

Original Paper

Genome-wide transcriptome analyses reveal p53 inactivation mediated loss of miR-34a expression in malignant peripheral nerve sheath tumours

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Abstract

Malignant peripheral nerve sheath tumours (MPNSTs) are aggressive soft tissue tumours that occur either sporadically or in patients with neurofibromatosis type 1. The malignant transformation of the benign neurofibroma to MPNST is incompletely understood at the molecular level. We have determined the gene expression signature for benign and malignant PNSTs and found that the major trend in malignant transformation from neurofibroma to MPNST consists of the loss of expression of a large number of genes, rather than widespread increase in gene expression. Relatively few genes are expressed at higher levels in MPNSTs and these include genes involved in cell proliferation and genes implicated in tumour metastasis. In addition, a gene expression signature indicating p53 inactivation is seen in the majority of MPNSTs. Subsequent microRNA profiling of benign and malignant PNSTs indicated a relative down-regulation of miR-34a in most MPNSTs compared to neurofibromas. *In vitro* studies using the cell lines MPNST-14 (NF1 mutant) and MPNST-724 (from a non-NF1 individual) show that exogenous expression of p53 or miR-34a promotes apoptotic cell death. In addition, exogenous expression of p53 in MPNST cells induces miR-34a and other miRNAs. Our data show that p53 inactivation and subsequent loss of expression of miR-34a may significantly contribute to the MPNST development. Collectively, our findings suggest that deregulation of miRNAs has a potential role in the malignant transformation process in peripheral nerve sheath tumours.

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Introduction

Malignant peripheral nerve sheath tumours (MPNSTs) are aggressive sarcomas that are associated with a 5-year survival rate of ~40% [1]. Approximately half of MPNSTs occur as sporadic cases; the remainder arise in patients with the autosomal dominant genetic disorder neurofibromatosis type 1 (NF1). NF1 is caused by inactivating mutations in the *NF1* gene and affects 1:3000 live births. It

is associated with a significant risk of developing malignancies, especially MPNSTs, which occur in NF1 patients with an incidence of ~10% [2–4]. In NF1 patients, MPNSTs most often develop from pre-existing neurofibromas. Screening for malignant transformation in NF1 patients is difficult, due to the large number and diverse anatomical sites of neurofibromas that occur in these patients. As a result, most MPNSTs are identified at a late clinical stage [1,2].

Table 1. Summary of clinical, histopathological, and molecular data for the PNST and SS cases used in this study

Clinical feature	MPNST	Neurofibroma	Schwannoma	Synovial sarcoma
Sample size (n)	20	37	27	13
Male gender (%)	55	35	28	42
Median age (years)	33	34	49	33
Median tumour size (cm)	9	6	4.1	21
NF1 (%)	55	67	nd	nd
SYT-SSX fusion (%)	0	nd	nd	100

nd = not done.

The development of MPNSTs from neurofibromas is a complex process and a number of studies have described different molecular findings in these lesions. Both NF1-associated and sporadic MPNSTs are characterized by loss of *NF1* expression [5] that leads to increased RAS signalling and increased cell proliferation [6]. Molecular events such as DNA amplification with gain of expression of *TOP2A* and *EGFR* [7,8], and inactivation of *CDKN2A* and *p53* [9–11] have been implicated in malignant transformation towards MPNSTs. Yang *et al* [12], using a mouse model of NF1, demonstrated that for neurofibroma formation, *Nf1* haploinsufficiency is required in the non-neoplastic cells of the tumour micro-environment and also implicated mast cells as critical mediators of neurofibroma initiation. Earlier studies have shown differences in the gene expression patterns between neurofibromas and MPNSTs, and between dermal and plexiform neurofibromas [13,14]. However, NF1-associated and sporadic MPNSTs could not be distinguished by gene expression profiling [15]. Miller *et al* [16] demonstrated down-regulation of Schwann cell differentiation markers in MPNST and showed that reduction of TWIST1 expression inhibited chemotaxis.

Regulation of gene expression can occur through post-transcriptional modification by microRNAs (miRNAs). These small non-coding RNAs are 18–22 nucleotides in length [17] and have been implicated in apoptosis, proliferation, and differentiation [18]. Using murine and human cell lines, it was recently shown that the tumour suppressor function of the transcription factor p53 involves up-regulation of a network of miRNAs that includes miR-34a [19]. Expression of miR-34a in turn regulates a large number of genes associated with the cell cycle and proliferation [20]. In order to understand the potential role of miRNAs in the malignant transformation process, we analysed the global mRNA and miRNA expression profiles of peripheral nerve sheath tumours using gene microarrays and used *in vitro* approaches to study the possible role of miR-34a in malignant transformation in MPNSTs.

Materials and methods

The reader is referred to the Supporting information, Supplementary Materials and methods section for details.

