# MicroRNAs

micro molecules
with macro clinical
& molecular
implications

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# MicroRNAs - Micro Molecules with Macro Clinical & Molecular Implications

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# Table of Contents

TABLE OF CONTENTS 4

**CHAPTER 1** **6**

GENERAL INTRODUCTION 6

**CHAPTER 2** **22**

EXPRESSION AND INHIBITION OF BRD4, EZH2 AND TOP2A IN NEUROFIBROMAS AND MALIGNANT PERIPHERAL NERVE SHEATH TUMORS 22

**CHAPTER 3** **46**

DEREGULATED MICRORNAS IN NEUROFIBROMATOSIS TYPE 1 DERIVED MALIGNANT PERIPHERAL NERVE SHEATH TUMORS 46

**CHAPTER 4** **76**

NON-CODING RNAS, A NOVEL PARADIGM FOR THE MANAGEMENT OF GASTROINTESTINAL STROMAL TUMORS 76

**CHAPTER 5** **108**

MOLECULAR COMPARISON OF IMATINIB-NAÏVE AND RESISTANT GASTROINTESTINAL STROMAL TUMORS: DIFFERENTIALLY EXPRESSED MICRORNAS AND MRNAS 108

**CHAPTER 6** **134**

GENERAL DISCUSSION & SUMMARY 134

*Discussion* *135*

*Summary* *145*

*Samenvatting* *147*

*Word of Thanks* *150*

*List of publications* *155*

*Curriculum Vitae* *158*

# Chapter 1

## General Introduction

# 1. Soft tissue sarcomas

Soft tissue sarcomas (STS) are rare malignant mesenchymal tumors, which occur in soft tissues of the body such as fat, deep skin tissues, muscles, blood vessels, and other connective tissues (Fig.1). They have a high diversity as the WHO categorizes more than 50 various subtypes based on their anatomical location, tissue of origin and biological potential [1]. Their rarity, STS account for 1% of all adult malignant tumors, and diversity within and across various subtypes classifies them among the more challenging malignant tumors. Each subtype represents unique and distinct clinical and biological features, which have significant impact on the prognosis and diagnosis of these malignant neoplasms as well as on their sensitivity towards chemotherapy. Moreover, the body location of STS is also variable making it even more challenging to reach optimal patient care.

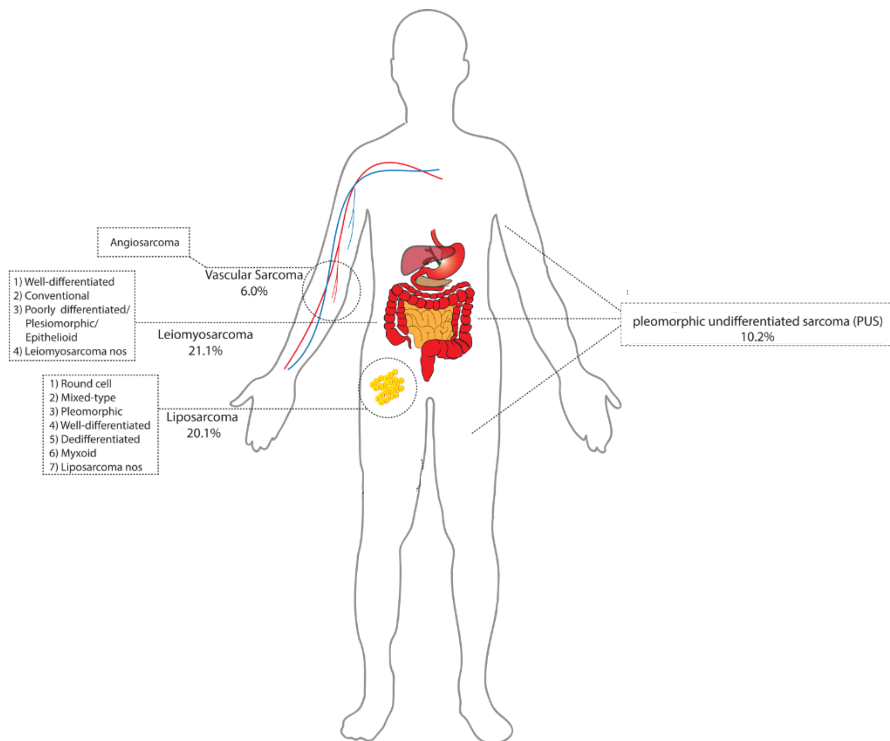


**Figure1. Most common soft tissue sarcomas in the Netherlands [2].** The depicted incidence percentages for each subtype are calculated from the 2006-2011 soft tissue sarcoma patient cohort (≥18 years, n = 3317) in the Netherlands.

## 1.1 Limitations in clinical care of STS

For patients with advanced STS who are not candidates for treatment approaches with a curative intent, systemic treatment with anti-tumor drugs remains the only option next to best supportive care. Concerning drug development, oncology has a higher failure in clinical trials compared to other disease areas and this holds true for drug development in advanced STS as well. There are multiple underlying reasons for this including suboptimal preclinical drug validations because of preclinical models that do not accurately represent the complexity of human cancers. Also tumor heterogeneity, even within a single STS subtype significantly impacts prognosis, diagnosis, and therapeutic responses of the patients. This brings major limitations and consecutive challenges for successful and effective treatment of these patients. The advent of molecular biology and emergence of advanced genetic approaches allows to get a better insight into the tumor biology of the diverse STS subtypes, and has -up to a certain extent- improved the accuracy of clinical care for some STS patients. Relevant molecular techniques include: fluorescent in situ hybridization (FISH), next-generation sequencing (NGS), gene (mRNA) expression analysis, miRNA expression profiling, karyotype analysis and polymerase chain reaction (PCR) [3]. For example, an improved understanding of the biology of gastrointestinal stromal tumors (GIST), a STS sub-entity that originates from interstitial cells of Cajal (ICC), revealed that the majority of these tumors are driven by gain-of-function mutations in c-KIT or in PDGFR-α. The introduction of imatinib, a tyrosine kinase inhibitor that targets these mutants [5, 6], has dramatically improved the outcome for GIST patients. Initially patients faced only a 9 months overall survival (OS) which increased, because of imatinib, to 5 years [4] with a median progression-free survival of about 2 years with a response rate of approximately 50% [5]. Despite the revolutionary role of imatinib therapy in these patients, the vast majority develops resistance due to the acquisition of several resistance mechanisms [6]. Resistance mechanisms may be multi-factorial and display intra- and intertumoral heterogeneity emphasizing the importance of identifying and molecularly characterizing the GIST subpopulations to define effective therapeutic regimens for these patients.

The chemotherapeutic drug pazopanib is a treatment option for patients with advanced non-adipocytic STS after failure to prior chemotherapy. In general, tumor progression after 4 to 6 months of pazopanib treatment is observed. However, it has been shown that the exact duration of pazopanib response varies remarkably between different histological STS subtypes as well as within the same tumor type [7]. Failure of pazopanib treatment in STS highlights the need to discover subsequent effective treatments for these patients. To this end, cell viability assays, using different experimental drugs, as well as genomic profiling experiments were conducted on cells and xenograft models generated from pazopanib resistant tumors. These analyses did show impaired tumor cell growth upon BEZ235 (Dactolisib, a PI3K inhibitor) and AZD2014 (Vistusertib, a mTOR inhibitor) treatments inhibiting the mTOR/AKT pathway in refractory undifferentiated pleomorphic sarcoma. These drugs have been suggested as an alternative treatment option for patients who did not respond to pazopanib in clinical trials [8]. Considering these reports, the importance of acquiring a better and deeper knowledge of tumor

biology as well as systematically testing alternative therapeutic strategies in a rational fashion is imperative to make progress and develop better and more effective treatments.

Another major hurdle in acquiring successful therapies for STS patients is the poor understanding of underlying mechanisms involved in tumorigenesis and tumor progression due to the lack of representative and reliable pre-clinical models. Malignant peripheral nerve sheath tumors (MPNST) are another example of a challenging malignancy. MPNST are highly aggressive STS with a high local recurrence rate as well as the propensity to metastasize and resistance to therapeutic interventions. About half of these tumors arise in the context of a pre-existing benign counterpart, plexiform neurofibromas as they occur in neurofibromatosis type 1 (NF1) patients [9]. Lack of knowledge of the underlying mechanisms of MPNST formation and metastasis in the NF1 setting is another deficiency in the field. A better understanding of MPNST biology and the molecular drivers of the malignant transformation from neurofibromas to MPNST is therefore needed to reveal new and effective treatment targets. Recent studies, using human cell line models and genetically engineered mouse models, have identified candidate genes and biochemical pathways that may be targeted for therapeutic purposes. However, as of yet none of these findings was translated into novel treatments for MPNST patients [10]. This might be due to crucial cross-species differences and biological variability that exist between the animal and human cell line models of MPNST on one hand, and the actual characteristics and behavior of these tumors in patients. Xenograft models only partly solve these problems (cross-species differences) as they usually simulate a single cancer stage and do not model early stages of tumor formation and progression. Apart from the above mentioned hurdles, tumor heterogeneity between different individuals should not be underestimated. In order to overcome these obstacles the generation of a reliable pre-clinical model that allows us to monitor the various stages of tumorigenesis and tumor progression is necessary. The analysis of the distinct cellular and molecular phenotypes of cells, going through distinct stages of tumor formation, may lead to the discovery of novel genes/pathways involved in carcinogenesis, the identification of reliable biomarkers for early diagnostic purposes and new therapeutic opportunities.

## 1.2 Future directions to improve STS patient’s outcome

Although great strides have been made in improving the clinical care for STS patients, a lot is still unknown about this heterogeneous group of tumors, which makes it challenging to develop effective therapies. The increasingly advanced and sensitive molecular techniques have already promoted our understanding of STS etiology and biology and bear great promise for the future. These developments will ultimately lead to novel and more effective ways of diagnosing and treating STS patients.

We owe it to the patients who suffer from rare STS to translate laboratory findings to the clinic and patients as quickly as possible. International collaboration is in this respect extremely important, sharing resources like patient samples and unpublished experimental and clinical data on a world-wide scale. This can only happen if all involved parties cooperate including medical oncologists, pathologists, biologists, surgeons and pharmacologists as well as

governments and patients. Only focus, hard work and setting aside personal interests will enable us to make progress for the benefit of the patient.

# 2. MicroRNAs

MicroRNAs (miRNAs) are involved in the pathobiology of many diseases. An improved understanding of their exact role in STS may yield the identification of specific miRNAs, which could serve as a prognostic or predictive biomarker or as a target for treatment. In 1993, the first small non-coding RNA, *lin-4,* was discovered through a forward genetic screen in the nematode *Caenorhabditis elegans* [11]. The negative regulatory role of this small RNA molecule on the expression/function of the protein coding *lin-14* was further established in the same year [12]. The field got a major impetus with the discovery of another small RNA involved in developmental timing in nematodes, *let-7*. In contrast to *lin-4*, *let-7* was phylogenetically highly conserved and could be detected in variety of animals including humans [13]. It soon became clear these small (18-25 nucleotides) RNA molecules represented a novel class of endogenous, evolutionarily conserved small RNA molecules, microRNAs, that negatively regulate gene expression at a post-transcriptional level [14]. They play pivotal roles in the regulation of fundamental cellular and physiological processes and are closely involved in the pathobiology of many disorders including cancer. Advances in next-generation sequencing methodologies [15] followed by computational/bioinformatics analyses have greatly facilitated research into the regulatory role of miRNAs and the subsequent effects on their mRNA targets [16]. In general miRNAs negatively regulate gene expression by binding, in the context of the RNA-induced silencing complex, to the 3' untranslated region (3' UTR) of target mRNAs. In some cases, however, the 5'UTR or open reading frame (ORF) are reported as binding site for miRNAs [17, 18]. The miRNA binding causes mRNA destabilization/degradation and/or inhibition of mRNA translation [19, 20].

## a. MiRNAs biogenesis canonical and non-canonical pathways

In mammalian cells mature miRNAs can be generated from endogenous transcripts that fold into hairpin structures by canonical and non-canonical processes. The majority of miRNAs are produced by the canonical pathway.

### i. Canonical pathway

Canonical miRNA biogenesis starts with the transcription of a long precursor molecule in the nucleus called primary-miRNA (pri-miRNA) (Fig.2). One (monocistronic) or multiple (polycistronic) hairpin loop structures are embedded within the pri-miRNAs, which have major roles in protecting the structural stability of the transcript and providing recognition sites

for RNA binding and processing proteins. Next, within the nucleus, the microprocessor complex, which includes an RNA-binding protein (DGCR8) and a nuclear RNase III enzyme (DROSHA) processes the pri-miRNA. The transcript is cleaved at the base of the hairpin loop, which leaves behind a 2-nt 3' overhang [21]. The resulting ~70-nt hairpin-like secondary transcript is known as the precursor miRNA (pre-miRNA). Pre-miRNAs are then translocated to the cytoplasm mediated by Exportin-5 and Ran-GTP [22]. The terminal loop of the pre-miRNAs is subsequently removed by another RNase III endonuclease Dicer releasing a ~22-nt double stranded RNA (dsRNA) [23]. Dicer binding to the pre-miRNAs is assisted by the help of TRBP, which is an RNA binding protein pivotal for increasing Dicer binding affinity and its cleavage accuracy [24, 25]. The dsRNA is bound by Argonaute2 (Ago2) protein, which ultimately forms part of the RNA-induced silencing complex (RISC) and has crucial roles in unwinding of the duplex and selecting the mature miRNA strand [26]. The duplex contains two partially complementary miRNAs (miRNA/miRNA*) of which in most cases the passenger or star(*) strand gets degraded. With the help of Ago2 and several other proteins, the mature miRNA strand is loaded into the RISC complex and guided towards specific binding sites on target messenger RNAs (mRNAs). Ago2 and glycine-tryptophan 182 (GW182) are among the key components for the assembly and function of the RISC complex, in which the GW182 acts as the downstream effector in repression. GW182 functions as a flexible bridge to make the interactions between RISC and other downstream proteins in the RISC complex. Depending on the complementarity region between the (seed) region of the mature miRNA and targeted mRNA, translational inhibition and/or mRNA degradation/destabilization will take place [27]. MiRNAs can act as potential translational repressors by partially pairing with the 3', 5' UTRs or coding sequences without any impact on the structure of the targeted mRNAs [28].

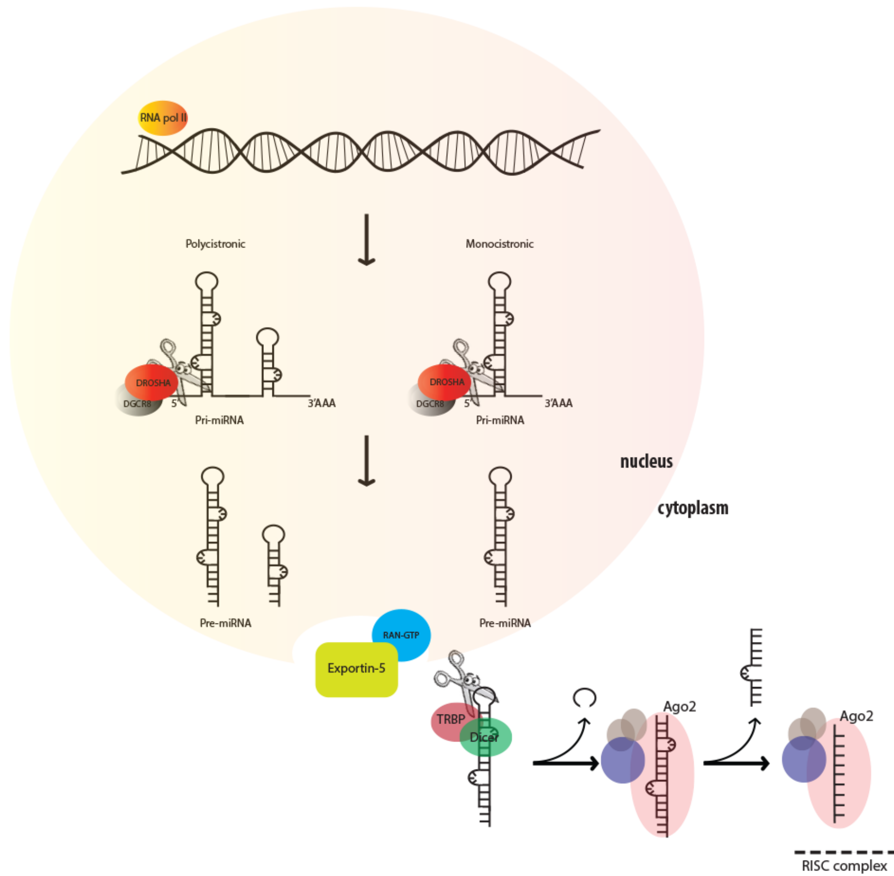


**Figure2. Canonical miRNA biogenesis.** The pri-miRNA is generated by the activity of RNA polymerase II. This long transcript is then processed by DROSHA-DGCR8 resulting in the production of the pre-miRNA in the nucleus. This ~70-nt hairpin-like structure is then transported to the cytoplasm with the help of Exportin-5 and Ran-GTP. Dicer and TRBP are involved in a second RNA processing step and consequently the production of a double stranded RNA molecule containing the mature miRNA and a passenger strand which may be degraded or also function as mature miRNA. The double stranded RNA and eventually the mature miRNA is attracted to the Argonaute proteins and eventually incorporated into the RNA-inducing complex.

### ii.Non-canonical pathway

Some miRNAs are formed by a non-canonical pathway, independent of DROSHA/DGCR8 activity [29]. This has been demonstrated by the deletion of *DROSHA* and *DGCR8*, encoding the two proteins that constitute the microprocessor complex, which resulted in a complete loss of canonical miRNAs. However, no impact was observed on the biogenesis of non-canonical miRNAs [30, 31]. So called miRtrons are among the first discovered non-canonical generated miRNAs, for which the presence of the cytoplasmic Dicer is still indispensable [31]. Their generation is initiated with the transcription of short introns of protein-coding genes, which are subsequently spliced out of the primary transcript by the spliceosome. The short intron sequences that contain a miRNA fold into characteristic hairpin-like structures. Most mammalian hairpins that form this way contain a relatively high GC content that increases the stability of these structures [32]. The resulting short-sized hairpins (pre-miRNAs) are next delivered to the cytoplasm by Exportin-5 in order to be cleaved by Dicer. In the miRtron pathway (non-canonical miRNA biogenesis pathway) the microprocessor process is replaced by the splicing activity, and further on, upon nuclear export merges with the canonical pathway.

Analysis of the genomic loci within the non-mirtronic regions have illustrated signs of Dicer-dependent/microprocessor-independent reads. MiR-320 and miR-484 are among these groups and their transcripts have elucidated the potential in forming the hairpin structures [33, 34].

## b. MiRNAs functions

A single miRNA is capable of targeting multiple, perhaps hundreds of mRNAs, thereby affecting their expression. At the same time a single mRNA may be targeted by several miRNAs. The picture that arises is that of a complex miRNA-mediated regulatory network that governs biochemical processes within cells and tissues that directly affect physiological and pathological processes. As these regulatory events must be carefully orchestrated both spatially and temporally miRNA activity is probably highly regulated. Binding of miRNAs in the context of RISC to target mRNA causes mRNA degradation and/or translation inhibition [35, 36]. MiRNA-mediated translational repression/silencing can occur by distinct processes: (i) deadenylation and mRNA degradation, (ii) 5'-decapping, and (iii) ribosome detachment [37]. These post-transcriptional silencing processes are all initiated by the association of miRNAs with the RISC components and the degree of sequence complementarity between the miRNA and the target mRNA determines the type of activity. MRNA destabilization/degradation takes place if there is a high level of complementarity. However, the presence of several mismatches within the miRNA/mRNA duplex facilitates translational repression, which is the most common event taking place in mammals [37, 38]. MiRNAs can act as potential translational repressors by partially pairing with the 3', 5' UTRs or coding sequences without any impact on the structural integrity of the targeted mRNAs [39]. The net effect of miRNA regulatory actions is that the protein levels encoded by the target mRNAs are reduced.

## c. MiRNAs in cancer

In 2001 reports described the presence of highly conserved small non-coding RNA molecules (miRNAs) in multiple eukaryotic organisms and mammalian species [40, 41]. Over the past decade it became clear that miRNAs fulfil a crucial role in cancer initiation, progression and metastasis [42-44]. In the context of cancer certain miRNA may be considered as oncogenes or tumour suppressors. A consistent finding in cancer is the tumor-specific dysregulation of miRNA expression with most miRNAs found to be downregulated in cancer [45]. The exact molecular reasons for aberrant miRNA expression are not always known and may include epigenetic silencing i.e. hyper-methylation of the promoter regions of miRNA genes or histone methylation [46-49]. Alternatively, defects within the miRNA biogenesis pathways can also modulate miRNA expression levels. Mutations within genes Dicer [50] or Exportin-5 [51], result in the accumulation of pri-miRNAs and the depletion of mature miRNAs [52]. The presence of single-nucleotide polymorphisms (SNPs) in both miRNA genes and/or their mRNA targets may increase the complexity. SNPs have been described that impair pri-miRNA processing [53] and SNPs within miRNA binding sites in mRNA may affect miRNA mediated regulation [54].

The aberrant miRNA expression profiles can be quantitatively determined in tumor samples using different methodologies and platforms including microarrays, RT-PCR and next generation sequencing approaches [55-58]. These profiling studies – often comparing cancer with relevant or adjacent normal tissues – have led to the identification and further characterization of miRNAs that initiate and/or contribute to the process of tumorigenesis in a variety of cancers [42, 59]. The expression profiles have also been instrumental for diagnostic purposes but have also been exploited for prognostic and predictive biomarker purposes [57, 60-62]. Currently much attention is being given to minimally-invasive liquid biopsies (whole blood, serum, plasma and urine) in which cancer–related miRNAs can be found to circulate and used as biomarker [63-65]. As miRNAs are small, often bound to proteins or associated with vesicular structures such as exosomes in the circulation they are relatively resistant to degradation and therefore suited as biomarker. Although many researchers report on miRNA biomarkers in different cancers, very few are actually used in the clinic. This may be due to the poor reproducibility of many studies because of limited sample numbers, sample heterogeneity, biased sample selection, poorly annotated samples, the use of different detection platforms and the use of poorly standardized protocols and normalization procedures [42, 66]. For many cancers the current literature implies the existence of relevant miRNA-based biomarkers, however, well-devised validation studies are needed to identify the most reliable and robust miRNA biomarkers.

In recent years interest arose to exploit miRNAs for their therapeutic potential in cancer. For example, a miRNA that is overexpressed in cancer may be inhibited through antisense miRNAs (antimirs) or alternatively, the expression of a miRNA that is downregulated may be restored. Therefore, miRNA-based treatment strategies can be conducted in two ways: miRNA reduction using antisense/antimiR inhibitors and miRNA replacement using miRNA mimics.

MiRNA mimics are chemically synthesized double-stranded RNAs that harbor the same sequence as the corresponding miRNA and functionally restore the loss of its expression. By contrast, antimiR inhibitors are single-stranded chemically modified antisense oligonucleotides which are designed to bind to the complementary sequence of the over-expressed miRNA in order to block its function. MiR-122, miR-103/107, miR-155, miR-29, miR-16 and miR-34 are among the most common tumor suppressor and oncogenic miRNAs that are currently being used as therapeutic molecules in clinical trials [42, 67]. Similar to other forms of systemic therapy adverse effects may occur of which the nature and severity may vary depending on the miRNA that is modulated. Of note, a phase I clinical trial involving liposomal miR-34a (MRX34, Mirna Therapeutics) was prematurely terminated due to multiple immune-related severe adverse events [68].

Despite the potential of miRNAs for cancer therapy, RNA-based therapeutic approaches (mimics and antimiRs) encounter considerable challenges. One of the major barriers in miRNA-based therapeutic delivery is the risk of RNA-degradation in the blood or endocytic compartment by RNases before reaching the target or target cells. To make miRNA-based therapeutic less prone to degradation, several nucleotide modifications in the RNA backbone have been incorporated: replacement of the phosphodiester group with a phosphorothioate (PS) linkage, introduction of an O-methyl or 2-methoxyethyl group, and the use of locked nucleic acids (LNAs) [69]. PS oligonucleotides exhibit dramatic increase in their half-life. However, chemical modifications may result in the production of toxic molecules as a result degradation processes that may lead to off-target gene silencing [70]. In addition to the biological stability issues, another concern when using these miRNA-based therapeutic molecules is the target-specific delivery and efficiency of cellular uptake [71]. Non-viral and viral strategies for delivery have been designed although the use of viral vectors in the clinic is still not widely encouraged due to the safety issues [72]. Non-viral carriers are safer, biodegradable and non-immunogenic delivery particles, which can be made up of synthetic polymers or lipids [71]. Encapsulating the candidate small-RNAs therapeutics using EnGenelC Delivery Vehicles (EDV) nanocells, poly (ethylene glycol) (PEG) and N-acetyl-D-galactosamine are among the most common delivery systems being used and evaluated in clinical trials [73-75]. Other delivery particles such as dendrimers, neutral lipid emulsions, chitosan, cyclodextrin, poly (lactic-co-glycolide) (PLGA), and synthetic polyethylenimine (PEI) are still mostly used in pre-clinical studies due to their dose-limiting toxicity, low efficiency in delivering to the tumor cells, and the low rate of loading small-RNA molecules in these particles [76-80]. A faulty or non-targeted delivery may lead to off-target and adverse effects through the modulation of gene expression in non-cancerous tissues or cause immune responses. In order to increase target-specificity, two main approaches are proposed: 1) utilizing synthetic oligonucleotide nanoparticles that are coated with antibodies specific for binding to the desired tumor cells, and/or 2) direct injection of these nucleotides into the tumor itself (examples; sarcomas and brain tumors) instead of a systemic administration [70, 71].

Despite all challenges, advanced discoveries on miRNAs-mediated regulatory processes have led to a better understanding of the underlying biological mechanisms operational in carcinogenesis [42, 66, 81, 82]. The potential of miRNAs in simultaneously regulating several mRNAs and multiple biological pathways as well as their relative stability in tissues and the

circulation make them suitable biomarker and therapeutic candidates for many disorders. Although the application of miRNAs in the clinic, either as biomarker or for therapeutic purposes, is still in its infancy, the strong involvement and determining roles of miRNAs in different aspects of carcinogenesis hold great promise to make an impact on patient care in the near future.

# 3. Thesis outline

In order to improve the clinical outcome of patients with STS, a better understanding of the molecular mechanisms involved in carcinogenesis is crucial. In **Chapter 2**, we studied the potential therapeutic role of targeting BRD4, EZH2 and TOP2A in a STS sub-group called MPNSTs.

De-regulated miRNAs, commonly observed in cancers, are known to contribute to tumorigenesis and cancer progression. Their use as diagnostic, prognostic and predictive biomarker as well as their therapeutic potential is widely studied. In **Chapter 3**, the involvement of miRNAs in key cancer-related processes in NF1-derived MPNST was investigated.

An overview of the aberrant expression and association with clinicopathological parameters of non-coding RNAs, particularly miRNAs, in GIST is provided in **Chapter 4**. GIST patients are effectively treated with the tyrosine kinase inhibitor imatinib but ultimately develop drug resistance causing tumor progression. In **Chapter 5** miRNAs and genes were identified potentially involved in the regulation of imatinib resistance.

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# Chapter 2

# Expression and inhibition of BRD4, EZH2 and TOP2A in neurofibromas and malignant peripheral nerve sheath tumors

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# Abstract

Malignant peripheral nerve sheath tumors (MPNST) are rare, highly aggressive sarcomas that can occur spontaneously or from pre-existing plexiform neurofibromas in neurofibromatosis type1 (NF1) patients. MPNSTs have high local recurrence rates, metastasize easily, are generally resistant to therapeutic intervention and frequently fatal for the patient. Novel targeted therapeutic strategies are urgently needed. Standard treatment for patients presenting with advanced disease is doxorubicin based chemotherapy which inhibits the actions of the enzyme topoisomerase IIα (TOP2A). Recent molecular studies using murine models and cell lines identified the bromodomain containing protein 4 (BRD4) and enhancer of zeste homolog 2 (EZH2) as novel targets for MPNST treatment. We investigated the expression and potential use of BRD4, EZH2 and TOP2A as therapeutic targets in human NF1-derived MPNSTs. The transcript levels of *BRD4*, *EZH2* and *TOP2A* were determined in paired formalin-fixed paraffin-embedded (FFPE) neurofibroma/MPNST samples derived from the same NF1 patient and in a set of plexiform neurofibromas, atypical neurofibromas and MPNST. We further examined the effect on cell viability of genetic or pharmacological inhibition of BRD4, EZH2 and TOP2A in an MPNST cell line panel. Our results indicated that in MPNST samples *BRD4* mRNA levels were not upregulated and that MPNST cell lines were relatively insensitive to the bromodomain inhibitor JQ1. We corroborated that *EZH2* mRNA expression is increased in MPNST but failed to confirm its reported pivotal role in MPNST pathogenesis as EZH2 knockdown by siRNA did not interfere with cellular proliferation and viability. Finally, the relation between TOP2A levels and sensitivity for doxorubicin was examined, confirming reports that *TOP2A* mRNA levels were overexpressed in MPNST and showing that MPNST cell lines exhibited relatively high TOP2A protein levels and sensitivity to doxorubicin. We tentatively conclude that the potential for effective therapeutic intervention in MPNST by targeting BRD4, EZH2 and TOP2A individually, may be limited. Clinical studies are necessary to ultimately prove the relevance of BRD4 and EZH2 inhibition as novel therapeutic strategies for MPNST.

# Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder which has a *de novo* incidence of one in 3000 individuals (1-3). This genetic disorder is caused by defects in the *NF1* gene located on chromosome 17q11.2. The *NF1* gene encodes a tumor suppressor called neurofibromin 1, which through its GTPase-activating protein (GAP) domain negatively regulates Ras signaling keeping cell proliferation in check. Inherited or sporadic mutations of *NF1* and the partial inactivation of neurofibromin, lead to an increased risk of developing various tumors. Almost all NF1 patients develop cutaneous neurofibromas and in many patients plexiform neurofibromas cause additional morbidity. All tumors exhibit biallelic inactivation of the *NF1* gene and consequently activated signaling through the Ras pathway driving cancer formation (1, 4). Plexiform neurofibromas may transform into malignant peripheral nerve sheath tumors (MPNST), the most common malignancy occuring in NF1 patients, at an incidence of 2% and a lifetime risk of 8-13% (5). MPNSTs are classified in the group of the soft tissue sarcomas (STS) and comprise approximately 5-10% of all STS. MPNST are a class of highly aggressive and clinically challenging sarcomas. High local recurrence rates, early metastasis and resistance to chemotherapy are common clinical phenotypes in this cancer. When metastasized, patients face a poor prognosis with only a limited number of systemic chemotherapeutic agents available (6, 7). Of these, doxorubicin is probably the most active one, targeting - through intercalation into the DNA - the activity of the enzym topoisomerase IIα (TOP2A) (8). Transcriptome data analyses have shown that *TOP2A* was among the most upregulated genes in MPNSTs when compared to benign neurofibromas (9, 10). However, despite the high expression of TOP2A, advanced MPNST patients do not respond well to doxorubicin given a 2 year overall survival rate of approximately 20%, which is roughly equivalent to the outcome of patients with metastatic STS other than MPNST (7). This poor outcome clearly underscores the need to get better insight into the exact relationship between TOP2A expression and doxorubicin sensitivity in MPNST and the necessity to reveal new leads for treatment.

A better understanding of the pathobiology of MPNST may lead to the identification of novel treatment targets. Recently, Patel *et al*. reported the upregulation of *Brd4* mRNA and protein levels in a newly developed murine MPNST model (11, 12) based on transplantation of $Nf1^{-/-}$ ,$P53^{-/-}$ skin-derived precursor cells into nerves of athymic nude mice (13). Further investigations inferred a critical role for Brd4 in MPNST pathogenesis as inhibition by shRNAs or by JQ1, a small molecule BET (bromodomain and extraterminal domain) inhibitor, severely impaired *in vitro* growth and *in vivo* tumorigenesis (13). It was demonstrated that inhibition of Brd4 induced expression of the pro-apoptotic molecule Bim leading to apoptosis in MPNST cells. The BET subfamily of bromodomain proteins to which BRD4 belongs has a role in regulating transcription by RNA polymerase II. The best studied member BRD4 recruits transcriptional regulatory complexes to acetylated chromatin and modulates transcriptional elongation of essential genes involved in cell cycle and apoptosis (14). In addition, also enhancer of zeste homolog 2 (EZH2) was found upregulated in MPNST compared to neurofibroma and normal nerves (15). EZH2 is a core element of the polycomb repressive complex 2 (PRC2) a well-known epigenetic modulator of gene expression (16) and is

frequently found overexpressed in malignancies or mutated in lymphomas (17). EZH2 involvement in MPNST pathogenesis was demonstrated by the transient *EZH2* knockdown using si/shRNA or EZH2 inhibition by 3-deazaneplanocin A causing cell cycle arrest and apoptosis in MPNST cells (15, 18). Evidence is provided for the existence of a novel signaling pathway in MPNST that mediates the effects of EZH2 via miR-30a/30d to karoypherin (importin) beta 1 (KPNB1) (15, 18). Both EZH2 and BRD4 can be targeted by selective and potent small molecule inhibitors (19, 20) that are currently being evaluated in clinical trials making them appealing targets for the treatment of MPNST.

To further investigate the potential role as treatment targets of the above-mentioned proteins, we investigated the expression level of the target genes in FFPE and fresh frozen sample sets of plexiform neurofibromas and MPNSTs as well as neurofibroma and MPNST cell lines in order to validate the obtained results from the previous studies.

# Materials and methods

## Patients and samples

From the Erasmus MC patient files, nine neurofibroma type 1 patients were selected of which resected plexiform neurofibroma material was present and who developed MPNST. Archival formalin-fixed paraffin-embedded (FFPE) tumor samples of both plexiform neurofibroma and MPNST from the same patient (paired samples) were recovered from the Erasmus MC tissue bank. Fresh frozen samples from plexiform neurofibroma (n=11), atypical neurofibroma (n=4) and MPNST (n=7) were also obtained from the Erasmus MC tissue bank. The FFPE and fresh frozen sample sets do not overlap and were derived from distinct patients. All patients and tumor characteristics are listed in Table 1. For the histopathological diagnosis of MPNST, atypical neurofibroma and plexiform neurofibroma criteria were used as described before (21, 22) in accordance with the 2016 WHO classification of Tumours of the Central Nervous System (23).

In short, for the diagnosis of MPNST we used morphological criteria. Immunostaining for S100 was used for identification of a Schwann cell component in the tumors. Atypical neurofibroma was defined by the presence of mitotic figures, and/or cytological atypia, and/or increased cellularity. The combination of all three features, however, defined low grade MPNST. Plexiform neurofibroma involved multiple nerve fascicles and lacked the above mentioned atypical features. Prior to our research the Daily Board of the Medical Ethics Committee Erasmus MC of Rotterdam, The Netherlands, reviewed the research proposal. As a result of this review, the Committee decided that the rules laid down in the Medical Research Involving Human Subjects Act do not apply to this research (MEC-2016-213).

