings constitute the final diagnosis, a case of juvenile high-risk NB stage IV.

3.4 Course of disease

Treatment started on the mid-2006 with a preoperative chemotherapy according to the protocol of high-risk Neuroblastoma of the Société Internationale D´Oncologie Pédiatrique (HR-NBL-1/SIOP). This beginning part of therapy consisted of 8 blocks with different combinations of antineoplastic and cytotoxic agents: oncovin (cincristine), carboplatin, VP 16 (etoposide), cisplatin and endoxan (cyclophosphamide) to achieve primary tumor size reduction to be followed by attempted complete excision of the primary tumor about 3 months after chemotherapy commencement. Priming for stem cell harvest via 3x peripheral stem cell apharesis happened from mid to late September of 2006. High dose chemotherapy was performed by following the carboplatin, etoposide and melphalan regime (CarEtoMel); the following autologous hematopoietic stem cell transplant (autoHSCT) went without complications. A residual tumor mass was found in post-operative imaging, an extirpation of the remaining tumor was therefore planned and performed. Due to an intraoperative complication the whole left kidney had to be removed. Radiation therapy of the tumor bed followed, together with administration of isotretinoin and anti-GD_2 antibodies.

Until September of 2008 the patient remained in remission, when a routine check-up revealed a multifocal tumor recurrence with multiple affected supraclavicular and thoraco-

abdominal lymph nodes. A bone marrow puncture further exposed a minimal involvement of the medulla. Therapeutic reaction was induction of a 4-block topotecan-vincristine-doxorubicin (TVD) treatment three days after the newly detected relapse. To suppress further disease progression before the planned paternal allogenic HSCT (alloHSCT), a combination of campto (irinotecan) and temodal (temozolomide) was introduced before the beginning of conditioning chemotherapy for the duration of eight days.

The conditioning for the alloHSCT consisted of fludarabin, thiotepa and melphalan. The alloHSCT took place in March of 2009. OKT3, a monoclonal antibody, was used to prevent acute rejection of the paternal cells and was given over 3-week course. At day +17 mycophenolat-mofetil (MMF) was further reduced; subsequently a cutaneous Graft versus Host Disease (GVHD) developed which responded well to treatment with prednisolone. Supportive cytokine stimulation with pegfilgastrim (a granulocyte colony stimulating factor) and darbpoetin alpha (a re-engineered form of erythropoietin) was implemented. Imaging showed tumor regression and lab work revealed negative tumor marker equating to a good partial remission.

At a routine check-up in November the same year, the MIBG scan showed increased tracer uptake mediastinal, at the base of the diaphragm and at the cranial pole of the right kidney. Thus, the patient was referred to the nuclear medicine department of the Universitäsklinik in Innsbruck for repeated ^{131}J-MIBG-therapy. Five cycles of radioiodine therapy were conducted, simultaneously the patient received Bevacizumab, a monoclonal antibody that inhibits neo-vascularization by blocking vascular endothelial growth factor (VEGF) receptors. This treatment led the patient into laboratory-chemical remission, yet in late November of 2011 multifocal bone infiltration was verified. An adaption of the chemotherapeutic approach was the result of this new turn by introducing sirolimus, dasatinib, temozolomid and irinotecan, according to the RIST-protocol, as well as a double SC boost with paternal HSTCs.

In consultation with the patient and her parents, the decision was made to perform a third HSCT, this time with the mother being the donor. The conditioning started in late January of 2013. With a combination of anti-thymocyte globulin (ATG), fludarabine, treosulfan and thiotepa a myeloablative approach was taken. The patient received the maternal donor STCs in February of 2013. Further, GVHD prophylaxis with MMF, donor lymphocyte infusions and anti-GD_2 antibodies were administered. GVHD affecting skin, liver and intestinal tract, yet emerged and was treated with prednisolone, methylprednisolone, MMF, sirolimus and thalidomide, a chronic development was unavoidable. A MIBG scan in

January 2014 showed massive tumor progression, the laboratory revealed immensely elevated tumor marker. From mid-January to early February a salvage therapeutic approach was taken with arsenic trioxide. Due to a suspected arsenic-induced capillary leak syndrome the treatment was stopped shortly after. With accelerated diuresis and dexamethasone, the pleural effusions could be dissipated and the patient was released into palliative care at home with the support of a mobile palliative team and support from the haemato-oncological department upon request at any time. Analgesic therapy was adapted with Transtec-patches (buprenorphin), liquid vendal (morphine) and dexamethasone. The tumor specific therapy at this point was completely retracted by the wish of the patient. If needed dronabinol drops (tetrahydrocannabinol) and haldol (haloperidol) were accessible for the patient. A patient controlled analgesia was implemented, after extensive conversation with the patient and her mother. In March of 2014 the 18-year-old patient passed away in the presence of her family.

