

Genetic basis of tumorigenesis in NF1 malignant peripheral nerve sheath tumors

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1. ABSTRACT

Malignant peripheral nerve sheath tumors (MPNSTs), often found associated with neurofibromatosis type 1 (NF1), are aggressive tumors that pose significant diagnostic and therapeutic challenges. About 10% of NF1 patients may develop an MPNST, exhibiting a poor prognosis. With no effective treatment available, radical surgery and chemo- and radiotherapy are required to reduce tumor recurrence, metastasis and prolong patient survival. MPNST pathogenesis is poorly understood due mainly to its complex histopathology, but biallelic *NF1* gene inactivation is essential for tumor development. There is also no defined molecular signature for MPNST development, although several cell-cycle and signalling regulation genes (*CDKN2A*, *TP53*, *RBI*, *EGFR*, *CD44*, *PDGFR*, *PDGFRA*, *HGF*, *MET* and *SOX9*) are deregulated. Constitutive activation of several critical cell signalling cascades also occurs in MPNSTs and these may define therapeutic targets. Both preclinical and clinical trials are proposed, most involving a combinatorial therapeutic approach. Multidisciplinary collaborative efforts are clearly essential to fully decipher both the complex molecular basis of MPNST development and to define potential therapeutic targets.

2. INTRODUCTION

Neurofibromatosis type 1 (NF1), a familial tumor predisposition syndrome, is characterised by the growth of benign and malignant tumors involving the peripheral and central nervous system (1,2). NF1 results from inactivating germline mutations of the *NF1* gene located at 17q11.2. Most NF1 patients develop multiple benign cutaneous neurofibromas, with some 30-50% of patients also developing larger plexiform neurofibromas (PNFs), and about 5-10% of patients eventually developing a malignant peripheral nerve sheath tumor (MPNST) in later life. Half of all MPNSTs diagnosed occur in association with NF1, with affected patients exhibiting a poor prognosis. Cutaneous neurofibromas develop as discrete outgrowths from nerve endings in the skin, while the larger PNFs usually develop along major nerve trunks, often involving multiple fascicles and nerve branches. NF1-associated MPNSTs (NF1-MPNSTs) usually develop in association with a pre-existing PNF or a focal subcutaneous neurofibroma, and represent the malignant transformation of benign tumor cells (3). Patients with multiple internal PNFs, identified by a whole-body MRI scan, exhibit a 20-fold increased risk of MPNST development, underlining the need for regular clinical surveillance in such individuals

(4). Sporadic MPNSTs not associated with NF1 appear to be *de novo* malignant tumors (5). Many MPNSTs are not diagnosed until they reach an advanced stage, or when primary tumors have metastasised, most commonly affecting the lungs, and less frequently the liver and brain.

The current diagnosis and clinical management of NF1-MPNSTs is unsatisfactory underlining the need to better define the causative molecular changes driving tumor formation and to use this information to identify potential therapeutic targets. This review elucidates our current knowledge of molecular basis of NF1-MPNST development, attempts to identify some of the possible key players and finally discusses some recent developments in potential therapies.

3. NF1-ASSOCIATED MPNST

3.1. Frequency and survival rate

MPNSTs represent about 10% of all soft tissue sarcomas, with 50% occurring in association with NF1. These highly aggressive malignant tumors cause significant morbidity and mortality in the 5-10% of NF1 patients affected. These tumors occur with an annual incidence of 0.16% in NF1 compared to only 0.001% in the normal population, and with a lifetime risk of 8-13% in NF1 individuals (3,6,7,8). NF1-MPNSTs usually develop in the second or third decade of life, may be present at birth and in infants (9), while sporadic MPNSTs occur only later in life, and multiple primary MPNSTs are rarely observed (5, 6). The five year survival rate for NF1-MPNST patients is less than half of that for sporadic cases (21% vs. 42% (5,7), with the survival rate often related to the grade and size of the tumor at diagnosis, a failure to radically remove all tumor cells and surrounding tissues during surgery resulting in frequent tumor recurrence and metastasis (6,8). MPNSTs most often develop on the upper and/or lower limb extremities and in the pelvis (10,11).

3.2. Symptoms and Risk factors

Clinical symptoms often associated with NF1-MPNST are persistent nocturnal pain occurring in the affected PNF or subcutaneous neurofibroma, a sudden increase in the size and/or texture of a PNF and the rapid onset of a neurological deficit, although similar symptoms can also occur in benign PNFs not undergoing malignant transformation. Indeed, the early detection of MPNST development in NF1 patients is seriously hampered by the lack of any definite clinical or pathological features associated with malignant transformation, emphasising the need for regular assessment of NF1 patients with internal PNFs (4, 8).

Several risk factors indicate an increase likelihood of MPNST development, such as having large numbers of cutaneous neurofibromas (3), the presence of multiple internal PNFs (4), any previous radiation therapy, presence of large genomic germline mutation that removes the entire *NF1* gene (12), the presence of neurofibromatous neuropathy (13), or a family history of NF1-MPNSTs. A major problem with these aggressive tumors is that they may not be diagnosed until appreciable growth of the

primary tumor has occurred, or until detection of the associated metastases.

3.3. Diagnosis

3.3.1. Radiological Diagnosis

The radiologic diagnosis of an MPNST can be challenging, with current MRI protocols often unable to discriminate a large benign PNF from a PNF that has undergone MPNST transformation. The recent introduction of fluoro-deoxyglucose-positron emission tomography (FDG-PET) is however significantly improving MPNST detection (5,14,15) and relates to the much higher uptake of fluoro-deoxyglucose by the actively growing malignant tumor cells compared to the less active cells of the associated benign PNF. Indeed, FDG PET/CT is proving to be a highly sensitive and specific imaging modality for MPNST diagnosis in NF1 patients (15), while whole-body imaging of young NF1 patients may allow those at highest risk for developing MPNST to be identified early in life (16).