**Table 1. Patient and tumor characteristics.**

| **Paired FFPE tumor samples (n = 9 pairs)** | |
|---|---|
| **Gender** | |
| Male | 6 (66.7%) |
| Female | 3 (33.3%) |
| **Age at biopsy/resection NF (years)** | |
| Median (range) | 28 (5-63) |
| **Age at biopsy/resection MPNST (years)** | |
| Median (range) | 27 (14-70) |
| **Plexiform neurofibroma** | |
| Head and Neck | 1 (11.1%) |
| Extremities | 3 (33.3%) |
| Trunk | 5 (55.6%) |
| **MPNST** | |
| Head and Neck | 1 (11.1%) |
| Extremities | 4 (44.4%) |
| Trunk | 4 (44.4%) |
| **Fresh frozen tumor samples** | |
| **Plexiform neurofibroma (n = 7)** | |
| **Gender** | |
| Male | 4 (57.1%) |
| Female | 3 (42.9%) |
| **Age at biopsy/resection (years)** | |
| Median (range) | 29 (10-63) |
| **Location** | |
| Head and Neck | 1 (14.3%) |
| Extremities | 4 (57.1%) |
| Trunk | 2 (28.6%) |
| **Atypical neurofibromas (n = 4)** | |
| **Gender** | |
| Male | 2 (50%) |
| Female | 2 (50%) |
| **Age at biopsy/resection (years)** | |
| Median (range) | 25.5 (15-43) |
| **Location** | |
| Head and Neck | - |
| Extremities | 4 (100%) |
| Trunk | - |
| **MNST (n = 11)** | |
| **Gender** | |
| Male | 5 (45.5%) |
| Female | 6 (54.5%) |
| **Age at biopsy/resection (years)** | |
| Median (range) | 36 (12-76) |
| **Location** | |
| Head and Neck | 3 (27.3%) |
| Extremities | 3 (27.3%) |
| Trunk | 5 (45.4%) |

MPNST; malignant peripheral nerve sheath tumor. NF; plexiform neurofibroma

# Cell culture

Human MPNST cell lines ST88-14, 90-8TL, T265 (NF1-associated MPNST) and STS26T (sporadic MPNST) were kindly provided by Dr. Eduard Serra (Institute of Predictive and Personalized Medicine of Cancer/IMPPC, Barcelona, Spain). sNF96.2 and HS53.T were obtained from the ATCC and derived from an NF1-associated MPNST and a cutaneous, NF1-derived, neurofibroma, respectively. Human embryonic kidney (HEK) 293T cells were a kind gift from the department of Genetics, Erasmus MC, Rotterdam, the Netherlands). All cell lines were cultured in DMEM (Gibco Life Technologies) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% $CO_2$ atmosphere. All cell lines were regulary monitored for mycoplasma infection and were subjected to authentication by performing a short tandem repeat (STR) DNA analyses and matched, when available, with STR databases. The absence of SUZ12 protein expression in ST88-14 and 90-8TL as reported by de Raedt *et al*. (24) was confirmed by Western blotting (S1 Fig). Similarly, the presence or absence of detectable NF1 protein in the various cell lines was examined (S2 Fig).

# RNA isolation

Total RNA was isolated from cell line pellets and fresh frozen tissues using RNAbee (Tel test Inc., Friendswood, Texas, USA) according to the manufacturer's instructions. RNA from FFPE tumor samples (5-6 20 µm sections) was isolated using the RecoverAll™ total nucleic acid isolation kit (Ambion/Life Technologies). RNA quality and quantity were checked using a Nanodrop-1000 (Nanodrop Technologies).

# Quantitative RT-PCR

cDNA was synthesized from 250 ng of total RNA using TaqMan® Reverse Transcription Reagents (ThermoFisher Scientific). The mRNA expression levels of target genes and housekeepers were determined by real time PCR using TaqMan® Universal PCR Master Mix and specific Assay-On-Demand products (ThermoFisher Scientific/Applied Biosystems) using an ABI 7500 Real-Time PCR machine. The following assays were used *EZH2* (Hs01016789_m1), *TOP2A*(Hs01032137_m1), *BRD4*(Hs04188087_m1). Expression of *EZH2*, *TOP2A* and *BRD4* were normalized using *PPIA* (Pedersen *et al*, 2014) (Hs99999904_m1) using the comparative $C_T$ method (25). Each tumor or cell line RNA sample was measured in duplicate after which the data were analyzed using SDS software (Applied Biosystems). Statistical significance ($p<0.05$) was determined on the normalized expression values of the paired FFPE samples using a paired Student t-test.

# Protein lysates, SDS-PAGE and Western blotting

Total protein was extracted from cells using lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10% glycerol, 1% NP-40, 0.5% Na-deoxycholate, 1 mM $Na_3VO_4$, 20 mM NaF, 1 mM Pefabloc) supplemented with a cocktail of protease inhibitors. Protein concentration was quantified using a Bradford assay (Bio-Rad, Veenendaal, The Netherlands). Equal amounts of total protein (15 – 20 µg/lane) were subjected to SDS-PAGE and subsequently transferred to a PVDF membrane by electroblotting. Remaining protein binding sites of the membrane were blocked in PBS, 0.05% Tween 20 (PBS/Tween) containing 5% non-fat dried milk. Primary antibody incubations were carried out in the same buffer with the following primary antibodies: mouse monoclonal anti-EZH2 (1:1000, NCL-L-EZH2, Leica Microsystems;), rabbit monoclonal anti-SUZ12 (1:1000, D39F6, Cell Signaling Technology), rabbit polyclonal anti-BRD4 (1:10000, A301-985A100, Bethyl Laboratories, Inc), rabbit monoclonal anti-TOP2A (1:1000, D10G9, Cell Signaling Technology), rabbit monoclonal anti-NF1 (1:1000, D7R7D, Cell Signaling Technology), rat monoclonal anti-tubulin (1: 4000, YL1/2, Abcam) and mouse monoclonal anti-β-actin (1:10000, A5441, Sigma-Aldrich). HRP conjugated goat-anti-rabbit, goat-anti-mouse and goat-anti-rat were used as secondary antibodies. Enhanced chemiluminiscence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA) was used to visualise the signal in a ChemiDoc MP Imager (Bio-Rad, Veenendaal, The Netherlands). Protein expression was quantitated using ImageJ, a public domain Java-based image processing program (26). Each Western blot was replicated at least three times, depicted are representative blots.

# In vitro cytotoxicity assay

*In vitro* cytotoxicity of the BET inhibitor JQ1 (BioVision Inc, Milpitas, CA, USA), and the anthracycline doxorubicin (Pharmachemie, Haarlem, The Netherlands) were determined by a sulforhodamine B (SRB) assay essentially as described by Keepers *et al*. (27). In brief, on day 0 cells were plated in 96-well flat bottom microtiter plates. On day 1 a ten-step, two-fold dilution series was prepared and added to the cells resulting in a highest concentration of 2500 nM for JQ1 and 500 ng/ml for doxorubicin. Every dilution was assayed in quadruplicate. After 48 -72 hours the assay was terminated, the cells fixed with 10% trichloroacetic acid in PBS for 1 h at 4°C. After at least four washes with tap water the cells remaining in the wells were stained with 0.4% SRB in 1% acetic acid for at least 15 min at RT. Subsequently the unbound stain was removed by 4 washes in 1% acetic acid. Plates were air-dried and bound stain was dissolved in 150 µl of 10 mM Tris-base. Staining was quantified by measuring the absorbance at 540 nm in a spectrophotometer. Concentration-response curves were generated and $IC_{50}$ values were calculated by the use of Deltasoft 3 software.

# *EZH2* siRNA mediated knockdown

Twentyfour hours prior to transfection the 90-8TL and T265 cell lines were plated in a 24-well plate in duplicate at such a concentration that the next day the wells reach 70-80% confluency. Cells were transfected with either a *EZH2*-specific siRNA (Qiagen, FlexiTube siRNA SI02665166) or a negative control scrambled siRNA (Qiagen, SI03650325) at a concentration of 50 nM using the DharmaFECT1 transfection reagent (Dharmacon/Thermo Scientific) as recommended by the manufacturer. Twentyfour hours post-transfection the medium was replaced with standard culture medium and cell density as a measure for proliferation was assessed by SRB staining at 24, 48 and 72 hours after transfection.

# Results

## Human *BRD4* mRNA levels are not increased in MPNST compared to neurofibromas

In the search for targetable alterations in MPNST Patel *et al*. reported a potential pathogenic role of a BET bromodomain family member (*Brd4*) in an MPNST mouse model. Inhibition of Brd4, which was found highly upregulated in MPNST, induced increased expression of the pro-apoptotic molecule Bim inducing apoptosis in MPNST cells and tumor shrinkage (13). We examined *BRD4* mRNA expression by qRT-PCR in a series of nine paired human MPNST and plexiform neurofibroma FFPE samples, each pair derived from the same patient (Fig 1A). To rule out that degradation of the total RNA isolated from the archival samples impairs accurate quantitation we also determined *BRD4* mRNA levels in a set of fresh frozen plexiform neurofibromas (n=7), atypical neurofibromas (n=4) and MPNST (n=11) (Fig 1B). Both in the neurofibroma-MPNST pairs as well as in the fresh frozen samples we did not detect *BRD4* overexpression in the MPNST samples (Figs 1A,B). The paired sample analyses indicated significantly higher *BRD4* mRNA levels in 6 of the neurofibromas compared to their corresponding MPNST whereas in most fresh frozen samples there was no significant difference in *BRD4* mRNA levels between (atypical) plexiform neurofibromas and MPNSTs. It must be noted, however, that mRNA levels may not be indicative for protein levels as in most MPNST cell lines *BRD4* mRNA levels were similar but BRD4 protein levels varied considerable (cf. Figs 1C and 1D). To further investigate whether BRD4 can serve as a target for treatment we determined the sensitivity of our cell line panel consisting of a neurofibroma and 5 MPNST cell lines, to the BET bromodomain inhibitor JQ1 (Fig 2A). In an *in vitro* cytotoxicity assay the cell lines were exposed to increasing concentrations of JQ1 for 72 hours. Most MPNST cell lines did not display a clearly increased sensitivity to JQ1 compared to the neurofibroma cell line. The MPNST cell lines sNF96.2, T265 and 90-8TL expressed approximately equal levels of BRD4 protein and displayed similar sensitivity to JQ1 (Fig 2B). ST88-14 another NF1-derived MPNST cell line expressed relatively low BRD4 protein levels and was accordingly found less sensitive to JQ1. In contrast the sporadic MPNST cell line STS26T harbors high levels of BRD4 protein but is relatively insensitive to JQ1. For these cell lines we were not able to calculate $IC_{50}$ values (Fig 2B).

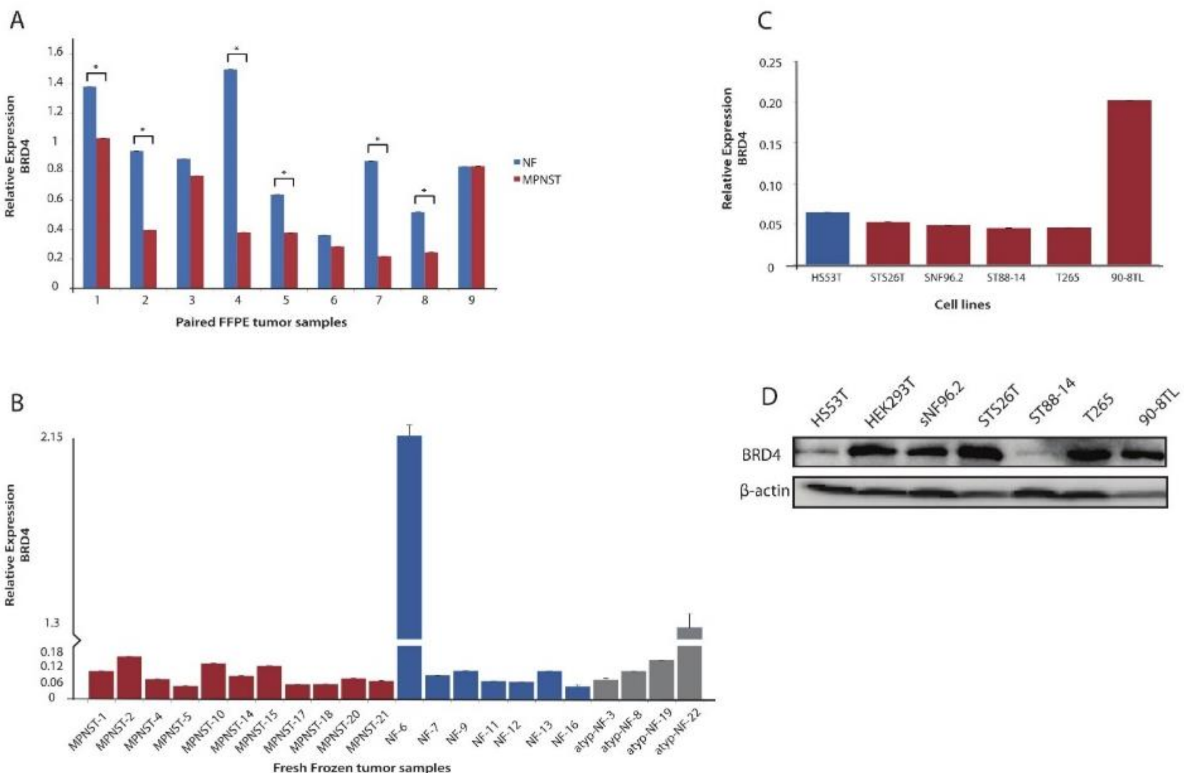
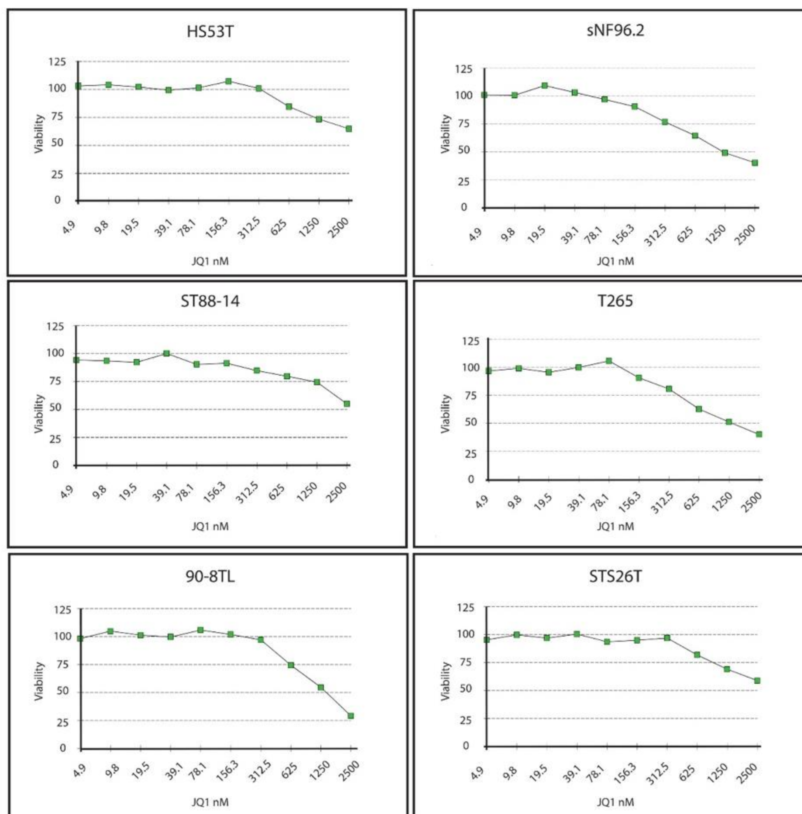



**Fig 1. Expression level of *BRD4* in human neurofibroma and MPNST samples and cell lines.** (**A**) qRT-PCR was used to determine mRNA levels of *BRD4* in paired plexiform neurofibroma (NF, blue, n=9) and MPNST (red, n=9) formalin-fixed paraffin-embedded tumor samples, each pair being derived from the same NF1 patient. Asterisk indicates $P<0.05$. (**B**) qRT-PCR was used to determine mRNA levels of *BRD4* in fresh frozen MPNST (red, n=11), plexiform neurofibroma (blue, n=7) and atypical neurofibroma (grey, n=4). (**C**) qRT-PCR was used to determine mRNA levels of *BRD4* in a cell line panel: Hs53.T neurofibroma cell line (blue) and STS26T, sNF96.2, ST88-14, T265 and 90-8TL MPNST cell lines (red) (**D**) Western blot displaying BRD4 protein expression in cell line panel and HEK293T. ß-actin levels are shown as a loading control.

A




B

| cells | IC50 |
|---|---|
| HS53T | - |
| SNF96.2 | 1215.5 nM |
| ST88-14 | - |
| T265 | 1364.2 nM |
| 90-8TL | 1422.0 nM |
| STS26T | - |

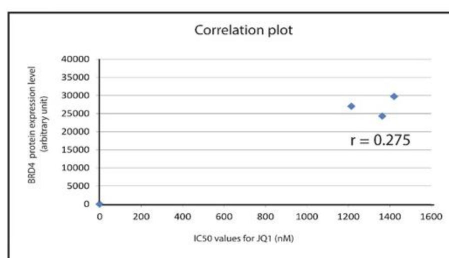



**Fig 2. Sensitivity of neurofibroma and MPNST cell lines to the BET bromodomain inhibitor JQ1.** (**A**) An *in vitro* cytotoxicity assay (SRB assay) was used to determine $IC_{50}$ values (nM) for the BET bromodomain inhibitor JQ1 of neurofibroma and MPNST cell lines after a 72h exposure to the drug. Graphs show cell viability as a function of JQ1 concentration.

Depicted is the average viability (n=4) of a representative experiment. (**B**) Listing of calculated $IC_{50}$ values and correlation plot, with BRD4 protein expression levels on the Y-axis and $IC_{50}$ values for JQ1 on the X-axis. Pearson correlation coefficient is depicted in the graph.

# EZH2 levels are increased in MPNST compared to neurofibromas but do not affect cellular proliferation.

Nuclear EZH2 levels were reported to be induced in MPNST compared to neurofibromas and normal nerves as measured by immunohistochemistry (15). Our observations support these results as the *EZH2* mRNA levels were significantly increased in the MPNST samples from 6 out of 9 plexiform neurofibroma/MPNST pairs (Fig 3A). Also in RNA isolated from fresh frozen neurofibroma and MPNST samples EZH2 mRNA levels appeared on average to be 8-fold higher in MPNST than in (atypical) plexiform neurofibromas (Fig 3B). Similarly, all the MPNST cell lines displayed relatively high EZH2 mRNA levels compared to the neurofibroma cell line (Fig 3C). At a protein level, as judged by Western blot, EZH2 also seems more highly expressed in the MPNST cell lines although it is clear that protein expression and mRNA levels do not always perfectly match (Fig 3D). Next, we investigated whether EZH2 inhibition exerts an anti-proliferation activity as was previously reported (15). Both T265 and 90-8TL MPNST cells were transiently transfected with an EZH2 siRNA and a scrambled siRNA control for comparison. EZH2 protein levels were significantly reduced by the EZH2 siRNA treatment at 48 – 72 h after transfection (Fig 4A). However, despite the clearly decreased EZH2 levels no significant inhibition of cell proliferation was observed (Fig 4B).

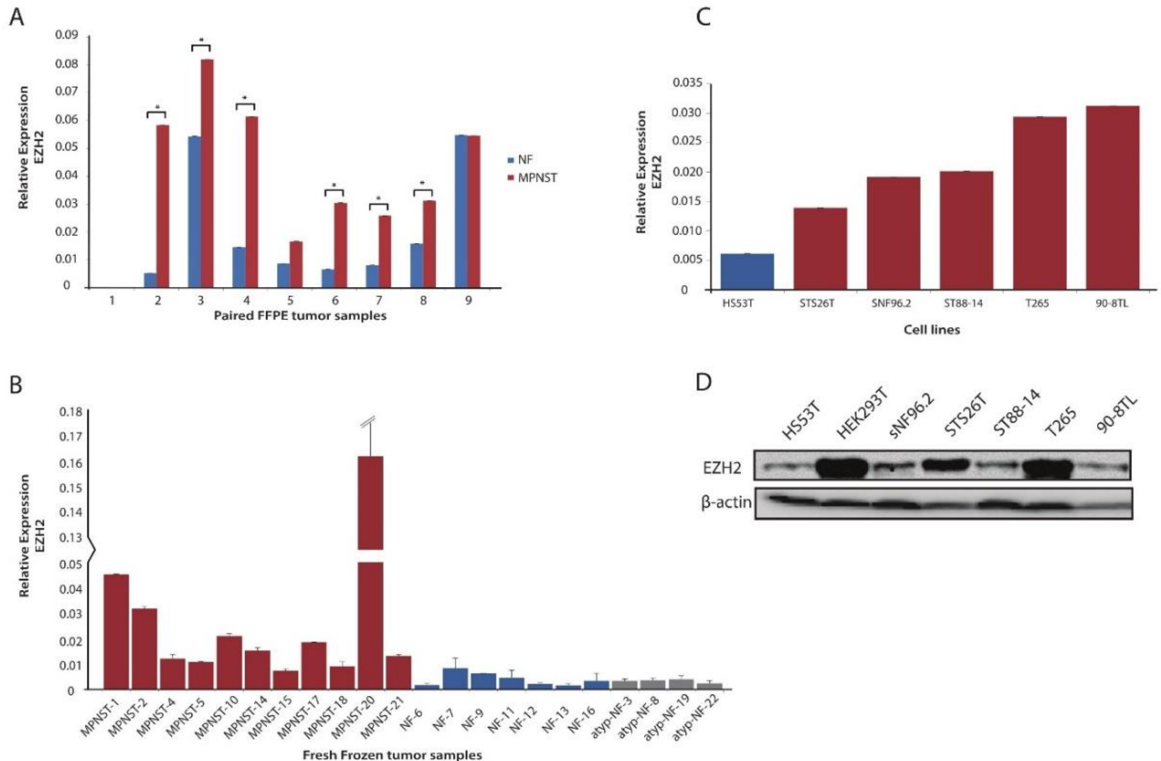



**Fig 3. Expression level of *EZH2* in human neurofibroma and MPNST samples and cell lines.** (**A**) qRT-PCR was used to determine mRNA levels of *EZH2* in paired plexiform neurofibroma (NF, blue, n=9) and MPNST (red, n=9) formalin-fixed paraffin-embedded tumor samples, each pair being derived from the same NF1 patient. Asterisk indicates P<0.05. (**B**) qRT-PCR was used to determine mRNA levels of *EZH2* in fresh frozen MPNST (red, n=11), plexiform neurofibroma (blue, n=7) and atypical neurofibroma (grey, n=4). (**C**) qRT-PCR was used to determine mRNA levels of *EZH2* in a cell line panel: Hs53.T neurofibroma cell line (blue) and STS26T, sNF96.2, ST88-14, T265 and 90-8TL MPNST cell lines (red). (**D**) Western blot displaying EZH2 protein expression in cell line panel and HEK293T. ß-actin levels are shown as a loading control.

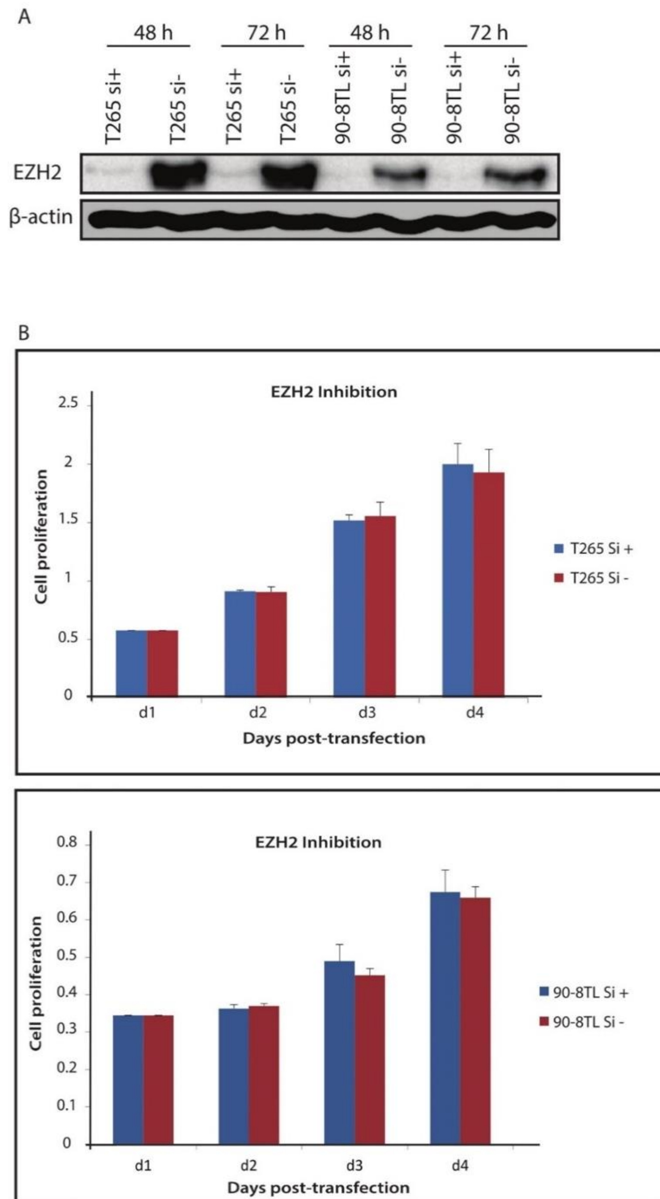



**Fig 4. siRNA mediated knockdown of EZH2 and its effect on cell proliferation.** (**A**) Western blot showing the effect of EZH2 siRNA (si+) or a scrambled control siRNA (si-) on EZH2 protein levels in T265 and 90-8TL at 48h and 72 h post-transfection. (**B**) Cell proliferation monitored in time after transfection of T265 and 90-8TL with *EZH2* siRNA (si+) or a scrambled control siRNA (si-). ß-actin levels are shown as a loading control.

# Relative high expression of TOP2A in MPNST is associated with doxorubicin sensitivity.

To verify whether TOP2A expression levels are increased in MPNST as was reported in the literature (9, 10) we determined the *TOP2A* mRNA levels in our paired FFPE and fresh frozen plexiform neurofibroma/MPNST sample sets. In both panels *TOP2A* mRNA expression was clearly induced in MPNST when compared to the levels detected in plexiform neurofibromas. In 7 out 9 paired FFPE samples *TOP2A* levels were significantly increased in the MPNST samples (Fig 5A). In the fresh frozen sample set *TOP2A* mRNA levels were on average 24-fold higher in the MPNST than in the plexiform neurofibromas (Fig 5B). In the cell line panel *TOP2A* mRNA levels in the MPNST cell lines were mostly equal or lower than the levels measured in the neurofibroma cell line Hs53.T, only the MPNST 90-8TL cell line exhibited relatively high *TOP2A* levels (Fig 5C). At the protein level, however, all MPNST cell lines displayed markedly higher TOP2A expression than the Hs53.T cells (Fig 5D). To examine whether the relatively high MPNST TOP2A levels translate into sensitivity to the TOP2A targeting chemotherapeutic drug doxorubicin we determined the sensitivity of the cell lines to this drug using an *in vitro* cytotoxicity (SRB) assay. All four NF1-associated MPNST cell line (sNF96.2, ST88-14, T265 and 90-8TL) and one sporadic MPNST cell line (STS26T) were more sensitive to doxorubicin than the neurofibroma Hs53.T cells, many of them displaying $IC_{50}$ values of less than 50 ng/ml (Figs 6A,B). A comparison of TOP2A protein expression levels and the calculated $IC_{50}$ values of the cell lines indicated a correlation, although not very strong, of TOP2A levels and doxorubicin sensitivity (Fig 6B).

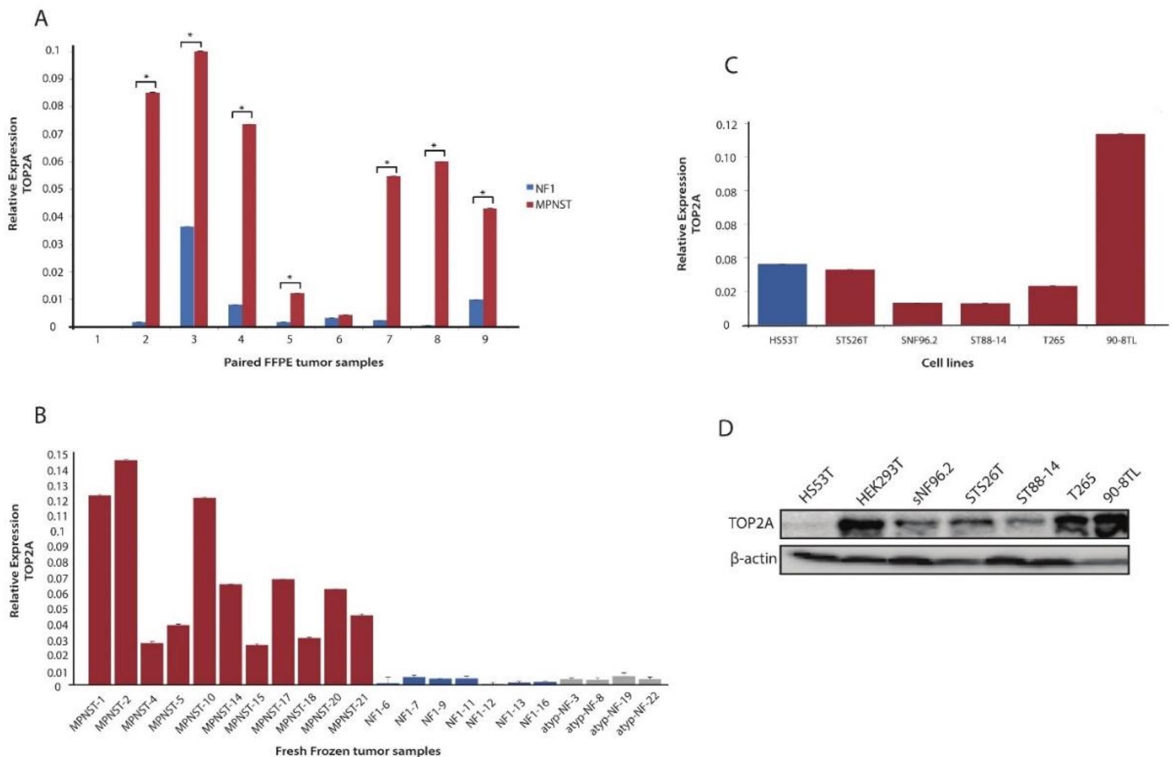



**Fig 5. Expression level of TOP2A in human neurofibroma and MPNST samples and cell lines.** (**A**) qRT-PCR was used to determine mRNA levels of *TOP2A* in paired plexiform neurofibroma (NF, blue, n=9) and MPNST (red, n=9) formalin-fixed paraffin-embedded tumor samples, each pair being derived from the same NF1 patient. Asterisk indicates P<0.05. (**B**) qRT-PCR was used to determine mRNA levels of *TOP2A* in fresh frozen MPNST (red, n=11), plexiform neurofibroma (blue, n=7) and atypical neurofibroma (grey, n=4). (**C**) qRT-PCR was used to determine mRNA levels of *TOP2A* in a cell line panel: Hs53.T neurofibroma cell line (blue) and STS26T, sNF96.2, ST88-14, T265 and 90-8TL MPNST cell lines (red). (**D**) Western blot displaying TOP2A protein expression in cell line panel and HEK293T. ß-actin levels are shown as a loading control.

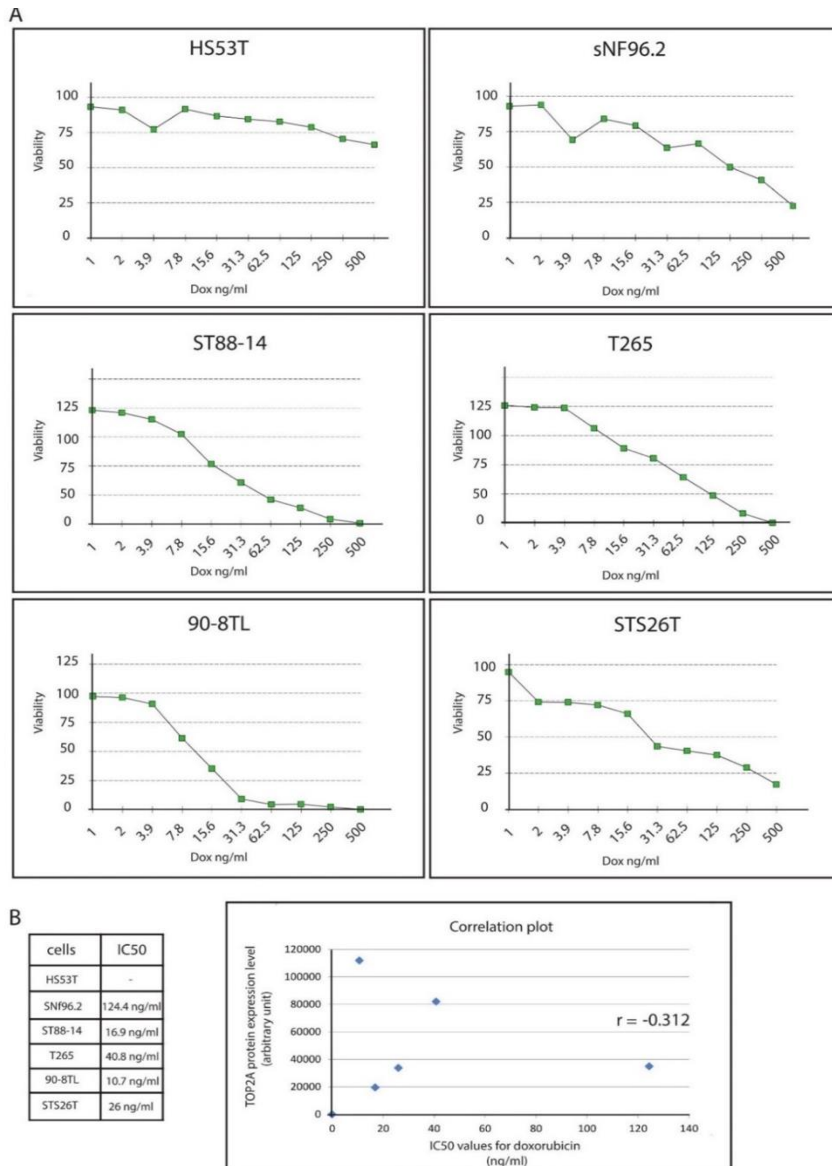



**Fig 6. Sensitivity of neurofibroma and MPNST cell lines to doxorubucin.** (**A**) An *in vitro* cytotoxicity assay (SRB assay) was used to determine $IC_{50}$ values (ng/ml) for doxorubucin of neurofibroma and MPNST cell lines after a 48h exposure to the drug. Graphs show cell viability as a function of doxorubucin concentration. Depicted is the average viability (n=4) of a representative experiment. (**B**) Listing of calculated $IC_{50}$ values and correlation plot, with TOP2A protein expression levels on the Y-axis and $IC_{50}$ values for doxorubicin on the X-axis. Pearson correlation coefficient is depicted in the graph.