In **Figure 5** below a timeline depicts the order of major events during the treatment period of the patient. In **Figure 6** selective laboratory markers are presented to better identify phases of disease regression, stagnation and progression.

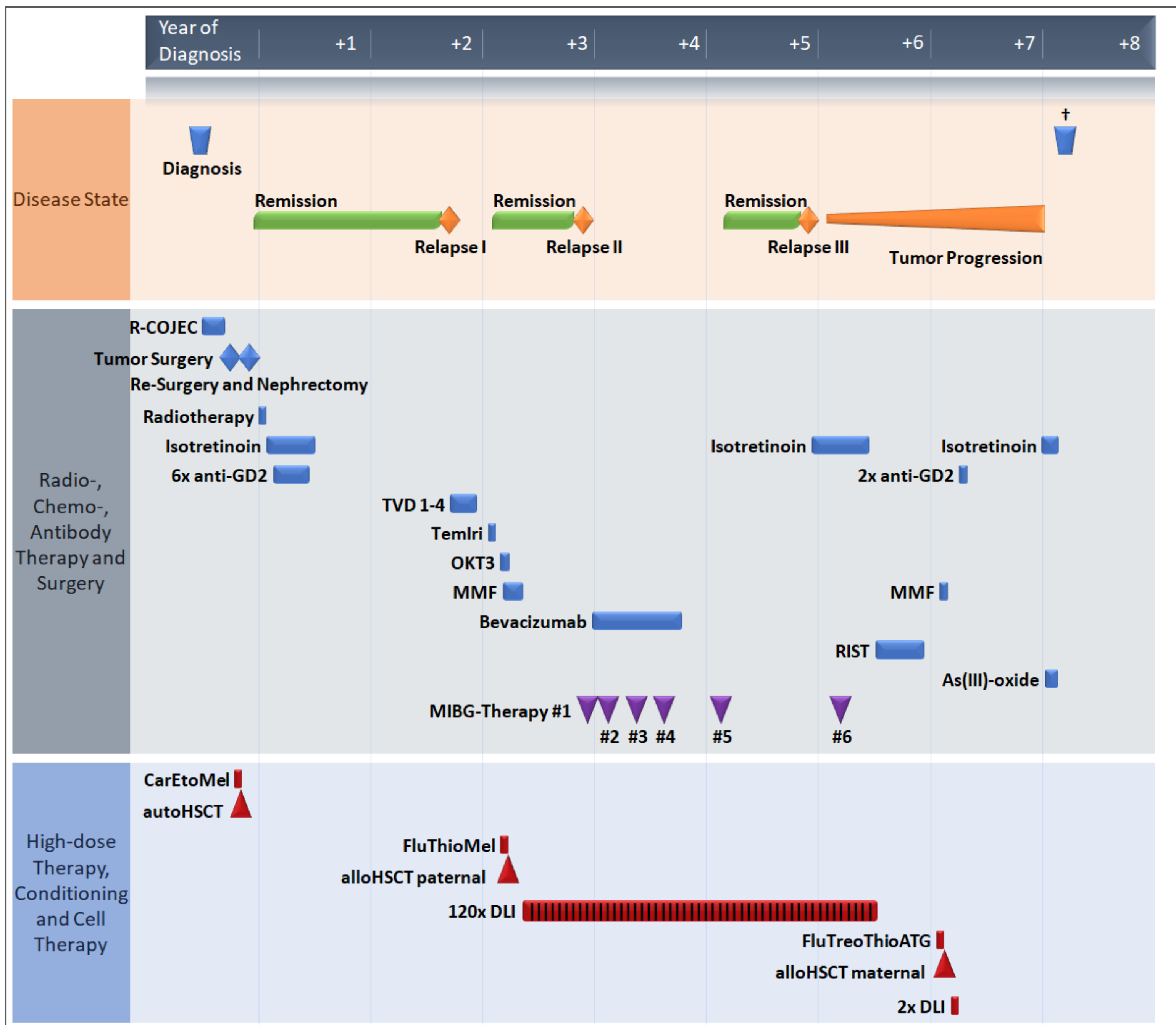


Figure 5 - Therapy of the index patient chronologically from the year of diagnosis to the patient's death. The portrayed information includes different therapy regimes through different involved paediatric departments over a time span of about 8 years. The position of the triangular, trapezoid and diamond shaped blocks indicate events that happened at the time corresponding to the time axis on the top, rectangular blocks are dedicated to repeated events or treatments for a certain period, conforming to the length of the block. Abbreviations: R-COJEC, rapid (R) - cisplatin (C), vincristine (O), carboplatin (J), etoposide (E) and cyclophosphamide (C); TVD, topodecan (T), vincristine (V) and doxorubicin (D); TemIri, temozolomide (Tem) and irinotecan (Iri); OKT3, Muromonab-CD3 (trade name Orthoclone OKT3); MMF, mycophenolat-mofetil; RIST, sirolimus (trade name Rapamycin, R), irinotecan (I), dasatinib (S) and temozolomid (T); MIBG, metaiodobenzylguanidine; CarEtoMel, carboplatin (Car), etoposide (Eto) and melphalan (Mel); FluThioMel, fludarabine(Flu), thiotepa (Thio); FluTreoThioATG, treosulfan (Treo), anti-thymocyte globulin (ATG), DLI, donor lymphocyte infusion.