3.3.2. Histopathology Diagnosis

The pathohistological diagnosis of MPNSTs can also be difficult, due mainly to a lack of well-defined histological criteria or specific antigenic markers and the considerable tumor tissue heterogeneity. While nerve sheath differentiation biomarkers, such as S100, often lack sufficient sensitivity and specificity, if used in conjunction with the nestin protein marker they provide increased MPNST tissue-specificity (17). Grading systems used to assess malignant tumors usually monitor tumor histology, type and degree of cellular necrosis, level of cellularity, presence of nuclear pleomorphism and the degree of mitotic activity (18, 19). NF1-MPNSTs often demonstrate a fascicular growth pattern with many spindle-shaped cells, nuclear hyperchromasia tissue necrosis and high mitotic activity (8,17). The variable tissue heterogeneity observed in MPNSTs relates to their development from benign PNFs, such that different areas of the tumor may exhibit features representative of different grades of malignancy. A region of the MPNST may therefore be designated as 'atypical', demonstrating histological features of both a benign PNFs and a low grade MPNST, with hypercellularity and hyperchromatic nuclei, however with relatively low mitotic activity. Atypical PNFs may be asymptomatic or can be associated with pain and neurological deficit. In regions of the tumor consistent with frank MPNST, based on high cellularity, nuclear pleomorphism, and mitotic activity, there can be further pathological heterogeneity as to grade of MPNST as well as degree of de-differentiation. One aspect of such de-differentiation of a high-grade MPNST into skeletal muscle, resembling rhabdomyosarcoma, is referred to as a malignant triton tumor (MTT) (20).

A histological and molecular analysis of a single MPNST tumor was recently carried out by our group (21). The tumor contained at least three histopathologically distinct regions, a benign PNF (region 1), an atypical PNF/low grade MPNST (region 2), and a high-grade MPNST (region 3). DNA from each tumor region was screened for *NF1* gene and other gene mutations and for

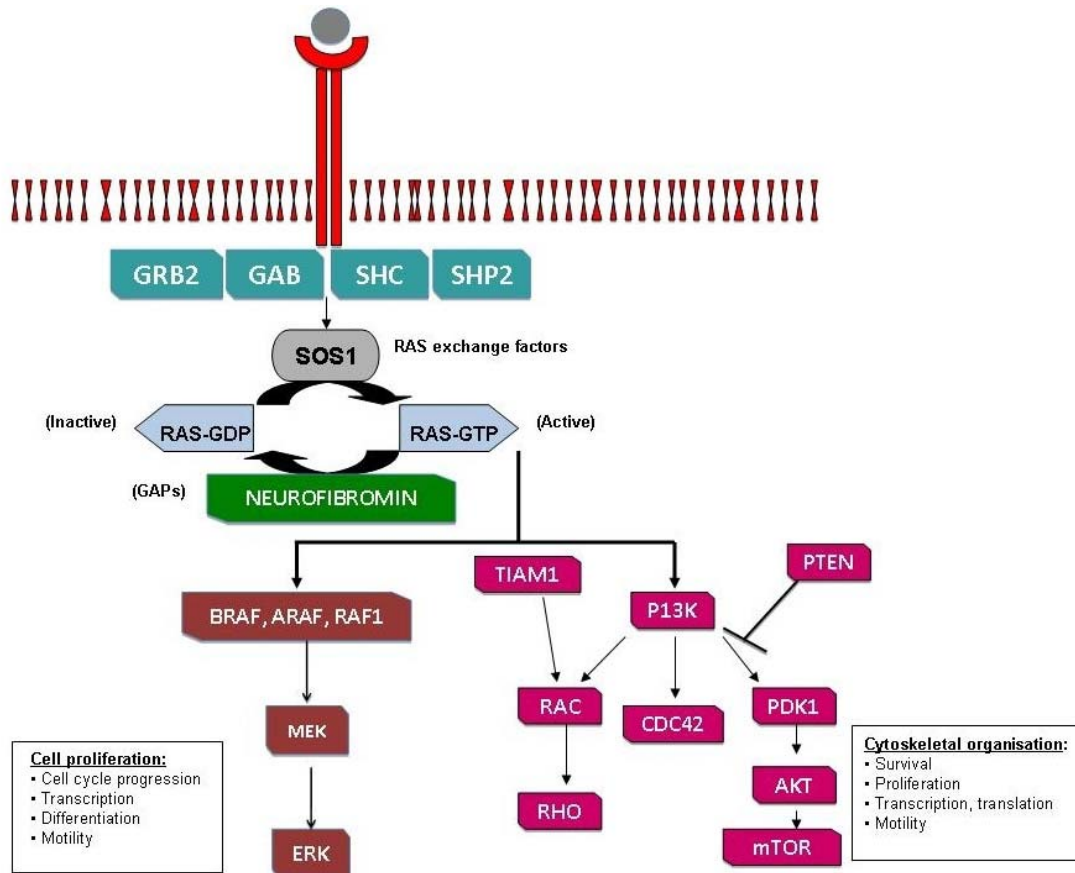


Figure 1. The complex Ras MAPK signalling pathway demonstrating the multiple cellular pathways regulated by activated Ras-GTP. Growth factor binding to cell surface receptors results in the formation of an activated receptor complex that contains several protein adaptors SHC, SHP2, Grb and Gab. These proteins recruit SOS1 which increases active Ras GTP levels by catalysing nucleotide exchange. Ras with bound-GDP is inactive and only when combined with GTP is activated. Activated GTP-bound Ras regulates signalling through a number of downstream effector pathways. The RAF/MEK/ERK cascade is involved in the regulation of cell growth and differentiation. Activated Ras can also send signal along PI3 kinase AKT and mTOR pathway resulting in cellular growth, proliferation and survival (adapted from Bennett *et al*, 2009).

evidence of loss-of-heterozygosity and it was found that the more malignant tumor regions correlated with increased levels of *NF1*-associated LOH (with 9%, 42%, and 97% LOH in regions 1, 2, and 3, respectively). Additional genetic changes were also identified but only in DNA from region 3 (high-grade MPNST) and included loss of the *TP53*, *RB1* and *CDKN2A* genes, as well as of several other oncogenes and cell-cycle genes. A microarray-based CGH study of the tumor DNA also only found genomic changes in DNA from region 3. This is the first study correlating the histological and molecular changes associated with MPNST development that demonstrates the significant cellular and genetic heterogeneity of such tumors and the considerable diagnostic and therapeutic challenges they pose.