Tumour samples

Ninety-seven fresh frozen tumour samples [20 MPNSTs, 37 neurofibromas, 27 Schwannomas, and 13 synovial sarcomas (SSs)] were obtained and centrally reviewed (CDMF). Clinicopathological features of the tumour samples are shown in Table 1 and the Supporting information, Supplementary Table 1.

Array analysis

The Stanford cDNA microarrays used in the study contain approximately 42 000 spots, representing about 28 000 genes or expressed sequence tags (<http://www.microarray.org/>). A total of 5229 genes showed significant variation in expression and were used for further analysis. Unsupervised hybrid hierarchical clustering, SAM analysis, and gene set enrichment analysis were performed as described in the Supporting information, Supplementary Materials and methods section.

The Stanford microRNA microarrays used in the study contained 482 known and predicted human miRNAs and 28 control probes (Ambion, Austin, TX, USA) spotted in duplicates (see the Supporting information, Supplementary Materials and methods section for details).

Cell culture and transfection, plasmid constructs, cell viability assays, and qRT-PCR

Two MPNST cell lines (JAF) were used for *in vitro* studies. MPNST-14 cells were established from a young NF1 male; the p53 is WT in exons 4–10. MPNST-724 cells were established from a non-NF1 primary tumour with p53 mutation (codon 254 deletion) and deletion of other p53 allele (see the Supporting information, Supplementary Materials and methods section, Supplementary Table 2, and Supplementary Figure 1 for details).

The complete raw data for mRNA and miRNA expression profiles will be made available through the Stanford Microarray Database (<http://smd.stanford.edu/>). Supplementary tables can be better viewed as Excel files.

Gene expression profiles of peripheral nerve sheath tumours

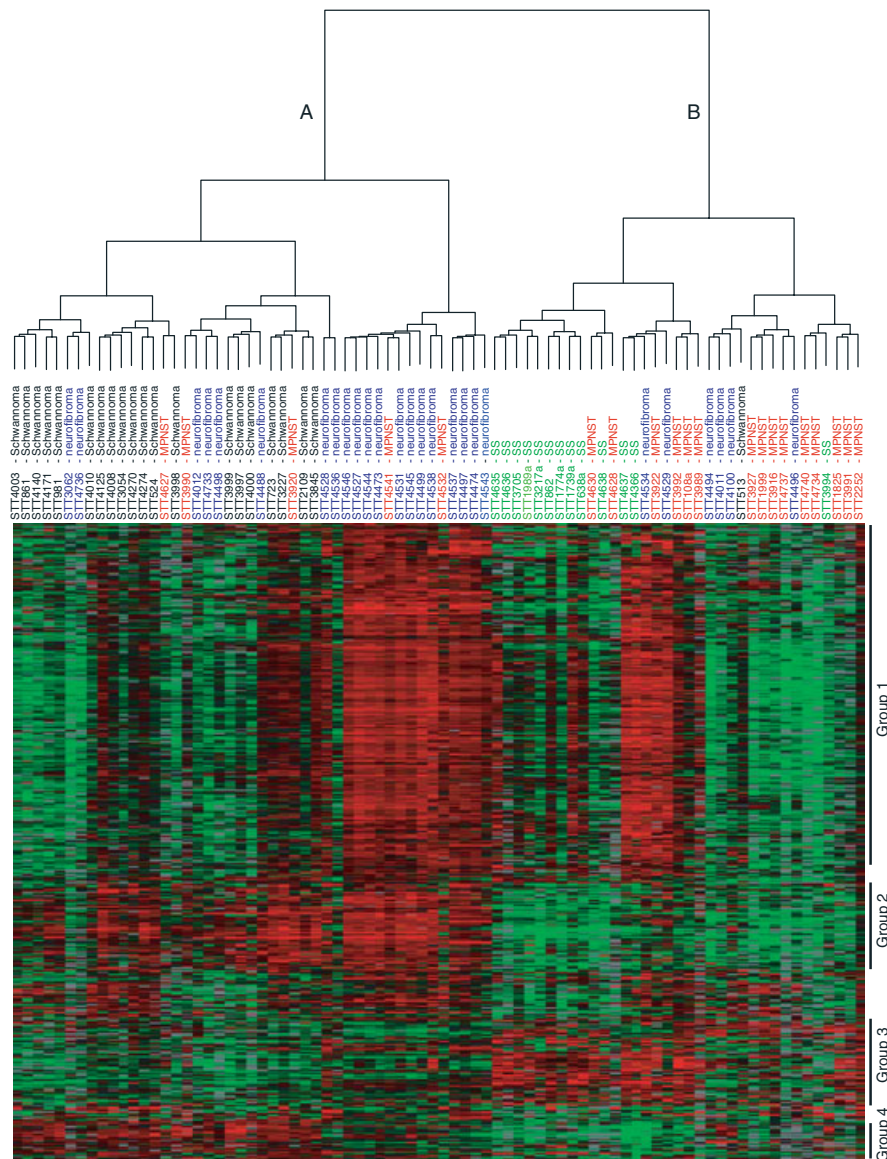


Figure 1. Unsupervised hierarchical cluster analysis of the gene expression profiles of 67 nerve sheath tumours and 13 synovial sarcomas using 5229 genes that passed the filter criteria. Each row represents the relative levels of expression for a single gene across the samples tested. Each column shows the expression levels for a single sample. The red and green colours indicate high and low expression, respectively. Grey indicates missing data. The two main branches of the dendrogram labelled A and B predominantly separate the benign tumours (neurofibroma and Schwannoma) from the malignant tumours (MPNSTs and SS)