# Discussion

Given the limited number of therapeutic options for advanced MPNST patients, the identification of novel drug targets and the development of new treatments and treatment strategies is urgently needed. In this study we analyzed the expression level of three potential drug targets BRD4, EZH2, and TOP2A in selected human MPNST and neurofibroma samples from the Erasmus MC tissue bank. Our sample set included both fresh frozen samples and a set of nine paired FFPE samples consisting of plexiform neurofibromas and MPNST that were resected from the same patient.

With respect to BRD4, it has been shown that inhibition of this protein profoundly suppresses MPNST tumorigenesis and tumor cell growth in a murine MPNST model (13). To confirm this putative key role of BRD4 in human MPNST pathogenesis, we evaluated the expression level of *BRD4* in plexiform neurofibromas and MPNST samples. In addition, we studied the effect of BRD4 modulation by JQ1 on the cell viability of MPNST cell lines. In contrast to what has been reported for the MPNST mouse model (13), we did not find evidence for an increased expression of *BRD4* in human MPNST samples when compared to plexiform neurofibromas. It must be noted, however, that we only examined a limited set tumor samples due to the rarity of MPNST. Additionally, in order to deal with tumor heterogeneity, it may be useful to examine multiple biopsies from the same tumor. Nevertheless our analyses of *BRD4* expression, either of FFPE or fresh frozen samples, do not indicate an overexpression in MPNST. In contrast, previously reported overexpression of *EZH2* and *TOP2A* in MPNST could be convincingly demonstrated in our sample sets, using similar RT-PCR assays, indicating RNA quality is good. Alternatively, our inability to confirm *BRD4* overexpression in the human MPNST setting may indicate that data acquired with genetically engineered animal models cannot always be easily translated to the human situation. It might be that these models do not recapitulate the full complexity of human cancers and/or there are unrecognized fundamental cross-species differences in the process of tumorigenesis (28, 29). Moreover, BRD4 inhibition by JQ1 treatment in our panel of MPNST cell lines indicated that they were less sensitive to JQ1 than the primary murine skin-derived precursors ($Nf1^{-/-}$, $P53^{-/-}$) and MPNST cells derived thereof which display $IC_{50}$ values of < 400 nM (13). Although Patel *et al*. did use the human S462 MPNST cell line they did not present a dose-response curve from which an $IC_{50}$ value could be deduced making a direct comparison with our results difficult. Likewise Patel *et al*. did not validate their findings regarding Brd4 overexpression in clinical tumor samples. Interestingly, de Raedt and colleagues provided evidence that BRD4 inhibition by JQ1 exerted only a modest, cytostatic effect on human MPNST cell lines and that only the combination of JQ1 with PD-901, a MEK-inhibitor, caused a tumor growth inhibition and regression (24).

Zhang *et al*. demonstrated that EZH2 is overexpressed in MPNST and fulfils a key role in tumorigenesis (15, 18). Both downregulation of *EZH2* by si/shRNA or pharmacological inhibition of EZH2 in the S462 (NF1-derived MPNST) and MPNST724 (spontaneous MPNST) cell lines severely affected cellular proliferation rates, induced apoptosis and

interfered with tumor formation in an MPNST724 xenograft model. We do confirm that *EZH2*, at least at the mRNA level, is more abundantly expressed in MPNST than in plexiform neurofibromas. However, when we examined the consequences of EZH2 downregulation on cellular proliferation in 90-8TL and T265 we did not observe any inhibitory effect, despite a significant EZH2 knockdown. It might be that the cell lines used by Zhang *et al*. respond differently to EZH2 knockdown or inhibition than the NF1-derived MPNST cell lines 90-8TL and T265 that we examined. It may be that knockdown of EZH2 is compensated for by other members of the PRC2 complex and/or the related EZH1. Our findings, however, do suggest that EZH2 functions may be dependent on cellular context. Importantly, it was recently reported that a substantial number of MPNST, irrespective of their origin (NF1-derived, spontaneous or radiation induced) exhibit an inactivated PRC2 complex due to somatic loss-of-function mutations in *SUZ12* and *EED* (24, 30, 31). Both SUZ12 and EED - just as EZH2 - are integral parts of the PRC2 complex. It is not yet known what the consequences of such a PRC2 inactivation are for the remaining unaffected PRC2 complex subunits like EZH2. Is EZH2 still present in a protein complex and is EZH2 capable of fulfilling a biological role in this context or on its own? Perhaps the discrepancy between our findings and those of Zhang *et al*. (15) can be explained by different levels of PRC2 complex inactivation in the cell lines used. Translated to the clinic this would imply that before targeting EZH2 in the context of MPNST it is imperative to verify whether the PRC2 complex is in fact inactivated e.g. by determining the absence of H3K27 trimethylation (H3K27$me_3$) in the tumor tissue. Only MPNST patients that display an active PRC2 complex may benefit from EZH2 inhibition.

The enzyme TOP2A functions in maintaining DNA topology after replication. The cellular abundance of TOP2A is reported to determine the efficacy of anthracycline based chemotherapy in various cancers (32-37). The anthracycline doxorubicin, a widely used anticancer agent, can interfere with the catalytic cycle of TOP2A either by preventing its binding to DNA or by trapping TOP2A cleavage complexes and blocking DNA religation generating double strand DNA breaks (8). *TOP2A* levels in MPNST were reported to be upregulated due to amplification of the *TOP2A* gene (9, 10). Our results verified the abundant expression of *TOP2A* in MPNST and may explain why doxorubicin is widely used in the treatment of advanced MPNST patients. Though in general outcomes are poor, some patients may derive durable benefit from doxorubicin based treatment (7). When we determined the sensitivity of our neurofibroma and MPNST cell line panel for doxorubicin we observed that the MPNST cell lines exhibited the highest sensitivity in agreement with their higher TOP2A levels. Still the outcome of doxorubicin treatment in the clinic is poor for most MPNST patients perhaps due to the rapid activation of drug resistance mechanisms that diminish the efficacy of this chemotherapy.

From this study, we tentatively conclude that the potential for effective therapeutic intervention in MPNST by targeting BRD4, EZH2 and TOP2A individually, is limited. However, this does not preclude the use of inhibitors in certain subpopulations of patients and/or in combination therapies. We strongly encourage other research groups to validate our findings and are in favor of clinical studies involving patients as only these will ultimately prove the true value of BRD4, EZH2 and TOP2A inhibitors in the MPNST setting. Last but not least further

investigations are needed into the biology of MPNST to identify additional druggable disease drivers for novel therapeutic strategies.

# Supplementary information

Supplementary data are provided online

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# Chapter 3

# Deregulated microRNAs in neurofibromatosis type 1 derived malignant peripheral nerve sheath tumors

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# Abstract

Malignant peripheral nerve sheath tumors (MPNST) are aggressive cancers that occur spontaneously (sporadic MPNST) or from pre-existing, benign plexiform neurofibromas in neurofibromatosis type 1 (NF1) patients. MPNSTs metastasize easily, are resistant to therapeutic intervention and are frequently fatal. The molecular changes underlying the transition to malignancy in the NF1 setting are incompletely understood. Here we investigate the involvement of microRNAs in this process. Using an RT-PCR platform microRNA expression profiles were determined from a unique series of archival paired samples of plexiform neurofibroma and MPNST. At least 90 differentially expressed microRNAs ($p<0.025$; FDR<10%) were identified between the paired samples. Most microRNAs (91%) were found downregulated and 9% of the microRNAs were upregulated in MPNST. Based on the fold changes and statistical significance three downregulated microRNAs (let-7b-5p, miR-143-3p, miR-145-5p) and two upregulated microRNAs (miR135b-5p and miR-889-3p) were selected for further functional characterization. In general their expression levels were validated in a relevant cell line panel but only partly in a series of unpaired fresh frozen tumor samples containing plexiform neurofibromas, atypical neurofibromas and MPNSTs. As part of the validation process we also determined and analyzed microRNA expression profiles of sporadic MPNSTs observing that microRNA expression discriminates NF1-associated and sporadic MPNSTs emphasizing their different etiologies. The involvement of microRNAs in tumorigenesis and cancer progression was examined in NF1-derived MPNST cell lines through modulating microRNA levels by transient transfection of microRNA mimics or inhibitors. The effects of microRNAs on cellular proliferation, migration, invasion and Wnt/ß-catenin signaling were determined. Our findings indicate that, some of the selected microRNAs affect migratory and invasive capabilities and Wnt signaling activity. It was observed that the functional effects upon microRNA modulation are distinct in different cell lines. From our study we conclude that miRNAs play essential regulatory roles in MPNST facilitating tumor progression.

# Introduction

Neurofibromatosis type 1 (NF1) is a relatively common autosomal dominant disorder which is caused by inherited or sporadic mutations in the *NF1* gene [1-3]. The *NF1* gene encodes the tumor suppressor neurofibromin 1 that functions as a negative regulator of Ras signaling by its GTPase- activating protein (GAP) domain. The partial inactivation of neurofibromin 1 seen in NF1 patients can cause variable symptoms affecting the skin, bone and the nervous system. Moreover, the disease is associated with an increased risk of benign and malignant tumor formation. Almost all NF1 patients develop cutaneous neurofibromas and in many instances also deeper seated plexiform neurofibromas. These benign tumors are believed to originate from the Schwann cell lineage i.e. mature Schwann cells or Schwann cell precursors [4,5] and are characterized by a biallelic inactivation of the *NF1* gene [1,6]. Approximately 10% of NF1 patients develop malignant peripheral nerve sheath tumors (MPNSTs) usually in the context of pre-existing plexiform neurofibromas. MPNST are highly aggressive tumors that are largely responsible for the reduced life expectancy these patients face [7-9]. Early metastasis, poor prognosis, and resistance to therapeutic interventions are common clinical features of this cancer. While patients with non-metastatic disease may benefit from surgical resection and radiation, many patients relapse. These patients, and also those initially presenting with advanced disease, face a poor prognosis as there are only a limited number of systemic agents available for these patients such as doxorubicin, ifosfamide and pazopanib. The relatively modest anti-tumor activity of these agents translates in a median overall survival of approximately one year [10,11]. A better understanding of the essential molecular mechanisms underlying plexiform neurofibroma transformation to MPNST is crucial to reveal NF1 patients who are at risk to develop MPNST and to identify new targets for treatment.

MicroRNAs (miRNAs) are a class of small non-protein coding RNAs of approximately 19-26 nucleotides in length that function in post-transcriptional gene regulation. They generally operate by binding in the context of the RNA induced silencing complex (RISC) to the 3' untranslated region of target mRNAs. MiRNA binding, through base pairing between the miRNA and mRNA, cause mRNA degradation and/or inhibition of translation [12,13]. Over the past two decades it became clear that miRNAs fulfil pivotal roles in a wide variety of biochemical and physiological processes and are intimately involved in numerous pathological processes including cancer [14-17]. A dysregulated miRNA expression profile is a key characteristic of cancer and can be exploited for diagnostic purposes. There is ample evidence that miRNAs can have oncogenic or tumor suppressive properties. However, in many instances the extent to which individual – aberrantly expressed – miRNAs contribute to carcinogenesis and cancer progression and/or affect treatment response is not fully understood. A limited number of miRNA profiling studies examined human neurofibroma and NF1-derived MPNST tumor samples and implicated the involvement of several miRNAs in the malignant transformation of plexiform neurofibroma to MPNST [18-21]. Although of interest, these studies are difficult to compare as different miRNA detection platforms were used, variable numbers of unpaired tumor samples were examined and only a few miRNAs were functionally characterized. Here we analyzed miRNA expression, using an established and highly reproducible RT-PCR procedure, in a unique series of paired human archival tumor samples

of plexiform neurofibroma and MPNST. Each individual neurofibroma/MPNST pair being derived from the same NF1 patient. The expression of a selected set of differentially expressed miRNAs was validated using a well-characterized neurofibroma/MPNST cell line panel as well as fresh frozen samples of plexiform neurofibromas, atypical neurofibromas and MPNST. To understand how these miRNAs affect carcinogenesis and/or MPNST progression we modulated their expression levels in MPNST cell lines and assessed their impact on cellular proliferation, migration and invasion and Wnt/β-catenin signaling.

# Materials and Methods

## Tumor samples

Neurofibromatosis type 1 patients from which both archival plexiform neurofibroma as well as MPNST resection samples were available were identified in the Erasmus Medical Center patient files. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected, from the Erasmus MC Tissue bank, of a set of nine neurofibroma-MPNST pairs (see Supplementary Table 1 for patient and tumor characteristics). In addition, ten FFPE tumor tissue blocks were collected that were derived from patients diagnosed with sporadic MPNST (Supplementary Table 2). Fresh frozen tumor samples from plexiform neurofibroma (n=7), atypical neurofibroma (n=4) and NF1-associated MPNST (n=11) (Supplementary Table 3) from the Erasmus MC tissue bank were included for validation purposes. Hematoxylin-eosin-stained sections of these samples were examined by an expert pathologist at the Erasmus MC (RMV) to confirm the initial histopathological diagnosis using criteria as described before [22,23] in accordance with the 2016 WHO classification of Tumours of the Central Nervous System [24]. The experimental protocol was submitted for review to, and approved by, the Medical Ethics Committee Erasmus MC of Rotterdam (MEC-2016-213). All experimental procedures, including the use of human tissues samples, were performed in accordance with the relevant guidelines and regulations, with all researchers adhering to the code of conduct for medical research as laid out by the council of the Federation of Dutch Medical Scientific Societies (https://www.federa.org/codes-conduct). The use of anonymous or coded left-over material for scientific purposes is part of the standard treatment agreement with patients and therefore informed consent was not required according to Dutch law.

## Cell culture

The human NF1-associated MPNST derived cell lines 90-8TL, ST88-14 and the sporadic MPNST derived STS26T cell line were a kind gift of Dr. Eduard Serra (Institute of Predictive and Personalized Medicine of Cancer/IMPPC, Barcelona, Spain). The sNF96.2 cell line (NF1-derived MPNST) and the HS53T cell line (cutaneous neurofibroma) were obtained from the American Type Culture Collection (ATCC). All cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% $CO_2$. All cell cultures were regularly screened for mycoplasma infection. Short Tandem Repeat (STR) profiles of the cell lines were established for authentication purposes (Supplementary Fig. 1) and were matched to source profiles at the ATCC, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) or the literature when available.

# RNA isolation

Total RNA was extracted from 5-6 20 µm sections from each FFPE tumor sample using the RecoverAll™ total nucleic acid isolation kit (Ambion / Life Technologies) according to the manufacturer's recommendations. RNAbee (Tel test Inc.) was used to isolate total RNA from cell pellets and fresh frozen tumor tissue samples following standard protocols. The quality and concentration of all the RNA preparations were examined using a Nanodrop-1000 (Nanodrop Technologies).

# MicroRNA profiling

The miRNA expression profiles were determined in FFPE samples using TaqMan® Low Density Array (TLDA) Human MicroRNA Cards (A card v2.0, B card v3.0; Applied Biosystems/Thermo Fisher Scientific) capable of detecting 756 distinct human miRNAs essentially as previously described [25]. In brief: Two pools of cDNA were prepared using Megaplex™ RT Primers Human Pools (pool A v2.1, pool B v3.0) and a Taqman® microRNA reverse Transcription kit (Applied Biosystems/ Thermo Fisher Scientific). Next, a pre-amplification step was carried out using Megaplex™ PreAmp Primers Human Pools (pool Av2.1, pool B v3.0) together with the Taqman™ PreAmp master-mix (Applied Biosystems/Thermo Fisher Scientific). The resulting products were further amplified using Taqman™ Universal PCR Master-Mix No AmpErase® on human microRNA A and B cards in a 7900HT Fast Real-Time PCR system (Applied Biosystems/Thermo Fisher Scientific). The expression ($C_T$ value ) of a specific miRNA in a sample was normalized to the median $C_T$ of all detectable miRNAs in that sample. Subsequently the normalized relative expression ($2^{-\Delta CT}$) was calculated for each miRNA. The normalized miRNA expression data were log 2 transformed and median centered to acquire the relative expression values that were used for hierarchical clustering analyses using Cluster-3.0 and Java TreeView for visualization. The clustering was based on the uncentered correlation as a distance metric using average linkage. A Student T-test (paired) was used to determine statistical significance between distinct groups of expression data and the Benjamini-Hochberg false discovery rate (FDR) was used to control for multiple testing.

# RT-PCR

The expression level of individual miRNAs was determined using the TaqMan® MiRNA Assays Technology (Applied Biosystems/Thermo Fisher Scientific) in a neurofibroma/MPNST cell line panel and fresh frozen tumor samples. In brief: Total RNA (50 ng) was reverse transcribed in a multiplex reaction using specific miRNA primers from the TaqMan® MiRNA Assays and reagents from the TaqMan® MiRNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific) according to the manufacturer's protocol. The resulting cDNA was used as input in a quantitative real-time PCR (qPCR) using a miRNA

specific primer/probe mix together with the TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems/Thermo Fisher Scientific) using the 7500 Fast Real-Time PCR systems (Applied Biosystems/Thermo Fisher Scientific). The qPCR data were analyzed using SDS software (version 2.4, Applied Biosystems/Thermo Fisher Scientific). A standard dilution series of a cDNA sample-pool was included on every plate allowing for the absolute quantification of the miRNA expression.

The mRNA expression of Wnt target genes was determined by RT-PCR using the TaqMan® Technology (Applied Biosystems/Thermo Fisher Scientific). In brief: Total RNA (1 µg) was used as input for a reverse transcription reaction using a high capacity cDNA reverse transcription kit (Applied Biosystems/Thermo Fisher Scientific) according to protocols of the manufacturer. The cDNA was used as input in a PCR reaction using primer/probe combinations from the following Taqman® gene expression assays (LEF1, assay ID: Hs01547250_m1; MSX2, assay ID: Hs00741177_m1; SOX9, assay ID:Hs00165814_m1; TWIST1, assay ID: Hs00361186_m1) and Taqman® Universal PCR master mix using the 7500 Fast Real-Time PCR system (all obtained from Applied Biosystems/Thermo Fisher Scientific) according to the manufacturer's recommendations. Three housekeepers (GAPDH, HPRT and PPIA) were used for normalization purposes using the comparative $C_T$-method [26]. The qPCR data were analyzed using SDS software (version 2.4, Applied Biosystems/Thermo Fisher Scientific).

# Transfections

Human MPNST cells were plated in 96-well plates at a concentration of 2 - 18 x $10^3$ cells/well (SNF96.2); 2 - 10 x $10^3$ cells/well (ST88-14) and 2 -10 x $10^3$ cells/well (90-8TL) in a total volume of 200 µl of standard cell culture medium without antibiotics. After 24 h cells were transfected with 50 nM MiRIDIAN microRNA mimics (Dharmacon) of let7b-5p, miR-143-3p, miR-145-5p and miR-29c-3p or 50 nM MiRCURY LNA™ inhibitors (Exiqon) of miR-135b-5p and miR-889-3p. As controls a scrambled miRNA mimic Negative control #1 (Dharmacon) and the miRCURY LNA™ inhibitor Negative Control (Exiqon) were used. DharmaFECT I was used as a transfection reagent. Transfection conditions were optimized (transfection efficiency > 90%) for each of the cell lines using a fluorescently labelled miRNA mimic (miRIDIAN mimic transfection control Dy547; Dharmacon) (Supplementary Fig.2).

# Proliferation assay

Twenty-four hours prior to transfection, cells were plated in a 96-well plate. The next day the cells were transfected at approximately 40-50% confluency with selected miRNA mimics/inhibitors or appropriate controls. Cell viability was assessed by a sulforhodamine B (SRB) assay at 72 h post-transfection essentially as described previously[27]. In short: cells were fixed by 10% TCA in PBS, washed and stained by 0.4% SRB in 1% acetic acid for 15 min,

washed in 1% acetic acid and dried. Color was dissolved in Tris-Base after which the $A_{540nm}$ was measured using a spectrophotometer.

# Migration assays

Wound healing kinetics: Twenty-four hours prior to transfection, cells were plated in a 96-well ImageLock™ plate (Essen BioScience Ltd.). The next day the cells were transfected with selected miRNA mimics/inhibitors or appropriate controls. At 24 h post-transfection, all 96 wells were scratched simultaneously in the central axis of the individual wells using the WoundMaker™ (Essen Bioscience Ltd.). A live-cell imaging system, IncuCyte (Essen BioScience Ltd.) was used to automatically monitor the kinetics of cell migration every 2 hours for a total duration of 26 h during which cells migrate from the scratch edges into the wound area.

Cell-speed measurements: Twenty-four hours prior to transfection, cells were plated in a 96-well CellCarrier™-96 Ultra microplate (PerkinElmer). The next day the cells were transfected with selected miRNA mimics or appropriate controls. At 24 h post-transfection cells were imaged at 2 h intervals in an Opera Phenix™ HCS system (PerkinElmer) for 40 h. Software (Harmony® High Content Imaging and Analysis Software, PerkinElmer) was used to calculate the average cell speed of the individual cells in the wells of the microplate.

# Invasion assay

Cells were cultured in a 6-well plate and transfected with selected miRNA mimics/inhibitors or appropriate controls. At 24 h post-transfection the cells were harvested by mild trypsinization, resuspended in DMEM supplemented with 2.5% FBS and plated into the IncuCyte™ ClearView 96-well insert (Essen BioScience Ltd.) at a concentration of $2 \times 10^3$ cells/well (SNF96.2); $2 \times 10^3$ cells/well (ST88-14) and $7.5 \times 10^3$ cells/well (90-8TL). Prior to plating the transfected cells, the IncuCyte ClearView insert membranes were coated with 50 µg/ml Matrigel (BD Biosciences). The inserts were subsequently placed in a 96-well plate containing DMEM supplemented with 10% FBS and incubated under standard cell culture conditions. An IncuCyte live-cell imaging system (Essen BioScience Ltd.) was used to capture cell invasion monitoring and quantifying invading cells through the matrigel coated membranes every two hours for a total period of 67 h.

# Wnt reporter assay

Wnt/β-catenin signaling activity was determined by a β-catenin/TCF reporter assay in a two-step transfection process. In brief: SNF96.2, ST88-14 and 90-8TL cell lines were plated in 24-

well plates in culture medium without antibiotics. When the cells reached 60 – 70% confluency they were transfected with MiRCURY LNA$^{TM}$ inhibitors (Exiqon) of miR-135b-5p, miR-889-3p or a miRCURY LNA$^{TM}$ inhibitor Negative Control (Exiqon) in a final concentration of 50 nM using Dharmafect I. After 24 h the cells were co-transfected with 250 ng of the TOP-Flash or FOP-Flash firefly luciferase reporter constructs [28] and 25 ng of a SV40-Renilla luciferase expression (for normalization purposes) using FuGene ®HD (Promega). Eight hours post-transfection the cells were stimulated with 25% L-control medium in DMEM or 25 % L-Wnt3A medium in DMEM and left to incubate for 16 h after which the cells were lysed and assayed for firefly and Renilla luciferase activities using the Dual-Luciferase reporter assay system (Promega).

# Results

## Plexiform neurofibromas can be distinguished from MPNST by their microRNA expression profile.

To study the involvement of miRNAs in the malignant transition of benign plexiform neurofibromas into MPNST we determined the miRNA expression profiles of a unique series of nine paired plexiform neurofibromas and MPNST samples. Each plexiform neurofibroma / MPNST pair was derived from the same NF1 patient. An unsupervised hierarchical clustering, based on the expression of all detectable miRNAs in these paired samples, already grouped most of the benign plexiform neurofibromas and MPNSTs in distinct clusters (Supplementary Fig. 3). A supervised clustering analysis using the 90 most significant differentially expressed miRNAs ($p<0.025$; FDR<10%) between neurofibromas and MPNSTs grouped the samples into clearly separate clusters (Fig. 1, Supplementary Table 4). The majority (82 out of 90; 91%) of the differentially expressed miRNAs were found to be downregulated in the MPNST group in comparison to the plexiform neurofibromas. The downregulated miRNAs include members of well-known cancer related miRNA clusters like the miR-23/27/24 clusters on chromosome 9-q22.32 and 19-p13.12, the miR-143/145 cluster on 5-q33.1, the miR-29b-1/29a and miR-29b-2/29c clusters on chromosome 7-q32.3 and 1-q32.2, respectively. In addition, 5 members of the let-7 family, let-7a/b/c/d/e were found downregulated in MPNST. Only 8 (9%) of the miRNAs exhibited a higher expression in the MPNST samples than in neurofibromas, these include miR-135b, miR-889, miR-493, miR-433 and miR-541, the last four all belonging to a large miRNA cluster on the long arm of chromosome 14 (14-q32.31). Particularly, miR-135b and miR-541 are aberrantly expressed in the MPNST setting with a 52-fold and 20-fold upregulation, respectively.

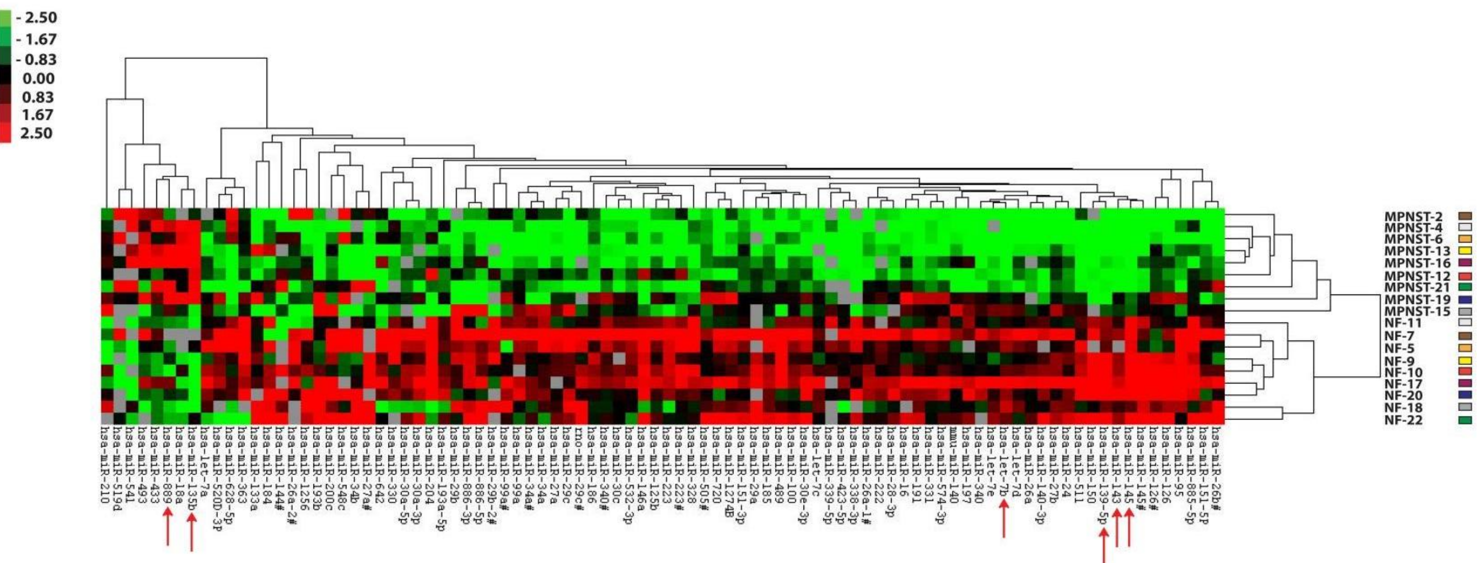
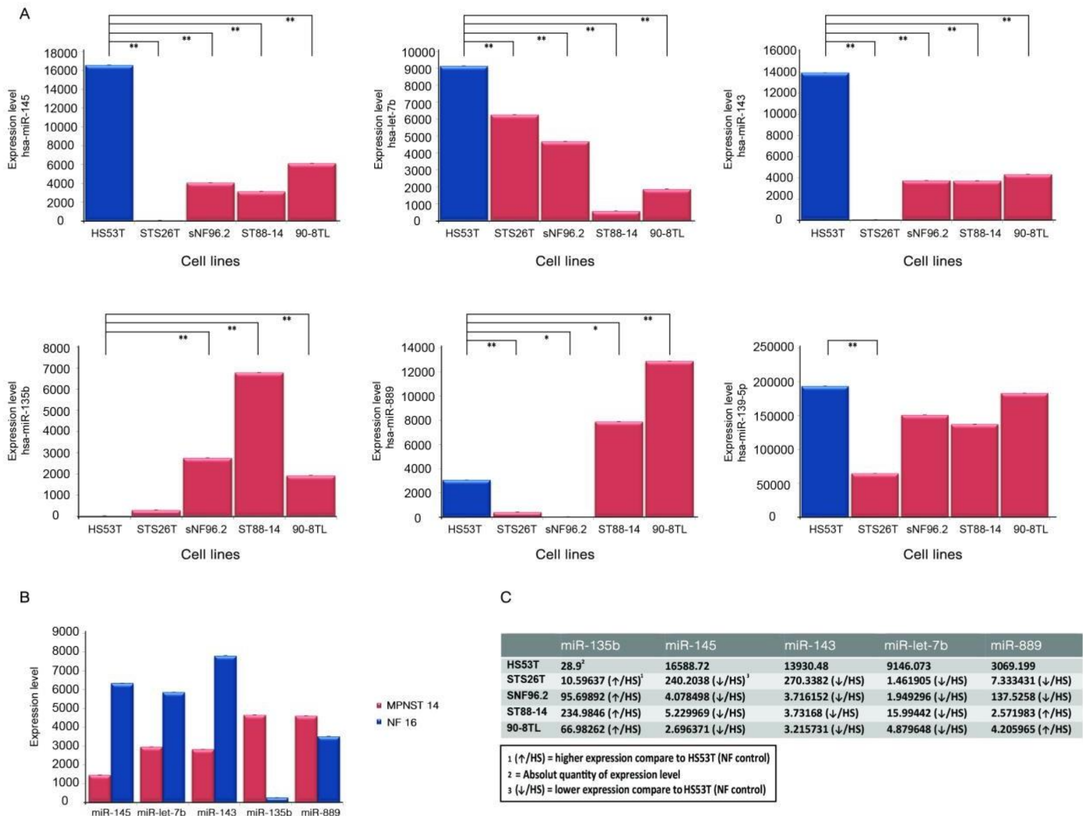



**Fig. 1 - Differentially expressed microRNAs between plexiform neurofibromas and MPNST**. The miRNA expression profiles were determined of FFPE sections from nine paired sets of plexiform neurofibroma (NF) and MPNST tumor samples, each pair derived from the same NF1 patient that initially presented with a plexiform neurofibroma and at a later stage developed an MPNST. Depicted are the results of a supervised hierarchical clustering using the most differentially expressed miRNAs ($p<0.025$, FDR <10%) in the analyses. The color code on top indicates the relative position of the neurofibroma-MPNST pairs in the cluster tree. The heat map lists the individual miRNAs and their relative expression levels in the MPNST and neurofibroma clusters. Red arrows indicate miRNAs that were selected for further validation and functional studies. In the heat map red indicates relative high expression, green relative low expression, grey designates missing expression values.

# Validation of differentially expressed microRNAs between plexiform neurofibromas and MPNST in a cell line panel and fresh frozen tumor samples.