4. NEUROFIBROMIN AND THE RAS/RAF/MAPK PATHWAY:

NF1 is an autosomal dominant progressive tumor predisposition syndrome (22). The disorder is

characterised by pigmentary changes of the skin (with café-au-lait spots and skinfold freckling), the development of benign neurofibromas, and hamartomatous nodules of the iris (Lisch nodules). Neurofibromin, the *NF1* gene product, is a ubiquitously expressed cytosolic protein, with its highest levels in central and peripheral nervous tissues, kidney, spleen and bone (23). Neurofibromin is a mammalian Ras-GTPase activating (GAP)-related protein; a highly conserved protein family, that dynamically regulates activated Ras protein levels and thereby down-regulates the important RAS/RAF/MAPK signalling pathway defining its role as a tumor suppressor protein. Most cellular Ras proteins are present in an inactive Ras-GDP form, with only very low levels of the functionally active Ras-GTP form usually available (Figure 1). The GTPase-activating domain within neurofibromin stimulates the inherent GTPase-activity of Ras greatly increasing conversion from active Ras-GTP to inactive Ras-GDP, resulting in the complete suppression of the RAS/RAF/MAPK signalling pathway, and its downstream effectors. Loss of neurofibromin function, usually due to

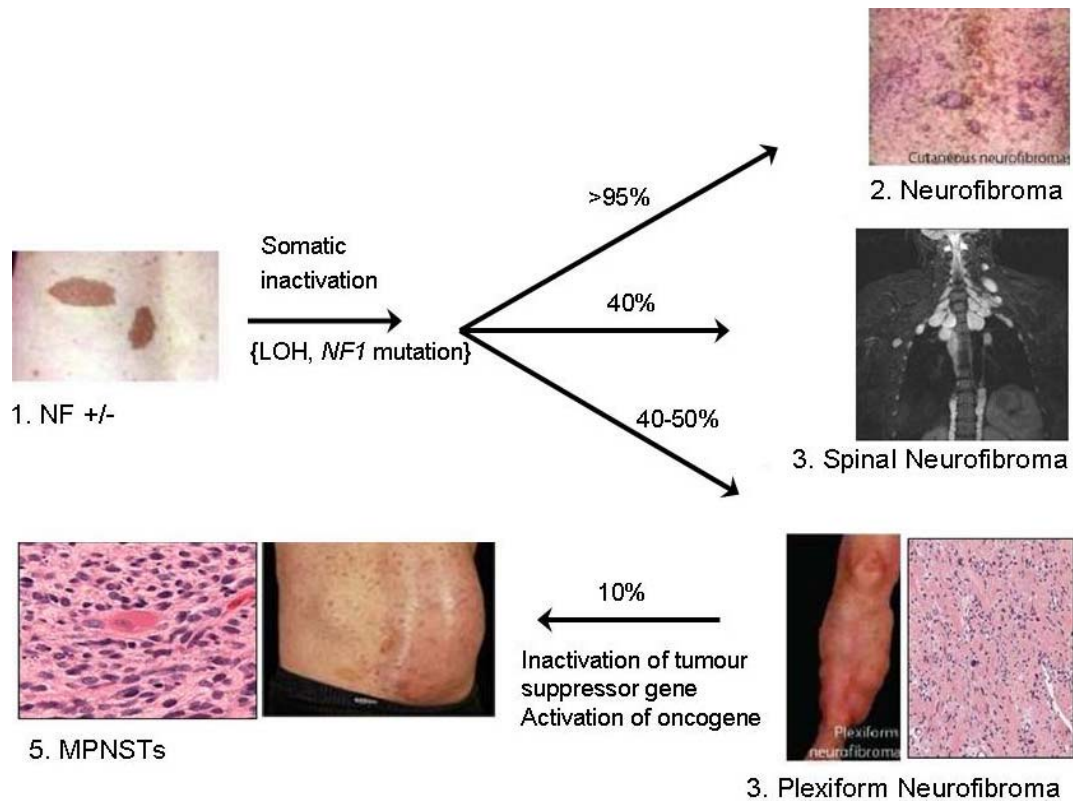


Figure 2. All NF1 patients carry a germline *NF1* mutation on one of their chromosomes 17. Benign neurofibromas (cutaneous, spinal and plexiform) develop as a result of somatic *NF1* mutation on the normal chromosome 17. This somatic event may occur either as LOH or as point mutation in the *NF1* gene. At least 95% of NF1 patients will develop cutaneous neurofibromas and these therefore represent the hall mark features of NF1. About 40% patients develop spinal neurofibromas and 40-50% will develop plexiform neurofibromas. Thus biallelic inactivation at the *NF1* locus results in the development of benign neurofibromas. About 10% of plexiform neurofibromas will go on to become malignant. For malignant transformation, mutations in additional genes (tumor suppressors and oncogenes) are required.

NF1 inactivating mutations, leads to the constitutive activation of Ras-GTP that increases cell proliferation and survival, often resulting in tumorigenesis.

4.1. Neurofibromin mTOR

Increased Ras-GTP levels also stimulate the central PI3K/AKT/mTOR signalling pathway that regulates cell proliferation and growth and also helps protect cells from apoptosis, this pathway is also constitutively activated in the absence of functional neurofibromin, resulting in increased cell proliferation and survival and eventually tumor development. Both the RAS/RAF/MAPK and PI3K/AKT signalling pathways activate mTOR signalling and this is highly regulated in neurofibromas and MPNST, with the pathway constitutively activated, even in the absence of growth factors, in both NF1 tumors and neurofibromin-deficient cultured Schwann cells (24).