Results

Global gene expression modules of peripheral nerve sheath tumours

Gene expression profiling was performed on 20 MPNSTs, 26 neurofibromas, 21 Schwannomas, and 13 SS cases. Using the gene filtering described in the Materials and methods section, 5229 genes passed the criteria and were used for analysis. Unsupervised hybrid clustering separated the 80 tumours into two main groups. The majority of the benign tumours (neurofibromas and Schwannomas) clustered in branch A of the dendrogram, leaving the majority of the malignant tumours (MPNSTs and SSs) in branch B (Figure 1). Within branch B, the MPNSTs and SSs formed discrete subgroups. Ten of the 13 SSs clustered together

on a small sub-branch with two other SSs on an adjacent sub-branch, a finding consistent with the relatively homogeneous gene expression profile reported for this tumour [21]. One SS (STT3994) was distinct from the other 12 SSs and had an unusual *SYT*–*SSX* fusion transcript (see below). The 20 MPNSTs showed a more complex clustering pattern, with nine of the 20 cases on a small sub-branch of branch B and six MPNSTs clustering along with the 12 SSs. The remaining five MPNST cases were distributed amongst the neurofibromas and Schwannomas in branch A (Figure 1). The three MPNSTs (STT3920, 4627, and 3990) that clustered with Schwannomas in branch A showed diffuse S100 reactivity as well as histological features indicative of Schwannian differentiation. Of the two MPNSTs (STT4532 and 4541) that clustered with neurofibromas, one case (STT4541) was

Table 2. Gene ontology terms associated with each group of genes in the expression analysis

Gene ontology term	Cluster frequency	Gene frequency in background	Corrected p value
Group 1			
Nucleic acid binding	339 out of 1881 genes, 18.0%	508 out of 3379 genes, 15.0%	1.41E-05
RNA binding	84 out of 1881 genes, 4.5%	108 out of 3379 genes, 3.2%	0.00056
Zinc ion binding	220 out of 1881 genes, 11.7%	332 out of 3379 genes, 9.8%	0.01315
Group 2			
Enzyme regulator activity	36 out of 468 genes, 7.7%	133 out of 3379 genes, 3.9%	0.01088
Group 3			
Cell division	21 out of 409 genes, 5.1%	44 out of 3379 genes, 1.3%	2.96E-06
Cell cycle	40 out of 409 genes, 9.8%	137 out of 3379 genes, 4.1%	1.96E-05
M phase of mitotic cell cycle	18 out of 409 genes, 4.4%	37 out of 3379 genes, 1.1%	2.68E-05
Mitosis	18 out of 409 genes, 4.4%	37 out of 3379 genes, 1.1%	2.68E-05
M phase	20 out of 409 genes, 4.9%	46 out of 3379 genes, 1.4%	5.04E-05
Mitotic cell cycle	19 out of 409 genes, 4.6%	45 out of 3379 genes, 1.3%	0.00018
Skeletal development	13 out of 409 genes, 3.2%	26 out of 3379 genes, 0.8%	0.00148
Microtubule cytoskeleton organization	8 out of 409 genes, 2.0%	11 out of 3379 genes, 0.3%	0.00326
Microtubule-based process	12 out of 409 genes, 2.9%	24 out of 3379 genes, 0.7%	0.00369
Regulation of mitosis	7 out of 409 genes, 1.7%	11 out of 3379 genes, 0.3%	0.04947
Group 4			
Receptor activity	31 out of 162 genes, 19.1%	211 out of 3379 genes, 6.2%	1.75E-06
MHC class II receptor activity	6 out of 162 genes, 3.7%	6 out of 3379 genes, 0.2%	2.63E-06
Transmembrane receptor activity	21 out of 162 genes, 13.0%	126 out of 3379 genes, 3.7%	7.20E-05
Signal transducer activity	40 out of 162 genes, 24.7%	375 out of 3379 genes, 11.1%	0.0001

Percentage is based on the background genes for each group.

histologically a low-grade nerve sheath tumour. Six neurofibromas clustered with MPNSTs in branch B; however, clustering of MPNSTs and neurofibromas was not associated with known NF1 status of the tumour and there were no histological or molecular features detected that distinguished these six cases from the other neurofibromas.