It was investigated whether the differential expression of a selected set of miRNAs could be validated in a well-characterized cell line panel and additional, unpaired fresh frozen neurofibroma and MPNST samples. Taking statistical significance ($p<5x10^{-4}$; FDR<1%), fold-difference (>3 in at least 75% of the sample pairs) into account as well as the reported involvement of miRNAs in cancer, we selected the following miRNAs for further validation and subsequent functional studies miR-145-5p, miR-143-3p, miR-139-5p and let-7b-5p all downregulated in MPNST and miR-135b-5p and miR-889-3p as representatives of the upregulated miRNAs (Fig. 1, Supplementary Tables 4 and 5). When considering the expression of the selected miRNAs in the individual NF-MPNST samples pairs it was noted that the fold-difference of the up or down-regulation varies considerable between different pairs (Supplementary Table 5). Using quantitative PCR, we could validate the expression of the selected miRNAs as shown in Fig. 2A. MiR-145, let-7b, miR-143 - and to a lesser extent miR-139-5p - were all downregulated in the MPNST cell lines STS26T, sNF96.2, ST88-14 and 90-8TL compared to their expression level in a cutaneous neurofibroma cell line Hs53.T. Conversely, miR-135b found upregulated in ~~most~~ MPNST cell lines. MiR-889 was clearly upregulated in ST-88-14 and 90-8TL but downregulated in sNF96.2 and the sporadic MPNST cell line STS26T. general, these results (Fig. 2C) confirm our miRNA profiling findings and identify the MPNST cell lines as representative models for this malignancy. As the expression distribution between neurofibroma and MPNST for miR-139-5p reflected our profiling results the least, we omitted this miRNA from further analyses. As additional validation we determined the expression levels of the selected miRNAs in fresh frozen samples from a plexiform neurofibroma / MPNST sample pair derived from the same patient (Fig. 2B). In agreement with our previous observations we demonstrated downregulation of miR-145, miR-143 and let-7b whereas miR-135b and miR-889 were upregulated in the MPNST sample. We also determined the expression levels of the selected miRNAs in a larger unpaired panel of fresh frozen tumor samples consisting of plexiform neurofibromas (n=6), atypical neurofibromas (n=4) and MPNSTs (n=10) (Fig. 3). The expression level of the miRNAs in atypical neurofibroma samples is not significantly different from the expression observed in plexiform neurofibromas. A comparison between the miRNA expression levels in MPNST and neurofibromas indicated a significant downregulation in the MPNST group of let-7b ($p<0.01$) and of miR-145 when the expression levels in MPNST were compared to levels in atypical neurofibromas ($p<0.05$). A comparison of miR-145 levels between MPNST and plexiform neurofibromas was borderline significant ($p=0.0572$). Likewise, the expression of miR-143 between MPNST and atypical neurofibromas was borderline significant ($p=0.0584$). No significant statistical difference between sample groups was observed for miR-889, miR-143 and miR-135b expression. The high variability observed in the expression levels of the selected miRNAs, particularly in the MPNST samples, most likely reflects tumor heterogeneity and may obscure differences. This problem may be partly overcome by analyzing paired samples.




| | miR-135b | miR-145 | miR-143 | miR-let-7b | miR-889 |
|---|---|---|---|---|---|
| HS53T | 28.9[2] | 16588.72 | 13930.48 | 9146.073 | 3069.199 |
| STS26T | 10.59637 (↑/HS)[1] | 240.2038 (↓/HS)[3] | 270.3382 (↓/HS) | 1.461905 (↓/HS) | 7.333431 (↓/HS) |
| SNF96.2 | 95.69892 (↑/HS) | 4.078498 (↓/HS) | 3.716152 (↓/HS) | 1.949296 (↓/HS) | 137.5258 (↓/HS) |
| ST88-14 | 234.9846 (↑/HS) | 5.229969 (↓/HS) | 3.73168 (↓/HS) | 15.99442 (↓/HS) | 2.571983 (↑/HS) |
| 90-8TL | 66.98262 (↑/HS) | 2.696371 (↓/HS) | 3.215731 (↓/HS) | 4.879648 (↓/HS) | 4.205965 (↑/HS) |

1 (↑/HS) = higher expression compare to HS53T (NF control)
2 = Absolut quantity of expression level
3 (↓/HS) = lower expression compare to HS53T (NF control)

**Fig. 2 – Expression of selected microRNAs in a neurofibroma/MPNST cell line panel and in fresh frozen plexiform neurofibroma, atypical neurofibroma and MPNST samples. (A)** Expression levels measured by quantitative RT-PCR of selected miRNAs (miR-145-5p, let-7b-5p, miR-143-3p, miR-135b-5p, miR-889-3p and miR-139-5p) in a well-characterized cell line panel consisting of a cutaneous neurofibroma cell line (HS53T), NF1-associated MPNST cell lines (SNF96.2, ST88-14, and 90-8TL) and a sporadic MPNST cell line (STS26T). Bars depict average values ± SD (n=3-4). A T-test was used to determine statistical significance; p-value <0.05 (*), p-value <0.01 (**). (B) Expression level of selected miRNA in a fresh frozen plexiform neurofibroma/MPNST sample pair derived from the same NF1 patient. (C) Summary of the expression level fold-changes of selected miRNAs in the MPNST cell lines compared to

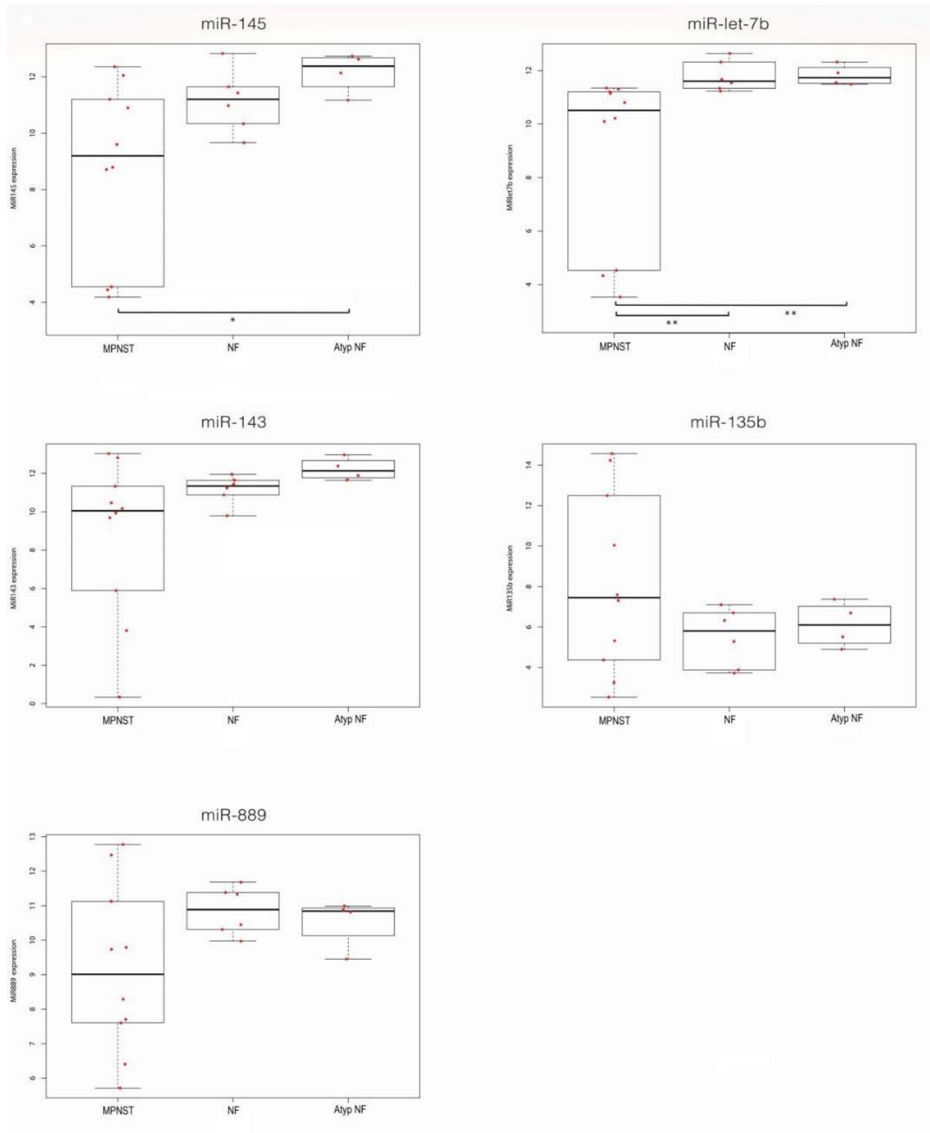



**Fig. 3 Expression level of selected microRNAs in unpaired fresh frozen plexiform neurofibroma, atypical neurofibroma and MPNST samples.** A quantitative RT-PCR was used to determine miRNA levels of miR-145-5p, let-7b-5p, miR-143-3p, miR135b-5p and miR-889-3p in unpaired fresh frozen NF1-derived MPNST (n=10), plexiform neurofibroma (NF; n=6) and atypical neurofibroma (Atyp NF; n=4). Relative expression is depicted using Box-Whisker plots with boxes showing 1st to 3rd quartile with the median marked by a horizontal line. A Mann Whitney U test was used to determine statistical significance; p- value <0.01 (**), p-value <0.05 (*).

# NF1-associated MPNST and sporadic MPNST display distinct miRNA expression profiles.

To assess whether the selected miRNAs are specifically dysregulated in NF1-derived MPNST, we examined miRNA expression in 10 archival sporadic MPNST samples. A comparison between the miRNA profiles observed in the sporadic MPNST and the NF1-derived MPNST revealed many differentially expressed miRNAs (Fig. 4, Supplementary Table 6, Supplementary Fig. 4) emphasizing these tumor types have a different etiology and possibly a different biology. A cluster analysis using the 45 most significantly differentially expressed miRNAs ($p<0.03$, FDR<10%) completely discriminated the two MPNST types (Fig. 4). Very few of the miRNAs identified in the plexiform neurofibroma-MPNST comparison, and none of the selected miRNAs, were detected in the sporadic MPNST – NF1-derived MPNST comparison. Apparently, the selected miRNAs were not differentially expressed between sporadic and NF1-derived MPNST. Therefore, we cannot rule out that the selected miRNAs are also aberrantly expressed in sporadic MPNST and play a role in carcinogenic processes in these tumors as well.

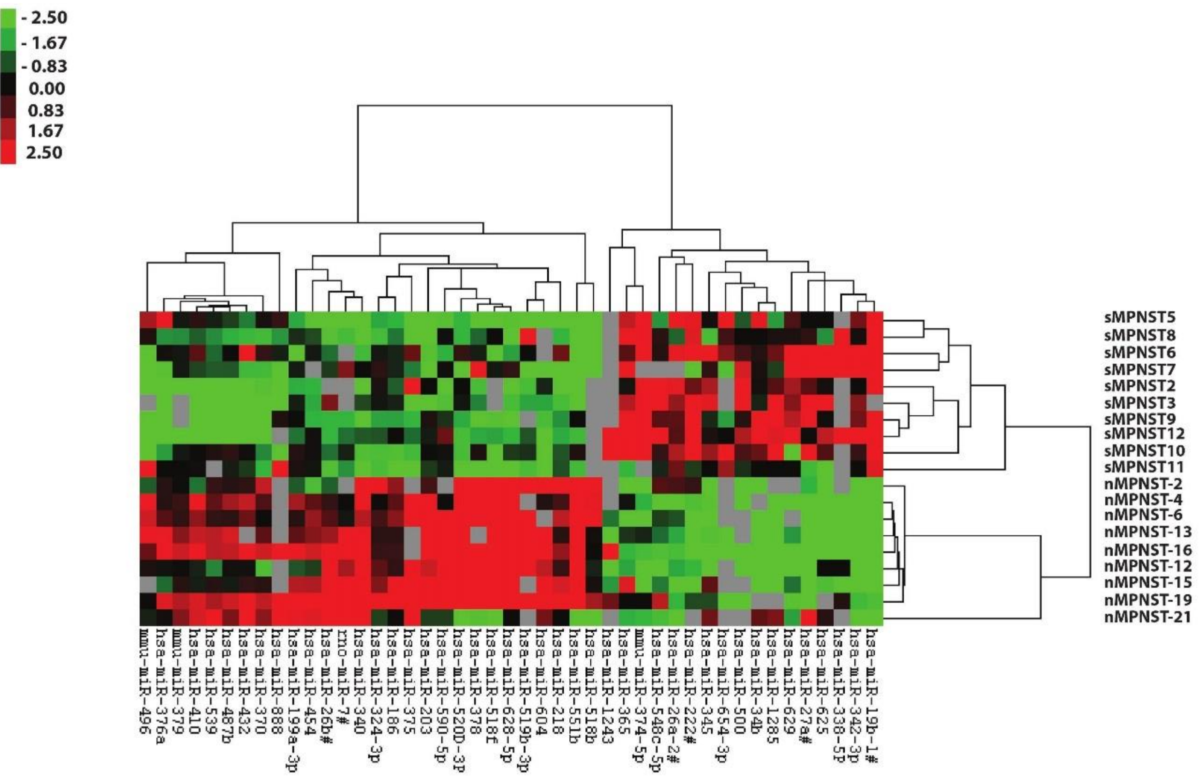



**Fig. 4 - Differentially expressed microRNAs between NF1-derived and sporadic MPNST**. The miRNA expression profiles derived from FFPE sections of sporadic MPNST (sMPNST) (n=10) are compared with the miRNA profiles from NF1-derived MPNST (n=9, see Fig. 1). Depicted is a supervised hierarchical clustering using the most significant differentially expressed miRNAs ($p<0.011$, FDR<10%). The heat map lists the miRNAs and their relative expression levels. The differences indicate the different etiology of sporadic and NF1 derived MPNST and may reflect a different biology.  In the heat map red indicates relative high expression, green relative low expression, grey designates missing expression values.

# MicroRNAs affect migratory and invasive capacity of MPNST cell lines.

The selected miRNAs that are dysregulated in NF1-associated MPNST may contribute to the process of tumorigenesis and metastasis. All have been linked to various aspects of carcinogenesis in other cancers. The clustered miR-143 and miR-145 genes are widely regarded as tumor suppressors in epithelial tumors [29-32] and were indicated as having a critical role in tumor stroma [33,34]. MiR-135b has been implicated in the progression of several cancers [35-37] and let-7b is considered a tumor suppressor miRNA [38]. *In vitro* experiments were conducted to examine the functional role of the selected miRNAs and their effect on cellular proliferation, migration and invasion. To that end, transiently, the expression levels of let-7b, miR-145 and miR-143 were restored and miR-135b and miR-889 levels were reduced, in MPNST cell lines. As a control we included a miR-29c mimic. This miRNA was reported by Presneau *et al*. to be reduced in MPNST and to affect migration and invasion, but not proliferation [20].

First, we focused on cellular proliferation using an SRB assay to assess the effects of miRNA modulation on cell viability. It was observed that none of the miRNA mimics or inhibitors significantly and consistently affected proliferation with the exception of miR-29c overexpression in sNF96.2 which stimulated cell proliferation (Supplementary Fig. 5). We next assessed whether the selected miRNAs affect the migratory and invasive capacity of the tumor cells. We performed a scratch assay to measure the migration potential of the transfected MPNST cells. Fig. 5 depicts representative results on the kinetics of migration in sNF96.2 and ST88-14 transfectants obtained by a live-cell imaging system. Most miRNA mimics and inhibitors did not significantly interfere with the migratory capacity of the MPNST cells (Fig. 5A, C). However, a clear reduction of the migration rate was observed in sNF96.2 cells transfected with let-7b (Fig. 5B) and in ST88-14 cells transfected with miR-29c (Fig. 5D). None of the miRNA mimics and inhibitors had an effect on the migration capacity of 90-8TL cells (Supplementary Fig. 6A). These observations were confirmed when we determined the average cell speed as a measure for migratory capacity using a different cell imaging system (Supplementary Fig. 7).

Next, we examined the effect of the selected miRNAs on the invasive capacity of MPNST cells in a cell invasion assay. The MPNST cell lines sNF96.2 and ST88-14 were transfected and seeded onto 96-well invasion plates containing a matrigel coated membrane. Invasion of cells was quantitatively monitored by live-cell imaging in time. A strikingly reduced invasive capacity was observed in ST88-14 transfected with miR-135b ($p=0.001$) and miR-889 ($p=0.028$) inhibitors (Fig. 6C). These effects, however, were not seen in sNF96.2 transfectants (Fig. 6A) or 90-8TL transfectants (Supplementary Fig. 6B). In sNF96.2 cells, transfection with let-7b ($p=0.007$) and miR-29c ($p=0.007$) mimics, and to a lesser extent with miR-145 ($p=0.047$) resulted in reduced invasiveness (Fig. 6B). In contrast, miR-143 ($p=0.00006$) and miR-145($p=0.005$) mimics appeared to boost invasion in ST88-14 (Fig. 6D). We conclude that miRNA modulation effects are cell line dependent.

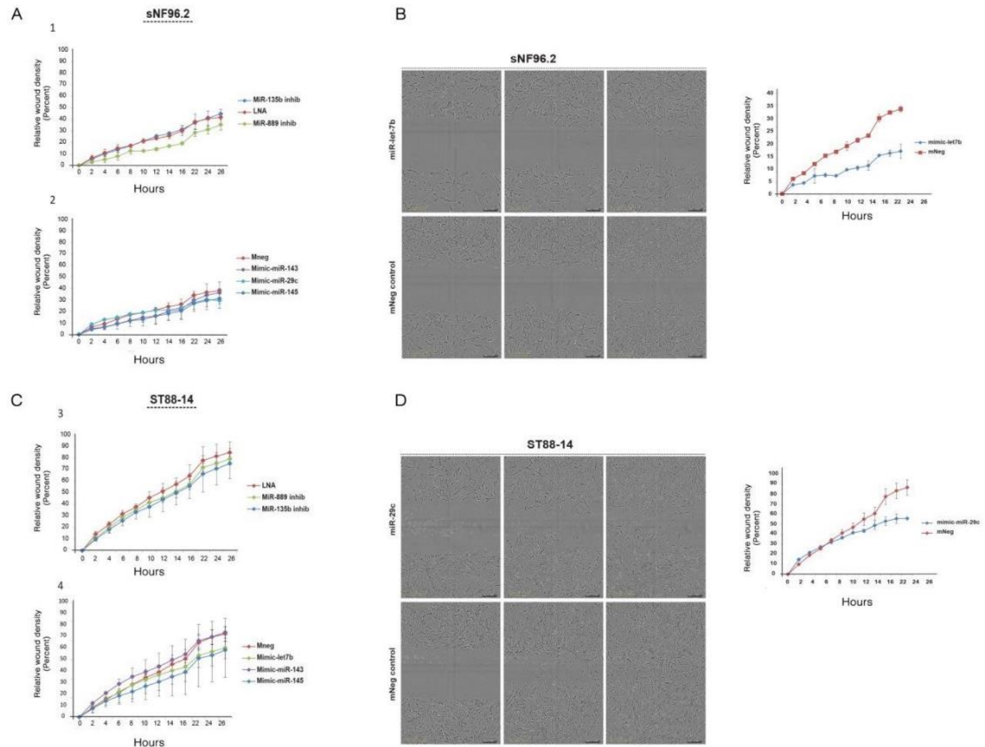



**Fig. 5 – Effects of let-7b and miR-29c on cell migration of the MPNST cell lines sNF96.2 and ST88-14.** The NF1-associated MPNST cell lines sNF96.2 and ST88-14 were transfected with scrambled (LNA control), miR-135b and miR-889 inhibitors or with a scrambled (mneg control), miR-143, miR-145, let-7b and miR-29c mimics. (A, C) Scratch assay after which cell migration is monitored every two hours for 26 h using a live-cell imaging system (IncuCyte; Essen Bioscience Ltd.). (B, D) Micrographs illustrating the effects of let-7b and miR-29c mimics on cell migration in sNF96.2 and ST88-14, respectively. The individual panels show the situation directly after scratching (left panels), at 14 h (middle panels) and after 26 h (right panels). Depicted are representative images and graphs of three independent experiments, in the graphs individual data points display average values ± SD (n=3).

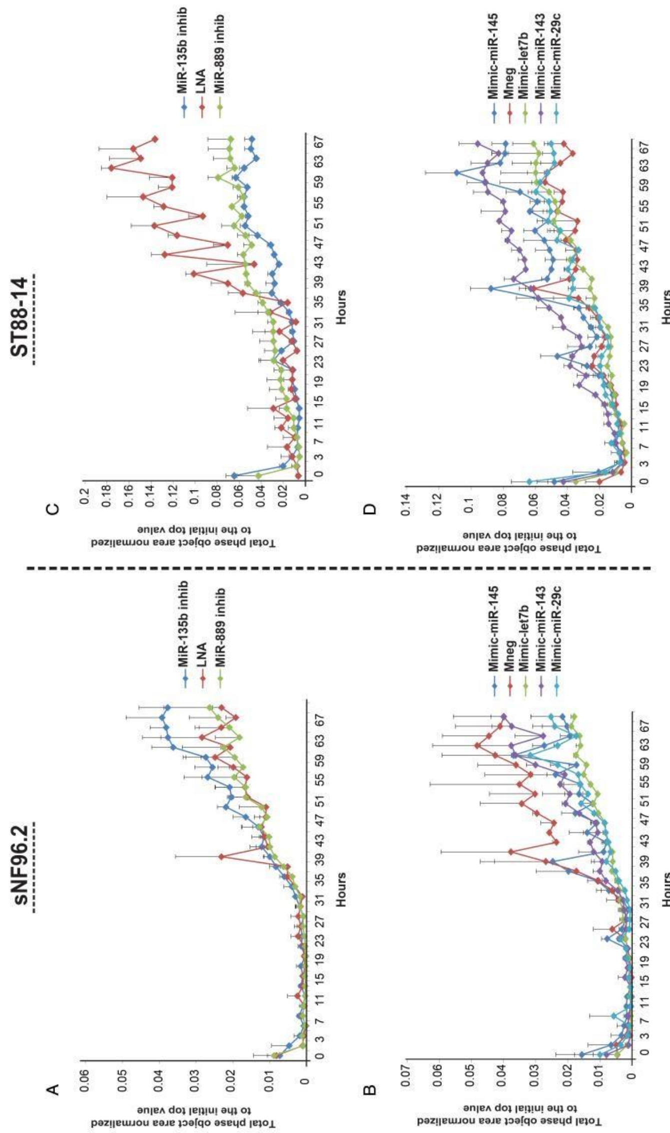



**Fig. 6 – Effects of selected miRNA mimics on invasive capacity of the MPNST cell lines sNF96.2 and ST88-14**. (A-D) The NF1-associated MPNST cell lines sNF96.2 and ST88-14 were transfected with scrambled (LNA control), miR-135b and miR-889 inhibitors or with a scrambled (mneg control), miR-143, miR-145, let-7b and miR-29c mimics. Invasive capacity was monitored every two hours for 67 h using a live-cell imaging system (IncuCyte). Y-axis indicates the “Total phase object area normalized to the initial top value” as a measure for the invading cell population. Depicted are representative graphs of xx independent experiments, points in graphs display average values ± SD (n=3). A Mann Whitney u test was used to determine statistical significance comparing the last 10 datapoints in each series.

# miR-135b and miR-889 modulate Wnt/β-catenin signaling in MPNST cells.

Recently the canonical Wnt/β-catenin signaling pathway has been identified as driver pathway of both benign neurofibromas and MPNST [39,40]. Moreover, miR-135b was reported to target multiple negative regulators of Wnt like Adenomatous Polyposis Coli (APC) [36]. Likewise, miR-889, another overexpressed miRNA in MPNST, was also predicted to target both *APC* according to Targetscan version 7.1. We therefore examined whether miR-135b (on average 52x higher in MPNST) and miR-889 (on average 3x higher in MPNST) (Supplementary Tables 4 and 5) are capable of modulating Wnt signaling activity in the MPNST setting. sNF96.2, ST88-14 and 90-8TL were transfected with miR-135b or miR-889 inhibitors. Next, we determined Wnt/ß-catenin signaling activity using a TCF/ ß-catenin reporter assay. It was noticed that Wnt/ ß-catenin activity upon induction with Wnt ligand was highest in ST88-14 (Fig. 7A) and 90-8TL (Supplementary Fig. 6C) with relatively low Wnt activity being measured in sNF96.2 (Fig. 7A). Transient reduction of miR-135b and miR-889 expression significantly impaired the induction of Wnt/ ß-catenin signaling activity in ST88-14 (Fig. 7A). No significant effects were observed in the 90-8TL despite the relatively high Wnt activity levels observed in this cell line (Supplementary Fig. 6C). A small but significant reduction of Wnt activation was seen in miR-889 inhibitor transfectants of sNF96.2 but not in the miR-135b inhibitor transfectant (Fig. 7A). To verify that Wnt is indeed affected the mRNA expression levels of Wnt target genes LEF1, MSX2, SOX9 and TWIST1, all genes expressed in MPNST and Schwann cells[39], were determined by quantitative PCR. ST88-14 cells that display active Wnt signalling (Fig. 7A) were transfected with control, miR-135b and miR-889 inhibitors. Figure 6B indicates that miR-135b inhibition consistently showed a trend of lowering the expression level of the Wnt target genes compared to a control transfection although without reaching statistical significance. Inhibition of miR-889 gave rise to similar results but the reduction in expression of the Wnt targets seemed stronger, more persistant and reached statistical significance. We conclude that both the overexpressed miR-135b and miR-889 in NF1-associated MPNST may augment Wnt signaling activity.

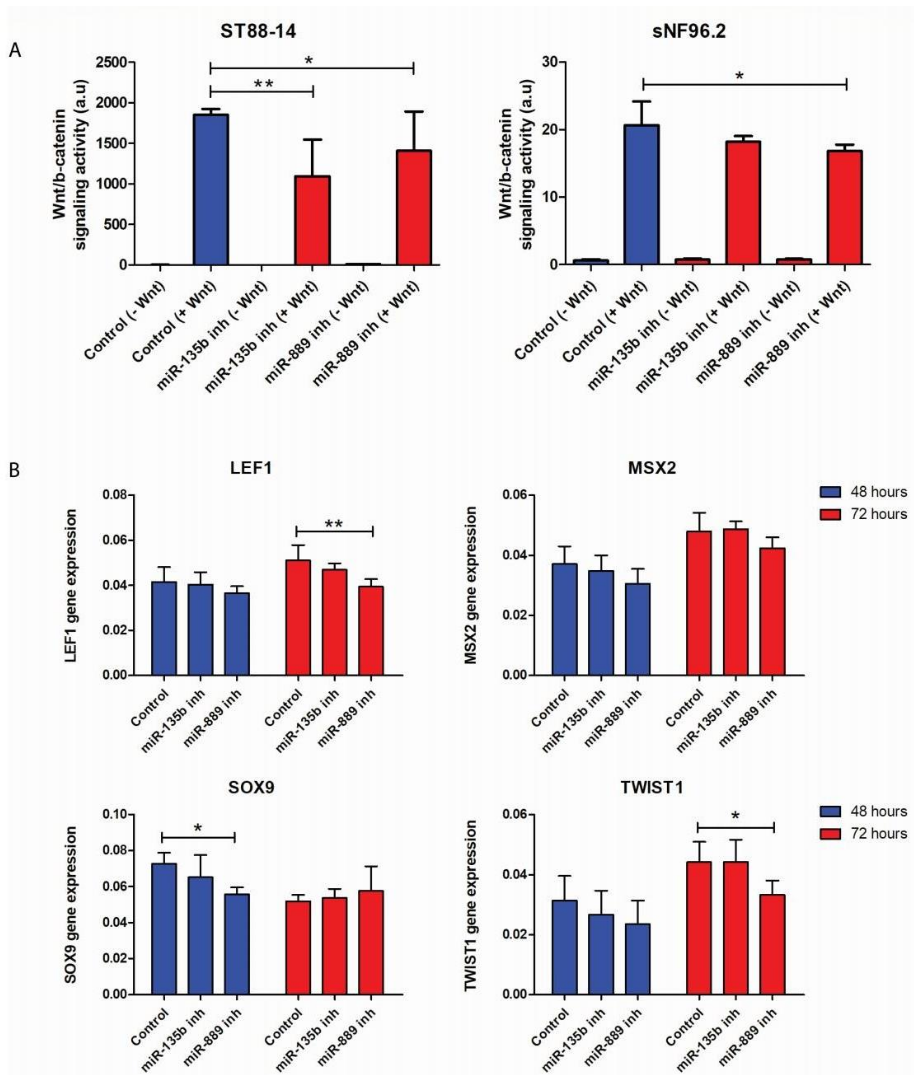



**Fig. 7 – Effect of miRNA modulation on Wnt signaling capacity in ST88-14 and 90-8TL cell lines**. The NF1-associated MPNST cell lines sNF96.2 and ST88-14 were transfected with scrambled (LNA control), miR-135b and miR-889 inhibitors. (A) Wnt/ß-catenin signaling activity upon induction by Wnt ligand was determined using a β-catenin/TCF reporter assay. Depicted are average values ± SD (n= 9) (B) mRNA expression levels measured by quantitative RT-PCR of Wnt target genes (LEF1, MSX2, SOX9, TWIST1). Depicted are normalised average expression values ± SD (n=4-6). In both (A) and (B) a Mann Whitney U test was used to determine significance, p-value <0.05 (*), p-value <0.01 (**).

# Discussion

MPNSTs are highly aggressive tumors with a dismal prognosis for those confronted with advanced disease [10,11]. Half of these tumors arise in the context of NF1 from benign pre-existing plexiform neurofibromas [7,41]. Genetic aberrations associated with this transformation include mutations in CDKN2A [42] and TP53 [43] and the recently disovered loss-of-function mutations in SUZ12 and EED, essential components of the PRC2 complex [44-46]. However, the precise molecular mechanisms underlying this maligant transition are still unclear. We investigated the involvement of miRNAs in the tumorigenesis and progression of MPNST.

MiRNAs are intricately connected to cancer and play critical roles in cancer gene regulation and diverse aspects of tumorigenesis [14-17]. Until now only few studies addressed the miRNA involvement in neurofibroma and MPNST biology [18-21]. All studies reported clear differences in miRNA expression between plexiform neurofibromas and MPNST when unpaired tumor samples were analyzed. However, the functional significance and pathological roles of dysregulated miRNAs in the context of MPNST are not, or poorly studied. In 2010, Chai *et al.* were the first to point out the downregulation of let7a/b in MPNST cells and their effect on MPNST cell invasiveness [18]. We as well observed the downregulation of multiple let-7 family members, including let7a/b in MPNST and noticed that let-7b expression interfered with cellular migration and invasion in NF1-derived MPNST cell lines. Let-7 family members are known to target Ras [47] it might therefore be that their relatively low levels in MPNST facilitate Ras signaling. Presneau *et al*. described a reduction of miR-29 members, most notably miR-29c, in MPNST [20]. They demonstrated that miR-29c played a role in tumor progression by controlling migration and invasion via the regulation of the matrix metalloproteinase-2 (MMP2) [20]. Our findings also indicated significantly lowered levels of miR-29a/b/c and we confirmed – although not in all NF1-derived MPNST cell lines – the effects of miR-29c on migration and invasion [48]. Supplementary Table 7 presents an overview of all miRNAs that were found dysregulated in NF1-associated MPNST in comparison to plexiform neurofibromas in multiple studies. Note that in general only few miRNAs were found upregulated in MPNST and that most differentially expressed miRNAs display a reduced expression level in MPNST.

By comparing the miRNA expression profiles of a unique series of paired samples of neurofibromas and MPNST, we defined a group of miRNAs that are aberrantly expressed in NF1-derived MPNST. From the 90 miRNAs that were identified we chose six miRNAs to examine their functional role in the pathogenesis of MPNST. MiR-143/145, let7b, miR-139-5p, miR135b, and miR-889 were among the top 15 of differentially expressed miRNAs. (Supplementary Table 4). All, with the exception of miR-889, were also reported as misexpressed in MPNST by other researchers (Supplementary Table 7). However, none of the miRNAs we examined, with the exception of let-7b, has been studied in MPNST. We were able to validate the reduced expression of let-7b, miR-143/145 and the increased expression of miR-135b in MPNST using a relevant cell line panel. The upregulation of miR-889 was only observed in two NF1 associated MPNST cell lines (ST88-14 and 90-8TL) and not in sNF96.2

and the sporadic MPNST cell line STS26T. The increased expression of miR-889 in MPNST, which is less striking than that of miR-135b, may be more variable and occur only in a subset of tumors. When we examined the expression of the selected miRNAs in an unrelated series of unpaired fresh frozen plexiform neurofibroma, atypical neurofibroma and NF1-derived MPNST samples the down or upregulation of most selected miRNAs could not be firmly established. This may be due to the limited number of samples, together with the highly variable expression of the miRNAs examined in the MPNSTs. These results, however, do emphasize the value of paired samples and the need to analyze well-characterized and adequately sized cohorts to account for tumor heterogeneity.

A direct comparison between sporadic MPNST and NF1-derived MPNST revealed that these two tumor types could be completely distinguished on the basis of their miRNA expression profiles. This result contrasts with the findings of Holtkamp *et al.* who reported that sporadic and NF1-derived MPNST could not be distinguished by their mRNA expression patterns [49]. However, this study only examined the expression of 558 genes comparing 6 sporadic MPNSTs with 4 NF1-derived MPNST, due to its limited set-up differences may have been missed.

Functional experiments initially focused on proliferation, migration and invasion, all key elements of carcinogenesis and cancer progression. It was uncovered that the selected miRNAs did not affect proliferation but their overexpression (miR-143, miR-145 and let-7b) or inhibition (miR-135b, miR-889) interfered with migration and invasion although not all cell lines responded in a similar fashion and/or with equal intensity (Figs. 4 and 5; Supplementary Figs. 6 and 7). Recently Watson *et al.* implicated the Wnt/β-catenin signaling pathway to fulfil an essential role in both neurofibromas and MPNST showing that inhibition of Wnt signaling by small molecules reduced viability and induced apoptosis [40]. The precise biological basis of Wnt/ β-catenin signaling activation is only partly known and involves the downregulation of members of the β-catenin destruction complex and the expression of R-spondin 2 potentiating Wnt signaling. When we measured Wnt/β-catenin signaling activity using a β-catenin/TCF reporter system we observed that cell lines do show a different Wnt-pathway activation upon exposure to Wnt ligand with high activity in ST88-14 and 90-8TL and low activity in sNF96.2. This difference may be caused by the variable expression levels of Wnt pathway genes in different cell lines as reported by Luscan *et al.* [39]. Transient inhibition of both miR-135b and miR-889 using antisense inhibitors reduced the capacity of ST88-14 to induce Wnt signaling upon stimulation. In agreement with this observation is the fact that miR-135b and miR-889 inhibitors impair invasion of ST88-14 cells as Wnt signaling has been shown to be involved in invasion in many cancer cells [50]. No clear effects were seen on proliferation and migration of this cell line upon miR-135b and miR-889 inhibition. It cannot be excluded that the inhibition of Wnt signaling by interfering with miR-135b and miR-889 levels is not potent enough to affect these processes.

An intriguing question is what causes the aberrant expression of miRNAs as seen in MPNST. It was recently reported that in about 60% of NF1-derived MPNST the PRC2 complex is inactivated [44-46]. The PRC2 complex is a well-known epigenetic modulator of gene expression

[51] by establishing di-and trimethylation of histon H3 lysine 27 (HeK27me2 and HeK27me3) both critical epigenetic silencing marks. Inactivation of PRC2 leads to aberrant gene expression due to the loss of these silencing marks. A list of genes differentially expressed in MPNSTs with loss of PRC2 and those with wild-type PRC2 is presented by Lee *et al.* [45]. Interestingly the expression of *LEMD1*, the gene that harbors miR-135b in one of its introns, is also induced upon PRC2 inactivation. This may explain the clearly increased levels of miR-135b observed in at least some of the MPNST samples (Fig. 1, Supplementary Tables 4 and 5) as it is co-expressed with its host gene *LEMD1*. Of note, the increased miR-135b levels may enhance Wnt signaling activity in the MPNST cells. Likewise, PRC2 inactivation may affect the miRNA cluster on chromosome 14 that contains four of the miRNAs, including miR-889, that were found upregulated in MPNST. However, PRC2 inactivation does not readily explain the downregulation of miRNAs observed in MPNST. Interesting in this respect are findings of de Raedt *et al.* who demonstrated that inactivation of PRC2 boosts the Ras signaling pathway which is already activated by the NF1 loss in these tumors [44]. Ras activation has been reported to downregulate the expression of miR143/145 cluster [31] thereby explaining their relatively low levels in MPNST.

Our findings indicate that miRNAs operate in a cell line specific manner as different NF1-associated MPNST cell lines respond to miRNA modulation with different intensities or in a different fashion. It could be that the cell lines that comprise our MPNST cell line panel differ at a molecular level, perhaps reflecting different chromosomal copy number alterations as commonly observed in MPNST samples [52]. This may cause the cell lines to respond differently to miRNA regulation. This is a highly relevant issue which is often overlooked, as usually only a limited number of cell lines is used in *in vitro* experiments to functionally characterize miRNAs. We have studied the cellular effects by modulating the levels of individual miRNAs transiently. It could very well be that miRNAs display additive, or even synergistic effects and give rise to more pronounced cellular phenotypes when their levels are modulated simultaneously.

# Conclusions

From our study we conclude that at least some miRNAs play essential regulatory roles in MPNST facilitating tumor progression. These, and other miRNAs that are aberrantly expressed in MPNST, may be exploited as biomarker, with miRNA presence and/or levels being measured in suspect plexiform neurofibroma biopsies or in the circulation where they may signal the presence of MPNST. These avenues should be explored and can be particularly valuable in the context of neurofibromatosis type 1, with patients having a 10 – 13% life time risk of developing MPNST. Finally, as miRNAs are powerful regulatory biomolecules their therapeutic potential should be investigated in the context of MPNST in addition to the exact biochemical pathways and genes they regulate. These investigations may identify novel drug targets and lead to more effective therapeutic strategies.

# Supplementary information

Supplementary data are provided online.

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# Chapter 4

# Non-coding RNAs, a novel paradigm for the management of gastrointestinal stromal tumors

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# Abstract

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal malignancies found in the gastrointestinal tract. At a molecular level, most GISTs are characterized by gain-of-function mutations in V-Kit Hardy-Zuckerman 4 Feline Sarcoma Viral Oncogene Homolog (*KIT*) and Platelet Derived Growth Factor Receptor Alpha (*PDGFRA*) leading to constitutive activated signaling through these receptor tyrosine kinases, which drive GIST pathogenesis. In addition to surgery, treatment with the tyrosine kinase inhibitor imatinib forms the mainstay of GIST treatment particularly in the advanced setting. Nevertheless, the majority of GISTs develop imatinib resistance. Biomarkers that indicate metastasis, drug resistance and disease progression early on could be of great clinical value. Likewise, novel treatment strategies that overcome resistance mechanisms are equally needed. Non-coding RNAs, particularly microRNAs, can be employed as diagnostic, prognostic or predictive biomarker and have therapeutic potential. Here we review which non-coding RNAs are deregulated in GISTs, whether they can be linked to specific clinicopathological features and discuss how they can be used to improve the clinical management of GIST.