4. MOLECULAR GENETICS OF MPNST DEVELOPMENT

5.1. Molecular studies at *NF1* locus

The recognized difficulty in the early clinical and pathological diagnosis of NF1-MPNSTs, emphasises the

need for a better definition of the molecular basis of NF1-associated malignancy (25). Somatic biallelic inactivation of both *NF1* alleles was first demonstrated in NF1-MPNSTs in 1989 (26) and is consistent with Knudson's two-hit hypothesis and *NF1* being a tumor suppressor gene. Homozygous *NF1* inactivation was subsequently detected in cutaneous and plexiform neurofibromas, as well as in MPNSTs, when analyzed for loss-of-heterozygosity (LOH) of the *NF1* gene region (27-30). This somatic LOH usually involves the normal chromosome 17 bearing the non-mutated *NF1* gene. A complete loss of neurofibromin also occurs in some sporadic MPNSTs (31), and NF1-MPNST cell lines also exhibit increased Ras activity concomitant with neurofibromin loss (32,33), with a 15-fold increase NF1-MPNST cells compared to non-NF1 associated schwannomas (34). The presence of biallelic *NF1* inactivation in benign neurofibromas indicates that while complete inactivation of the *NF1* gene is almost certainly the initiating event in neurofibromagenesis, it fails to fully explain the subsequent malignant transformation of these tumours (Figure 2). Somatic *NF1* mutation studies in humans and mice both found that LOH of the *NF1*-gene region only occurs in Schwann cells (35) and while human tumor studies indicate that only the cells within the

Schwann cell lineage are required to undergo *NF1*-LOH to allow tumor development there is still no defined timeline for either tumor development or progression. Mouse NF1 model studies have shown that while mice without neurofibromin in Schwann cell precursors develop neurofibromas (36), they fail to develop neurofibromas in an wild type (*Nf1*^{+/+}) cellular microenvironment, demonstrating that neurofibroma development requires a heterozygous *Nf1*^{+/-} cellular background (37,38).

Cancer genes are somatically inactivated by many different mechanisms, from single base pair changes to large genomic microdeletions and insertions, to loss-of-heterozygosity of the gene region, or even gene inactivation by hyper-methylating the gene promoter. Loss-of-heterozygosity may result from large genomic deletions, from aberrant mitotic recombination between alleles, or due to complete chromosomal loss, with or without reduplication. Few studies have attempted to characterise the somatic *NF1* mutation spectrum associated with NF1 tumorigenesis, and make comparisons with the *NF1* germline mutations found in NF1 patients. Our studies have shown that the main types of somatic *NF1* mutation found in the majority of NF1-MPNSTs are large genomic deletions (39), as recently confirmed by Bottila and colleagues (40). This differs significantly from the somatic mutations in benign neurofibromas possibly indicating different mutational mechanisms. The large genomic deletions found as the somatic mutation in MPNST are also significantly different from the spectrum of *NF1* germline mutations found in most NF1-MPNST patients, that are mostly point mutations with few microdeletion and insertion mutations. Indeed *NF1* germline mutational spectrum in patients with either cutaneous neurofibromas or PNF or MPNST appear similar (41, 42). Despite the marked differences in somatic *NF1* mutational spectra in benign and malignant NF1-associated tumors, comparison with the associated germline *NF1* mutations have failed to detect any obvious influence of the initial germline mutation on the subsequent somatic mutations. This indicates that other genetic or biological factor(s) are likely to be responsible for the observed biological differences.

While the majority of *NF1* germline mutations are intragenic point mutations, some 5-10% of NF1 patients have large genomic deletions that remove the entire *NF1* gene and also delete a variable number of immediately flanking genes (43). NF1 patients with the recurrent 1.4-Mb deletion, resulting from non-allelic homologous recombination between homologous low-copy repeats (*NF1-REP-a* and *NF1-REP-c*) flanking the *NF1* gene (12, 43), exhibit an increased risk of MPNST development. Indeed, the large number of genomic somatic deletions found in NF1-MPNSTs does suggest that it is the inactivation of one or more of the 13 co-deleted genes that might be involved in regulating MPNST development (43). Although the 1.4-Mb deletion is the most frequent somatic mutation found in NF1-MPNSTs, several different sized genomic deletions have also been found that encompass the *NF1* gene and also involve a variable number of flanking genes. For example, we have recently identified 3 unrelated MPNST tumors with genomic deletions of at least 2.2 Mb

in size that have variable deletion breakpoints (Pasmant *et al*, unpublished data) that might suggest additional genes on 17q may also be involved in MPNST development.

5.2. Cytogenetic/molecular

In contrast to the specific chromosomal translocations found in many synovial and other sarcomas, no similar consistent pathogenic chromosomal aberrations are associated with MPNSTs, although a number of small recurrent chromosomal alterations have been reported. Cytogenetic studies have reported that MPNSTs have complex karyotypes with a wide spectrum of chromosomal aberrations including translocations, duplications and numerical gains and losses. Frequent deletions of the 9p21 region, associated with down-regulation of the *CDKN2A* (p16) gene located at 9p21 is reported in several NF1-MPNSTs (44-48). At least two other regions on chromosome 17, in addition to the *NF1* gene at 17q11.2, appear to be involved in tumorigenesis, with gains at 17q21-q22 region, possibly explaining the up-regulation of the *TOP2A* gene from this region in several NF1 MPNST patients with a poor disease outcome (49). Loss of the 17p13 region, that encompasses the *TP53* gene, found in many MPNSTs (50), and in soft sarcomas and in many other cancers. While the *TP53* gene is often inactivated by allelic loss, or by more subtle lesions, biallelic *TP53* inactivation rarely occurs in MPNSTs. Functional loss of p53 leads to an overall increase in genomic instability, manifesting as increased gene amplification, aneuploidy or other chromosomal rearrangements. Mouse NF1 models confirm an active role for *TP53* loss, in conjunction with *NF1* inactivation, in MPNST development (51, 52), although it is still not known what other genetic modifiers may also predispose to MPNST development.