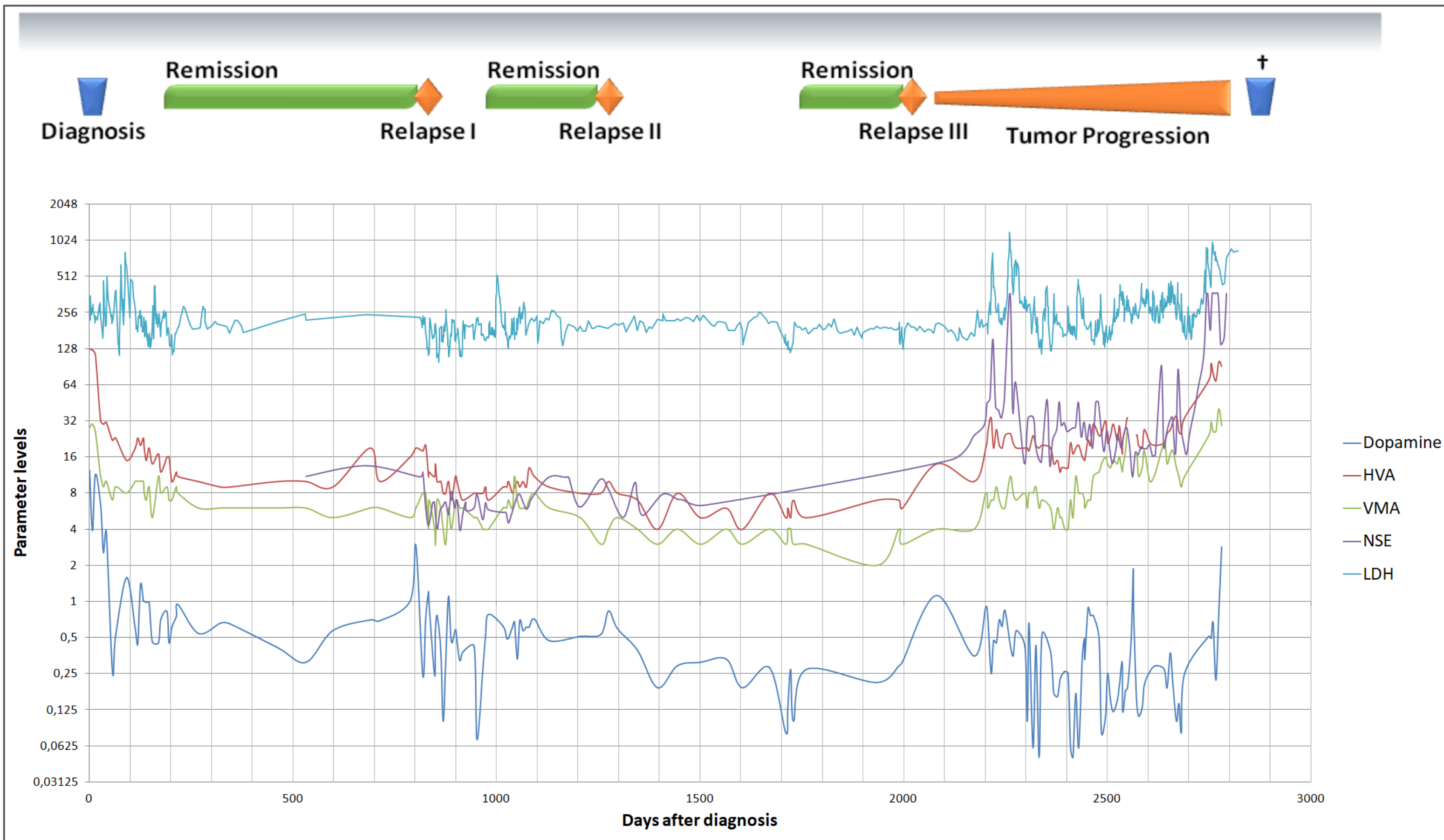


Figure 6 - Laboratory parameters of the index patient throughout the course of NB treatment. On the x-axis the days after diagnosis are plotted, the level of the different tested parameters on the y-axis. Testing stop the last weeks before death, as the patient stayed at home in palliative supportive care. Abbreviations: HVA, homovanillic acid; VMA, vanillylmandelic acid; NSE, neurone specific enolase; LDH, lactate dehydrogenase.

3.5 *Extended translational scientific results of the index patient and family members*

The described case of the index patient represents an instance of a highly aggressive and -despite intensive therapy- progressing NB. Tumor samples from patients sent to the CCRI for investigation of therapy relevant molecular-genetic modifications are also in focus of ongoing research regarding tumor-genesis and