The major distinction between neurofibromas and MPNSTs is the lower level of expression of a large number of genes in MPNSTs rather than a widespread increase in expression levels. The 5229 differentially expressed genes separated into four major transcriptional gene groups. The gene ontology terms associated with each transcriptional gene group are given in Table 2 and the complete gene list is given in the Supporting information, Supplementary Table 3.

SAM analysis of gene expression in MPNST versus neurofibroma

SAM analysis was performed to identify genes that are differentially expressed between MPNST samples and benign peripheral nerve sheath tumours. *CRABP1*, *BEX1*, *CPA3*, *DLK1*, *HIST1H2BD*, *CYTL1*, *LOC388152*, and *IGFBP2* were the eight genes that were highly expressed in all MPNSTs relative to the benign nerve sheath tumours (FDR 1%). Large numbers of genes (~2200) were expressed at lower levels in MPNSTs, compared with benign peripheral nerve sheath tumours. The genes with low levels of expression in MPNSTs included *LICAM*, *CDH1*, *ERBB3*, *MAL*, *SOX10*, *CRYAB*, *CD44*, *S100B*, and *PMP22*

(Figure 2). These genes have significant SAM negative scores. Genes such as *LICAM*, *CRYAB*, *SOX10*, and *S100B* were previously reported to be down-regulated in MPNSTs [16], and MPNSTs often show only focal reactivity for S100B in immunohistochemistry. The complete list of genes differentially expressed in MPNSTs relative to neurofibromas is given in the Supporting information, Supplementary Table 4.

MPNSTs are known to display a variety of histological appearances and the diagnosis of MPNST is among the most difficult in sarcoma histopathology. In an effort to discover more genes that are differentially expressed between the malignant and benign nerve sheath tumours, we considered the 15 MPNSTs in branch B as a group distinct from the five cases that clustered with the benign peripheral nerve sheath tumours in branch A. We compared the gene expression levels of these 15 'core' MPNSTs with 'core' neurofibromas (neurofibromas clustered in branch A of Figure 1). This SAM analysis identified over 300 genes that are significantly up-regulated in the 'core' MPNSTs (FDR 1%). Several genes such as *CRABP1*, *CRABP2*, *IGF2*, *TPX2*, *ADNP*, *PBK*, *PTK7*, *FOXM1*, *TOP2A*, *TWIST1*, and *NEK2* (Figure 2) were up-regulated in this group and these have been previously implicated in various cancers [22]. The complete lists of high ranking positive and negative genes in 'core MPNSTs' and neurofibromas are listed in the Supporting information, Supplementary Tables 5 and 6, respectively.