5. MOLECULAR SIGNATURES ASSOCIATED WITH MPNST DEVELOPMENT

Although functional loss of neurofibromin represents the primary event in tumorigenesis, the exact role of other genetic lesions required to promote malignant transformation of these benign tumors is poorly understood. The recent introduction of microarray-based technologies however now allows us to simultaneously analyse multiple genes and many different genomic regions in RNA or DNA from cancers in a single experiment. Such array-based analyses give us a tremendous opportunity to globally-profile all gene expression and copy number changes that may be associated with genetic disease and cancer. Several studies have already analysed tumor DNA and RNA to search for potential molecular signatures able to differentiate between MPNSTs and benign neurofibromas (Table 1).

5.1. Copy number changes (CNC)

Fluorescent *in situ* hybridisation (FISH)-based studies were initially used to identify large genomic alterations in NF1-MPNSTs (49, 56,58)(Table 1), with subsequent studies using chromosomal comparative genomic hybridisation and more recently, high-resolution oligonucleotide microarray analysis to screen for specific DNA copy number changes in the NF1-tumors. Many of

Table 1. Copy number changes and expression alteration in MPNSTs

Amplified chromosome location	Deleted chromosome Location	Gene at the amplified locus	Gene at the deleted locus	Methodology	Ref
17q24	13q14-q21			CGH-microarray	57
	9p21		INK4	Southern blot; mPCR	53
7q, 8q, 15q, and 17q	17p, 19q			CGH	54
17q and X	17p11.2-p13, 17q24-25, 19p13.2			CGH	55
7,8,	1p,10q,9,15,22			FISH analysis	56
17q21		TOP2A		FISH analysis	49
7/7q, 8/8q	1p36,3p21-pter,9p23-pter,10,11q23-qter,16/16q24,17,22/22q			FISH analysis	58
		MKI67,BIRC5,SPP1,MMP13, TERT, TOP2A	ITGB4,CMA1,L1CAM,MPZ, DHH,S100B,ERBB3,PTCH2, RASSF2,TP	q-PCR	66
17q25		TOP2A,ERBB2,PTDSR,BIRC5, SEP T9,SOCS3		FISH analysis	63
		TRIO,IRX2,NKD2		BAC-microarray	65
		SOX9,TWIST1	SOX10,CNP,PMP22,NGFR	Affymetrix array	69
		KRT18,BIRC5,TNC,ADA,VLA3, VLL	IGFBP-6	Microarray	62
17q		TOP2A,ETV4,BIRC5		CGH-microarray	64
		TGB4,PDGFRA,MET,TP73,HGF, PDGFRA	NF1,HMMR/RHAMM,MMP 13,L1CAM2	CGH-microarray	59
7p14,5q14.1,7q 36.1	9p21.3	NEDL1,AP3B1,CUL1	CDKN2A,CDKN2B,MTAP	BAC-microarray	60
		SOX9		Affymetrix array	70

these earlier DNA dosage studies however had either limited resolution, or only assessed a few gene regions (49, 53-58). We initially used a targeted gene array to screen for possible gene alterations in MPNST DNA, and found concomitant amplification of the *HGF*, *MET*, and *PDGFRA* genes (59). Hepatocyte Growth Factor (*HGF*) binds to the c-Met membrane receptor (*MET*) thereby activating the tyrosine kinase signalling cascade involved in regulating cell growth, motility and morphogenesis. Hemizygous deletion of the *HMMR* gene was also found in about half of the MPNSTs analysed. *HMMR* over expression has been implicated in several cancers, including NF1-MPNSTs, although its copy number status in other cancers is not known so the identification of MPNST-specific *HMMR* deletions therefore advocates this to be a strong candidate gene for the initiation and/or development of malignancy in NF1 individuals. A subsequent analysis of MPNST DNA with a 32K BAC array identified a common ~540 kb deletion at 9p21.3, the region containing the *CDKN2A*, *CDKN2B* and *MTAP* genes (60). Biallelic loss of all three genes was found in our study in 3 tumor samples, with single copy deletions in 8 MPNSTs (60). Holtkamp *et al* (61) have previously reported that the likely gene candidates involved in the malignant transformation of plexiform neurofibromas included *PDGFRA*, *PDGF*, *KIT*.

An up-regulation of both *BIRC5* and *TNC* has been described in MPNST but was not found in neurofibromas (62). Survivin, the protein encoded by *BIRC5* inhibits apoptosis and *BIRC5* up-regulation is a frequent alteration in many cancers, including MPNSTs (59, 63). Amplifications of two apoptotic genes, *BIRC5* and *TP73* were also identified in our study (59). Similarly, the *TP73* gene amplification observed in MPNSTs implies that the encoded DNp73 protein, an apoptosis inhibitor may also be up-regulated in malignant Schwann cells (59). The

topoisomerase II alpha (*TOP2A*) gene is also up-regulated due to the amplification of the relevant 17q21-q22 region in many MPNSTs (49, 64). Amplification of *TRIO*, *NKD2* and *IRX2* has been reported in soft tissue sarcomas including MPNSTs (65).

5.2. Gene and protein expression analysis

Evidence that DNA copy number changes can directly affect genes involved in these genomic changes, has led to application of array-based gene expression studies to many different cancers. In NF1, a quantitative RT-PCR study measured the expression level of 489 selected genes in NF1-MPNSTs and plexiform neurofibromas and found that 28 genes exhibited significantly different expression levels in MPNSTs compared to PNFs, with 16 genes being upregulated and 12 downregulated (66). Of the altered genes two were involved in the Ras signalling pathway (*RASSF2* and *HMMR*) and two in the Hedgehog-Gli signalling pathway (*DHH*, and *PTCH2*), while the other genes were variously involved in cell proliferation (*MKI67*, *TOP2A*, *CCNE2*), senescence (*TERT*, *TERC*), apoptosis (*BIRC5*, *TP73*) and extracellular matrix remodelling (*MMP13*, *MMP9*, *TIMP4*, *ITGB4*). Active growth of MPNSTs requires increased angiogenesis and activated Ras is known to upregulate *VEGF* (vascular endothelial growth factor) expression and both *VEGF* expression and tumor vascularisation are significantly increased in MPNSTs (67,68). While a similar comparison of the RNA 'transcriptomes' of sporadic and NF1-associated MPNSTs failed to detect any differences, this gene expression profiling study did identify a 159-gene molecular signature that distinguished MPNST cell lines from normal Schwann cells (69). Schwann cell differentiation markers (*SOX10*, *CNP*, *PMP22* and *NGFR*) all down-regulated in MPNSTs, while the neural crest stem cell markers (*SOX9* and *TWIST1*) were over-expressed. The