tumor-evolution. The following shows results of different analytic approaches to gain insight into the genomic situation of the family.

3.5.1 Single Nucleotide Polymorphism (SNP) Array Analysis

The SNP array analysis was conducted by the Ambros group of the CCRI.

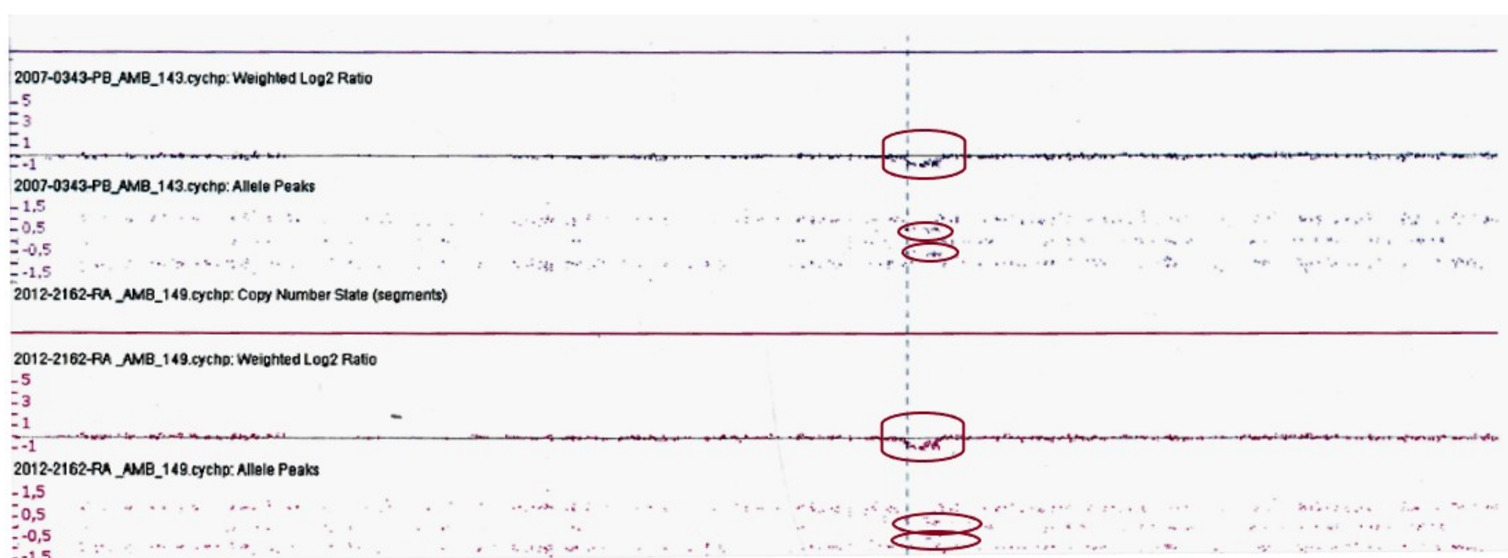


Figure 7 - Affymetrix cytoscan HD SNP array analysis including weighted log2 ratio (upper dotted lines) and allele peaks (lower dotted lines) presentation. The upper sample (dark blue) is peripheral blood (PB) from the index patient, the lower sample (pink) are disseminated tumor cells (DTC) from the bone marrow of the index patient. Both samples show loss of signal intensity in the weighted log2 ratio (red boxes). In the red ovals, two shifted tracks in the middle can be seen for the extent of the microdeletion, indicating the presence of only a single allele.