Gene expression profiles of peripheral nerve sheath tumours

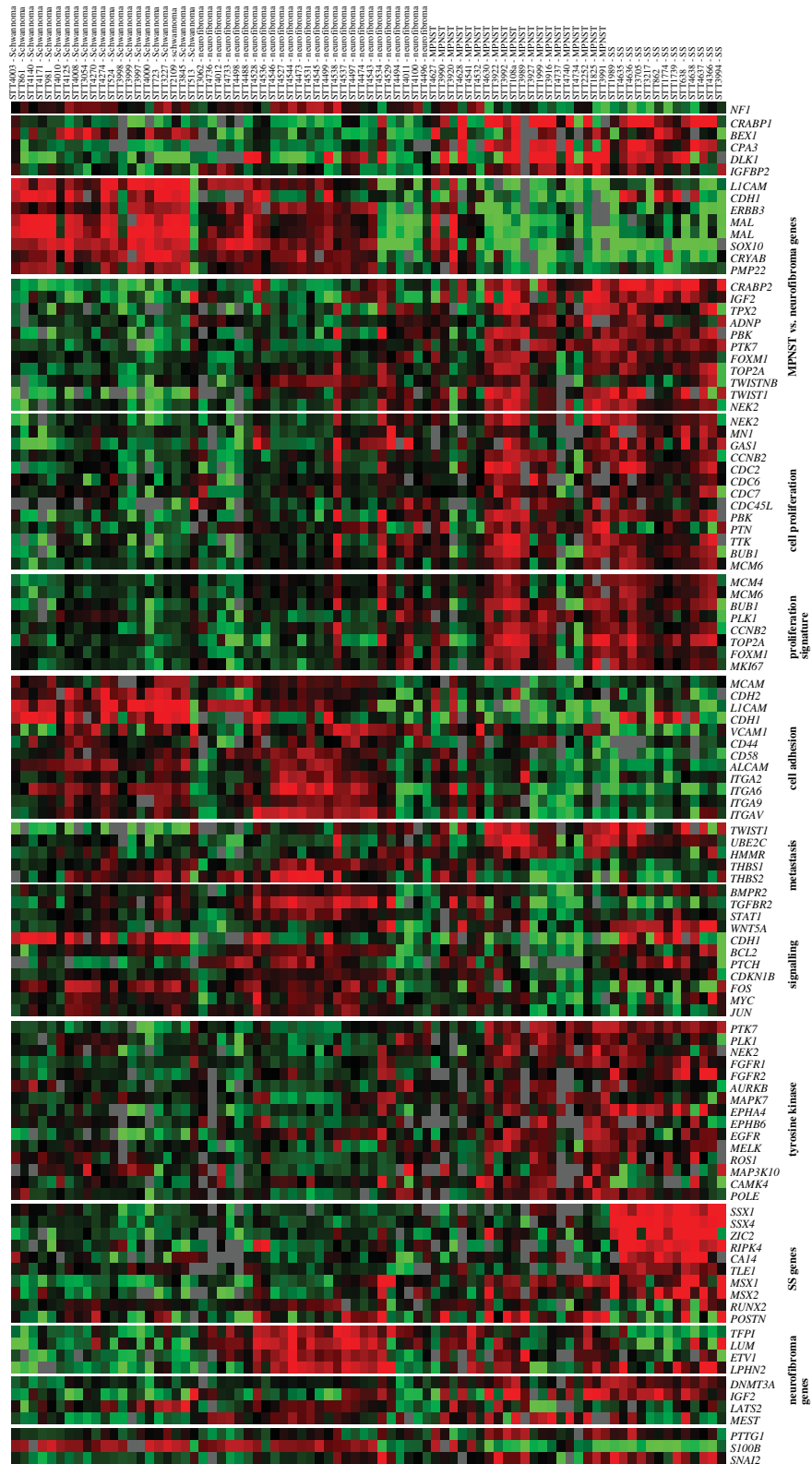


Figure 2. Overview of several expression patterns of groups of genes described in the text involved in cell cycle regulation, cell adhesion, metastasis, and other gene networks. Some genes that are associated with more than one gene grouping appear more than once

Expression profiles in MPNST associated with known function

MPNSTs showed lower levels of expression of the *NFI* gene, with a fold change of 0.237 compared with neurofibromas. While numerically most genes were

expressed at lower levels in the majority of MPNSTs compared with benign nerve sheath tumours, a significant number of genes involved in cell division, cell proliferation, migration, and malignant transformation were expressed at higher levels in MPNSTs (Figure 2).

Cell proliferation signature

A large number of genes known to be involved in cell division and cell proliferation (*NEK2*, *MNI*, *GAS1*, *CCNB2*, *CDC2*, *CDC6*, *CDC7*, *CDC45L*, *PBK*, *PTN*, *TTK*, *BUB1*, and *MCM6*) were up-regulated in the majority of MPNSTs compared with benign nerve sheath tumours. Whitfield *et al* [23] described a 'core set' of genes (*MCM4*, *MCM6*, *BUB1*, *PLK1*, *CCNB2*, *TOP2A*, *FOXMI*, and *MK167/MIB1*) as a signature for cell proliferation in proliferating cells. Evaluation of the expression profiles for these 'core sets' of genes in MPNSTs and other tumours revealed that the cell proliferation signature was a characteristic feature of MPNSTs and SSs but not of neurofibromas or Schwannomas.

Loss of cell adhesion

Several cell adhesion genes, such as *MCAM*, *CDH2*, *LICAM*, *VCAMI*, *CD44*, *CD58*, *ALCAM*, and *ITGAs* (integrins), were expressed at lower levels in MPNSTs relative to neurofibromas. E-cadherin (*CDH1*) was down-regulated in both neurofibromas and MPNSTs compared with Schwannomas and SSs, whereas *CDH2*, a neuronal cell adhesion molecule, was down-regulated in MPNSTs but expressed at higher levels in neurofibromas.

Metastatic signature

TWIST1, *UBE2C*, and *HMMR* are implicated in malignant progression and metastasis [24,25]. Compared with the benign PNSTs, most MPNSTs and SSs showed elevated expression of *TWIST1* and *UBE2C*, while increased expression of *HMMR* was more frequently seen in MPNSTs alone. In MPNSTs, there was a significant down-regulation of thrombospondin (*THBS1* and *THBS2*, inhibitors of angiogenesis). The repression of thrombospondin leads directly to angiogenesis and tumour formation in mammary epithelial cells and kidney cells [26].