TWIST1 gene is implicated in apoptosis inhibition, resistance to chemotherapy, and to metastasis, however, silencing *TWIST1* expression in MPNST cells, with small interfering RNA, did not affect either apoptosis or chemoresistance, but cell chemotaxis was inhibited. Another related gene expression profiling study analysed normal Schwann cells, Schwann cells from primary benign neurofibromas, (both dermal and plexiform), and cell lines from MPNST and other solid tumors (70). Neurofibromas were found to repress gene programmes normally upregulated in late-developing immature Schwann cells, while MPNST cells activated gene programmes usually expressed early in the development, at the neural crest stage. Strong expression of the *SOX9* transcription factor was found in both neurofibroma and MPNST tissue, schwannomas expressed low levels of *SOX9* expression, and synovial sarcomas, tumors histologically similar to MPNSTs, were completely negative for *SOX9*. The *in vitro* reduction of *SOX9* expression in MPNST cell lines was found to result in rapid cell death.

A major problem with all microarray-based analyses of cancers is the very large number of genes and genomic regions often found to be altered, making the identification of the few causative 'driver' gene changes amongst the mass of alterations to other 'passenger' genes, difficult, emphasizing the need for rigorous functional analyses of these changes to support the microarray data.

A sarcoma-based study, that included MPNST of affected patients with moderate to strong expression of *HIF1A* in their tumors, exhibited a significantly shorter survival rate compared to patients whose tumors showed little *HIF1A* expression (71). What seems to come out from these molecular studies is that, no other single gene, besides *NF1*, appears to be consistently altered in all NF1-associated MPNST. However, the Ral A (Ras-like protein A), a Ras-GAP protein might be an exception, as RalA is reported to be strongly activated in both human and mouse MPNST cell lines and tumor samples, as compared to non-transformed Schwann cells (72). RalA has a pivotal role in tumorigenesis and its inactivation in MPNST cells was found to significantly reduce cell proliferation and tumor invasiveness. This interesting single study showing consistent over-activation of RalA in mouse and human NF1-MPNSTs needs confirmation in a much larger NF1 patient study.

Another possible MPNST-specific marker is the up-regulation of telomerase, a reverse transcriptase enzyme that functions by adding TTAGGG repeats to the ends of chromosome telomeres that shorten with each cell replication. Cells with telomeres that are too short eventually stop dividing and die. Although telomerase activity is fully repressed in most normal somatic tissues it often becomes reactivated in cancer cells. Our study of telomerase activity in MPNSTs and neurofibromas found high enzyme activity in 14 of the 18 high-grade MPNSTs assayed, whereas there was negligible telomerase activity in 6 low-grade MPNSTs and 17 benign tumors (73).

A recent report showed that cathepsin K expression was found in cutaneous MPNSTs, but absent

from adjoining neurofibroma tissues, leading to speculation that cathepsin K activation in MPNSTs could mediate extracellular matrix degradation, thus promoting tumor cell invasiveness, and therefore might provide a useful therapeutic target (74).

A study by Miller *et al* (2009) (75) reported a decrease in *DACHI* expression and an increase in *PAX6*, *EYA1*, *EYA2*, *EYA4* and *SIX1-4* expression in MPNSTs and that these were all normalised following the exogenous expression of the NF1-GAP domain in MPNST cell lines. *EYA4* expression showed a 20-fold elevation in the tumor cell lines, and following its suppression with *EYA4*-specific siRNA, the treated cells exhibited reduced adhesion and migration and increased cell death, without apparently affecting either cell proliferation or apoptosis, an indication that the *EYA4* pathway may be a potential therapeutic target.

Many protein-coding genes are known to be regulated by microRNAs (miRNAs) in the cell, with many miRNAs able to silence multiple target mRNAs (76). An increasing number of pathological conditions are reported to be associated with miRNA profile alterations (77,78), with many cancers exhibiting consistent modifications to their miRNA expression patterns. It is therefore likely that assessment of miRNA profiles in neurofibromas and MPNSTs may also provide useful diagnostic markers and identify additional therapeutic targets. Several different histological sub-types of sarcoma are known to exhibit distinct miRNA expression profiles (79), and recent studies in MPNSTs have shown miRNA-34a to be down-regulated, but not in the associated neurofibromas (80), while miRNA-10b was found to be upregulated in primary MPNST tissues and derived cell lines, as well as in neurofibroma-derived Schwann cells (81). It is to be expected that other miRNAs may also be deregulated in NF1-associated tumors.

7. THE CANCER STEM CELL

The recently proposed cancer stem cell (CSC) model for tumor development is receiving much attention, the basic premise being that all tumors derive from a small specific subset of mutated stem cells that exhibit similarity to normal tissue stem cells, including the ability to self-renew and to differentiate into all the cell types within an actively proliferating tumors (82). Cancer stem cell model has obviously profound implications for potential therapies aimed at preventing tumor growth, because if most cancers follow this stem cell pattern then elimination of all the relevant cancer stem cells will be required to ensure cessation of tumor growth. This has indeed been demonstrated in testicular cancer, these tumors have a small number of rapidly dividing undifferentiated cancer cells amidst a much larger population of differentiated cancer cells that have little capacity to proliferate. Elimination of the rapidly dividing pool of undifferentiated cells completely prevented tumor growth (83).