At region chr22:26,247,460 - 26,282,048 (hg19) a deletion including 4 exons was found. The missing exons 22, 23, 24 and 25 of *Myo18B*, consisting of 419 nucleotides, constitute an out of frame shift of the remaining nucleotide sequence.

Figure 8 - Affymetrix cytoscan HD SNP array analysis including weighted log2 ratio (dotted lines) and smooth signal (continuous lines) presentation. From top to bottom the samples for this array were index patient (DTC, purple), father of index patient (PB, pink), brother of index patient (PB, light blue), brother of index patient (PB, green), brother of index patient (PB, orange) and mother of index patient (PB, blue). The white arrows point to the region of hemizygous deletion at locus chr22:26,247,460 - 26,282,048; the black squares are used to cover patient identification marks added by the laboratory. The index patient, two brothers and the father are affected; one brother and the mother show no sign of microdeletion in this tested region.

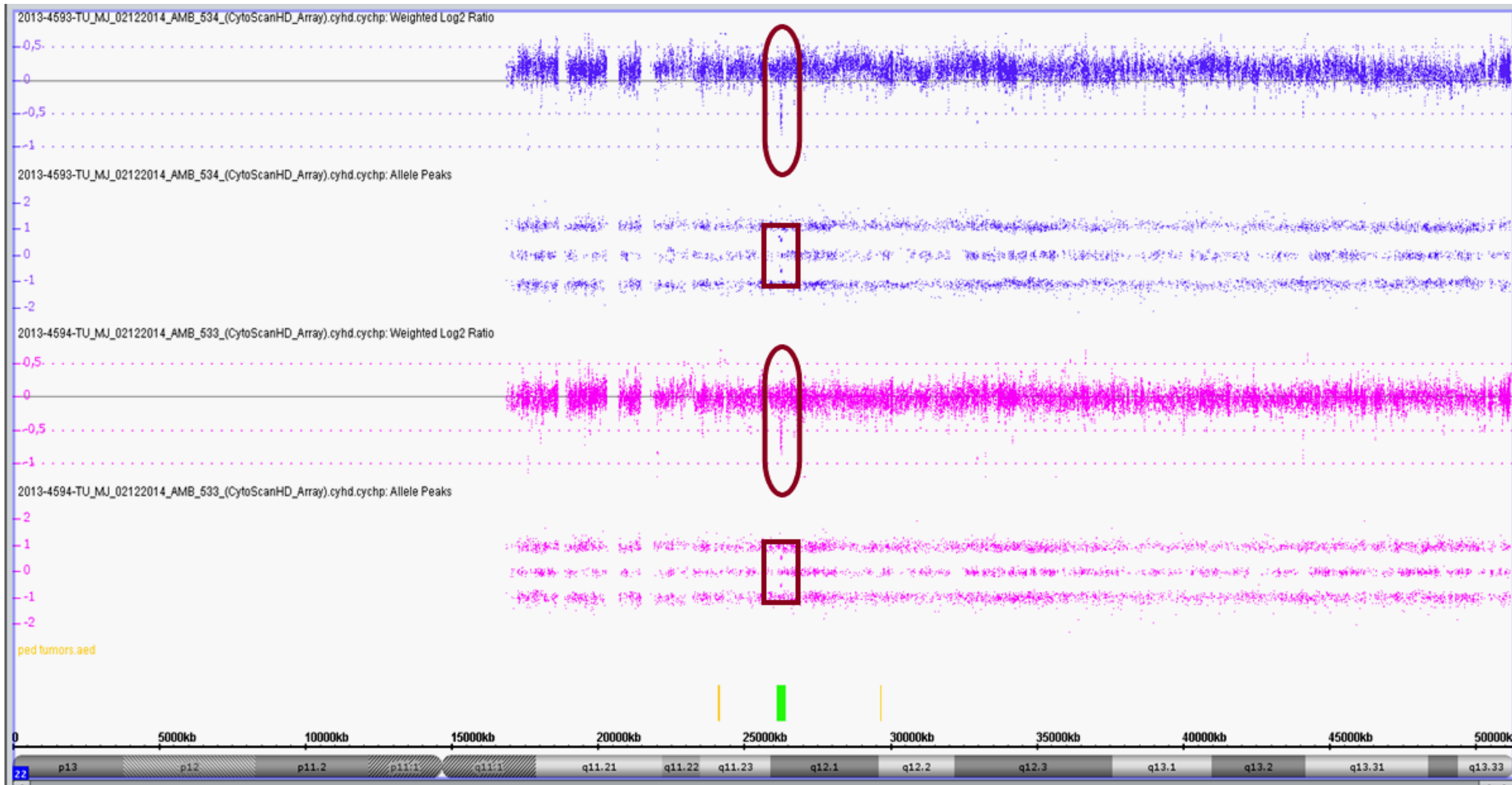


Figure 9 - Affymetrix cytoscan HD SNP array analysis of tumor tissue of the index patient (pink) and tumor tissue of one affected brother (blue, testicular cancer). Both tumor samples show loss of signal intensity in the weighted log2 ratio (red oval). In the rectangular red boxes, two tracks in the middle can be seen for the extent of the microdeletion, indicating the presence of only a single allele.