Signalling pathways affected in MPNST

Gene set enrichment analysis [27] was used to identify signalling pathways affected in MPNSTs. By comparing 'core' MPNSTs and 'core' neurofibromas, several signalling pathways were identified that appear to be associated with malignant transformation. Genes associated with signalling pathways [such as the extracellular matrix (ECM) pathway], cancer-related genes involved in immune function, and genes involved in cell death were significantly down-regulated in MPNSTs. The list of affected pathways ranked based on their enrichment score is given in Table 3. As an example, the data for the ECM pathway are shown in the Supporting information, Supplementary Figure 2. The *TGF β* pathway-related genes, such as *TGFBR2* and *STAT1*, are down-regulated in

Table 3. Gene set enrichment analysis of core neurofibromas and MPNSTs

Gene set	Size	ES	NES	NOM p value
PIP3 signalling in B lymphocytes	37	0.6385	1.8572	0.0000
Extracellular matrix pathway	26	0.5802	1.7145	0.0189
Cancer immune function	37	0.5731	1.6910	0.0370
Cancer death	55	0.5039	1.6773	0.0182
Glycerolipid_metabolism	28	0.5840	1.6758	0.0000
HTERT_DOWN	64	0.4757	1.6565	0.0526
GPCRs class A rhodopsin-like	33	0.4980	1.6518	0.0169
mcalpain pathway	26	0.5314	1.6491	0.0167
Keratinocyte pathway	39	0.4620	1.5182	0.0308
JNK-MAPK pathway	51	0.4566	1.5166	0.0357

ES = enrichment score; NES = normalized enrichment score; NOM = normalized.

MPNSTs (Figure 2). We noticed relative low expression of several tumour suppressor genes (*BMPR2*, *TGFBR2*, *CDH1*, *WNT5A*, *ITGAs*, *NF1*, *BCL2*, *PTCH*, and *CDKN1B*) in MPNSTs. In addition, MPNSTs were characterized by increased expression of several kinase family genes, as shown in Figure 2. Although *EGFR* was highly expressed in a subset of MPNST cases (Figure 2), the SAM analysis did not rank *EGFR* as one of the high ranking genes in MPNSTs, as this gene was also highly expressed in a subset of neurofibromas.

Tissue microarray validation of EGFR expression

To confirm gene microarray findings, we evaluated the protein expression of EGFR in peripheral nerve sheath tumours and SSs by immunohistochemistry. About 67% (46 of 68 cases) of MPNSTs and 64% (27 of 42 cases) of neurofibromas showed positive staining for EGFR. This is similar to the high levels of mRNA expression found on gene arrays in 60% (12 of 20 cases) and 57% (15 of 26 cases) of MPNSTs and neurofibromas, respectively. Synovial sarcomas stained for EGFR in 93% (14 of 15 cases) of the cases, with high levels of mRNA found in 84% (11 of 13 cases) on gene array analysis. Finally, expression of EGFR was absent in all 22 Schwannomas on the TMA (Figure 3A). By gene array analysis, only one of 21 Schwannomas showed high levels of *EGFR* expression. This was the only Schwannoma that clustered with the malignant cases in branch B of Figure 1. Of the 68 MPNST cases represented on the tissue microarray, 44 were NF1-associated and 24 were sporadic cases. EGFR staining showed no significant correlation with NF1 status (66% of NF1-associated MPNSTs and 71% of sporadic MPNSTs showed EGFR staining) (Figure 3B). Representative examples of EGFR staining on peripheral nerve sheath tumours and synovial sarcoma are shown in Figures 3C–3H.