**Keywords:** microRNA; long non-coding RNAs; GIST; biomarker; therapy.

# Gastrointestinal stromal tumors: a brief introduction

Gastrointestinal stromal tumors (GISTs) are rare tumors of mesenchymal origin from the gastrointestinal (GI) tract with an estimated annual incidence of 10-20 per 1.000.000 in the population [1-3]. They can be found anywhere along the GI-tract, but occur most commonly in the stomach (60-70%) and small intestine (20-30%) [4]. GISTs are believed to originate from the interstitial cells of Cajal (ICC) or their precursor cells [5, 6]. In the GI-tract ICC operate as pacemaker cells responsible for peristaltic movement. That GIST originate from ICC is exemplified by shared immune-phenotypical features such as the expression of KIT (CD117) [5, 6], anoctamin 1 (ANO1 / DOG1) [7] and ETV1 [8] that are currently used a diagnostic biomarkers for GIST. Activating mutations in KIT or PDGFRA were identified as oncogenic drivers in GIST [9-11] (Figure 1A). The gain-of-function mutations in these receptor tyrosine kinases are mutually exclusive and cause constitutive kinase activity in the absence of growth factor binding. Activated KIT and PDGFRA signaling stimulate downstream pathways such as the RAS-RAF-MAPK, PI3K-AKT and JAK/STAT pathways inhibiting apoptosis and promoting cellular survival and proliferation [12] (Figure 1B).

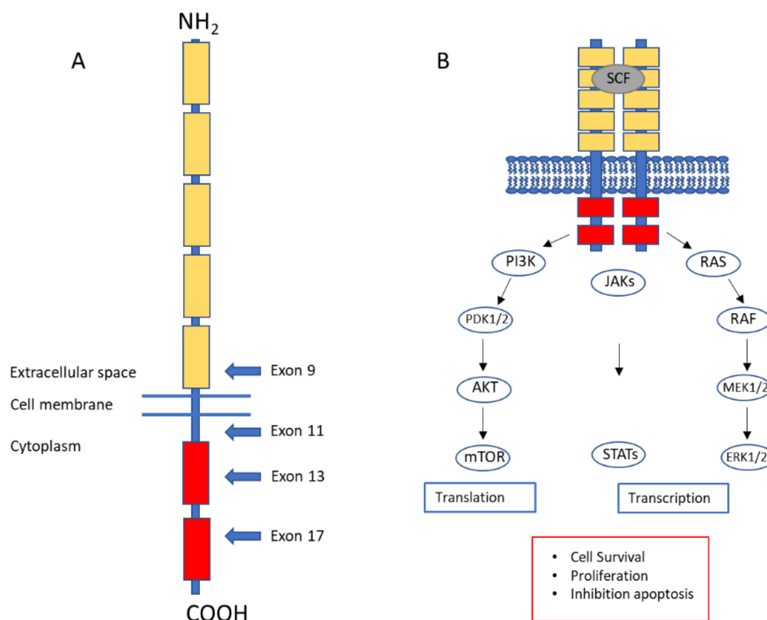


**Figure 1. KIT receptor structure and KIT signaling.** (A). The KIT proto-oncogene codes for a 145 kDa transmembrane receptor tyrosine kinase KIT (CD117). KIT, together with PDGFRA, belongs to the type III tyrosine kinase receptor family and consists of 5 extracellular immunoglobulin (Ig)-like domains involved in KIT ligand (Stem Cell Factor, SCF) binding, a transmembrane domain, a juxtamembrane region and an intracellular kinase domain. Mutations in GIST occur in exons that encode functional domains (arrows). (B). Constitutive KIT signaling as observed in GIST is transduced through the PI3K/AKT/mTOR, RAS-RAF-MAPK and JAK/STAT pathways thereby inhibiting apoptosis, promoting cell survival and proliferation.

Approximately 80% of GISTs contain a mutation in KIT at specific locations in exon 11 (90%), exon 9 (8%) or - less often - in exon 13 (1%) or exon 17 (1%). KIT exon 11 encodes the juxtamembrane region, and mutations in this protein domain impair the autoinhibitory activity of the receptor. The mutations detected in exon 9 are supposed to imitate the conformational changes following ligand binding leading to receptor dimerization and activated signaling. Mutations in exon 13 act on the ATP-binding region of KIT while mutations in exon 17, which codes for the activation loop of the kinase, stabilize the receptor in its active conformation. PDGFRA mutations occur in 10-15% of GISTs, most commonly in exons 12, 14 or 18. The specific mutations in KIT and PDGFRA, with the exception of PDGFRA D842V, make GISTs amenable to treatment with the tyrosine kinase inhibitor imatinib. This drug selectively inhibits the kinase activity of KIT and PDGFR through competitive binding at the ATP binding site of these enzymes [13-15]. In a minority of GIST cases (5-10%) no mutations in KIT or PDGFRA can be detected. In these so-called wild-type GISTs (WT-GIST), other mutated genes like NF1, BRAF and succinate dehydrogenase subunits (SDHB, SDHC, SDHD) can drive tumorigenesis [16-20].

# Current treatment of gastrointestinal stromal tumors

Surgical excision is the preferred treatment modality for localized GIST aiming for resection margins devoid of tumor cells [21]. Prior to surgery, imatinib may be administered if complete resection is difficult without downsizing the tumor. If routine risk assessment, which is usually based on parameters like mitotic rate, tumor size and tumor location, indicates a significant chance of relapse after surgery adjuvant imatinib may be prescribed for up to 3 years [22]. The efficacy of imatinib treatment may vary and is partly dependent on the *KIT* or *PDGFRA* mutational status of the tumor [23]. For example, GISTs that harbor *KIT* exon 11 mutations generally respond well to imatinib [24, 25] whereas patients with an exon 9 *KIT* mutation frequently need an increased daily dose of 800 mg/day instead of the regular 400 mg/day to exhibit a treatment response [26]. Furthermore, PDGFRA D842V mutants are resistant to imatinib [27, 28], just like WT-GISTs and GISTs with mutations in genes other than *KIT* and *PDGFRA* that display insensitivity to imatinib and other tyrosine kinase inhibitors [29].

Imatinib is listed as first-line treatment for locally advanced, unresectable and metastatic GIST. In this context imatinib is usually prescribed indefinitely as treatment pausing generally leads to tumor progression [21]. Unfortunately, the vast majority of GIST patients treated with imatinib eventually presents with tumor progression due to the development of drug resistance [23]. The precise molecular changes and mechanisms underlying imatinib resistance are not completely clear. In about half of the patients, secondary mutations arise in KIT, normally in exon 13, 14, 17 or 18 that cause resistance [30-32]. In the remaining half of resistant patients other, less defined, resistance mechanisms are operational [30, 33-37]. Standard second-line treatment is currently sunitinib [38, 39] with regorafenib as third line option [40].

Other tyrosine kinase inhibitors are under development of which avapritinib and ripretinib are the most promising and tested in phase III studies [41, 42]. These drugs were shown to have inhibitory activity in advanced GISTs resistant to approved treatments and in GISTs with a PDGFRA D842V mutation.

# Clinical needs regarding the management of gastrointestinal stromal tumors

GIST is routinely diagnosed on specific morphological features, immunostaining for KIT and ANO1 (DOG1) and the presence of *KIT* or *PDGFRA* mutations. A risk assessment is being made by the pathologist based on the mitotic count observed in a tumor biopsy, the tumor size and tumor location. Additional biomarkers that can be quantitatively measured in a standardized fashion, may be very useful to further fine-tune the grading procedure. Additionally, one can think of biomarkers that highlight metastasis and can be determined in the patient's tumor and/or circulation. Although effective treatments exist for GIST, most notably imatinib, almost all patient ultimately develop resistance. Biomarkers that indicate the development of resistance may not only provide insight into the specific mechanisms of resistance, leading to the development of novel strategies to overcome resistance, but also enable the clinician to adjust treatment before overt progression occurs. Last but not least, novel therapeutic approaches are needed that target the oncogenic pathways in GIST differently than the tyrosine kinase inhibitors, give rise to lasting responses while circumventing resistance.

# *Non-coding RNAs*

Novel classes of RNA transcripts, including microRNAs and long non-coding RNAs, have recently been discovered in eukaryotic cells. Their tissue specific expression, role in gene regulation and their intricate, often essential, involvement with normal- and pathological physiology makes them particularly suitable as biomarker and endows them with therapeutic potential.

The sequencing of the human genome initially indicated the presence of approximately 30.000 protein coding genes [43, 44], a number that over the years was adjusted to about 20.500 protein coding genes [45]. GENCODE (www.gencodegenes.org) lists in its most recent version (release 34) 19.959 protein coding genes. This number of genes is comparable to that found in other – quite often less complex – organisms [46] implicating that organismal complexity is not determined by protein coding gene numbers alone. In fact, the protein coding genes constitute only 1.5% of the human genome but, intriguingly, about two-third of the genome is transcribed into RNA [47-49]. This vast transcriptional output cannot be all considered as transcriptional noise as that would be an utter waste of cellular energy. Based on

these facts it is proposed that organismal complexity is driven by the expansion of the regulatory potential of the non-coding portion of the genome [50]. There is growing evidence that non-coding transcripts exercise diverse biological functions that are still ill-defined or, more often, not yet assigned in most cases. Several classes of RNA transcripts have been recognized and a start has been made to functionally annotate these biomolecules. This review will focus on the rather well-defined subset of microRNAs (miRNAs), small regulatory RNAs of 19-26 nucleotides, and briefly touch upon long non coding RNAs (lncRNAs) and its subclass circular RNAs (circRNAs) in the context of GIST. MiRNAs were first described in the mid-nineties of the last century in the nematode Caenorhabditis elegans [51]. Initially miRNAs were considered a peculiarity of these worms until was realized that many miRNAs are evolutionarily conserved suggesting a functional relevance for miRNAs[52, 53]. Currently, there are 2654 mature human miRNAs listed in miRBase (version 22.1; http://www.mirbase.org/) and it is well established that miRNAs play pivotal roles by regulating many fundamental developmental and cellular processes [54]. Although exceptions have been reported [55, 56] miRNAs most commonly operate by binding in the context of the RNA induced silencing complex (RISC) to the 3' untranslated region (3' UTR) of target mRNAs. The miRNA-mRNA interaction in the context of RISC causes translation inhibition and/or mRNA degradation. In this way miRNAs are capable of regulating gene expression. Interestingly, any given miRNA may target multiple mRNAs and conversely a single mRNA can be targeted by multiple miRNAs. In this way a refined regulatory network is created which itself again can be modulated in various ways and at different levels. It is estimated that two third of all genes are under regulation by miRNAs [57, 58] by inference it is safe to state that miRNAs are small riboregulators involved in almost all – if not all – biochemical and cellular processes. Just as miRNAs are intimately related to normal cellular, tissue and organismal physiology they also play essential roles in diseases including cancer [59-61].

A common feature of cancer is the dysregulation of miRNA expression caused by the genomic alterations, amplification and deletions, that are frequently encountered [62]. Alternatively, epigenetic mechanisms may underlie the aberrant expression of miRNAs. It is well established that miRNAs can carry out essential oncogenic and tumor suppressive roles in the tumorigenic process. Additionally, miRNAs are also known to play a driving role metastasis [63] and drug resistance [64] thereby affecting the outcome of drug treatment. The close involvement of miRNAs with many biological and clinical aspects of cancer, their tissue-specific expression and quantitative detection methods defines miRNAs as suitable biomarkers. Advantageous in this respect is that miRNAs are stable present in many tissues and body fluids such as urine, saliva and blood [65, 66]. Driven by academic progress that highlights the key roles miRNAs play in all kinds of disorders, the pharmaceutical industry and biotech developed an interest in miRNA-based therapeutics. Despite significant initial technical challenges related to safety, stability and delivery numerous clinical trials are ongoing [67, 68].

Recently other classes of RNA transcripts such as long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) gained a lot of attention. LncRNAs are broadly defined as transcripts > 200 nucleotides in length that are transcribed from independent pol II promoters and not translated into protein. LncRNAs comprise a rather heterogeneous class of transcripts

that includes intergenic and intronic transcripts, enhancer RNAs, pseudogenes, circular RNAs (circRNAs) and sense and antisense transcripts that overlap with other genes. Currently GENCODE v34 annotates 48.479 lncRNA transcripts from 17.960 lncRNA genes. LncRNA genes can consist of multiple exons, that upon transcription are subjected to regular splicing resulting in transcripts that contain a 5'CAP structure and 3'poly (A) tail. The majority of lncRNAs are not highly conserved between species and many lncRNAs display a lineage and/or cancer specific expression [69]. LncRNAs are found capable of regulating gene expression by diverse mechanisms operating at epigenetic, transcriptional or post-transcriptional levels [68, 70-73]. They either function in *cis*, mediating effects nearby, or in *trans* at distant genomic or cellular locations. LncRNA have been reported to direct chromatin modifying complexes to specific gene promoters, to bind transcription factors or RNA binding proteins, often involved in creating scaffolds facilitating interactions between different biomolecules. They are also known to bind directly to DNA or function as competitive endogeneous RNA (ceRNA) acting as miRNA sponges. Some lncRNAs have been functionally characterized as essential actors in tumorigenesis and tumor maintenance either in oncogenic or tumor suppressive roles [68, 74, 75]. However, the relevance and precise functions of the vast majority of lncRNAs and their integration in normal or diseased states remains to be elucidated.

Although the presence of circRNAs was already reported a few decades ago, a publication by Salzman *et al*. in 2012 renewed the interest in these transcripts by emphasizing their abundance and variety of in mammalian cells [76]. CircRNAs are single-stranded, covalently closed circular RNA molecules produced by precursor mRNA back-splicing of exons in which a downstream 5'splice site is linked with an upstream 3'splice site [77]. The process of back-splicing is facilitated the canonical spliceosomal machinery and regulated by complementary sequences in introns flanking the circularized exons and RNA binding proteins [78]. It appears circRNAs are found throughout the eukaryotic kingdom and are usually expressed in lineage specific patterns. Their circular nature endows them with increased stability providing a distinct advantage for use as biomarker. Initially considered the results of aberrant splicing it is now recognized that at least some circRNAs fulfil important biological functions [78]. However, so far only few circRNAs have been functionally characterized, a process that is hampered by technical hurdles as circRNAs resemble their linear counterparts [78]. CircRNAs have been implicated in carcinogenesis. Using an exome capture RNA sequencing protocol a comprehensive catalogue (MiOncoCirc) was generated of circRNAs detected in more than 2000 cancer samples derived from >40 cancer sites and included primary and metastatic tumors as well as rare tumor types [79]. MiOncoCirc lists >125.000 species of cancer-related circRNAs. In general, it is believed that circRNAs can function as ceRNAs capable of sequestering miRNAs and/or RNA binding proteins [68]. Future research will shed more light on the functional significance of circRNAs in physiological and pathological circumstances and see a further development of their potential as biomarker.

# Dysregulated miRNAs in GIST

Several research groups examined which miRNAs are aberrantly expressed in GIST as a first step in identifying miRNAs essential for the tumorigenesis, maintenance and progression of GIST (see Table 1 for an overview). Subramanian *et al.* discovered, analyzing the miRNA expression profiles in various sarcomas, that each sarcoma subtype, including GIST, was characterized by its own unique miRNA expression signature [80]. In addition to *KIT* or *PDGFRA* mutations, GIST displays characteristic genomic alterations most notably a loss of the long arm of chromosome 14 [81] and deletions of chromosome 1p and 22q [82, 83]. Loss of chromosome 14q is seen in approximately 70% of GISTs. Interestingly, Choi *et al.* reported on the existence of miRNA expression patterns linked to 14q loss. Many miRNAs that are actually located on chromosome 14q appear downregulated [84]. Haller *et al.* described localization and mutation dependent miRNA expressions patterns in GIST focusing on miR-132, miR-221, miR-222 and miR-504 [85]. Particular attention, by several research groups, has been given to miR-221/222 as these miRNAs were reported to regulate KIT receptor expression [86]. It was shown that miR-221/222 were downregulated in GIST and correlated to KIT expression [87] and also in GIST cells target *KIT* [88, 89]. Enhanced expression of miR-222 in GIST cells by miRNA mimics inhibited cell proliferation, affected cell cycle progression and induced apoptosis [88]. Also, miR-218 and miR-375-3p were mentioned to regulate *KIT* as well as miR-494, a miRNA associated with 14q loss [90-92]. Transient modulation of miR-494 in the GIST882 cell line led to inverse responses in KIT protein levels. Moreover, miR-494 overexpression provoked apoptosis, impaired cellular proliferation and affected the cell cycle. Interestingly, in a subsequent paper the research group reported that miR-494 also targets survivin (*BIRC5*) [93]. These findings led the authors to propose that miR-494 synergistically suppresses GIST when expressed by targeting both survivin and *KIT*. These *KIT* targeting miRNAs as well as the *ETV1* targeting miR-17 and miR-20a [88] may be of therapeutic value particularly in drug resistant disease in which GISTs still rely on KIT signaling. Yamamoto *et al.* noted that miR-133b was among downregulated miRNAs in high-grade GIST compared to intermediate and low-grade GISTs and further demonstrated that fascin-1 (*FSCN1*) expression was regulated by miR-133b [94]. It was subsequently shown that overexpression of FSCN1 correlated to shorter disease-free survival time and aggressive pathological factors. Tong *et al.* reported miRNA expression profiles that distinguish between malignant and more benign GISTs and between malignant and borderline GISTs [95]. Comparing GISTs with leiomyomas Fujita *et al.* described the upregulation of miR-140 in the GIST samples [96] but do not indicate potential mRNA targets. The epigenetic silencing of miRNAs in GIST was investigated by Isosaka *et al.* [97]. An *in* vitro screen using the cell line GIST-T1 revealed at least 21 miRNAs whose expression was associated with the methylation of an upstream CpG-island. MiR-34a and miR-335, miRNAs found silenced in GIST were further functionally characterized and were shown to suppress cellular proliferation of GIST-T1 cells when overexpressed. In addition, miR-34a, but not miR-335, affected migratory and invasive processes and was demonstrated to regulate PDGFRA. Using novel high-throughput sequencing methods Gyvyte *et al.* uncovered and validated miRNAs deregulated in GIST in comparison to adjacent normal tissue [98]. It was found that miR-215-5p levels were negatively correlated with the risk-grade of GIST and that miR-509-3p is upregulated in epitheloid and mixed cell type GIST compared to the spindle type. In a subsequent study the

same group focused on miR-200b-3p and miR-375-3p, both found reduced in GIST compared to normal adjacent tissue [91]. These miRNAs negatively affected cell viability and cellular migratory capability when overexpressed in GIST-T1 cells. MiR-200b-3p was demonstrated to directly target *EGFR* and indirectly affected ETV1 protein levels whereas miR-375-3p targeted *KIT*. A cell line study by Lu *et al*. revealed that miR-152 is downregulated in GIST cells, its overexpression inhibited tumor cell proliferation and induced apoptosis [99]. Interestingly, the miR-152 phenotype is mediated through the regulation of cathepsin L (*CTSL*). In search of new miRNA-based treatments for GIST Long *et al*. identified the overexpression of miR-374b in GIST and provided evidence that this miRNA targets the tumor suppressor *PTEN* [100]. It is suggested that miR-374b enhances survival, migration and invasion and inhibits apoptosis by stimulating the PI3K/AKT signaling pathway through downregulation of PTEN. The authors tentatively conclude that inhibition of miR-374b constitutes a novel therapeutic strategy for GIST. A different group highlighted that miR-4510 downregulation, as normally is observed in GIST cells, promotes GIST progression including tumor growth, invasion and metastasis through the increase of apolipoprotein C-II (*APOC2*) shown to be a miR-4510 target [101].

Some studies investigated both mutant GISTs and WT GISTs describing differentially expressed miRNAs between these GIST subtypes [102, 103]. Bioinformatic analyses led Pantaleo *et al*. to propose the existence of mRNA/miRNA regulatory networks that may be therapeutically targeted in WT GIST [103]. Bachet and co-workers examined miRNA expression profiles in murine NIH3T3 cells expressing either human wild-type *KIT*, hemizygous *KIT* mutants del 557-558 (D6) or del 564-581 (D54), heterozygous *KIT* mutants wild-type/D6 or wild-type/D54 and, for validation purposes, in human GIST samples [104]. Importantly, the authors concluded that miRNA, as well as mRNA, expression profiles depend on the homozygous/heterozygous/hemizygous status of the *KIT* mutations and the deletion/presence of TYR568 and TYR570 residues. These results appear to suggest different oncogenic pathways are activated and should be further validated using well-characterized GIST samples.

The various screens comparing tumor tissue with adjacent non-cancerous tissue usually indicate many miRNAs that are deregulated in GIST. However, it is often not clear which of the listed miRNAs fulfil a key oncogenic or tumor suppressive role in cancer-related processes and which miRNAs are not. In-depth, and often time-consuming, functional studies are necessary that first should establish which miRNAs affect cancer-related processes when modulated. Ideally these experiments should be performed both in vitro using well-characterized cell lines and in relevant in vivo models. Once a miRNA is singled out in this way its target genes and pathways should be identified in the GIST context. To this end bioinformatics may be used as well as unbiased biochemical approaches e.g. PAR-CLIP [105]. Once a miRNA target has been defined and validated it is important to verify that modulation of the target(s) e.g. by RNAi and/or overexpression experiments phenocopies the miRNA related cellular phenotype. The findings should be linked to the situation seen in the clinic so some sort of validation using clinical samples is needed to corroborate the clinical relevance. In this respect much work still needs to be done.

**Table 1.** Dysregulated miRNAs in gastrointestinal stromal tumors.

| miRNAs up/downregulated in GIST[a,c] | Comparison / number of samples | Platform | Validated miRNAs; Targets and/or pathways; Association with clinicopathological parameters | Ref. |
|---|---|---|---|---|
| *Upregulated*: Let-7b; miR-10a; miR-22; miR-29a; **miR-29b**; **miR-29c**; **miR-30a-5p**; miR-30c; miR-30d; miR-30e-5p; miR-99b; miR-125a; miR-140*; miR-143; miR-145<br><br>*Downregulated*: miR-1; miR-92; **miR-133a**; **miR-133b**; **miR-200b**; **miR-221**; **miR-222**; **miR-368**; **miR-376a** | Snap-frozen tumor and tissue samples Primary GIST (n=8) vs SS (n=7); LMS (n=6); DDLPS (n=1); RMS (n=6); NSM (n=5); skeletal muscle (=2) | Microarray Sequencing | | [80] |
| *Downregulated*: miR-127; miR-134; miR-136; miR-154; miR-154*; miR-299-5p; miR-299-3p; **miR-323**; miR-329; miR-342; **miR-368**; miR-369-5p; miR-369-3p; **miR-376a**; **miR-376a***; miR-376b; miR-377; miR-379; miR-381; **miR-382**; **miR-409-3p**; **miR-409-5p**; miR-410; miR-411; miR-431; miR-432*; miR-433; miR-485-3p; miR-487a; miR-487b; miR-493-3p; miR-493-5p; **miR-494**; miR-495; miR-539; **miR-625**; miR-654; miR-758 | Snap-frozen tumor samples Primary GIST (n=20) comparing 14q loss (n=14) vs 14q presence (n=6) | Microarray | • Association with 14q loss | [84] |
| *N.A.* | Snap frozen tumor tissue Discovery: Primary GIST (n=12) Validation: Primary GIST (n=49) | Microarray RT-PCR | miR-132; miR-221; miR-222; miR-504<br>• High miR-132 expression level associated with gastric PDGFRA-mutated GIST cf. gastric KIT-mutated GIST<br>• High miR-221 and miR-222 expression levels associated with wild-type GIST cf. GIST with KIT or PDGFRA mutation | [85] |

| | | | | |
|---|---|---|---|---|
| | | | • High miR-504 expression associated with gastric GIST with KIT mutation cf. intestinal GIST with KIT mutation | |
| <u>*Downregulated*</u>: **miR-221**; **miR-222** | FFPE samples Primary GIST and adjacent normal tissue (n=54 pairs) | RT-PCR | • Association with KIT positivity | [87] |
| <u>*Downregulated*</u>: **miR-494** | Snap-frozen tumor samples Primary GIST (n=31) | | miR-494; *KIT*<br>• Association with 14 q loss | [84, 92] |
| <u>*Upregulated*</u>: **miR-29c**; **miR-30a**; miR-330-3p; miR-497; miR-603<br><br><u>*Downregulated*</u>: miR-21; **miR-221**; **miR-222**; **miR-382**; miR-938 | Snap-frozen tumor samples Primary GIST (n=50) vs intestinal LMS (n=10) | Microarray RT-PCR | miR-17, miR-20a; *ETV1*<br>miR-222; *KIT* | [88] |
| See publication | FFPE samples Adult KIT/PDGFRA mutant GIST (n=30) vs adult WT GIST (n=25) vs pediatric WT GIST (n=18) | RT-PCR | • Distinct miRNA signatures for GIST subtypes correlating with clinicopathological parameters. | [102] |
| <u>*Upregulated*</u>: miR-330-3p; miR-455-5p; **miR-455-3p**; miR-886-3p<br><br><u>*Downregulated*</u>: miR-129-1-3p; miR-129-5p; miR-214-5p; miR-424; miR-450a; miR-491-5p | Snap-frozen tumor samples Discovery: KIT/PDGFRA mutant GIST(n=9) vs WT GIST (n=4) Validation: Mutant GIST (n=13) vs WT GIST (n=3) | Microarray RT-PCR | miR-139-5p; miR-148a; miR-193-3p; miR-330-3p; miR-455-5p; miR-129-1-3p; miR-129-2-3p; miR-876-5p<br>• miR-139-5p and miR-455-5p predicted to target *IGF1R*<br>• miR-139-5p predicted to target *CDK6*<br>• miR-330-3p predicted to target *CD44* | [103] |
| <u>*Downregulated*</u>: **miR-133b** | Snap-frozen tumor samples Primary GIST (n=19) comparing high grade vs intermediate and low grade | Microarray RT-PCR | miR-133b; inverse correlation between fascin-1 and miR-133b<br>• Downregulated in high-grade GIST | [94] |
| <u>*Downregulated*</u>: miR-218 | Snap-frozen tumor and tissue samples, primary GIST (n=10), normal adjacent tissue (n=5) | RT-PCR | miR-218; *KIT* | [90] |

| | | | | |
|---|---|---|---|---|
| *Upregulated*: miR-140-3p; miR-483-5p; miR-3151-5p<br><br>*Downregulated*: miR-28-3p; **miR-133a-3p**; **miR-133b**; miR-195-5p; miR-378f; miR-3135b; miR-4535 | Fresh tumor samples Primary GIST (n=9) vs leiomyomas (n=7) | Microarray RT-PCR | miR-140-5p, miR-140-3p | [96] |
| *Downregulated*: **miR-221; miR-222** | FFPE samples Primary GIST (n=24) vs smooth muscle (n=6) | RT-PCR | miR-221/222; *KIT* | [89] |
| *Downregulated*: miR-9-3p; miR-34a; **miR-152**; miR-155; miR-203; miR-335; **miR-375**; miR-489; miR-582; miR-615; miR-618 | GIST-T1 (n=1), snap-frozen primary GIST samples (n=39), FFPE primary GIST samples (n=98) | RT-PCR | miR-34a, *PDGFRA*<br>miR-335<br>• Association with CpG island methylation | [97] |
| *Upregulated*: miR-34c-5p; miR-4773<br><br>*Downregulated*: Let-7c; miR-218; miR-488*; miR-4683 | Snap-frozen tumor samples Primary GIST (n=53): malignant GIST (n=30) vs benign GIST (n=9)[b] | RT-PCR | • Association with malignant GISTs | [95] |
| *Upregulated*: **miR-196a**<br><br>*Downregulated*: Let-7c; **miR-29b-2***; **miR-29c***; miR-204; miR-204-3p; miR-218; **miR-625**; miR628-5p; miR-744; miR-891b | Snap-frozen tumor samples Primary GIST (n=53): malignant GIST (n=30) vs borderline GIST (n=14)[b] | RT-PCR | • Association with malignant GISTs | [95] |
| *Upregulated*: **miR-455-3p**; miR-483-5p; miR-509-3p; miR-675-3p<br><br>*Downregulated*: miR-141-3p; **miR-133a-3p**; **miR-133b**; miR-182-5p; miR-192-5p; miR-200a-3p; **miR-200b-3p**; miR-200c-3p; miR-203a-3p; miR-215-5p; **miR-375**; miR-429; miR-451a; miR-486-5p; miR-490-3p | FFPE tumor and tissue samples Discovery: Pairs (n=15) primary GIST and adjacent tissue Validation: Pairs (n=40) primary GIST and adjacent tissue | RNA-seq RT-PCR | All listed miRNAs validated<br>• miR-215-5p expression levels are negatively correlated to risk grade<br>• miR-509-3p expression levels associated with histological subtype | [98] |
| *Downregulated*: **miR-152** | Cell lines GIST48; GIST430; GIST882; GIST-T1 | RT-PCR | miR-152; *CTSL* | [99] |

| | | | | |
|---|---|---|---|---|
| *Upregulated*: miR-374b | FFPE samples Pairs (n=143) of Primary GIST and adjacent tissue | RT-PCR | miR-374b; *PTEN*<br>• Association of miR-374b levels with tumor diameter and pathological state | [100] |
| *Downregulated*: **miR-494** | Snap-frozen tumor samples Primary GIST (n=35) | Microarray | miR-494; *BIRC5* | [93] |
| *Upregulated*: **miR-29b-1-5p**<br><br>*Downregulated*: miR-134-5p; **miR-323b-3p**; **miR-382-5p**; **miR-409-3p**; miR-1185-1-3p; miR-3187-3p; miR-4510 | Discovery: Pairs (n=6) primary GIST and adjacent tissue Validation: Pairs (n=64) primary GIST and adjacent tissue | RNA-seq RT-PCR | miR-4510; *APOC2*<br>• Association of miR-4510 levels with tumor location, tumor size, mitotic index and risk classification. | [101] |
| *Downregulated*: **miR-200b-3p**; **miR-375-3p** | FFPE tumor and tissue samples Discovery: Pairs (n=15) primary GIST and adjacent tissue Validation: Pairs (n=40) primary GIST and adjacent tissue | RNA-seq RT-PCR | miR-200b-3p; *EGFR*<br>miR-375-3p; *KIT* | [91, 98] |

[a] In case multiple miRNAs have been detected only the 10 most significant differentially expressed miRNAs are listed / or miRNAs with the highest fold-change /or miRNAs of which de deregulation is validated.
b Classification into benign, borderline and malignant GIST according to [106, 107]
c The miRNAs listed in bold were detected in two of more independent studies.
Abbreviations: FFPE, formalin-fixed paraffin-embedded; SS, synovial sarcoma; LMS, leiomyosarcoma; DDLPS, dedifferentiated liposarcoma; RMS, rhabdomyosarcoma; NSM, normal smooth muscle.

# MiRNAs associated with Gastrointestinal Stromal Tumor metastasis

When GIST metastasizes, treatment becomes more difficult as complete surgical resection is not an option anymore. Biomarkers that indicate whether metastasis is about to occur or has already occurred are therefore useful. A limited number and/or small metastatic lesions may be more susceptible to systemic treatment. Several researchers identified miRNAs present in tumors of which the expression levels are associated with metastasis (Table 2). At least 27 miRNAs were found downregulated in high-risk GISTs when 10 high-risk GISTs were compared to 4 low-risk tumors [84]. Niinuma *et al.* identified miR-196a as being positively correlated with high-risk grade GIST but also with poor clinical outcome, tumor size, mitotic count and metastasis [108]. MiR-196a is known to be expressed from the *HOX* gene clusters in mammals. Intriguingly, *HOXC* and the lncRNA HOTAIR were coordinately expressed with

miR-196a. MiR-196a inhibition, however, did not affect HOTAIR levels and conversely knockdown of HOTAIR had no effect on miR-196a levels, the authors suggest an epigenetic mechanism underlies the linked expression. In a later paper the same group demonstrated that downregulation of miR-186 was observed in tumors that exhibit metastatic recurrence. Analysis of a large validation cohort of 100 primary GISTs uncovered that miR-186 expression is correlated to metastatic recurrence and poor prognosis. It was further shown that inhibition of miR-186 in a GIST cell line promoted cell migration, most likely by upregulation of multiple genes implicated in cancer metastasis [109]. Akçakaya *et al*. identified 44 miRNAs that could distinguish between metastatic and non-metastatic tumors with 19 overexpressed and 25 underexpressed in metastatic GISTs [110]. Unfortunately, none of these miRNAs were further functionally characterized. MiR-137, a miRNA found downregulated in GIST, was reported to modulate epithelial-mesenchymal transition (EMT) in GIST. Follow-up experiments involving GIST cell lines indicated that miR-137 expression enhanced epithelial cell morphology, possibly by reducing TWIST1 levels. Increased miR-137 levels led to reduced cell migration, activated a G1 cell cycle arrest and induced apoptosis [111]. Similarly, Ding *et al*. revealed that miR-30c-1-3p, miR-200b-3p and miR-363-3p may modulate EMT and hence invasiveness and consequently metastasis by regulation of *SNAI2*, a member of the snail $C_2H_2$-type zinc finger transcription factor family [112].

**Table 2.** MicroRNAs associated with metastasis in gastrointestinal stromal tumors.

| miRNAs[a,b] | Up/down regulation | Functional role | Ref. |
|---|---|---|---|
| miR-146b; **miR-150**; miR-132; miR-342; miR-16; miR-500; miR-212; miR-335; miR-21; miR-199a | Downregulation in high-risk GIST | | [84] |
| **miR-196a** | Upregulation in high-risk GIST | | [108] |
| miR-137 | Downregulation in GIST vs normal adjacent tissue | Regulation of EMT through targeting *TWIST1* | [111] |
| miR-30c-1-3p; miR-200b-3p; miR-363-3p | Downregulation in SNAI2 high GISTs | Regulation of invasion and migration through targeting *SNAI2* | [112] |
| miR-186 | Downregulation in primary GISTs that exhibit metastatic recurrence | miR-186 is linked to migration and genes implicated in metastasis | [109] |
| miR-301a-3p;<br>**miR-150-3p**; miR-1207-5p; miR-1915 | Upregulation in metastatic GIST<br>Downregulation in metastatic GIST | | [110] |

[a] In case >10 miRNAs were identified only the 10 miRNAs with the most significant expression or highest fold-changes are listed.
[b] The miRNAs listed in bold were detected in two of more independent studies.

# MiRNAs related to imatinib resistance

Imatinib has been a truly groundbreaking drug for the majority of GIST patients prolonging overall survival and quality of life [113]. Unfortunately, most GIST patients eventually become insensitive to imatinib and present with a tumor that is progressing and requiring other treatments. Several groups have investigated whether miRNAs can be linked to imatinib resistance (Table 3). These miRNAs can either be used as biomarker signaling drug resistance and possibly tumor progression or alternatively be exploited to obtain insight into the molecular mechanisms of resistance. Goa *et al*. compared the miRNA expression profiles of primary – imatinib naïve – and imatinib resistant GIST. MiR-320a, downregulated in imatinib-resistant GIST, was found associated with imatinib resistance although its mode of operation is not further investigated [114]. A cell line study by Fan *et al*. described that miR-218 is downregulated in resistant GIST contributing to the phenomenon of resistance by regulating PI3K/AKT signaling [115]. Akçakaya and coworkers, direct attention to the upregulation of miR-107, miR-125a-5p, miR-134, miR-301a-3p and miR-365 in association with imatinib resistance. A single miRNA, miR-125a-5p, is functionally characterized and shown to regulate *PTPN18* and consequently pFAK levels [110, 116]. Zhang et al. performed an *in silico* analyses, using GO function and KEGG pathway enrichment as well as lncRNA-miRNA-target gene regulatory network build, of the microarray datasets deposited by Akçakaya *et al* [110]. These studies highlighted miR-28-5p and – not surprisingly – miR-125a-5p both of which displayed a significant correlation to imatinib resistance and imatinib sensitivity [117]. Also, Shi *et al*. uncovered a series of up- or downregulated miRNAs by comparing imatinib-naïve with imatinib-resistant GIST samples [118]. A single miRNA, miR-518a-5p downregulated in imatinib resistant GIST, was further investigated and demonstrated to bind to the 3'UTR of *PIK3C2A*. It is proposed that the increased PIK3C2A expression affects the cellular response to imatinib and causes resistance. Kou *et al*. examined the miRNA expression profiles of serum samples derived from GIST patients having an imatinib responsive tumor or a tumor that progresses on the drug [119]. Receiver operating characteristics (ROC) curves demonstrated that miR-518e-5p levels could discriminate serum samples of imatinib-resistant GIST patients from imatinib-sensitive ones with high sensitivity (99.8%) and specificity (82.1%). Thirty-five differentially expressed miRNAs were detected comparing primary, imatinib-naïve and imatinib-resistant GISTs [30]. An accompanying mRNA profiling of a smaller subset of the same samples uncovered 352 differentially expressed mRNAs, subsequent pathway and network analyses implicated cell cycle and cell proliferation genes as involved in imatinib resistance.