We found multiple affected family members that have an identical heterozygous microdeletion on chromosome 22. SNP array analysis revealed the same deletion to be present in PB of all affected, in tumor tissue of the index patient (NB) and her brother (testicular cancer) and in DTC, obtained from bone marrow samples, of the index patient.

3.5.2 RNA Sequencing

The RNA sequencing analysis was conducted by the Ambros group of the CCRI.

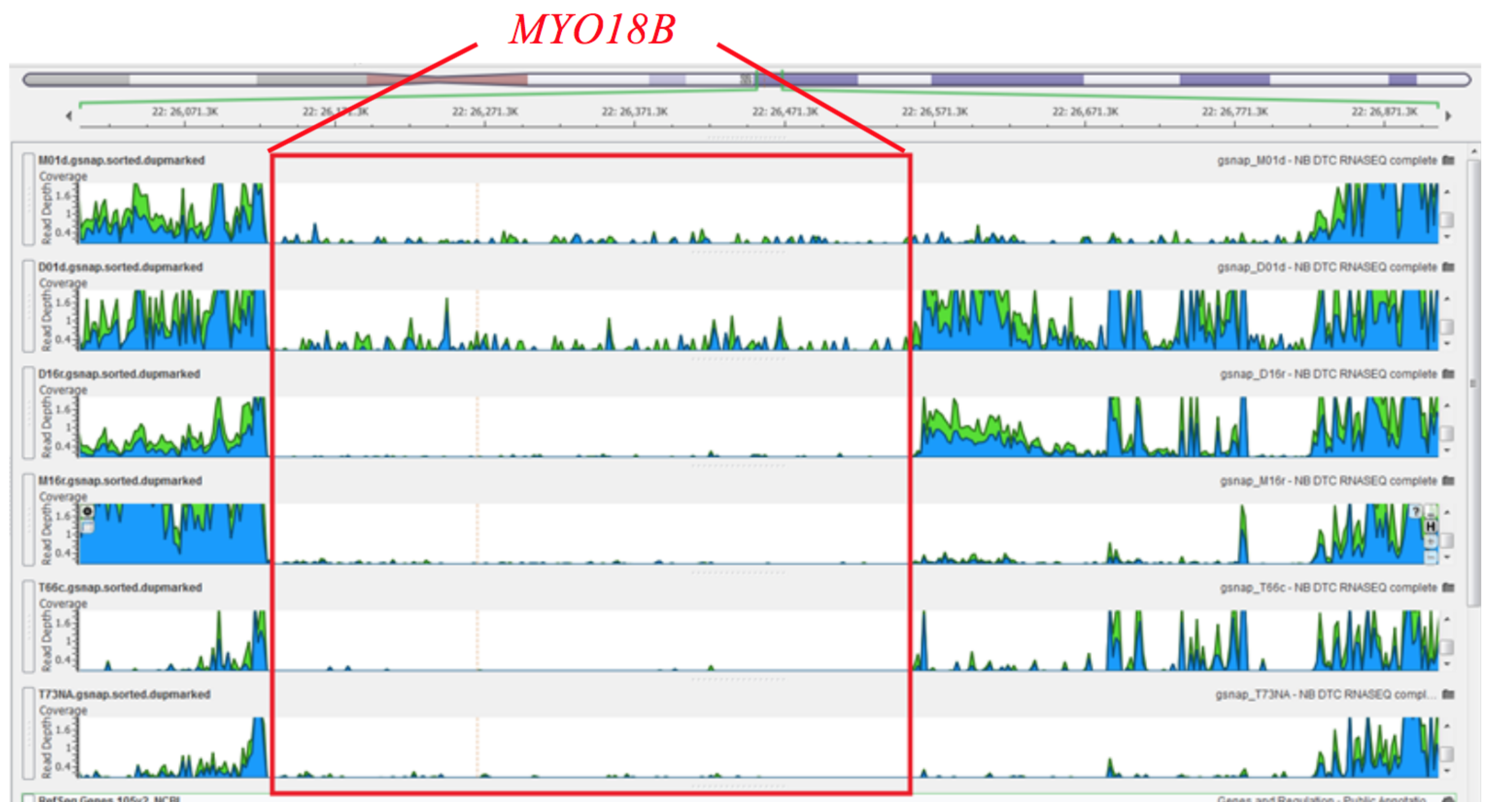


Figure 10 - RNA sequencing profile of *Myo18B* (NM_032608). The top two plots represent two samples of another NB patient (upper mononuclear cells; lower DTC) with intact *Myo18B*. The third plot is sampled from DTC of the index patient; the fourth plot is the expression profile of mononuclear cells of the index patient; the fifth represents tumor tissue from the primary NB of the index patient; the bottom plot's sample is tumor tissue from the index patient's brother (testicular cancer).

3.5.3 Whole Exome Sequencing and Filtering

Whole Exome Sequencing (WES) was performed by the Boztug Laboratory on seven different samples (six affected, one healthy) from peripheral blood (PB) and if available from tumor tissue. People included in WES analysis are the index patient, the affected father, two affected brothers and one not-affected brother. Two approaches in data filtering were taken. The first one presumed a germline alteration and investigated whether loss of function of another tumor suppressor gene or gain of function of an oncogene is present to shine light on a potential modifying effect of *Myo18B* with another oncogenic alteration. This was performed by merging the PB data sets of affected subjects and subtracting healthy PB exome data sets to reveal common homozygous or heterozygous variants. The second approach presumed a somatic alteration. Here all PB data sets from affected and unaffected were subtracted from the tumor data sets, to identify which variants are exclusively found in tumor tissue and common in both affected siblings with tumors to reveal a potential somatic 2nd hit. Unfortunately, the applied data processing pipeline was not specialized in identifying somatic variants; therefore, this step is being adapted and currently repeated for both approaches by the Department of Human Genetics and Molecular Biomedicine of the Medical University of Graz in collaboration with C. Windpassinger.

4 Discussion

Mutated, deleted, methylated and otherwise epigenetically modified Myo18B has been found in tumor cell lines (23–29,71,72) as well as in primary tumors (23,25–29,71). To our knowledge, we presented the first instance of a patient with high-risk NB and a deletion at locus chr22:26,247,460 - 26,282,048 (hg19), which led to complete absence of *Myo18B* RNA. Phenotypically, our patient family report (case series) demonstrated two main points: First, humans with a heterozygous germline mutation in *Myo18B* are viable and can have unimpaired heart and skeletal muscle