Gene expression profiles of peripheral nerve sheath tumours

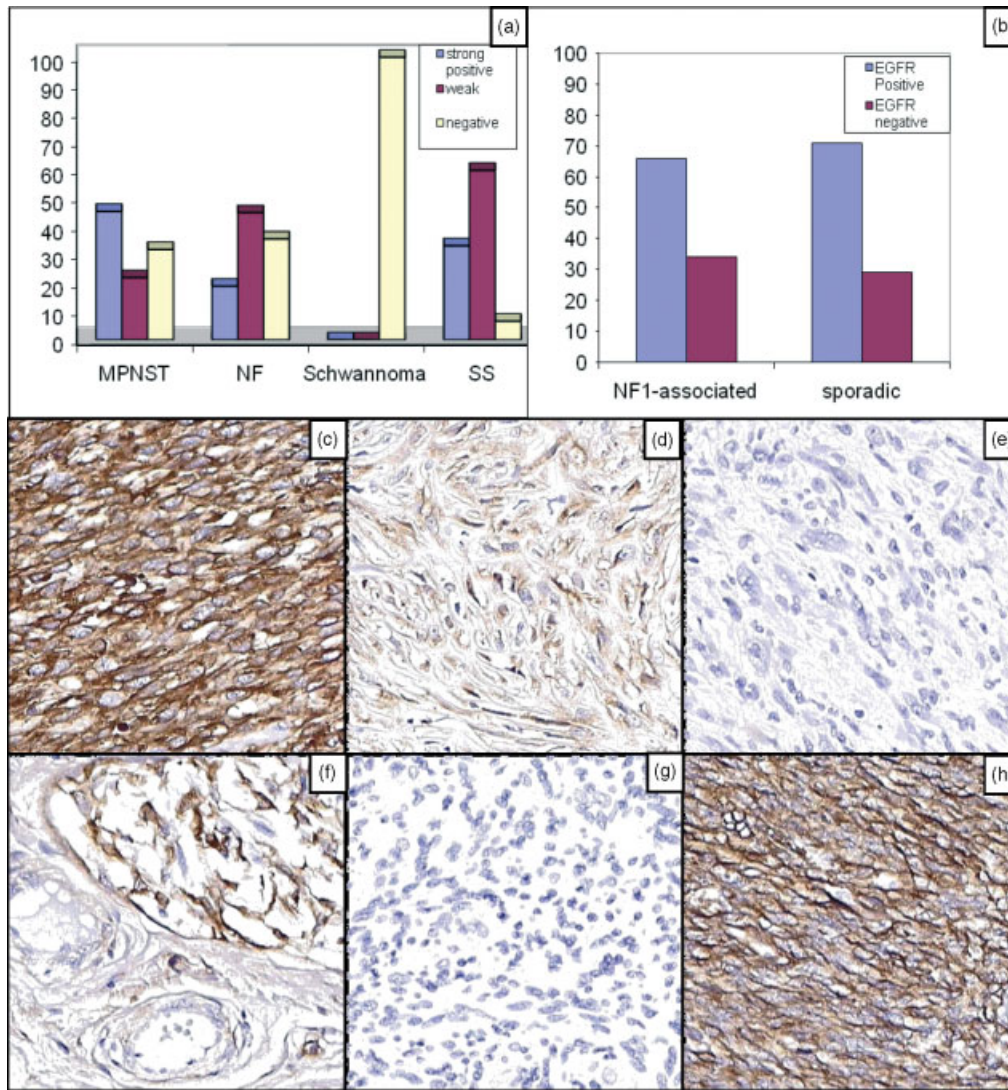


Figure 3. Graphical representation of the percentage of positive IHC for EGFR staining in (A) PNSTs and synovial sarcomas and (B) NF1-associated and sporadic MPNSTs. Representative TMA cores showing immunohistochemical staining of EGFR: (C) strong expression in MPNST; (D) weak expression in MPNST; (E) negative staining in MPNST; (F) staining in neurofibroma; (G) absence of staining in Schwannoma and (H) strong expression in a synovial sarcoma

Loss of p53 function

Loss of p53 function has been associated with increased cell proliferation. We analysed the expression patterns of the 38 genes that represent the 'inactivated p53-associated proliferation signature' [28] in the neurofibromas and MPNSTs. The analysis revealed that several of these 'p53 inactivation'-associated genes such as *TOP2A*, *TTK*, *CDC2*, *HMMR*, *PTTG1*, and *UBE2C* were up-regulated in MPNSTs (Figure 4). Though most MPNSTs included in the analysis showed the signature for p53 inactivation, four MPNST tumour cases (STT4734, STT4737, STT4541, and STT3920) did not show the signature for p53 inactivation. Of these four cases, two (cases STT3920 and STT4541) clustered with the benign tumours in the unsupervised clustering shown in Figure 1. Only a single neurofibroma (case STT4538) showed the expression signature for p53 inactivation. Conversely, genes that were down-regulated due to

inactivation of p53 (*PDE5A*, *TAGLN*, *CUL4B*, and *UGCG*) showed elevated expression in the majority of neurofibromas compared with MPNSTs (Figure 4).

MicroRNA expression signature in PNSTs

miRNA expression profiling was performed for 482 known and predicted human miRNAs on 23 peripheral nerve sheath tumours (six MPNSTs, 11 neurofibromas, and six Schwannomas). The six MPNSTs had also been used for mRNA profiling (Figure 1). Distinct miRNA expression profiles clearly distinguished MPNSTs from the benign tumours in unsupervised clustering using the 64 miRNAs that met the filtering criteria. Among the benign tumours, neurofibromas and Schwannomas clustered separately (Figure 5a). Using significance analysis of microarrays (SAM) analysis, we identified the miRNAs that were differentially expressed in each peripheral nerve sheath tumour class. In comparison with benign tumours, five