Cancer stem cells do however appear to be more resistant to the usual tumor therapies than do most other

cells in the tumor and this may explain the difficulty in preventing many metastatic cancers as they reflect this inherent resistance. The question is, does malignant transformation in NF1 also follow a cancer stem cell model? If so, this would indicate that MPNSTs develop from only a few tumor cells capable of extensive proliferation that could be targeted. Interestingly, Joseph and colleagues (36) have recently constructed several mouse models with mutations affecting their *Nf1*, *Cdkn2a* (*Ink4/Arf*) and *Tp53* genes and found that the MPNSTs that they developed arose from differentiated glial cells and not from neural crest stem cells. It was also found that many different cells isolated from these primary mouse MPNSTs were able to transfer disease when transplanted into genetically identical mice, an indication that these MPNSTs did not follow the CSC model.

8. TREATMENT

MPNST development in NF1 patients results in significant morbidity and mortality and these patients generally have a very poor prognosis. There is still uncertainty of the exact cell-of-origin for MPNST development and it therefore remains difficult to specifically target any particular cell type for effective treatment. It has also been suggested that different NF1-associated tumors may each have a unique cell-of-origin. The most effective treatment for an MPNST involves complete surgical excision, ensuring clear tissue margins, usually in combination with an initial radiotherapy and subsequent chemotherapy. Due to the considerable difficulties in totally excising deep-seated high-grade MPNSTs, extensive radiotherapy is often applied to the tumor region to provide local control and to delay tumor recurrence, although this has little effect on long-term survival of patients. Chemotherapy is often restricted to treatment of any subsequent metastatic disease, with ifosfamide and doxorubicin frequently used to treat patients with widespread disease.

9. PRECLINICAL AND CLINICAL TRIALS

9.1. Preclinical studies/ mouse models

Several potential therapeutic avenues for treating NF1 have been explored. The MPNST-derived cell lines have been useful for testing possible drug treatments and several recently developed mouse NF1 models have also helped to facilitate preclinical testing in NF1 (84, 85). Mouse NF1 models have proved to be an essential resource to define the underlying biology of the disease, the pathogenesis of its associated tumors, and identifying potential diagnostic and therapeutic targets (85). The initial transgenic mouse models generally proved to be unsuitable tools as heterozygous *Nf1*^{+/-} mice failed to develop any neurofibromas and homozygous *Nf1*^{-/-} mice died *in utero* from cardiac developmental abnormalities (1, 2). The development of chimeric NF1 mice, that have only a few *Nf1*^{-/-} cells, has however generated a mouse model that develops multiple plexiform neurofibroma-like tumors (85). The problem of establishing which cell type(s) required complete *Nf1* gene inactivation to induce tumor formation, led to a mouse model in which the *Nf1* gene was

conditionally inactivated only in Schwann cells (85). This model first demonstrated that the neurofibroma development has an absolute requirement for neurofibromin-deficient (*Nf1*^{-/-}) Schwann cells to interact with a surrounding a haplo-insufficient (*Nf1*^{+/-}) cellular microenvironment (37, 85). Such mouse models have also helped to demonstrate that de-granulated mast cells are also directly involved in neurofibroma development, possibly indicating that a cellular inflammatory response may precede NF1 tumorigenesis. Indeed, if such mutant mice are given a bone marrow transplant from a normal mouse, introducing *Nf1*^{+/-} mast cells, then very few tumors now develop, confirming neurofibroma development involves interplay between heterozygous (*Nf1*^{+/-}) mast cells with neurofibromin-deficient Schwann cells (38). Mast cells need to express the c-KIT receptor for normal development and function and this study also showed that treatment with imatinib, a specific inhibitor of the c-KIT receptor, can also reduce growth of neurofibromas.

Another transgenic mouse model that is doubly heterozygous at both the *Nf1* and *Tp53* genes (*Nf1*^{+/-}, *Tp53*^{+/-}) develop tissue sarcomas, including MPNSTs, derived from neural crest cells, and these mice are also useful for MPNST intervention studies (51, 52). Recently it was found that mice with constitutive *Kras* gene activation and *Pten* gene inactivation, also developed NF1 lesions that eventually became MPNSTs, demonstrating that *Pten* gene dosage, and its associated cellular pathways, may also be intimately involved in malignant transformation and represent therapeutic targets (86). This important finding was confirmed, using different genetically engineered mouse models it was demonstrated (87) that loss of tumor suppressor *Pten* in combination with over-expression of *Kras* oncogene, is an important step in MPNST development.

Drug treatment of several potential candidate targets are under consideration for preclinical and clinical NF1 studies. These targets include both the Ras signalling pathway and the PI3K and the PAK1 pathways, as well as their various downstream effectors, and also a number of growth factors and their receptors (3). Rapamycin, an immunosuppressant drug that inhibits activation of the critical mTOR pathway, reduces cell proliferation in primary MPNSTs and their derived cultured cells (24, 88, 89). A recent *in vivo* assessment of the effect of rapamycin treatment on human NF1-MPNST explants grown subcutaneously in mice found that tumor growth was significantly diminished without any obvious systemic toxicity (90). However it was found that although rapamycin effectively reduced MPNST cell proliferation and associated angiogenesis, it failed to increase apoptosis, an indication that while the mTOR pathway was clearly down-regulated, AKT, serine/threonine kinase that activates the mTOR pathway, appeared to be activated by rapamycin, although previous studies had failed to find such AKT activation by rapamycin (24). It would appear therefore that rapamycin, and its various analogues, may function as tumor stasis drugs rather than tumoricidal agents. Rapamycin may also be exerting its anti-tumorigenic effect by directly inhibiting angiogenesis (88-

91). Given the apparent activation of AKT by rapamycin, future studies might consider using dual kinase inhibitors targeting both mTOR and PI3K signalling to optimally suppress tumor growth in NF1.