development and function; and second, the putative tumor suppressor function of Myo18B is supported, given the high tumor predisposition observed in this family. In fact, the tumor predisposition phenotype cosegregated with the genotype insofar, as those individuals carrying the mutation who have not yet developed a malignancy are still younger than the average age of tumor diagnosis (II.2, myofibrosarcoma diagnosed at 48 years; II.3, testicular cancer diagnosed at 28 years; II.4, testicular cancer diagnosed at 31 years; III.5, metastasized scrotal germ cell tumor diagnosed at 21 years; III.6, index patient with neuroblastoma diagnoses at 11 years) as observed in the affected individuals.

The aggressive disseminating course of the index patient's NB accords with earlier publications on characteristics of cell cultures with inhibited function of Myo18B, by enabling anchorage independent growth and therefore constituting a metastasis and cancer promoting factor.(22,71)

No live births of Myo18B deficient mice were reported, because defective heart development impeded further embryonic life (21). Heterozygous mice did not develop malignancies (*personal communication with Rieko Ajiima*), which might be attributed, at least in part, to a shorter life under laboratory conditions, as compared to humans, in whom we allegedly propose to have observed a tumor predisposition. However, four human individuals with homozygous nonsense mutations of *Myo18b* and one patient with a constitutional compound heterozygous frameshift mutation have been reported.(21,30,31,33,34) The four patients with homozygous nonsense mutations were all delivered to consanguineous parents.(30,31,34) The one patient with compound heterozygous mutation was delivered to non-consanguineous parents.(33) Interestingly, the published homozygous variants cluster in the C-terminal region of the gene, raising the question whether an earlier occurring stop codon could be incompatible with life in

humans. One patient was diagnosed with large-cell anaplastic medulloblastoma and nemaline myopathy. (33) In his genome a frameshift variant of *Myo18B* was found without signs of NMD. The medulloblastoma surprisingly showed elevated mRNA expression *Myo18B* when compared to other tumors of central nervous origin and also compared to varying developmental stages of the brain. (33) Another patient that exhibited loss of a C-terminal exon due to a premature stop codon expressed the truncated protein with no sign of NMD. This patient also showed clinical signs of nemaline myopathy together with cardiomyopathy.(34) Alazami et al. presented two patients from different families, both from consanguineous parents. Each one of them was diagnosed with Klippel-Feil anomaly (cervical segmental spine fusion) and myopathy. A truncating muatation in Myo18B was found, resulting in a near complete loss of *Myo18B* transcript in patient lymphoblasts, thus confirming NMD and concluding a null nature of functioning protein.(31)