It is noted that the observed differences in miRNA expression between imatinib sensitive and resistant GIST tumors are relatively small. Nevertheless, even small miRNA differences may still have a significant impact as diverse miRNAs may act synergistically and the regulation of multiple targets within the same pathway may amplify biological effects [120, 121]. Despite a comparable set-up there is little overlap in imatinib-resistance linked miRNAs between the different studies. Of interest in this respect are miR-518a-5p, miR-518e-5p and miR-518d-5p that all derive from a large cluster of miRNAs on chromosome 19q13.42 a chromosomal region that may function in imatinib resistance. However, more extensive research is needed,

investigating the expression of other miRNA cluster members as well as chromosomal alterations that affect chromosome 19q.

**Table 3.** MicroRNAs associated with imatinib resistance in gastrointestinal stromal tumors.

| miRNAs up/downregulated in imatinib-resistant GIST[a] | Comparison / number of samples | Platform | Validated miRNAs; Targets and/or pathways | Ref. |
|---|---|---|---|---|
| Upregulated: miR-15a; miR-16; miR151-5p; miR-195<br><br>Downregulated: miR-140-5p; miR-140-3p; miR-320a; miR-483-5p; miR-574-3p; miR-1280 | *Tumor samples*<br>Discovery: primary GIST (imatinib naïve) (n=3) vs imatinib resistant GIST(n=4)<br>Validation: primary GIST (imatinib naïve) (n=16) vs imatinib resistant GIST(n=12) | Microarray RT-PCR | miR-320a | [114] |
| Downregulated: miR-218 | *Cell lines*<br>GIST882 vs GIST430 | RT-PCR | miR-218; PI3K/AKT signaling | [115] |
| Upregulated: miR-107; miR-125a-5p; miR-134; miR-301a-3p; miR-365 | *Tumor samples*<br>GIST responsive on imatinib (n=9-16) vs GIST progressive on imatinib (n=4-14) | Microarray RT-PCR | miR-125a-5p; *PTPN18* (modulation pFAK levels) | [110, 116] |
| Upregulated: miR-491-3p; miR-1260b; miR-2964a-5p; miR-3907<br><br>Downregulated: miR-221-3p; miR-518a-5p; miR-595; miR-3145-3p; miR-3655; miR-4466 | *Tumor samples*<br>Paired (n=20) primary GIST (imatinib naïve) vs imatinib resistant GIST | Microarray RT-PCR | miR-518a-5p; *PIK3C2A* | [118] |
| Upregulated: miR-518e-5p; miR-548e | *Serum samples*<br>Imatinib sensitive GIST(n=37) vs Imatinib resistant GIST(n=39) | Microarray RT-PCR | miR-518e-5p | [119] |
| Upregulated: miR-28-5p; miR-125a-5p | *Tumor samples*<br>GIST responsive on imatinib vs GIST progressive on imatinib | In silico analyses of microarray data[b] | miR-28-59; miR-125a-5p | [117] |
| Upregulated: miR-92a; miR-118-5p; miR-335; miR-526a/miR-520c- | *Tumor samples*<br>GIST imatinib-naïve (n=33) vs | Microarray RT-PCR | | [30] |

| | | |
|---|---|---|
| 5p/miR-518d-5p; miR-708*<br><br>*Downregulated*: miR-24; miR-186; miR-455-3p; miR-675; miR-1296 | GIST imatinib resistant (n=20) | |

[a] In case multiple miRNAs have been detected only the 10 most significant differentially expressed miRNAs are listed.
[b] miRNA expression data used are from public repository described in Akçakaya et al. (2014) [110]

# Additional non-coding RNAs in Gastrointestinal Stromal Tumors

Recently the association of lncRNAs with GIST and GIST pathological features was investigated (see Table 4 for an overview). In 2012, Niinuma and coworkers observed that the lncRNA HOTAIR expression was associated with high-risk grade GIST, metastasis and poor clinical outcome [108]. RNAi mediated knockdown of HOTAIR was shown to inhibit invasiveness, a surrogate for metastatic potential, of the GIST-T1 cell line. Basically, these findings were confirmed and expanded by others [122, 123]. Lee *et al*. demonstrated that HOTAIR in GIST cells suppressed apoptosis, was associated with cell cycle progression and controlled both invasion and migration [123]. Evidence is presented that HOTAIR through binding of PRC2 complex components, an epigenetic regulator of gene expression [124], affects the expression of distinct proteins, like protocadherin 10 (PCDH10), thereby mediating the HOTAIR phenotype. Bure *et al*. observed that HOTAIR depletion resulted in aberrant DNA methylation patterns through an unknown mechanism, causing either hypo- or hypermethylation patterns that affect gene expression [122]. Hu *et al*. reported a relative high expression of amine oxidase copper containing 4, pseudogene (AOC4P) in high-risk GIST and noted that also the epithelial-mesenchymal transition (EMT) related proteins ZEB1, SNAIL and Vimentin were highly expressed [125]. Knockdown of AOC4P affected the migratory and invasive capabilities of GIST cells, induced apoptosis and reduced EMT. Two reports examined the lncRNA CCDC26 in GIST indicating its link with imatinib-resistance through interacting with KIT and IGF-1R proteins [126, 127]. Badalamenti *et al*. investigated the expression levels of the well-known lncRNAs H19 and MALAT1 in GIST. MALAT1 expression appeared to be associated with KIT mutation status. Interestingly, H19 and MALAT1 expression was significantly higher in patients that respond poorly to imatinib i.e. a time-to-progression of < 6 months that perhaps indicates intrinsic resistance [128]. It is concluded that both H19 and MALAT1 expression levels hold prognostic potential to stratify GIST patients for first-line treatment with imatinib with high expressors indicating poor response to imatinib. H19 was also detected to be upregulated – together with FENDRR – in GIST samples compared to adjacent normal tissue by Gyvyte *et al*. [129]. The expression of multiple lncRNAs was analyzed by Yan *et al*. using a commercially available platform capable of detecting 63.542 lncRNAs and 27.134 mRNAs [130]. Most interestingly, differentially expressed lncRNA and mRNAs between primary GIST and imatinib-resistant GIST were identified. Further, *in silico* pathway- and network analyses implicated the hypoxia-inducible

factor 1 pathway as a mediator of imatinib resistance. The role of the lncRNA prostate cancer associated transcript 6 (PCAT6) was examined by Bai *et al*. [131]. First, PCAT6 was found to be upregulated in GIST in comparison with adjacent non-cancerous tissue. Follow-up *in vitro* studies revealed PCAT6 facilitated cancer by repressing apoptosis, enhancing cellular proliferation and – notably - by increasing GIST cell stemness and activating Wnt/β-catenin signaling. Further experiments showed miR-143-3p is a tumor suppressive miRNA in GIST as its expression levels are reduced in GIST cell lines in comparison to ICC. An RNA pull-down assay using biotinylated PCAT6 provided evidence that miR-143-3p is sequestered by PCAT6 causing the miR-143-3p target gene peroxiredoxin 5 (*PRDX5*) to be upregulated. Rescue experiments revealed that PCAT6 regulates GIST cell proliferation, apoptosis and stemness by reducing miR-143-3p and enhancing PRDX5.

CircRNAs are a recently recognized class of cellular transcripts that potentially have the capability to affect cellular processes and contribute to pathological processes including cancer [132]. A first study was performed by Jia and co-workers who used ceRNA microarrays that can monitor the expression of 88,371 circRNAs and 18.853 mRNAs [133]. When comparing 3 pairs of GIST and normal adjacent tissue a total of 5770 differentially expressed circRNAs and 1815 mRNAs were detected. Three circRNAs (circ_0069765; circ_0084097; circ_0079471) that localized to the host genes *KIT*, *PLAT* and *ETV1* and were upregulated in GIST were further investigated. The circRNAs contained 3 – 6 exons of their host genes and their upregulation was confirmed by RT-PCR in a relatively large validation cohort (n= 68). Next, miRNAs predicted to bind to the circRNAs were identified and a circRNA-miRNA-mRNA regulatory network was created. From these studies the authors concluded that the circRNAs, host genes and miR-142-5p, miR-144-3p and miR-485-3p may be key regulators in GIST.

**Table 4.** Long non-coding RNAs in gastrointestinal stromal tumors.

| Lnc RNA | Up/Down regulation | Functional role | Ref. |
|---|---|---|---|
| HOTAIR | Upregulation in high-risk GIST cf. low and intermediate GIST | • Repression apoptosis<br>• Stimulation invasion and migration<br>• Stimulation cell proliferation<br>• Hypo- and hypermethylation (e.g. PCDH10; DDP4; RASSF1; ALDH1A3) | [108, 122, 123] |
| AOC4P | Upregulation in high-risk GIST cf. low and intermediate GIST | • Repression apoptosis<br>• Stimulation invasion and migration<br>• Induction EMT | [125] |
| CCDC26 | Low expression linked to imatinib resistance | • CCDC26 interacts with c-KIT and IGF-1R<br>• CCDC26 knockdown upregulate c-KIT and IGF-1R | [126, 127] |
| FENDRR, H19 | Upregulation in GIST cf. adjacent normal tissue | • Positive correlation between H19 and ETV1 | [129] |

| | | | |
|---|---|---|---|
| | | • Positive correlation between H19 and miR-455-3p | |
| H19 | High expression in advanced GIST with TTP<6 months | | [128] |
| MALAT1 | High expression in advanced GIST with TTP<6 months | • Correlation with c-KIT mutational status | [128] |
| TERT-2, OMD-1, ATP7A-2, RERE-4, TCP1-5, FAM108B1-3, C15orf54-4, ATP7A-1<br><br>TCF4-6, SNRPN-2 | Upregulation in imatinib resistant GIST<br><br>Downregulation in imatinib resistant GIST | • HIF1 pathway regulation | [130] |
| PCAT6 | Upregulation in GIST cf. adjacent normal tissue | • Repression apoptosis<br>• Stimulation cell proliferation<br>• Promotion GIST stemness<br>• Activation Wnt/β-catenin signalling<br>• Sponging of miR-143-3p | [131] |
| circ_0069765, circ_0084097, circ_0079471 | Upregulation in GIST cf. adjacent normal tissue | • Role in predicted network of circRNAs, host genes (*KIT*, *PLAT*, *ETV1*, resp.) and miR-142-5p, miR-144-3p and 485-3p. | [133] |

Abbreviations: EMT, epithelial-mesenchymal transition; TTP, time to progression.

# Biomarkers

It is evident that non-coding RNAs can be exploited as diagnostic, prognostic and predictive biomarkers. The investigations carried out with GIST report diagnostic miRNA classifiers that distinguish GIST from other sarcomas [80, 88, 134], identify histological and molecular subtypes [85, 98] and define location specific markers [85]. Of particular clinical interest are the miRNAs associated with relapse risk [84, 94, 95, 98, 101, 108-110, 112, 135] that may be used to predict tumor recurrence and metastasis. These prognostic biomarkers may be further developed into a more quantitative risk evaluation for GIST which is now based on mitotic index, tumor size and tumor location. Finally, miRNAs associated with imatinib resistance [30, 110, 114, 115, 117-119] may be used to signal evolving imatinib resistance enabling early clinical intervention. Interestingly, also lncRNAs have been identified that could be used for diagnostic purposes [129, 131, 133] or are specifically linked to high-risk / advanced GIST [108, 122, 123, 125, 128] and imatinib resistance [126, 127, 130]. However, more research is necessary to select the miRNA classifiers that are most promising for validation in prospective clinical studies. Most studies so far provide proof-of-principle that biomarkers can be

identified but do so on a limited number of samples (Table 1). To end up with reliable biomarkers, future studies should avoid caveats and be aware of the critical steps in miRNA related translational research [136-138]. First, appropriately sized sample cohorts should be analyzed taking tumor heterogeneity into account. In addition, the tumor samples must be well-characterized, preferably come from different laboratories and meet certain defined and stringent quality criteria. Ideally, an unbiased, robust and reliable screening procedure should be used that can be standardized and easily executed in different laboratories. For miRNAs one could consider an RNA-seq approach adapted to suit the class of small RNAs one is interested in. The data should be analyzed using appropriate statistics and the biomarkers should display a defined sensitivity and specificity. It can very well be that a robust classifier needs to be based on the expression of an miRNA panel. For relatively rare tumors such as GIST- but also for more abundant tumor types - it unavoidable to carry out these studies in international consortia particularly if one intends to bring biomarkers to the clinic [139].

The majority of biomarkers studies on GIST were carried out using tumor samples acquired by invasive biopsies or after tumor resection (Tables 1, 3). The exploitation of liquid biopsies, often simple blood draws in a minimally invasive way, have not yet been extensively investigated in GIST patients. Only few investigators examined the miRNA profiles in serum samples. Distinct serum miRNA expression patterns were observed between GIST patients and healthy controls [134] and miR-518e-5p was identified a classifier for imatinib resistance [119]. Circulating miRNAs or other non-coding RNAs - either packaged in extracellular vesicles or not - may signal tumor recurrence, development of drug resistance and tumor progression or indicate metastasis. Particularly, frequent sampling in high-risk patients may indicate disease progression early on, enabling early clinical intervention.

# Therapeutic potential of non-coding RNAs

The mere fact that non-coding RNAs play key roles in carcinogenesis, displaying either oncogenic or tumor suppressive functions, and cancer progression implies they have therapeutic potential. This has also been demonstrated in various laboratories for GIST. Of interest in this respect are the miRNAs that target the *KIT* receptor: miR-218 [90], miR-221/222 [88, 89] and miR-494 [84, 92]. Alternative targets with therapeutic potential are *PDGFRA*, reported to be targeted by miR-34a [97], *PTEN* [100], *BIRC5* [93] and *APOC2* [101]. Also amenable for therapeutic modulation are the lncRNAs HOTAIR [108, 122, 123], AOC4P [125] and PCAT6 [131]. In principle, one could restore expression of non-coding RNAs that display reduced levels in cancer using mimics. Conversely, overexpressed non-coding RNAs may be inhibited using antisense approaches. RNA targeting therapeutic approaches have been discussed in the literature since the discovery of RNAi in the nineties but encountered significant challenges related to stability, delivery, importantly tissue specificity, tissue penetrance and intracellular trafficking, and toxicities [140, 141]. Although many of these issues have not been completely solved significant advances have been made as exemplified by FDA approved oligonucleotide drugs aimed to induce cleavage of a target mRNA or alter the splicing pattern [142]. RNA oligonucleotides are chemically modified to

increase stability, providing protection against nucleases, and improve target binding affinity [143, 144]. Most importantly, the use of 2'-O-methy substitutions in the sugar backbone of the RNA, 2'-fluoro- or locked nucleic acid (LNA) bases and the use of oligoribonucleotides with phosphorothioate linkages replacing the regular phosphodiester bonds [145]. Moreover, oligonucleotides with a peptide backbone have been generated giving rise to increased stability and binding affinities, additional modifications e.g. cholesterol conjugation and cell penetrating peptides may improve cell uptake. Currently nanoparticles, notably lipid-based nanocarriers and polymer-and peptide particles are being generated. The packaging of oligoribonucleotides in nanoparticles partly overcomes the stability issue and allows for innovative ways to direct the particles to the target tissue [145]. Particularly the progress made in delivery technologies have enabled clinical trials in which non-coding RNA-based therapeutic agents are tested in patients [67, 68]. Finally, as the functional significance of the vast majority non-coding RNAs, especially lncRNAs in specific cancers, remains unknown it is virtually impossible to select the best candidate for therapeutic intervention. This problem may be solved by the use of large-scale CRISPR-CAS9 based screens to rapidly determine the therapeutically actionable lncRNAs [146].

# Future Directions

With the ongoing functional annotation of the non-coding genome comes the realization that non-coding transcripts constitute a central and essential element of eukaryotic biology and as such are intimately involved in all kinds of pathological processes including cancer. The clinical relevance of non-coding RNAs is emphasized by many studies listed in the clinical trial database (http://clinicaltrials.gov) that evaluate non-coding RNAs. Frequently these trails concern oncological patients in which non-coding RNA expression levels are determined and linked to clinicopathological data for biomarker purposes [68].

For GIST, non-coding RNA biomarkers associated with high-risk GISTs and imatinib resistance may be particularly relevant and obtain a place in the clinical management of this disease. As current biomarker discovery studies are based on relatively small sample cohorts additional research is required to validate the found biomarker signatures. At the same time the specificity and sensitivity of the biomarker signatures should be determined and how they relate to the traditional clinical and pathological classifiers.

In current clinical practice advanced GISTs are being effectively treated with imatinib and other small molecule inhibitors targeting the receptor tyrosine kinases KIT and PDGFRA. It seems that for GIST therapeutic targeting of key non-coding RNAs is less relevant. However, eventually all patients develop (multi)drug resistance yielding the GISTs unresponsive to drugs. In this instance additional drug targets are needed that, when inhibited or stimulated, affect the ongoing signaling through KIT or PDGFRA. signaling. MiRNA or lncRNAs based therapeutic approaches can be of use in this setting. Challenges, however, remain and mainly involve drug safety and targeted delivery issues [142]. Nevertheless, the future will see the

enormous potential of the noncoding genome unleashed revealing new biology followed – undoubtedly - by clinical applications in the form of specific and sensitive biomarkers or the introduction of novel therapeutic strategies.

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# Chapter 5

# Molecular Comparison of Imatinib-Naïve and Resistant Gastrointestinal Stromal Tumors: Differentially Expressed microRNAs and mRNAs

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# Abstract

Despite the success of imatinib in advanced gastrointestinal stromal tumor (GIST) patients, 50% of the patients experience resistance within two years of treatment underscoring the need to get better insight into the mechanisms conferring imatinib resistance. Here the microRNA and mRNA expression profiles in primary (imatinib-naïve) and imatinib-resistant GIST were examined. Fifty-three GIST samples harboring primary KIT mutations (exon 9; n = 11/exon 11; n = 41/exon 17; n = 1) and comprising imatinib-naïve (IM-n) (n = 33) and imatinib-resistant (IM-r) (n = 20) tumors, were analyzed. The microRNA expression profiles were determined and from a subset (IM-n, n = 14; IM-r, n = 15) the mRNA expression profile was established. Ingenuity pathway analyses were used to unravel biochemical pathways and gene networks in IM-r GIST. Thirty-five differentially expressed miRNAs between IM-n and IM-r GIST samples were identified. Additionally, miRNAs distinguished IM-r samples with and without secondary KIT mutations. Furthermore 352 aberrantly expressed genes were found in IM-r samples. Pathway and network analyses revealed an association of differentially expressed genes with cell cycle progression and cellular proliferation, thereby implicating genes and pathways involved in imatinib resistance in GIST. Differentially expressed miRNAs and mRNAs between IM-n and IM-r GIST were identified. Bioinformatic analyses provided insight into the genes and biochemical pathways involved in imatinib-resistance and highlighted key genes that may be putative treatment targets.

# Introduction

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal malignancies associated with the gastrointestinal tract that originate from the interstitial cells of Cajal (ICC) or precursors thereof [1]. GISTs and ICC share morphological and immunophenotypic features, notably the expression of KIT and CD34. Molecularly, GISTs are characterized by the presence of oncogenic gain-of-function mutations in *KIT* (~80% of cases) or *PDGFRA* (~10% of cases) [2,3]. *KIT* and *PDGFRA* mutations are absent in the so-called wild-type GISTs (~10% of cases) that may contain mutations in *BRAF*, *NF1*, or defects of the succinate dehydrogenase (SDH) complex [4]. The constitutive activation of KIT and PDFGRA signaling in the majority of GISTs drives tumor growth through the activation of downstream signaling cascades such as the RAS–RAF–MAPK, PI3K–AKT, and STAT3 pathways facilitating cell proliferation and survival [5]. The advent of the tyrosine kinase inhibitor imatinib mesylate, which targets both KIT and PDGFRA, has dramatically improved the outcome of patients with advanced disease [6,7]. Despite this great progress in GIST treatment and the fact that approximately 10% of the patients benefit for more than 10 years from imatinib [8], the majority of patients eventually develop imatinib resistance (acquired resistance) [8] with about 10% of GIST patients experiencing progression already within 6 months of start of therapy (intrinsic resistance) [6,7]. Where in intrinsic resistant cases in particular *KIT* exon 9 mutations or *PDGFRA* D842V mutations are involved [9], acquired resistance may occur because of secondary mutations within *KIT* that interfere with the binding of imatinib [10–14]. These resistance-causing secondary mutations cluster in two regions: (i) ATP-binding pocket (encoded by exons 13 and 14), and (ii) kinase catalytic regions/activation loop (encoded by exons 17 and 18). Such secondary mutations leading to acquired resistance are observed in approximately 50% of GIST patients. The remaining cases with acquired resistance display alternative resistance mechanisms that are much less defined and include *KIT* and *PDFRA* amplification [11,13] and receptor tyrosine kinase switches from KIT to activation of FAK, FYN, or AXL [15–17].

A better understanding of the causes yielding imatinib resistance is necessary to improve treatment and outcomes. Here we performed a molecular comparison between a unique set of imatinib-naïve (IM-n) GIST samples (n = 33) and imatinib-resistant (IM-r) GIST samples (n = 20) focusing on microRNA and mRNA expression to reveal molecular pathways associated with imatinib resistance.

# Results

## Differentially Expressed microRNAs between Imatinib-Naïve and Imatinib-Resistant GIST Samples

To investigate the molecular events underlying the acquisition of imatinib resistance in GIST we first determined the miRNA expression profiles in fresh frozen IM-n (n = 33) and IM-r (n = 20) GIST samples (Table 1). All imatinib resistant GIST patients displayed resistance after more than 6 months of imatinib treatment implicating acquired resistance mechanisms. Thirty-five significantly ($p < 0.01$ and False Discovery Rate (FDR) < 20%) differentially expressed miRNAs were detected between the two groups (Figure 1, Table S1). Figure 1 depicts the heat map from a supervised hierarchical clustering. Two main clusters were discerned, one cluster contained 82% of the IM-n samples and the other cluster included 85% of all IM-r samples. A number of samples of both IM-r and IM-n GISTs were found to miscluster, a fact that could not readily be explained by differences in malignancy risk or tumor location.

Secondary mutations in *KIT* are a frequent cause of imatinib-resistance in GIST. In the 20 IM-r samples that we analyzed, nine displayed secondary mutations in *KIT* exon 13 (n = 3) and *KIT* exon 17 (n = 6), whereas in the remainder (n = 11) no secondary mutations were observed (Table 1). When we compared the miRNA expression profiles of IM-r samples with and without secondary mutations, we identified 22 miRNAs that were significantly ($p < 0.01$) differentially expressed and almost completely separated the two groups (Figure 2, Table S2). This suggests miRNA biomarker profiles may be associated with the presence/absence of secondary mutations.

**Table 1.** Patient and tumor characteristics.

| Gastrointestinal Stromal Tumors Imatinib-Naïve (IM-n) | | | | | |
|---|---|---|---|---|---|
| Male | n = 23 | | | | |
| Female | n = 10 | | | | |
| Median age (range) | 65 (41–85) | | | | |
| **Sample code** | **KIT mutation status** | **Location** | **Risk of malignancy *** | **miRNA** | **mRNA** |
| X1KIT11 | p.V560D/KIT11 | Small intestine | intermediate | ✓ | ✓ |
| X4KIT11 | p.W557_V559delinsF/KIT11 | Small intestine | high | ✓ | ✓ |
| X6KIT11 | p.W557R/KIT11 | Small intestine | overtly malignant ** | ✓ | |
| X8KIT11 | p.L576_R588dup/KIT11 | Stomach | intermediate | ✓ | ✓ |
| X9.2.KIT11 | p.W557_V559delinsF/KIT11 | Stomach | high | ✓ | |
| X10KIT11 | p.W557R/KIT11 | Stomach | intermediate | ✓ | |
| X12KIT11 | p.K550_V555del/KIT11 | Stomach | high | ✓ | |
| X14KIT11 | p.581_590insKWEFPRNRLS/KIT11 | Stomach | intermediate | ✓ | ✓ |
| X23KIT11 | p.W557_K558del/KIT11 | Stomach | intermediate | ✓ | |
| X24KIT11 | p.V554D/KIT11 | Stomach | intermediate | ✓ | ✓ |
| X25KIT11 | p.W557_G592dup (c.1669_1774 + 2dup)/KIT11 | Stomach | high | ✓ | |
| X26KIT11 | p.K558_V559delinsN (AAT) homo/KIT11 | Mediastinum | high | ✓ | |
| X34KIT11 | p.W557_V560delinsF/KIT11 | Stomach | high | ✓ | |
| X35KIT11 | p.V560D/KIT11 | Stomach | overtly malignant | ✓ | ✓ |
| X39KIT11 | p.L576P/KIT11 | Duodenum | intermediate | ✓ | ✓ |
| X40KIT9 | p.A502_Y503dup/KIT9 | Colon | overtly malignant | ✓ | |
| X45KIT11 | p.K550_K558delinsG/KIT11 | Small intestine | overtly malignant | ✓ | |
| X47KIT11 | p.V559A/KIT11 | Stomach | low | ✓ | |
| X48KIT11 | p.V560A/KIT11 | Duodenum | intermediate | ✓ | ✓ |
| X53KIT11 | p.Q556_V559delinsH; c.1668_1676del9/KIT11 | Stomach | overtly malignant | ✓ | ✓ |
| X55KIT11 | p.W557_K558del/KIT11 | Stomach | intermediate | ✓ | |
| X64KIT11 | p.V560D/KIT11 | Stomach | high | ✓ | ✓ |
| X78KIT11 | p.W557_K558del; c.1669_1674del/KIT11 | Stomach | overtly malignant | ✓ | ✓ |
| X82KIT11 | p.W557_P573delinsFQ/KIT11 | Stomach | overtly malignant | ✓ | |
| X86KIT9 | p.A502_Y503dup/KIT9 | Small intestine | overtly malignant | ✓ | |

| X89KIT17 | p.N822K/KIT17 | Small intestine | overtly malignant | ✓ | ✓ |
|---|---|---|---|---|---|
| X95KIT11 | p.T574_R586insK/KIT11 | Stomach | intermediate | ✓ | |
| X100KIT9 | p.A502_Y503dup/KIT9 | Small intestine | overtly malignant | ✓ | |
| X101KIT11 | p.E554_K558del/KIT11 | Stomach | low | ✓ | |
| X102KIT11 | p.W557R/KIT11 | Stomach | high | ✓ | ✓ |
| X108KIT9 | p.A502_Y503dup/KIT9 | Small intestine | overtly malignant | ✓ | ✓ |
| X.KIT11 | p.M552_E554delinsK/KIT11 | Small intestine | overtly malignant | ✓ | |
| X119KIT11 | p.Q556_I563del/KIT11 | Stomach | low | ✓ | |

**Table 1.** *Cont.* Patient and tumor characteristics.

| **Gastrointestinal stromal tumors Imatinib-Resistant (IM-r) ***** | | | | | | |
|---|---|---|---|---|---|---|
| Male | n = 14 | | | | | |
| Female | n = 6 | | | | | |
| Median age (range) | 49.5 (22–67) | | | | | |
| **Sample code** | **KIT mutation status** | **KIT secondary mutation** | **Location** | **Risk of malignancy*** | **miRNA** | **mRNA** |
| X2KIT9 | p.A502_Y503dup/KIT9 | Not detected | Colon | overtly malignant | ✓ | ✓ |
| X27KIT11 | p.L576P; c.1727 T > C/27KIT11 | p.D820Y; c.2458G > T | Small intestine | overtly malignant | ✓ | ✓ |
| X36KIT11 | p.Q556_E561delinsQ/KIT11 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X42KIT9 | p.A502_Y503dup/KIT9 | KIT: p.V654A | Small intestine | overtly malignant | ✓ | ✓ |
| X44KIT9 | p.A502_Y503dup/KIT9 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X54KIT11 | p.K550_K558delinsQ/KIT11 | KIT: p.D820Y | Small intestine | overtly malignant | ✓ | ✓ |
| X58KIT11 | p.I563_Q575del/KIT11 | KIT: p.D820Y | Stomach | overtly malignant | ✓ | ✓ |
| X70KIT11 | p.E554_D572del/KIT11 | KIT: p.V654A | Small intestine | overtly malignant | ✓ | ✓ |

| | | | | | | |
|---|---|---|---|---|---|---|
| X71KIT11 | p.V559D/KIT11 | KIT: p.D820G | Small intestine | overtly malignant | ✓ | ✓ |
| X73KIT11 | p.N567_L576delinsI/KIT11 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X74KIT9 | p.A502_Y503dup/KIT9 | Not detected | Small intestine | overtly malignant | ✓ | |
| X77KIT9 | p.A502_Y503dup/KIT9 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X85KIT9 | p.A502_Y503dup/KIT9 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X91KIT11 | p.K550_K558del/KIT11 | KIT: p.D820Y | Small intestine | overtly malignant | ✓ | |
| X104KIT11 | p.W557_K558del/KIT11 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X105KIT9 | p.A502_Y503dup/KIT9 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X112KIT11 | c.1654_1671del18 (p.M552_W557del)/KIT11 | Not detected | Small intestine | overtly malignant | ✓ | ✓ |
| X116KIT11 | p.557_558del homo/KIT11 | Not detected | Small intestine | overtly malignant | ✓ | |
| X117KIT11 | p.K550_V555delinsL; c.1648_1663delinsT/KIT11 | p.D820Y; c.2458 G > T | Small intestine | overtly malignant | ✓ | |
| X118KIT11 | p.V559D; c.1676 T > A/KIT11 | KIT p.V654A; c.1961 T > C | Small intestine | overtly malignant | ✓ | |

* Tumor risk assessment was performed using AFiP criteria (Miettinen M. & Lasota, J. *Semin. Diagn. Pathol*. **2006**, 23,70–83); ** Recurrent or metastatic disease during clinical follow-up; *** Patients were only treated with imatinib, progression occurred after 6 months.

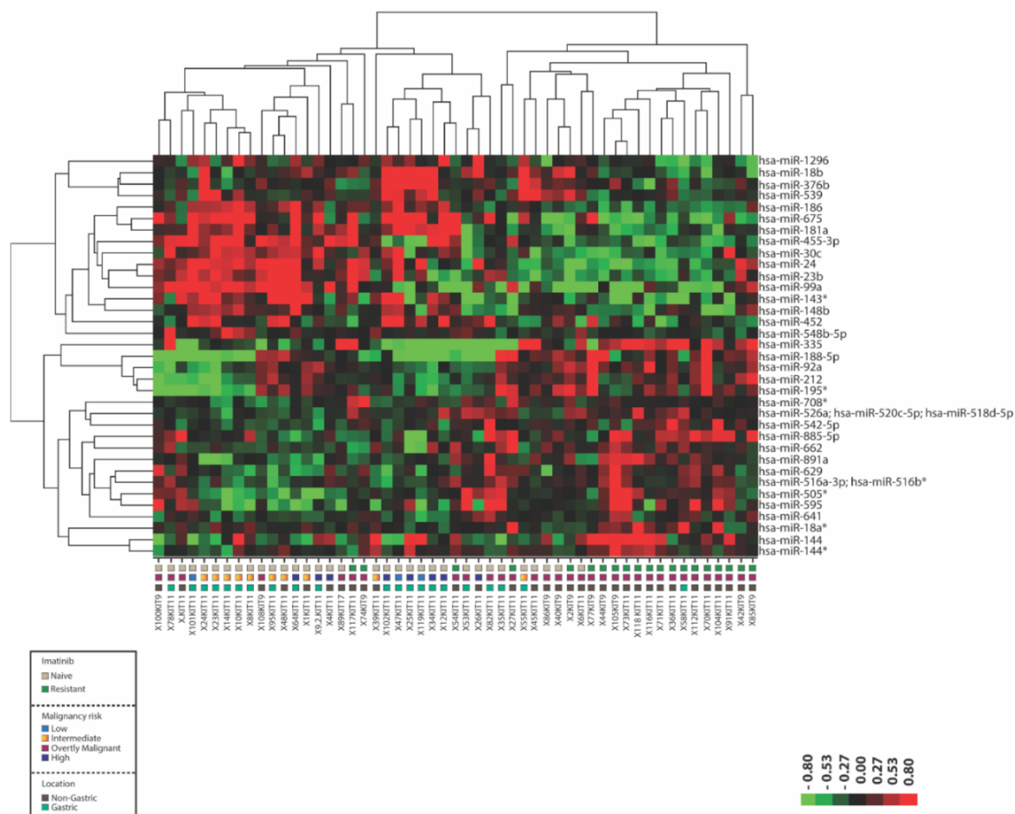



**Figure 1.** MicroRNA expression distinguishes imatinib-naïve (IM-n) from imatinib-resistant (IM-r) gastrointestinal stromal tumors (GIST). Fresh frozen tumor samples of IM-n and IM-r GIST patients were subjected to miRNA expression profiling. Depicted is the heat map of a supervised hierarchical clustering based on the 35 most significant ($p < 0.01$ and False Discovery Rate (FDR) < 20%) differentially expressed miRNAs. In the heat map red indicates relative high expression and green indicates relative low expression. The colored squares beneath the graph designate IM-n and IM-r samples, the malignancy risk and location of the tumors. Note that the sample codes below also indicate which KIT exon is mutated.

# mRNA Expression Profiling and Ingenuity Pathway Analyses Reveal Differentially Expressed Genes and Pathways in Imatinib-Naïve and Imatinib-Resistant GIST Samples

In order to better understand the genes and molecular pathways involved in imatinib resistance in GIST we performed mRNA expression analyses on a subset (IM-r, n = 15 vs IM-n, n = 14) of our GIST samples. At least 352 genes were identified to be significantly differentially expressed ($p < 0.008$, FDR < 10%) between the two groups (Figure S1; Table S3). Figure 3 shows the cluster tree of a supervised cluster analysis based on the expression of the 352 differentially expressed genes represented by 475 different Affymetrix probe sets (Figure S1). All IM-r samples cluster together as do all IM-n samples except one. A molecular pathway analysis, focusing on canonical pathways and using the Ingenuity platform, was performed with the 352 differentially expressed genes as input. Among these genes, regulators of estrogen-mediated S-phase entry ($p = 8.29 \times 10^{-8}$), cyclins and cell cycle regulators ($p = 3.09 \times 10^{-6}$), as well as checkpoint regulators of G2/M DNA damage ($p = 8.64 \times 10^{-6}$) were overrepresented (Figure S2). Of note, the cyclins A2, B1, B2, D2, and E2, as well as *CDK1* and the E2F transcription factors *E2F7* and *E2F8* were among the most differentially expressed genes found in two or more deregulated pathways (Table S4). Except for *CCND2* (5.4 fold lower in IM-r), all the other seven genes displayed increased expression in IM-r with the fold changes of 2.6 for *CCNA2*, 2.9 for *CCNE2*, 2.3 for *CCNB1*, 2.1 for *CCNB2*, 3.0 for *CDK1*, 2.1 for *E2F7*, and 2.0 for *E2F8* in comparison to the imatinib-naïve setting.