A number of individual functional components of the complex RAS/RAF/MAPK pathway are being considered as possible drug targets. The two mitogen-activated protein (MAP) kinase kinases, MEK1 and MEK2, lie downstream of RAF in this pathway, with both kinases activated by Raf-directed phosphorylation, and can be inhibited with MEK-blocking agents. Treatment of both primary MPNSTs and derived cells lines with MEK-blocking drugs is shown to significantly suppress tumor cell proliferation (92). Inhibition of several growth factor receptors is being studied, especially EGFR as this receptor is over-expressed in MPNSTs (93). The drugs erlotinib and gefitinib inhibit EGFR and erlotinib treatment was found to inhibit both tumour cell growth and the invasion potential of MPNST cell lines (93). PDGFR, another related growth factor receptor, is also over-expressed in MPNSTs where it is reported to have an active role in tumor proliferation and invasion (17, 25, 59 and 61) and preclinical studies using PDGFR inhibitors have shown promising results (94). The level of VEGF also appears to be elevated in MPNSTs compared to neurofibromas (17) and treatment with sorafenib, a VEGF inhibitor, also resulted in a significant anti-MPNST effect (95).

The use of statins to inhibit HMGCoA reductase and thus prevent the synthesis of mevalonate, farnesyl and geranylgeranyl pyrophosphate is also being investigated (3). Treatment of MPNST cells with a combination of a farnesyl transferase inhibitor and a statin (lovastatin) was found to significantly reduce cell proliferation and also stimulate tumor cell apoptosis (96). Previous studies have indicated that aberrant Ras, and the upregulation of downstream effectors, are often only found in some MPNST cell lines and primary tumors (59, 60, 75). It was found that silencing RalA, another Ras-GAP protein, in these tumours reduced the proliferation, invasiveness, and *in vivo* tumorigenicity of MPNST cells, and also reduced the expression of epithelial-mesenchymal transition markers. The forced expression of the NF1-GAP-related domain was able to diminish Ral activation levels implicating neurofibromin as a RalA activation regulator (72).

9.2. Clinical Trials

A better understanding of the molecular pathogenesis of MPNSTs is starting to identify possible targeted agents with which to initiate clinical trials (98). Unfortunately, possible treatments, for MPNSTs are hindered by a lack of knowledge of the exact cell-of-origin. However, multiple lines of evidence now identify the critical importance of the RAS/RAF/ MAPK and PI3K/AKT/mTOR pathways in controlling the proliferative capacity of both sporadic and NF-MPNST (22,85).

Clinical trials targeting PNF and MPNST with farnesyl transferase inhibitors shown to prevent the *in vitro* growth of NF1-derived MPNST cells have proved disappointing as it was found that the KRAS and NRAS

proteins can both be activated by geranylation, as well as by farnesylation, and thus escape functional inactivation following drug treatment. Pirfenidon an inhibitor of PDGFRA, has also not been effective. in phase-2 clinical trial (98). A phase-2 trial of the EGFR inhibitor erlotinib has also been undertaken in patients with NF1-associated and sporadic MPNSTs, but the drug failed to demonstrate inhibitory activity in MPNSTs (99).

A phase-2 clinical trial with sorafenib, an inhibitor of Raf kinase, other receptor tyrosine kinases, and VEGFR also proved ineffective (100). The recent *in vitro* studies indicating that deregulation of the mTOR pathway is involved in MPNST progression has led to the Phase II NF Clinical Trials Consortium (101) assessing the effect of rapamycin on NF1 progressive plexiform neurofibromas. Neurofibromin loss results in activation of both the RAS and PI3K pathways that induces phosphorylation and inactivation of the TS complex via AKT resulting in mTOR activation (88-90). It is therefore likely that the combined inhibition of the PI3K/AKT and mTOR pathways should provide novel therapy for NF1-MPNSTs, underlining the need to evaluate more complex combinational approaches (102).

Given the overall complexity of the RAS/RAF/MAPK and PI3K/AKT/mTOR signalling pathways, it is however unlikely that any single drug strategy will prove effective, and that a combinatorial drug approach will be required (2).

10. CLOSING REMARKS

MPNSTs are relatively rare tumors that represent some 5-10% of all sarcomas, and are frequently found in association with NF1. The lack of treatment effectiveness of most chemo- and radio therapeutic approaches is shifting the focus towards more biologically relevant drug approaches. A better understanding of the specific molecular changes associated with development of MPNSTs should allow us to more accurately predict tumor behaviour and its likely response to therapy. While the introduction of microarray-based analyses has led to the identification of alterations to many genes and specific genomic regions associated with MPNST development, problems arise in defining the few causative 'driver' genes from among the many 'passenger' gene changes. It is also hoped that combined clinical and molecular studies will better identify NF1 patients with an increased risk of developing MPNSTs. The application of biologically-relevant targeted therapies via extended preclinical and clinical trials and the application of a 'personalised medicine' approach, should also aid better management of these aggressive malignant tumors. Evaluation of the many new developments in MPNST biology will require a multiple 'omics' approach, that combines extensive patient, tumor DNA and RNA resequencing, in conjunction with comparative transcriptomic, proteomic and metabolomic studies.

The infrequency of multiple MPNSTs from NF1 patients also underlines the need to develop large

multidisciplinary collaborative efforts able to fully evaluate and analyse this limited sample pool. Such collaborations will almost certainly involve the combined efforts of clinicians, geneticists, epidemiologists, genetic diagnosticians, technologists and bioinformaticians to try to ensure the identification of effective diagnostic and prognostic markers. Much cancer research is directed at establishing the causative genes directly involved in cancer aetiology, mainly by identifying which genes consistently undergo somatic mutation in different tumor types and at what stages during tumorigenesis. Several comprehensive genome-wide analyses are currently being directed against a number of common malignant tumor types, such as glioblastoma, adenocarcinoma, ovarian, lung tumors and leukaemia (103-106). Many studies have identified that somatic mutations of the *NF1* gene are often directly involved in tumorigenesis in these tumors, underlining a much wider biological role for neurofibromin in tumor development. Thus, a comprehensive genome-wide analyses of NF1-MPNST is clearly warranted to elucidate the role of other genes in tumor development and to be able to better manage and treat these affected NF1 patients.

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