This raises the question, if a truncated protein is capable of retaining some of its tumor suppressing capabilities, but falls short of carrying out its physiological function of muscle cell alignment and orientation, hence resulting in clinical signs of myopathies and cardiomyopathies. Whether a faulty protein is decayed or not might be due to truncation of the C-terminally located Sug1 binding site, which targets the protein for degradation by the ubiquitin-proteasome pathway. (18,73)

Attempts of Myo18B protein detection were conducted at the Ambros laboratory at the CCRI but were reported unsuccessful (data not shown/ not available). Although we showed that no RNA was traceable, to fully validate this claim, however, a re-sampling of tissue in which Myo18B has the highest expression (skeletal muscle, heart) needs to be assayed and the RNA profile then compared to neighbouring housekeeping genes, as in peripheral blood little mRNA can be expected according to tissue specific expression charts.(74)

A central question of this study was to answer whether the 22q microdeletion that gave rise to absence of *Myo18B* RNA and, consequentially, of functional protein was alone sufficient to cause the tumor predisposition, or whether (and which) additional factors were necessary to cause the observed malignancies. None of the classical, known hereditary cancer predisposition syndromes were detected, corroborating that the absence of Myo18B that cosegregated with the phenotype was infact disease-causing. This search was

complicated by the facts that the types of tumours and the ages at presentation were entirely different (relapsing, refractory high-risk neuroblastoma at XX years of age; rapidly progressing myofibrosarcoma at yy years of age; metastasising testicular germ cell tumours at ZZ and QQ years of age), and that no other Myo18B-dependent phenotypical abnormality that was shared by the affected individuals could be detected. We performed WES of tumour tissue, normal tissue, and blood cells of affected subjects and a control family member to elucidate the potential involvement of second tumourigenic hits. Although we could not identify another, common, candidate oncogenic germline mutation, unfortunately, we cannot yet exclude the existence of additional genetic factors, because our data processing pipeline using MuTect® was not specialized in identifying somatic variants according to the Boztug Laboratory. Thus, at this point in time, we were not successful in seeking out a potential common second hit, which could be either a shared or a variable germline variant in a proto-oncogene, a tissue- and tumour-specific variable somatic variant in a tumor sample, or a "private" disease-modifying genetic or even epigenetic event, different in each individual. This process is being revised and taken care of by C. Winpassinger of the Medical University of Graz, who is currently reviewing WES and RNA sequencing data to plan further molecular investigations.

Taken together, the findings in the presented patients suggest that LOH of *Myo18B* is a dominant factor for the development of a malignancy.

5 Conclusions

We proposed the deletion at gene locus 22q12.1 plays a role in a tumor-suppressing pathway for tumors of diverse etiology. By introducing our case, we extended the pool of tumors associated with defective Myo18B by the NB.

In retrospect, a rework of the data analysis of WES data needs to be conducted, potential second hits elaborated and investigated for integrity regarding their role in carcinogenesis and oncogenesis.

Our observation of different tumor types in literature and in our presented pedigree suggests that varying driver mutations and/or second hits are involved. To gain insight into the role of Myo18B in cancer cells, its intracellular pathways, binding partners and protein-interactions need to be elucidated.

The carriers of the microdeletion of our investigated Austrian family receive an intensified medical screening regime according to oncological common sense. Basic medical screening once a year carried out by the general practitioner for affected family members, combined with a more selective examination in a clinical setting, is in accordance with already established screening regimes for other tumor predisposition syndromes.

6 Perspectives

A forward-looking approach on verifying the tumor suppressive role of Myo18B includes reanalysing the SNP, WES, and RNA sequencing data with updated bioinformatic criteria. Reanalysis of RNA-sequence data from samples with high Myo18B expression profile, put into proportion with neighbouring housekeeping genes, will allow a more precise and definite testimony of the patients individual RNA expression.

A search in the comparison of tumor DNA with peripheral blood samples, stringently with the goal of isolating tumor specific somatic mutations, yields the greatest potential of success.

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