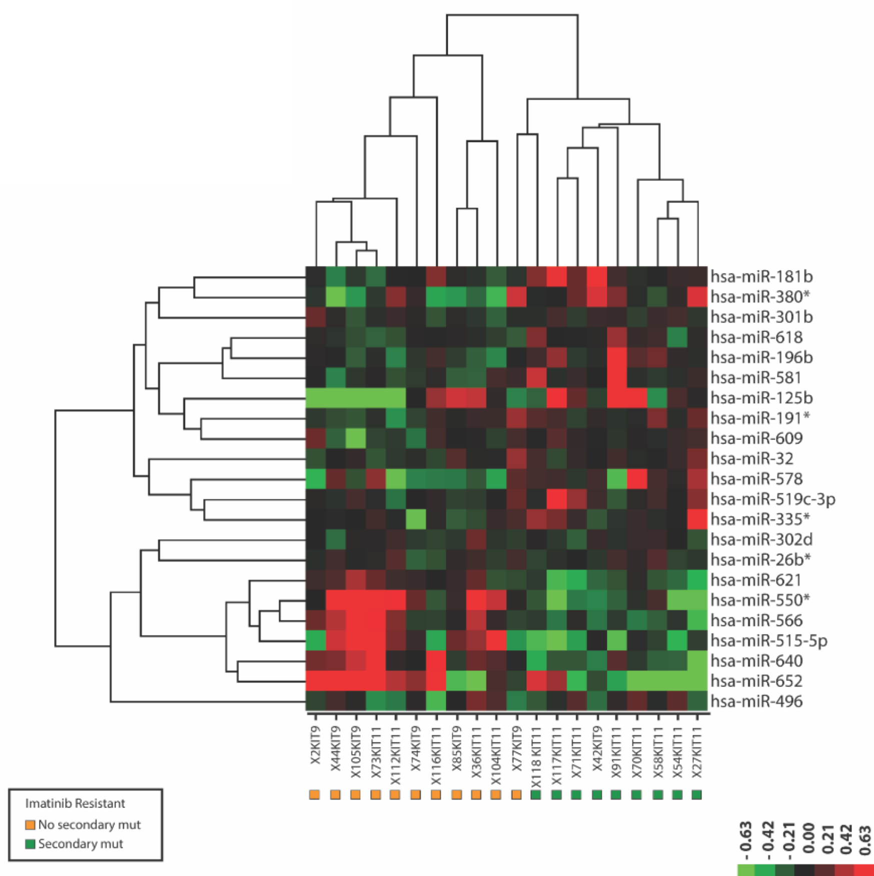



**Figure 2.** MicroRNAs differentially expressed between imatinib-resistant gastrointestinal stromal tumor (GIST) samples with and without secondary mutations in KIT. Depicted is a heat map of a supervised hierarchical clustering based on the 22 most significant ($p < 0.01$) differentially expressed miRNAs in fresh frozen GIST samples with (green squares) and without (orange squares) secondary imatinib resistance causing mutations in KIT. In the heat map red indicates relative high expression and green indicates relative low expression. Note that the sample codes below also indicate which KIT exon is mutated.

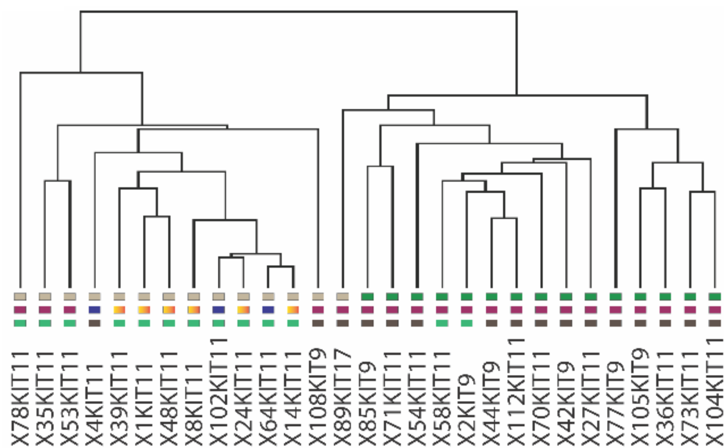
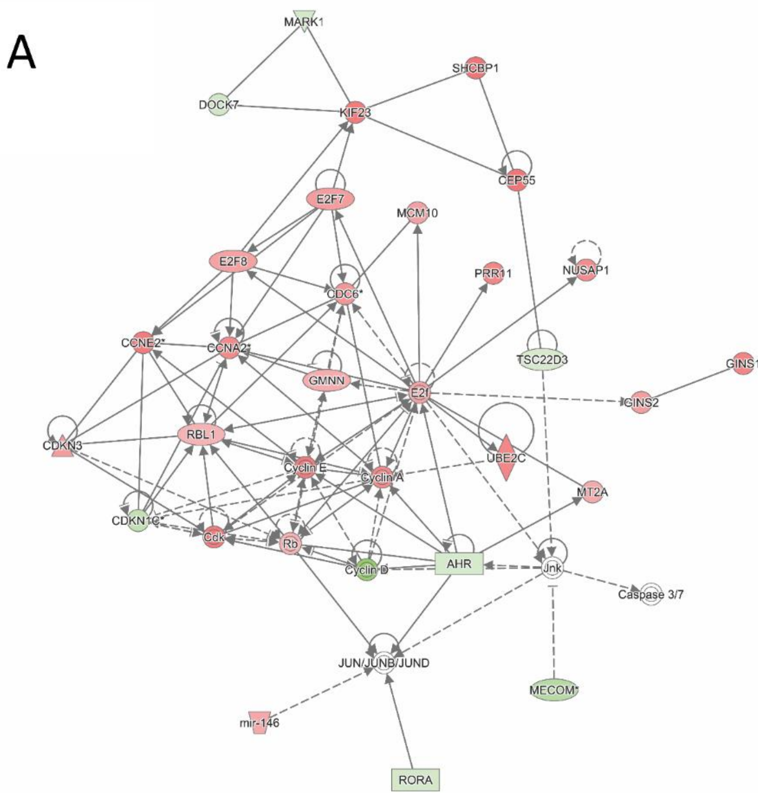



**Figure 3.** Supervised hierarchical clustering based on differential gene expression discriminates imatinib-naïve (IM-n) and imatinib-resistant (IM-r) gastrointestinal stromal tumor (GIST) samples. Transcript expression profiles were determined using the Affymetrix platform (U133 plus 2) of 29 fresh frozen samples derived of IM-n(n = 14) and IM-r(n = 15) GISTs. Depicted is the cluster tree of a supervised hierarchical clustering based on 352 significant ($p < 0.008$, False Discovery Rate (FDR) < 10%), differentially expressed transcripts. Note that 100% of the IM-r samples are clustered together with a single IM-n GIST sample. The colored squares beneath the graph designate imatinib-naïve and imatinib-resistant samples, the malignancy risk and location of the tumors (see Figure 1). Note that the sample codes below also indicate which KIT exon is mutated.

An ingenuity pathway analysis (IPA) was also conducted to examine interactions within the 352 most differentially expressed genes between IM-r and IM-n GIST samples. Figures 4A and 4B depict two of the largest and most significant interaction networks revealed by IPA (see for a symbol legend Table S5). Figure 4A displays associations between genes involved in cell cycle regulation and consequently cell proliferation. Cyclin A and cyclin E appear as central hubs in the gene network. The interaction network shown in Figure 4B also supports cell cycling and cell proliferation judged by the overexpressed central genes cyclin dependent kinase 1 (CDK1), aurora kinase B (AURKB), and forkhead box protein M1 (FOXM1). CDK1 plays a key role in cell cycle regulation, AURKB regulates the segregation of chromosomes and the spindle checkpoint in mitosis and FOXM1 is a transcription factor essential for cell cycle regulation.

# Integration of Differentially Expressed microRNAs and mRNAs into Networks

Using IPA, we investigated whether interaction networks between mRNAs and miRNAs could be defined to identify and better understand the possible regulatory role of miRNAs-mRNAs interactions in imatinib resistance. To be able to directly compare mRNA with miRNA expression in the same GIST samples, we considered only the differentially expressed miRNAs in the subset of GIST samples that were analyzed by mRNA expression profiling. We identified 88 differentially expressed miRNAs ($p < 0.03$, FDR < 30%) (Table S6). Note that almost 70% of the differentially miRNAs reported in Figure 1 and Table S1 were present in this miRNA selection.



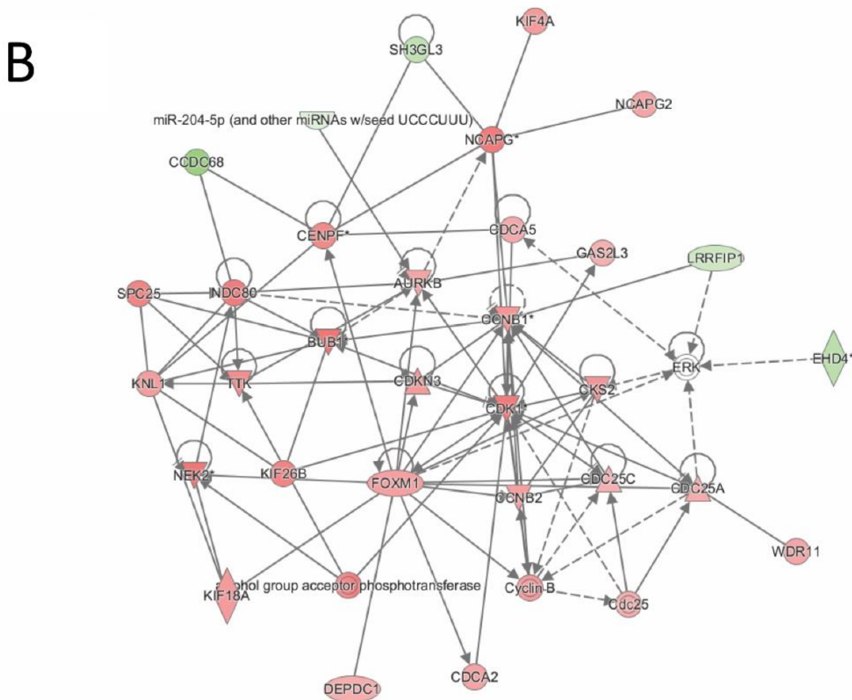



**Figure 4.** Ingenuity Pathway Analysis indicates the relation between genes differentially expressed between imatinib-naïve (IM-n) and imatinib-resistant (IM-r) gastrointestinal stromal tumor (GIST) samples. The 352 significant differentially expressed genes between IM-r and IM-n GIST samples were used as input for an Ingenuity Pathway Analysis (IPA). The depicted IPA networks illustrate and visualize associations between the genes. (**A**) IPA network highlighting cell cycle related, differentially expressed genes. (**B**) IPA network highlighting CDK1, AURKB, and FOXM1 interactions. Green and red shading indicates relatively low and high expression in the IM-r samples. See Table S5 for an extended symbol legend.

The gene-miRNA network presented in Figure 5 included most regulatory gene-miRNA interactions and related to cell cycle regulation. The network highlights regulation by miR-92a-3p, miR-99a-5p, and miR-101-3p. The differential expression of selected miRNAs and mRNAs, which were indicated in the text and IPA analyses, were verified by RT-PCR, thereby confirming our findings with the miRNA and mRNA array platforms (Figure S3).

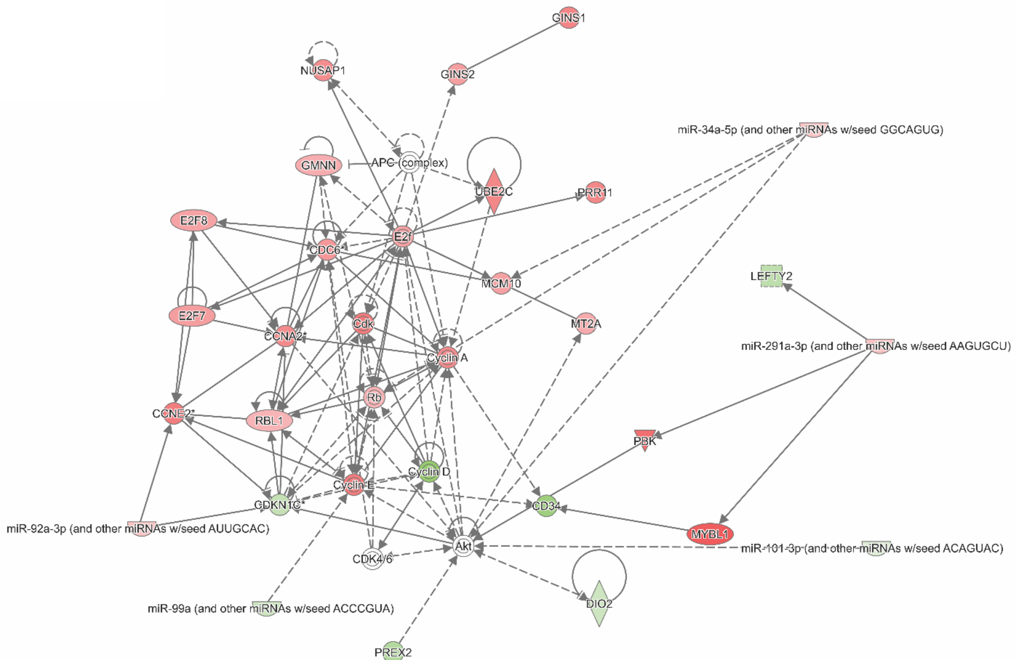



**Figure 5.** Ingenuity Pathway Analysis integrating differentially expressed genes and microRNAs between imatinib-naïve (IM-n) and imatinib-resistant (IM-r) gastrointestinal stromal tumor (GIST) samples. As input for an Ingenuity Pathway Analysis (IPA) the significantly differentially expressed transcripts (352 genes, $p < 0.008$, False Discovery Rate (FDR) < 10%) and miRNAs (88 miRNAs, $p < 0.03$, FDR < 30%) from the same set of IM-r (n = 15) and IM-n (n = 14) GIST samples were used. The depicted network indicates miRNA–gene interactions relevant in context of the cell cycle. Green and red shading indicates relatively low and high expression in the IM-r samples. See Table S5 for an extended symbol legend.

# Discussion

To better understand the mechanisms that account for imatinib resistance, here we molecularly characterized at an mRNA and miRNA level a unique set of IM-n and IM-r GIST samples. Bioinformatic approaches were used to identify signaling pathways and gene networks modulated in imatinib-resistant GISTs. The reason to look for differentially expressed miRNAs between IM-n and IM-r samples is based on the observations that miRNAs are intimately involved in GIST pathobiology [18,19] and well-known actors in drug resistance mechanisms occurring in cancer types other than GIST [20]. Indeed, we identified miRNAs that distinguished IM-r from IM-n GIST samples. Although the fold changes observed were relatively small, they can still have a significant impact on protein levels because the regulation of multiple targets within the same pathway can amplify their biological effect [21] and different miRNAs may cooperate and have synergistic effects [22]. Previously few other groups studied miRNA expression in relation to imatinib-resistance in GIST as well [23,24]. Akaçakaya et al. compared miRNA expression profiles of 17 GISTs of which 10 responded to imatinib (imatinib-sensitive) and seven progressed on imatinib (imatinib-resistant) [23]. They identified ten differentially expressed miRNAs a.o. miR-125a-5p that were found to be overexpressed in IM-r GIST and of which the expression was inversely correlated to levels of protein tyrosine phosphatase, non-receptor Type18 (PTPN18) [23]. The lowered PTPN18 levels conferred imatinib resistance in GIST822 cells. In a recent follow-up paper evidence was provided that the miR-125a-5p and PTPN18 effects on imatinib resistance were mediated through phosphorylated FAK levels [25]. Shi et al., reported downregulation of miR-518a-5p in IM-r that targets PIK3C2A [24]. Unfortunately, PIK3C2A levels were not modulated to validate its levels affecting imatinib sensitivity in GIST cells. Similarly, no evidence for an inverse correlation between miR-518a-5p and PIK3C2A expression in clinical samples was presented. The overlap in miRNAs detected between these studies and ours is limited, most likely due to different experimental set-ups, including the exact nature and number of GIST samples analyzed and the use of different miRNA detection platforms. In chronic myeloid leukemia (CML), another malignancy that is treated with imatinib, miRNAs have also been linked to imatinib resistance [26–28]. A number of miRNAs, e.g., miR-99a, miR-30c, and miR-101, which were all found downregulated in the IM-r samples (Table S6), have been previously associated with imatinib resistance in GIST or chronic myeloid leukemia [26,27]. The observed downregulation of miR-30c and miR-181a in our IM-r samples corresponded to findings in CML in which lowered expression of these miRNAs was also found in imatinib resistant cells [27,28]. In most cases dysregulated miRNAs in the IM-r setting were not further functionally characterized to substantiate their roles and involvement in imatinib resistance.

Interestingly, the miRNA expression profiles were able to distinguish IM-r GIST samples with and without secondary KIT mutations. This observation may reflect a different biology underlying the resistance phenotype in the two groups. However, the accompanying fold differences in miRNA expression are small. To verify our findings larger sample cohorts should be examined using an RT-PCR platform.

Pathway and network analyses using differentially expressed transcripts and mRNAs as input indicated the upregulation of multiple cell-cycle related genes in the IM-r GIST samples. The cyclins A and E are well-known regulators of G1/S, S, and G2/M transition phases. Their increased expression levels, as well as those of most other genes in the network, most likely facilitates cell cycling and consequently cell proliferation. In this context the reduced expression of cyclin dependent kinase inhibitor 1 C (*CDKN1C*), a negative regulator of cell proliferation, also makes sense. However, the reduced expression of cyclin D2 (CCND2) does not seem to fit as its expression was found downregulated in the IM-r samples. It is unclear to what extent these findings are merely a reflection of the progressive nature of the IM-r GISTs. Aberrant expression of the majority of these genes is known to be involved in drug-resistance in various cancer types [29–31]. Of interest is the increased expression of the atypical E2F transcription factor family members E2F7 and E2F8. The precise function of these E2F family members in GIST and other cancers is still ill-defined. E2F7 overexpression has been linked to tamoxifen and anthracycline resistance in breast cancer and head and neck squamous cell carcinoma, respectively [32,33]. E2F8 promotes cell proliferation and tumorigenicity in breast cancer [34] and cisplatin resistance to estrogen receptor positive breast cancer cells [35]. The increased cell cycle activity may render IM-r GIST sensitive to cell cycle inhibitors.

The other highlighted gene interaction network is also conducive of cell cycle progression. This network points to central roles for AURKB and FOXM1 of which the expression was increased in IM-r GISTs. AURKB, together with AURKA, which is also upregulated in IM-r samples (Table S3), are serine/threonine kinases that regulate mitosis. These genes are found upregulated in many cancers and targeted inhibitors have been developed [36]. In GIST AURKA expression has been identified as a negative prognostic factor [37,38] and has recently been implicated as a therapeutic target [39]. The significance of FOXM1 in GIST was recently emphasized by reporting its role in GIST progression [40]. Furthermore the FOXO3a–FOXM1 axis has been implicated in cancer related processes like proliferation, survival, drug resistance, angiogenesis, migration, and DNA repair in other cancers [41]. Perhaps FOXM1 overexpression can be therapeutically exploited, e.g., by using thiazole antibiotics.

The integrative network analyses implicated some of the differentially expressed miRNAs as regulators of cell cycle related genes. Of special interest is miR-92a-3p, which is predicted to target *CDKN1C* through a highly conserved binding site in its 3’UTR, as predicted by TargetScan v7.2 (http://www.targetscan.org).The downregulation of miR-99a-5p affects mTOR levels [42,43]. The upregulation of mTOR stimulates cell cycle progression through its cell growth effectors S6K1 and eIF4E [44]. Finally, miR-101-3p has been implicated in imatinib sensitivity in CML with high levels sensitizing to imatinib through the downregulation of JAK2 and inhibition of NF-κB target genes [26]. So conversely miR-101-3p downregulation might cause imatinib resistance. Furthermore miR-101-3p regulates the PI3K/AKT/mTOR pathway [45,46] mediating AKT activation, which may reduce CDKN1C levels [47].

Our findings demonstrated that IM-r GIST samples can be distinguished from IM-n GIST samples based on their miRNA and mRNA expression profiles. In addition, we identified

several miRNAs that discriminated between IM-r GIST samples with or without secondary KIT mutations. Pathway and network analyses highlighted cell cycle related genes/gene networks in IM-r GISTs and identified overexpressed proteins that may be pharmacologically targeted using small molecule inhibitors. Further, our data implicated at least three miRNAs, miR-92a-3p, miR-99a-5p, and miR-101-3p, as potential effectors of imatinib resistance. Future experimental in vitro and in vivo studies are needed to further substantiate and validate these findings.

# Materials and Methods

## Patient Samples

Fresh frozen GIST samples (n = 53) were obtained from the tissue bank of the Department of Pathology of the University Hospitals Leuven, Belgium and the Department of Soft Tissue/Bone Sarcoma and Melanoma, Marie Skłodowska-Curie Institute, Oncology Center, Warsaw, Poland. The initial GIST diagnosis was based on histological features as assessed by an expert pathologist, immunostaining for CD117/KIT and anoctamin (ANO1 or DOG1), and the presence of *KIT* mutations. All tumor samples that were analyzed contained >80% tumor cells, contained *KIT* activating mutations, and were derived from both IM-n (n = 33) and IM-r (n = 20) GISTs. The pathological and initial diagnostic molecular evaluation were all performed in a single institution (KU Leuven). Clinicopathological characteristics concerning patients and tumors are listed in Table 1. The majority of the patients from whom the GIST samples were derived were diagnosed and treated from 2000 to 2011 according to the applicable guidelines in that time-period. All patients consented to use their tissues for research purposes and approval was obtained from the Ethics Committee of the University Hospitals Leuven (ML7481) and the Oncology Center, Warsaw, Poland. The study was carried out in the context of a research protocol "Translational research in soft tissue sarcomas", which was reviewed and approved by the Medical Ethical Review board of the Erasmus Medical Center (MEC-2016-213) on 11th April 2016. The study was performed in accordance with the Declaration of Helsinki.

## RNA Isolation and microRNA Profiling

Total RNA was isolated from fresh frozen tumor samples using RNAbee (Tel Test Inc., Friendswood, TX, USA) following the standard extraction protocol recommended by the manufacturer. RNA concentration and quality were examined using a Nanodrop-1000 (Nanodrop Technologies, Wilmington, DE, USA). MiRNA expression profiles were determined using miRNA microarrays, essentially as described before by Pothof et al. [48]. In brief, using the Kreatech ULS$^{TM}$ aRNA labeling Kit (Kreatech Diagnostics/Leica Biosystems, Amsterdam, the Netherlands), 1 µg total RNA was labeled with Cy3. The Cy3-tagged RNA was hybridized overnight to LNA™ modified oligonucleotide capture probes (Exiqon, Vedbaek, Denmark) spotted in duplicate on Nexterion E slides. Of the 1344 capture probes on the slides, 725 were specifically designed to detect human miRNAs. After hybridization, slides were scanned, and median spot intensity was determined using ImaGene software (BioDiscovery Inc., El Segundo, CA, USA). After background subtraction, expression values were Quantile normalized using R-software, bad spots were deleted, and duplicate spots averaged. The normalized miRNA expression data were log 2 transformed and median centered to acquire the relative expression values that were used for hierarchical clustering analysis using the open source software Cluster 3.0 [49] and Java Tree View [50]. A two-

sample t-test was used to determine statistical significance ($p < 0.05$) between imatinib-naïve and imatinib-resistant samples and the Benjamini–Hochberg false discovery rate (FDR) was used to control for multiple testing. The miRNA expression datasets generated and analyzed during the current study are presented in Table S7.

# mRNA Expression Analysis

Gene expression analysis using the Affymetrix HG-U133_Plus_2 platform (Affymetrix, Santa Clara, CA, USA) was carried out according to standard operating procedures by the VIB MicroArray Facility of the KU Leuven. Raw. cel files were processed using fRMA parameters (median polish) after which batch effects were corrected using ComBat. [51,52]. BRB-Array tools (Biometric Research Branch Array Tools (http://brb.nci.nih.gov/BRB-ArrayTools/) was used for analyzing the transcript expression data and a two-sample t-test was used for statistical testing. The mRNA expression datasets generated and analyzed during the current study have been deposited to the Gene Expression Omnibus (GEO) data repository under accession number GSE132542.

# Quantitative RT-PCR

The differential expression of selected miRNAs in IM-n (n = 33) and IM-r (n = 20) GIST samples, as detected by the LNA™ modified oligonucleotide platform, was validated by RT-PCR using the TaqMan® MiRNA Assays Technology (Applied Biosystems/ThermoFisher Scientific, Bleiswijk, the Netherlands). In brief, total RNA (50 ng) was reverse transcribed in a multiplex reaction using specific miRNA primers from the TaqMan® MiRNA Assays and reagents from the TaqMan® MiRNA Reverse Transcription Kit (Applied Biosystems/ThermoFisher Scientific) according to the manufacturer's protocol. The resulting cDNA was used as input in a quantitative real-time PCR (qPCR) using a miRNA specific primer/probe mix together with the TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems/ThermoFisher Scientific) using the 7500 Fast Real-Time PCR systems (Applied Biosystems). The qPCR data were analyzed using SDS software (version 2.4, Applied Biosystems/ThermoFisher Scientific). A standard dilution series of a cDNA sample-pool was included on every plate allowing for the absolute quantification of the miRNA expression.

The differential expression of selected mRNAs in IM-n (n = 33) and IM-r (n = 20) GIST samples, as detected by the Affymetrix platform, was validated by RT-PCR using the TaqMan® Technology (Applied Biosystems/ThermoFisher Scientific). In brief, total RNA (1 µg) was used as input for a reverse transcription reaction using a high capacity cDNA reverse transcription kit (Applied Biosystems/ThermoFisher Scientific) according to procedures by the manufacturer. The cDNA was used in a PCR reaction using primer/probe combinations from

the following Taqman® gene expression assays (AURKA, assay ID: Hs01582072_m1; AURKB, assay ID: Hs00945858_g1; CCND2, assay ID:Hs00153380_m1; CCNE2, assay ID: Hs00180319_m1; CDK1, assay ID: Hs00938777_m1; CDKN1C, assay ID: Hs00175938_m1; E2F7, assay ID: Hs00987777_m1; FOXM1, assay ID: Hs01073586_m1) and Taqman® Universal PCR master mix using the 7500 Fast Real-Time PCR system (all obtained from Applied Biosystems/ThermoFisher Scientific) according to the manufacturer's recommendations. Three housekeepers (GAPDH, HPRT, and PPIA) were used for normalization purposes using the comparative $C_T$-method. The qPCR data were analyzed using SDS software (version 2.4, Applied Biosystems/ThermoFisher Scientific).

# Pathway Analysis

For pathway analyses, a commercial software application, Ingenuity® Pathway Analysis (IPA®) (Qiagen, Hilden, Germany), was used. IPA calculates and visualizes the known pathway associations and interactions between sets of transcripts. mRNAs and/or miRNAs that were significantly differentially expressed between IM-n and IM-r samples were selected and accompanying identifiers and fold changes were uploaded into the IPA. The mRNA data were used to identify canonical signaling and metabolic pathways that were predicted to be activated or inhibited (canonical pathway analysis). The miRNA and mRNA data together were used to construct interaction networks, networks based on molecular relationships between differentially expressed mRNAs and/or miRNAs. These networks were matched to and derived from a ''global molecular network'' developed from the available online information in the IPA. The pathway and network analyses were performed using filtering of ''Human'' and ''uncategorized'' for species as well as ''direct and indirect relationships'' for general settings. The presented networks were representations of molecular relationships between mRNA–mRNA and miRNA–mRNA interactions.

# Supplementary Materials

The following are available online at www.mdpi.com/xxx/s1: Table S1, Differentially expressed microRNAs between imatinib-naïve and imatinib-resistant gastrointestinal stromal tumors; Table S2, Differentially expressed microRNAs between imatinib-resistant gastrointestinal stromal tumors with and without secondary KIT mutations; Table S3, List of 352 differentially expressed genes between imatinib-resistant and imatinib-naïve GIST samples; Table S4, Differentially expressed genes associated with the top deregulated canonical pathways; Table S5, Ingenuity Pathway Analysis Symbols; Table S6, Differentially expressed microRNAs between imatinib-naïve and imatinib-resistant gastrointestinal stromal tumors in the samples that were used for mRNA profiling; Table S7, Overview of the microRNA expression levels measured in the imatinib-naïve and imatinib-resistant gastrointestinal stromal tumor samples; Figure S1, Differentially expressed mRNAs between

imatinib-naïve and imatinib-resistant GIST samples; Figure S2, Top deregulated canonical pathways between imatinib-naïve and imatinib-resistant gastrointestinal stromal tumors; Figure S3, Quantitative RT-PCR validation of differentially expressed microRNAs and mRNAs in imatinib-naïve and imatinib-resistant gastrointestinal stromal tumors.

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# Chapter 6

## General Discussion & Summary

# Discussion

Cancer is globally one of the leading causes of death ranking second after cardiovascular disorders. As cancer is often diagnosed at a late stage, scientists and clinicians aim to define prevention and early detection methodologies. At the same time ways to reliable stratify patients and novel treatments strategies are being developed. These ongoing efforts will ultimately improve patient care. Soft tissue sarcomas (STS) comprise approximately 1% of all adult tumors and 10% of all pediatric cancers. STS are a group of rare and heterogeneous tumors, mainly of mesenchymal origin that currently include more than 50 different histological subtypes [1]. Roughly this group can be divided into three main categories; small blue round cell tumors (SBRCTs), gastrointestinal stromal tumors (GIST) and (adult) STS. Different STS have a different etiology, diverse genomic aberrations and different morphologies and clinical features. This and their rarity, makes STS a challenging group of tumors to study in the laboratory and treat in the clinic.

In particular for patients with advanced-non-GIST-STS where cure by surgery or radiotherapy is not possible, the prognosis is dismal. Chemotherapy is the only remaining treatment option for these patients. Despite the advent of several new treatment strategies such as the second-line treatment options, trabectedin for leiomyosarcomas and liposarcomas, pazopanib for non-adipocytic STS, and eribulin for liposarcomas [2], the survival of advanced non-GIST STS patients is often less than one year. Therefore, there is a pressing medical need to further improve the existing treatment strategies and to generate innovative and more effective therapies.

# Tumor biology

In order to meet the challenges outlined above, a better and more thorough understanding of STS tumor biology is indispensable. Modern molecular technologies have greatly advanced our knowledge of cancer with the identification of genes, including non-protein coding genes, and signaling pathways involved in carcinogenesis [3]. However, due to their rarity and heterogeneity, insight into the biology of STS is somewhat lagging behind.

MicroRNA (miRNA) expression profiling is one of the approaches that can be used to molecularly characterize tumors. MiRNAs are small (18-25 nucleotides in length) non-protein coding RNA molecules, which exert regulatory roles on the expression of various genes. Binding of miRNAs, facilitated by the RNA induced silencing complex, to the untranslated regions (3’ or 5’ UTR) or the open reading frame (ORF) of target mRNAs leads to mRNA degradation and/or translation inhibition [4-6]. Numerous studies have already reported the involvement of the de-regulated miRNAs in tumorigenesis and tumor progression in multiple cancer types. These miRNA molecules can act as tumor suppressors or oncogenes [7, 8]. The

molecular reasons why miRNAs are aberrantly expressed, how they interfere with biological processes and contribute to tumor biology are topics actively being studied in many cancer types. Currently miRNAs are used, or being used, as biomarkers for diagnostic, prognostic and predictive purposes. Another intriguing exploitation of miRNAs in the context of cancer is their use as therapeutic agents that is currently being investigated [9]. Using antisense oligonucleotides (anti-miRNAs) or miRNA mimics dysregulated miRNA expression levels can be normalized for therapeutic purposes.

In view of the importance of miRNAs in tumor biology, their involvement in sarcomagenesis, use as diagnostic and prognostic biomarker and their therapeutic potential, miRNAs are increasingly being studied in various STS entities [10].

# (I) Malignant peripheral nerve sheath tumors

One of the STS entities in which molecular characterization, including unraveling the role of miRNAs, hopefully will lead to better outcomes is the group of malignant peripheral nerve sheath tumors (MPNSTs). MPNSTs are aggressive and chemo-resistant STS with a propensity to metastasize. About half of these tumors arise from a benign counterpart named plexiform neurofibromas in neurofibromatosis type 1 (NF1) patients while the rest develops sporadically. Available treatment options for patients with unresectable and advanced disease include doxorubicin-based chemotherapy in the first line setting and pazopanib in the second-line [11, 12]. An overall poor response to chemotherapeutic regimens and the aggressive nature of these tumors, make their treatment challenging. This translates into poor prognosis and high mortality rates for MPNST patients. A better understanding is required of the biology of these rare tumors to more clearly define the molecular mechanisms driving tumorigenesis, which will subsequently aid in the development of novel and more effective therapies.

Topoisomerase 2-α (TOP2A) is a direct molecular target of doxorubicin. This drug forms the basis of the current first-line treatment for advanced MPNST patients. It is known that TOP2A is more abundantly expressed in MPNST than in the benign counterpart i.e. plexiform neurofibromas [13, 14]. Despite the high level of TOP2A, advanced MPNST patients do not respond well to doxorubicin and face a dismal prognosis with a 2 year overall survival of 20%. To improve the efficacy of doxorubicin one could adjust the doxorubicin dose relative to the expression level of TOP2A in a patient-specific manner. We therefore investigated the relation between TOP2A level and sensitivity for doxorubicin in a panel of MPNST cell lines [14]. To overcome the drug resistance displayed by MPNST we need to understand which resistance mechanisms underlie the observed drug insensitivity. In general this is not an easy task as drug resistance in cancer appears to be a multifactorial phenomenon with multiple resistance mechanisms operating at the same time. The involvement of several factors such as epigenetics, drug efflux, drug target alterations, cell death inhibition, cancer cell heterogeneity, etc. has made the understanding of the underlying resistance mechanisms and ways to overcome them challenging [15, 16].

We also investigated the expression of other potential targetable molecules in MPNST. A bromodomain family member (*BRD4*) [17] and a PRC2 core element (*EZH2*) [18, 19] were both reported to be up-regulated and the therapeutic use of inhibitors specific for BRD4 and EZH2 was suggested. In fact, specific small molecules targeting these proteins are being assessed in clinical trials for several cancer types [20-22], making BRD4 and EZH2 indeed potential drug targets in MPNST treatment. However, we could not confirm the upregulation of *BRD4* in human MPNST samples nor could we demonstrate that inhibition of the of BRD4 or EZH2 interfered with cellular proliferation in our MPNST cell line panel. On the basis of our findings, we concluded that the clinical benefits of using inhibitors against BRD4 and EZH2 individually for MPNST treatment, is limited.

The apparent discrepancy between our findings and former investigations [17-19] could be due to the utilized laboratory models (i.e. cell lines and more advanced genetically engineered mouse models), which may not be appropriate representatives of the tumors encountered in the patient. In a subsequent study to identify driver miRNAs in MPNST we have attempted to address this issue by examining paired human samples comparing plexiform neurofibromas and MPNST that arose in the same patient over time. Using paired samples may remove some of the interpatient heterogeneity encountered when comparing non-paired tumor samples. Our study revealed miRNAs that were differentially expressed between plexiform neurofibroma and MPNST samples. When we functionally characterized selected miRNAs using an MPNST cell line panel we noted that not all cell lines responded equally to miRNA modulation. After ruling out technical explanations we concluded that these observations may be due to tumor heterogeneity that affects the way and intensity with which MPNST cells respond to miRNAs (**Chapter 3**).

Due to the rarity of MPNST its genomic landscape is incompletely known [3]. Of note, in about 60% of NF1-associated MPNST mutations and deletions are observed in SUZ12 or EED [23-25]. These proteins are core components of the PRC2 complex, which is an epigenetic regulator. The genomic aberrations in MPNST cause inactivation of the PRC2 complex thereby affecting the gene expression profile.

The observations made in our two studies regarding MPNST [14]( **Chapter 3**) highlight the importance of incorporating relevant laboratory models for both exploratory and confirmatory aspects of the pre-clinical studies. In addition, sample sizes must be able to capture inter- and intratumoral heterogeneity present and appropriate controls must be included in the study. This is not always evident when working with rare and relatively ill-defined tumors such as MPNST. Limitations in the available pre-clinical tools are considered a challenge in obtaining reproducible and reliable research findings in oncology, which is a major concern and has been addressed repeatedly [26, 27]. As patients are ultimately the main focus of all efforts within cancer research, investigators must consider and include robust and representative pre-clinical tools in order to make reproducible discoveries. This will improve the translation of laboratory findings into the clinic.

## (II) Gastrointestinal stromal tumors

Gastrointestinal stromal tumors (GIST) are rare tumors of the gastrointestinal tract predominantly associated with the stomach (~60%) or small intestine (~30%) [28]. Activating mutations in KIT are recognized as one of the most common and crucial events in GIST pathogenesis [29]. On the clinical level, imatinib mesylate (a drug targeting KIT) has dramatically improved the outcome of advanced patients from a median overall survival (OS) of 9 months before the drug treatment to 5 years [30] and a progression-free survival (PFS) of about 2 years with a response rate of approximately 50% [31]. The GIST patient's response to imatinib treatment is known to be correlated with the mutational status of the KIT gene. For instance those patients harboring a KIT exon 9 mutant are more likely to show progression within 6 months of the initial imatinib treatment than patients with other mutations. [32, 33]. Although, most patients respond favorable to imatinib, the vast majority of patients eventually develop resistance leading to tumor progression. Despite second and third line options such as sunitinib and regorafenib, the prognosis for advanced GIST patients who failed to imatinib remains poor. Therefore, to overcome drug resistance one needs to decipher and understand the underlying resistance mechanisms.

In GIST – similar to the situation encountered in many other cancers – miRNAs are intricately involved in the process of tumorigenesis. Furthermore miRNAs expression profiles can be used to classify stage of the tumors, to identify tumor location, and indicate GIST mutational status (**Chapter 5**). Also miRNAs have been implicated as therapeutic agents as their modulation interfered with GIST cell line proliferation and induced apoptosis or re-sensitized imatinib resistant (IM-r) tumors. For instance, *in vitro* inhibition of the expression level of miR-125-5p increased the sensitivity of GIST882R (IM-r) cells for imatinib [34]. In this context, in 2013, our group examined the therapeutic potential of restoring the expression of the dysregulated miR-221/222 and miR-17-92 clusters. The re-expression of downregulated miR-17, mir-20a and miR-222 in GIST cells affected cellular proliferation, induced apoptosis through targeting c-KIT and ETV1 [35].

In the context of imatinib-resistance in GIST we molecularly analyzed 53 fresh frozen GIST samples derived from imatinib-naïve (n=33) and imatinib-resistant (n=20) GISTs. We identified differentially expressed genes and miRNAs and performed an Ingenuity Pathway (IP) Analysis to reveal gene-miRNA interaction networks associated with acquired imatinib-resistance. At least three miRNAs (miR-92a-3p; miR-99a; miR-101-3p) were highlighted that directly or indirectly affect the expression of cell cycle regulators. In addition a number of genes were revealed, some of which targetable by small molecules, that appeared to fulfil hub-like function within the gene-gene and gene-miRNA interaction networks. In follow-up experiments using imatinib-sensitive and imatinib-resistant cell line and PDX models, it should be experimentally verified whether miRNA and gene modulation influences imatinib sensitivity.

# Challenges in soft tissue sarcoma pre-clinical studies

Many pre-clinical studies have focused on the elucidation of biological processes that drive STS tumorigenesis. However, potential effective treatment strategies coming forth from these pre-clinical studies could not be easily translated to the clinic and/or have not yet reached clinical routine. Many different factors contribute to this unwanted gap between laboratory and clinic.

## Tumor heterogeneity

One of the hallmarks of cancer is genomic instability [36] which leads to intra- and intertumoral diversity within and between individuals. Therefore, a single biopsy of a lesion does probably not fully capture the heterogeneity present in a patient's cancer. To overcome this hurdle, biopsies should be taken from multiple lesions in a patient and each lesion should be biopsied more than once. Ideally, but this may be difficult to implement in the clinical setting, biopsies should be taken at multiple time-points during the disease. This will enhance the precision and provide a better reflection of the heterogeneity within a single lesion and between lesions in the same patient. This holds true for GIST patients as often the complex nature of this tumor is not adequately reflected in the currently available pre-clinical studies due to the use of a single biopsy of one lesion. Despite the rarity, multiple primary GIST (MPG) harboring different KIT/*PDGFR* mutations within one individual have been detected. These mutational diversities have substantial impact on defining suitable treatment profile for the patient [37].

Moreover, variation across patients diagnosed with the same tumor type also makes investigations on tumor biology and the subsequent discovery of effective/novel therapeutic molecules challenging. Available animal models used for pre-clinical research are known to mainly carry a combination of limited genetic aberrations presented in a subset of tumors and may not represent the full genomic complexity present in tumors. To elaborate further on this statement, the available MPNST xenografts animal models most likely represent very late stages of MPNST only, whereas genetically engineers animal models for MPNST usually only reproduce part of the malignant transformation process as it occurs in patients [38]. In NF1 patients MPNSTs arise from plexiform neurofibromas via a distinct intermediary form called atypical neurofibromas. To our knowledge no genetically engineered animal model faithfully reproduces this sequence of events. This could be due to the absence of crucial mutations in these models, necessary for tumorigenesis in humans. As an example, the loss of SUZ12 in a subset of MPNSTs [23], which influences the obtained outcome from (pre)clinical studies, is among the vital mutations being neglected. SUZ12 is an essential element of the polycomb repressive complex 2 (PRC2), which is a well-known complex involved in epigenetic modulation of gene expression [39]. Inactivation of PRC2 upon SUZ12 loss will result in aberrant genes expressions and ultimately diversities in the (pre)clinical outcomes. On the

basis of these findings, in our effort to functionally characterize the chosen differentially expressed miRNA candidates, we detected that distinct NF-associated MPNST cell lines respond with diverse intensities or in a different fashion to miRNAs modulations. It could be that the MPNST cells lines in our cell line panel differ at a molecular level, perhaps due to variable chromosomal copy number alterations as frequently observer in MPNST. Moreover, our inability to recapitulate outcomes obtained from prior studies could also be explained by the existence of this genetic diversity (**Chapter 2**). For example, the discrepancy in validating the outcome of the expression level of *BRD4* and *in vitro* modulation of this potential drug-target upon JQ1 induction were among the challenges we faced [14].

In addition to the above-mentioned hurdles, the tumor micro-environment may also significantly contribute to heterogeneity and affect the response to clinical interventions. Of importance are also processes like hypoxia and inflammation that modulate the tumor micro-environment. Therefore, to obtain translatable outcomes from pre-clinical oncogenomic studies, which result in significant patient benefit, the use of patient-specific (personalized) model in laboratories is needed. These models can enhance our knowledge on individual needs and will introduce new possibilities in diverse field of regenerative medicine/healthcare.

# Future perspective

Despite the wide spectrum of studies being conducted on STS, these tumor types still remain a major challenge to medical oncologists and research scientists. The genomics of STS remain understudies therefore the development of STS, particularly the underlying genomic aberrations, are not thoroughly understood, which poses difficulties in obtaining successful treatment outcome. Thus, extra focus the mechanisms underlying disease onset and progression is necessary. To this end, utilizing reliable laboratory models for generating reproducible and translatable findings for the clinic is strongly warranted. However, as more has been learned from the use of the currently available models, the relevance and reliability of such models are extensively debated [26, 27].

## (I) Induced pluripotent stem cells

### *Pro's*

The recently developed induced pluripotent (iPS) technology can not only be utilized for disease modeling purposes [40-42] but is also as an attractive technique to create new pre-clinical models for cancer [43, 44] (Fig.1). First, induced pluripotent stem cells (iPSCs) can be derived from normal cells or patients cells e.g. skin fibroblasts from NF1 patients. Subsequently cancer-specific genomic aberrations ca be introduced e.g. by CRIPS-CAS9 and their effects studied at a molecular level by differentiating the cells towards the cell type in which the cancer phenotype will be fully revealed. Alternatively, iPSCs can be generated from cancer cells. It is likely that tumorigenicity is lost upon reprogramming and will reappear at a

certain cell-stage during differentiation when the tissue-specific epigenetic pattern allows the already present cancerous mutations in oncogenes and/or tumor suppressors to exert their effect. This process gives insights into the complex genetic aberrations from the initial phases of disease onset to a full-blown cancer. It will not only facilitate understanding tumor biology but will help scientist and clinicians to find reliable treatment approached. Therefore, cancer-derived iPSCs can be differentiated (*in vitro* & *in vivo*) towards the cell lineage the cancer originated from, enabling researches to probe the biology of tumor initiation, early progression and metastasis of human tumors. This approach can lead to the discovery of the molecular networks underlying tumor initiation and tumor progression, the detection of novel biomarkers indicating the early stages of cancer development and gives the possibility for high-throughput drug screens. As well, the recent advance in CRISPR-CAS9 genome editing technology adds another dimension to the use of iPSCs as treatment for human disease [45].

### *Con's*

Although, despite the great advances in the use of patient-derived iPSCs as cancer models, it is worth noting that also this technology is far from perfect due to genomic instabilities that are introduced during reprogramming, the heterogeneity in differentiation potency of iPSCs (transcriptional and epigenetic variabilities), challenging reprogramming and differentiation approaches for modeling cancer cells, and difficulties in monitoring tumorigenesis upon iPSC-derived cancer cells re-differentiation (*in vitro* or *in vivo*). Moreover, these models do not fully represent the tumor microenvironment. Some might suggest the injection of patient-derived iPSCs in an animal model to compensate for the absence of tumor microenvironment in 2D culture; however, the cross-species and biological variabilities in the process of tumorigenesis between animals and human should not be overlooked [46, 47].

## (II) Organoids & spheroids

### *Pro's*

To compensate for many of these deficiencies, other approaches such as tumor organoid and spheroid models are also among the new promising pre-clinical modeling systems [48]. For instance, one of the substantial advantages of using 3D culture systems over the traditional 2D monolayer cultures, is the feasibility of co-culturing/incubating patient-derived organoids with immune cell suspensions (ideally derived from the same patient) [49]. As inflammation is among the major causes of complex tumorigenesis, this approach will provide a more robust pre-clinical model for investigating inflammation-related carcinogenesis mechanisms as well will mimic patient-specific immune responses upon applying immunotherapeutic anti-cancer drugs. This model simulates the complex nature of cancer and its relation with the microenvironment. In addition, to having the capability to introduce components of the tumor microenvironment, the study of drug penetration and cell-cell interaction are also an asset of organoid and spheroid models.

### *Con's*

Similar to iPS technology, developing patient-specific organoid models also face challenges as for instance these models lack the ability to fully capture tumor microenvironment and its biological features. The interaction between the tumor cells and surrounding stromal cells, as well as cells from blood and lymphatic vessels are known to be crucial for immuno-therapy [50]. Therefore more effort in optimizing, improving and developing optimal growing conditions for these 3D models are expected in the future.

# (III) Tumor material

Apart from the aforementioned difficulties within cancer research, in sarcoma studies acquiring enough materials for developing novel pre-clinical models requires a considerable amount of effort due to the rarity of these tumors. Often affected individuals are treated across various hospitals as a consequence of which there is limited accessibility to tumor materials for research purposes. Therefore, to collect sufficient high-quality materials, a closer cooperation between various (inter)national sarcoma centers of expertise as well as scientists and clinicians is strongly required. This will aid and accelerate the accessibility to a substantial collection of samples. Moreover, more global efforts need to be made in order to expand an online data-base from standardized data reports of the available pre-clinical STS models. This will facilitate the access to basic and translational cancer research as well and will optimize the exchange of information.

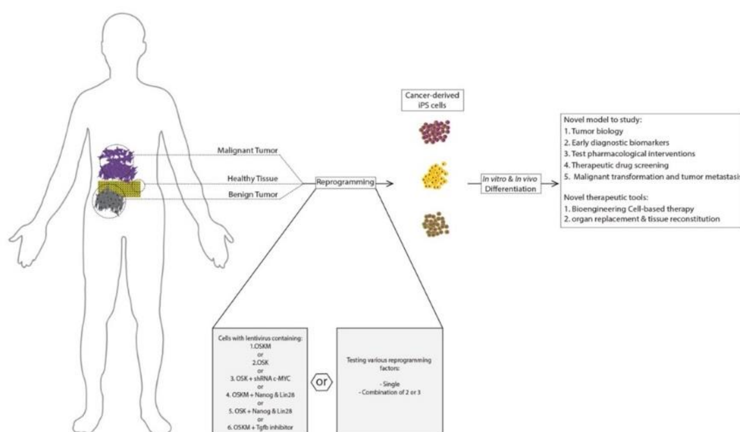


**Figure 1 – Potential of cancer-derived iPSCs in research and clinic.** Cancer cells(benign & malignant) may be reprogrammed to induced pluripotent stem cells (iPSC) using the appropriate combination of reprogramming factors. These iPSCs can be differentiated along the developmental lineage they arose from, enabling scientists to study the different stages of tumorigenesis, identify early diagnostic biomarkers and perform drug screens. Cells derived from healthy tissues and, if available, benign counterparts are the controls for the experimental procedures.

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# Summary

Soft tissue sarcomas (STS) are a rare and heterogenous group of cancers of predominantly mesenchymal origin. More than 50 subtypes are recognized mainly on basis of distinct morphological and pathological features. STS include local aggressive and malignant tumors arising in or from connective tissues such as synovial tissue, fat, muscle, peripheral nerves and fibrous or related tissues. Together the STS comprise about 1-2% of all adult malignancies and about 10% of all childhood malignancies.

Treatment modalities of STS are surgery, radiotherapy and chemotherapy. Systemic chemotherapy is usually offered to patients with advanced, not amendable for curative local treatments and metastasized disease and is usually based on doxorubicin or ifosfamide in combination with docetaxel or gemcitabine. Prognosis varies and depends on histological subtype, tumor size, tumor grade and location. Despite the recent introduction of novel systemic treatments with drugs like pazopanib and trabectedin the outcome for patients with advanced disease remains poor with a median overall survival of approximately 12 months stressing the need for novel therapeutic approaches.

The research described in this thesis concerns two STS subtypes, malignant peripheral nerve sheath tumors (MPNST) and gastrointestinal stromal tumors (GIST). The overall aim was to gain a better understanding of the biology of these tumors in order to explain their clinical behaviour and ultimately to improve treatment of these malignancies. In this context, special emphasis was given to a specific class of small non-coding RNAs i.e. microRNAs (miRNAs). These miRNAs capable of regulating gene expression, are commonly found dysregulated in cancer and fulfil essential roles in carcinogenic processes. Functional characterization of aberrantly expressed miRNAs in cancer may reveal interesting biology. Further, miRNAs can be exploited as diagnostic, prognostic or predictive biomarker and may have therapeutic potential.

MPNST are highly aggressive cancers that occur spontaneously or arise from benign plexiform neurofibromas in the context of neurofibromatosis type 1 (NF1) with the latter process of malignant transformation, via an intermediary atypical neurofibroma, poorly understood. MPNST are prone to metastasize, have high local recurrence rates and are relatively resistant to therapeutic intervention. Metastatic MPNST almost always proves fatal. Novel, more effective treatment strategies are urgently needed. In **Chapter 2** we examined known, topoisomerase IIα (*TOP2A)*, and new drug targets, bromodomain containing protein 4 (*BRD4)* and zeste homolog 2 (*EZH2*). The expression of these genes was studied in plexiform and atypical neurofibromas, NF1-derived MPNST tissue samples and MPNST cell lines. We subsequently investigated whether expression levels were associated with sensitivity to specific pharmacological or genetic inhibitors of these genes. We showed that *BRD4* transcript levels were not upregulated in MPNST compared to plexiform and atypical neurofibromas and that consequently MPNST cell lines were relatively insensitive to the bromodomain inhibitor

JQ1. Although *EZH2* levels were consistently found upregulated in MPNST, genetic knockdown of *EZH2* did not interfere with cellular proliferation and cell viability. It was verified that *TOP2A* is overexpressed in MPNST samples and that a relative high expression of TOP2A in MPNST cell lines correlates to sensitivity to doxorubicin. It was concluded that therapeutic effects of targeting of BRD4, EZH2 and TOP2A, individually, may be limited. **Chapter 3** focussed on the biology of MPNST particularly the involvement of miRNAs in cancer-related processes. A unique set of paired plexiform neurofibroma – MPNST samples, with each pair of tumors derived from the same patient, was used in miRNA expression profiling studies. Ninety miRNAs were found differentially expressed between MPNST and plexiform neurofibromas. Three downregulated (let-7b-5p; miR-145-5p and miR-143-3p) and two upregulated (miR-135b-5p and miR-889-3p) miRNAs in MPNST were selected for further validation and functional characterization in additional neurofibroma and MPNST samples and cell lines. Using *in vitro* experiments in which miRNA levels were transiently modulated it was established that the selected miRNAs generally did not interfere with cellular proliferation of MPNST cells. However, some miRNAs did affect the migratory and invasive capabilities, surrogates for metastasis, and Wnt signaling activity of MPNST cells but the effects differed depending on the cell line used. It was concluded that dysregulated miRNAs fulfil key roles in MPNST development and progression although in a cell context dependent fashion.

The next two chapters deal with GIST. These tumors are found along the gastrointestinal tract and are believed to originate from the interstitial cells of Cajal, pacemaker cells responsible for the peristaltic movement. At a molecular level the majority of these tumors are characterized by mutually exclusive activating mutations in the tyrosine kinase receptors KIT and PDGFRA that drive the pathogenesis. The tyrosine kinase inhibitor imatinib plays a central role in the treatment of both localized and advanced GIST. Unfortunately, most patients eventually become resistant this drug and present with progressive disease. In **Chapter 4** GIST was briefly introduced including its current treatment and the clinical needs regarding its management. In addition, different relevant classes of non-coding RNAs were presented. Next, the current literature on non-coding RNAs, with an emphasis on miRNAs, that are expressed in GIST and can be linked to distinct clinicopathological features like risk classification, imatinib resistance, metastasis, was reviewed. The need for and potential clinical use of non-coding RNAs as biomarkers was critically evaluated. Finally, certain non-coding RNAs whose expression was deregulated in GIST and that played key roles in GIST biology were discussed in the light of their therapeutic potential. **Chapter 5** addressed the phenomenon of imatinib-resistance as observed in GIST. Our analyses highlighted miRNAs and mRNAs that were differentially expressed in imatinib-resistant GISTs compared to imatinib-naïve GISTs. At least thirty-five miRNAs and 352 mRNAs were identified in this manner and used as input for further pathway and network analyses that highlighted cell cycle related genes/gene networks in imatinib-resistant GIST. Some overexpressed proteins e.g. AURKA, AURKB and FOXM1 in the resistant setting were considered amenable for inhibition by small molecules and three miRNAs, miR-92a-3p, miR-99a-5p and miR-101-3p were indicated as potential effectors of imatinib resistance. We concluded that further experimental *in vitro* and *in vivo* studies are necessary to further substantiate and validate our findings.

# Samenvatting

Weke delen tumoren (sarcomen) vormen een zeldzame en heterogene groep kankers van mesenchymale origine. Op basis van morfologische en pathologische kenmerken worden meer dan 50 verschillende subtypen onderscheiden. De groep sarcomen bevat locaal agressieve en maligne tumoren die onstaan in of uit bindweefsel zoals synoviaal weefsel, vet, spier, perifere zenuwen en fibreus of gerelateerde weefsel. Samen omvatten de weke delen tumoren ongeveer 1 a 2% van alle maligniteiten bij volwassenen en circa 10% van de maligniteiten die bij kinderen voorkomen.

Belangrijk voor de behandeling van sarcomen zijn chirurgie, radiotherapie en chemotherapie. Systemische chemotherapie wordt gewoonlijk gegeven aan patiënten met gevorderde of gemetastatseerde ziekte die niet meer in aanmerking komen voor een locaal curatieve behandeling. De chemotherapie is veelal gebaseerd op de middelen doxorubicine of ifosfamide in combinatie met docetaxel of gemcitabine. De prognose voor de patiënt varieert en is afhankelijk van het precieze sarcoom subtype, de tumor grootte, tumor gradering en de locatie van de tumor. Ondanks de recente introductie van nieuwe antikanker middelen, zoals pazopanib en trabectedine, blijft de uitkomst voor patiënten met gevorderde ziekte slecht met een mediane overleving van ongeveer 12 maanden. Dit benadrukt de noodzaak van de ontwikkeling van nieuwe therapeutische strategieën.

Het onderzoek beschreven in dit proefschrift richt zich op twee verschillende typen sarcomen, kwaadaardige perifere zenuwschede tumoren (MPNST) en gastrointestinale stromale tumoren (GIST). Het doel was om een beter begrip te krijgen van de biologie van deze tumoren om zo hun klinische gedrag te kunnen verklaren en uiteindelijk te komen tot een betere behandeling van deze kankers. Speciale aandacht ging uit naar een specifieke klasse niet-coderende RNAs, de microRNAs (miRNAs). miRNAs zijn in staat genexpressie te reguleren, komen in kankers verstoord tot expressie en kunnen een essentiële rol vervullen in kankerontwikkeling en progressie. De functionele karakterisering van microRNAs in kanker kan interessante biologie onthullen. Verder kunnen miRNAs worden gebruikt als diagnostische, prognostische of predictieve biomarker en hebben ze mogelijk therapeutisch potentieel.

MPNST zijn agressieve tumoren die spontaan kunnen ontstaan of uit benigne plexiforme neurofibromen in neurofibromatose type 1 (NF1) patiënten. Het proces dat ten grondslag ligt aan deze maligne transitie, dat verloopt via intermediaire atypische neurofibromen, wordt niet goed begrepen. MPNST metastaseert en recidiveert makkelijk en is relatief ongevoelig voor chemotherapie. Gemetastaseerde MPNST zijn bijna altijd dodelijk. Nieuwe en effectievere behandel mogelijkheden zijn nodig. In **Hoofdstuk 2** onderzochten we bekende en nieuwe therapie aangrijpingspunten zoals topoisomerase II alpha (TOP2A), het bromodomein bevattend eiwit 4 (BRD4) en zeste homoloog 2 (EZH2). De expressie van deze genen werd bestudeerd in plexiform- en atypisch neurofibroom, NF1- gerelateerde MPNST en MPNST cellijnen. Vervolgens werd bepaald of de expressie geassocieerd was met gevoeligheid voor

specifieke farmacologische of genetische remmers. Aangetoond werd dat *BRD4* mRNA niveaus niet verhoogd waren in MPNST in vergelijking tot plexiforme- en atypische neurofibromen en dat MPNST cellijnen relatief ongevoelig waren voor de bromodomein remmer JQ1. Hoewel *EZH2* mRNA niveaus consequent verhoogd waren in MPNST, interfereerde een verlaging van EZH2 eiwit niet met cel proliferatie en cel viabiliteit. Het werd geverifieerd dat *TOP2A* verhoogd tot expressie komt in MPNST en dat een relatief hoog TOP2A niveau in cellijnen correleert met een gevoeligheid voor doxorubicine. Uit deze studie werd geconcludeerd dat het therapeutische effect van individuele BRD4, EZH2 en TOP2A remming gering is. **Hoofdstuk 3** richtte zich op de biologie van MPNST, in het bijzonder de betrokkenheid van miRNAs bij kanker-gerelateerde processen. Hiertoe werd het miRNA expressie profiel bepaald van een unieke set gepaarde plexiform neurofibroom - MPNST monsters, waarbij elk afzonderlijk paar tumoren afkomstig is van een zelfde NF1 patiënt. Negentig miRNAs kwamen differentieel tot expressie in MPNST en plexiforme neurofibromen. Drie miRNAs (let-7b-5p; miR-145-5p en miR-143-3p) die verlaagd tot expressie kwamen in MPNST en twee miRNAs (miR-135b-5p en miR-889-3p) die verhoogd waren, werden geselecteerd voor verdere validatie en functionele karakterisering in aanvullende neurofibromen en MPNST weefsels en cellijnen. Gebruik makend van *in vitro* experimenten waarin miRNA niveaus tijdelijk werden verhoogd of verlaagd werd vastgesteld dat de geselecteerde miRNAs de cel proliferatie van MPNST cellen niet beïnvloeden. Daarentegen, hadden sommige miRNAs wel een effect op de migratie en invasie van MPNST cellen, beide surrogaat processen voor metastasering, echter de effecten waren niet in alle cellijnen hetzelfde. Ook werd de activiteit van het Wnt signalerings pad door miRNAs beïnvloed. Geconcludeerd werd dat tenminste een aantal miRNAs een belangrijke rol spelen bij MPNST ontwikkeling en progressie maar op een cel context afhankelijke wijze.

De volgende twee hoofdstukken behandelen ons onderzoek van GIST. Deze tumoren worden gevonden langs het gastrointestinale stelsel en worden verondersteld te ontstaan uit de interstitiële cellen van Cajal; pacemaker cellen verantwoordelijk voor de peristaltiek. Op moleculair niveau wordt de meerderheid van deze tumoren gekarakteriseerd door activerende mutaties in de tyrosine kinase receptoren KIT of PDGFRA die ten grondslag liggen aan de pathogenese. De tyrosine kinase remmer imatinib vervult een centrale rol in de behandeling van zowel locaal als gevorderde GIST. Deze meeste patiënten ontwikkelen na verloop van tijd resistentie voor imatinib dat leidt tot tumor progressie. GIST werd bondig geïntroduceerd in **Hoofdstuk 4** waarin ook de huidige behandeling van deze tumoren en de klinische behoeften voor verdere optimalisatie van de behandeling werden meegenomen. Verder werden verschillende, relevante klassen niet-coderende RNAs besproken. Vervolgens werd de wetenschappelijke literatuur handelend over niet-coderende RNAs, met de nadruk op miRNAs, in GIST systematisch doorgenomen met daarbij vooral aandacht voor niet-coderende RNAs die zijn geassocieerd met klinische- en pathologische kenmerken zoals risico evaluatie, imatinib resistentie en metastasering. De behoefte aan, en potentieel gebruik van, niet-coderende RNAs als biomarker in de klinische praktijk werden kritisch bediscussieerd. Ten slotte werd het therapeutische potentieel van niet-coderende RNAs beschouwd met name van RNAs die aberrant tot expressie komen en die een sleutelrol vervullen in de GIST biologie. Het fenomeen imatinib resistentie zoals dat wordt waargenomen in GIST werd in **Hoofdstuk 5** onderzocht. Onze analyses identificeerden miRNAs en mRNAs die differentieel tot expressie komen in imatinib-naïve en imatinib-resistentie GIST. Tenminste 35 miRNAs en 352 mRNAs

werden gedetecteerd en gebruikt als input voor biochemische pad en netwerk analyses. In imatinib-resistente GIST werden celcyclus genen/gen netwerken gedetecteerd. Sommige van de verhoogd tot expressie komende genen in imatinib-resistente GIST zoals *AURKA*, *AURKB* en *FOXM1* kunnen mogelijk worden geremd met specifieke doelgerichte medicijnen. Verder werd een drietal miRNAs, miR-92a-3p, miR-99a-5p en miR-101-3p, in verband gebracht met imatinib-resistentie. Geconcludeerd werd dat verdere experimentele *in vitro* en *in vivo* studies noodzakelijk zijn om de bevindingen verder te valideren en te onderbouwen

# Word of Thanks

''I have missed more than 9000 shots in my career. I have lost almost 300 games. Twenty-six times I have been trusted to take the game winning shot and missed. I have failed over and over and over again in my life and that is why I succeed''. –Michael Jordan

And that is how I would like to close the most challenging chapter of my life. Being able to finalize this period successfully would have not been attainable without the support of many people, who I would like to express my sincere and deepest gratitude to.

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ارومی و گرم محیط شما حمایت همیشه و نکردم نگرانی احساس هیچوقت زندگیم طول
،کردین من برای زندگی در که فداکاریهایی و ها حمایت تمام برای .کرد فراهم برام رو

مرحله این به تونستم نمی وجه هیچ به من ،شما پشتیبانی و حمایت بدون .ممنونم نهایت بی
.شمایین زندیگیم نعمت بزرگترین ،هستین مادرم و پدر که کنم می شکر همیشه .برسم
بتونم امیدوارم و ،هست و بوده من الگوی همیشه هردوتون اکادمی و شخصی زندگی
.بگیرم پیش خوب رو شما زندگی روش

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ی زندگی، تن و توانم همه تو

جانی و دلی ای دل و جانم همه تو

تو هستی من شدی از انی همه من

من نیست شدم در تو... از انم همه تو

# List of publications

1. **Azadeh Amirnasr**, Stefan Sleijfer, Erik A.C. Wiemer, Non-coding RNAs, a novel paradigm for the management of gastrointestinal stromal tumors. *International Journal of Molecular Sciences.* 2020; 21(18): 6975.

2. **Azadeh Amirnasr**, Rob M. Verdijk, Patricia F. van Kuijk, Walter Taal, Stefan Sleijfer, Erik A.C. Wiemer, Expression and inhibition of BRD4, EZH2 and TOP2A in neurofibromas and malignant peripheral nerve sheath tumors. *PLoS One.* 2017; 12(8): e0183155.

3. **Azadeh Amirnasr**, Robert M. Verdijk , Patricia F. van Kuijk, Pinar Kartal, Anne L.M. Vriends, Pim J. French, Martin E. van Royen, Walter Taal, Stefan Sleijfer, Erik A.C. Wiemer. Deregulated microRNAs in neurofibromatosis type 1 derived malignant peripheral nerve sheath tumors. *Scientific Reports*. 2020; 10: 2927.

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**General Courses:**

| Course | Year | ECTS |
|---|---|---|
| Biomedical Research Techniques | 2014 | 1.5 |
| Partek course on Microarrays and NGS | 2015 | 1.3 |
| Ensemble Gene Browsing workshop | 2015 | 0.6 |
| Basic and Translational Oncology | 2015 | 1.8 |
| The workshop writing successful grant proposal | 2016 | 0.5 |
| Research Integrity | 2017 | 0.5 |
| Biomedical Scientific English Writing | 2017 | 2.0 |
| SPSS | 2017 | 1.0 |
| R (statistical package) | 2017 | 2.0 |
| Programing with Python | 2017 | 1.2 |

**Specific Courses:**

| Course | Year | ECTS |
|---|---|---|
| Genetics course (MGC PhD) | 2015 | 1.0 |

**(Inter)national conferences/meetings:**

| Conferences/meetings | Year | ECTS |
|---|---|---|
| Nederlandse Sarcomenstudiedag (NKI) | 2014 | 0.5 |
| Molecular Medicine day (2X) (Rotterdam) | 2015-2016 | 1.0 |
| Winter School for the Collaborative Research Center TRR81 (Austria) | 2015 | 2.0 |
| MGC workshop (2x) (Netherlands, Germany) | 2015-2016 | 2.0 |
| American Association for Cancer Research (2X) (USA) | 2017-2018 | 0.5 |
| Innovation for Health (Rotterdam) | 2018 | 1.0 |
| JNI meetings (EMC) | 2014-2018 | 5.0 |
| Medical Oncology Department meetings (EMC) | 2015-2018 | 3.0 |
| Sarcoma meeting (Daniel Den Hoed) | 2016-2018 | 2.0 |

**Posters and Presentations:**

| Presentations | Year | ECTS |
|---|---|---|
| Nederlandse Sarcomenstudiedag (Oral) | 2014 | 0.1 |
| Molecular Medicine day (Poster) | 2015 | 0.3 |
| Winter School for the Collaborative Research Center TRR81 (Oral) | 2015 | 0.3 |
| MGC workshop (Poster) | 2015 | 0.3 |
| MGC workshop (Oral) | 2016 | 0.3 |
| American Association for Cancer Research (Poster) | 2017-2018 | 0.5 |
| Innovation for Health (Poster and Pitch) | 2018 | 0.5 |
| JNI meetings (Oral) | 2015-2018 | 1.0 |
| Medical Oncology Department meetings (Oral) | 2016-2017 | 0.5 |
| Sarcoma meeting (Oral) | 2016-2017 | 0.5 |

**Supervision:**

| Project | Year | ECTS |
| --- | --- | --- |
| MicroRNAs in STS (first year master student for 6 months) | 2017 | 15.0 |
| MicroRNA in MPNST (second year master student for 6 months) | 2018 | 15.0 |

**Other:**

| Event | Year | ECTS |
| --- | --- | --- |
| Organizing PhD day (Committee member for the ‘’healthy PhD’’) | 2018 | 1.0 |

# Curriculum Vitae

Azadeh (Azi) Amirnasr was born in Babol (Mazandaran), Iran, on September 1989. After receiving high school diploma from Isfahan University of Technology (IUT), she got accepted to Shahid Beheshti University of Tehran (SBUT) to pursue her Bachelor of Science degree in microbiology. During the second year of her education at SBUT, she moved to Isfahan to follow her BSc at Isfahan University. During this period she had the chance to join a biochemistry lab in the United states (UC Santa Barbara) and gain some experiences by studying the effective function of Proline-Rich protein 4 (PRP4) on the growth of medicago truncatula. After returning back to Iran, she continued her BSc education and participated in multiple research projects at the University of Isfahan. Finishing her BSc in Iran in 2012, she moved to the Netherlands, same year, to pursue her scientific education. She got admitted to Molecular Medicine master program, which is a two years research program at Erasmus Medical Center, Rotterdam. In September 2014, she received her master diploma and in November she started her PhD in the lab of Dr. Erik Wiemer, at the department of Medical Oncology, Erasmus Medical Center. During her PhD training, she focused on the role of miRNAs in the tumorigenesis of soft tissue sarcomas.