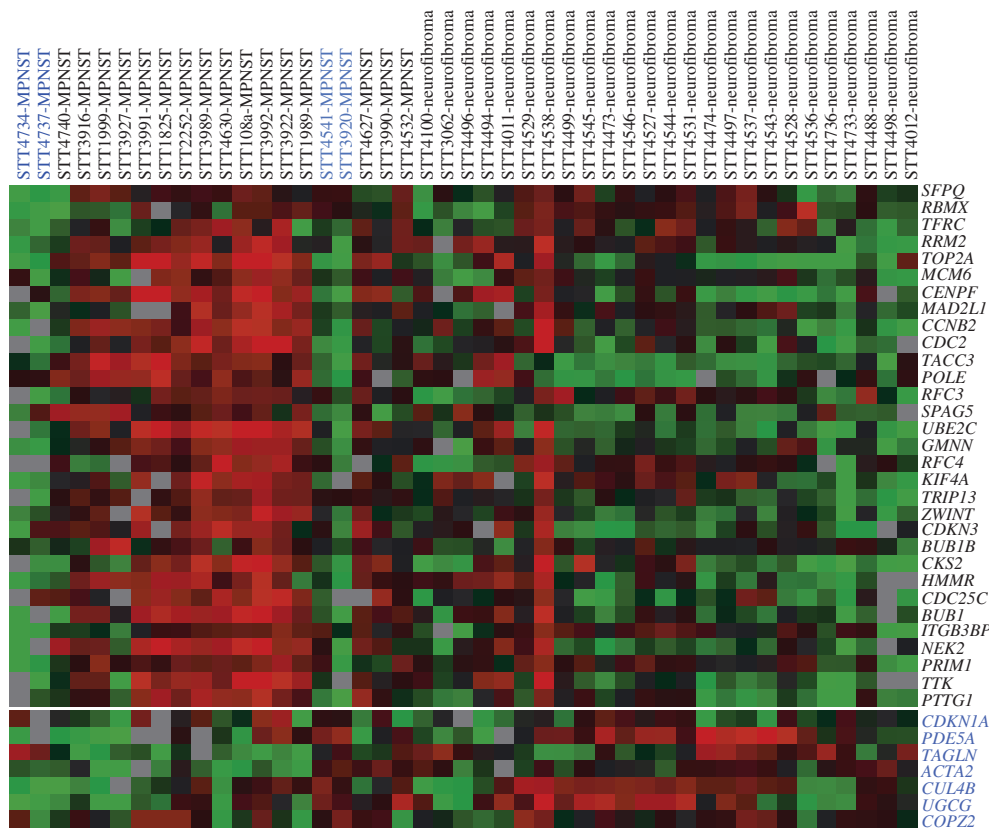


Figure 4. Expression levels of MPNSTs and neurofibromas for the gene signature that is associated with the inactivation of p53. Thirty-eight of the 168 genes described by Milyavsky *et al* [28] to be differentially expressed after p53 inactivation were available for analysis in our study. The genes in the upper part of the figure are expressed at higher levels after p53 inactivation and show a higher level of expression in MPNST than in neurofibroma. The genes in the lower part of the figure (shown in blue) are a group of eight genes that are down-regulated due to p53 inactivation in the study of Milyavsky *et al* [28] and are expressed at low levels in MPNSTs. A few MPNST cases (four cases in total) did not show the p53 inactivation signature and are highlighted in blue; two of these (STT4541 and 3920) clustered with the benign PNSTs in branch A of Figure 1

miRNAs (miR-214, miR-377, miR409-3p, miR-487b, and miR-99b) were relatively up-regulated and miR-517, miR-34a, miR-29a, miR-30e-5p, and miR-27a were down-regulated in MPNSTs (FDR 1%). The gene expression profiling data showed the gene signature for p53 inactivation in the majority of MPNSTs as shown in Figure 4. Since p53 is a transcription factor and miR-34a is one of the direct transcriptional targets of p53 [19], we selected miR-34a for further functional characterization. As a confirmation of the miRNA array data, quantification of miR-34a along with nine other differentially expressed miRNAs by qRT-PCR in five neurofibroma and MPNST patients' tumour samples validated the microRNA array data (Figure 5b).

Down-regulation of miR-34a in MPNST is due to p53 inactivation

We determined the expression levels of ten known p53-induced miRNAs [29] including miR-34a, miR-34b, miR-34c, miR-638, miR-373*, miR-492, miR-126, miR-140, miR-491, and miR-296 in the MPNST cell lines. Comparison of the expression levels of these miRNAs in MPNST-14 and MPNST-724 cells with HEK-293 cells (p53 positive) shows that both of these

MPNST cell lines have at least two to ten times lower levels of these p53-dependent miRNAs (Figure 5c).

Our mRNA and miRNA data analysis suggested that miR-34a transcript levels may depend on p53 activation in MPNSTs; to confirm this, we determined the transcript levels of miR-34a and other p53-dependent miRNAs by qRT-PCR after overexpressing wild-type p53 in both of the MPNST cell lines by transfection with wt-p53-GFP-containing plasmid. We found a significant increase in the levels of miR-34a and nine other p53-dependent miRNAs in these transfected cells (Figure 5d), confirming that in MPNST, miR-34a expression is dependent on p53 activation status.

Ectopic expression of p53 or miR-34a promotes apoptotic cell death in MPNST cells

The MPNST-14 cell line is deficient in miR-34a expression and we hypothesized that exogenous expression of either p53 or its direct transcriptional target miR-34a in this cell line would induce apoptosis in these MPNST cells. To test this, we transfected MPNST-14 cells with miR-34a expression construct and subjected the transfected cells to live/dead assay (Figure 6a, ii). We noticed approximately 36.23% of cell mortality in MPNST-14 cells, which was similar

Gene expression profiles of peripheral nerve sheath tumours

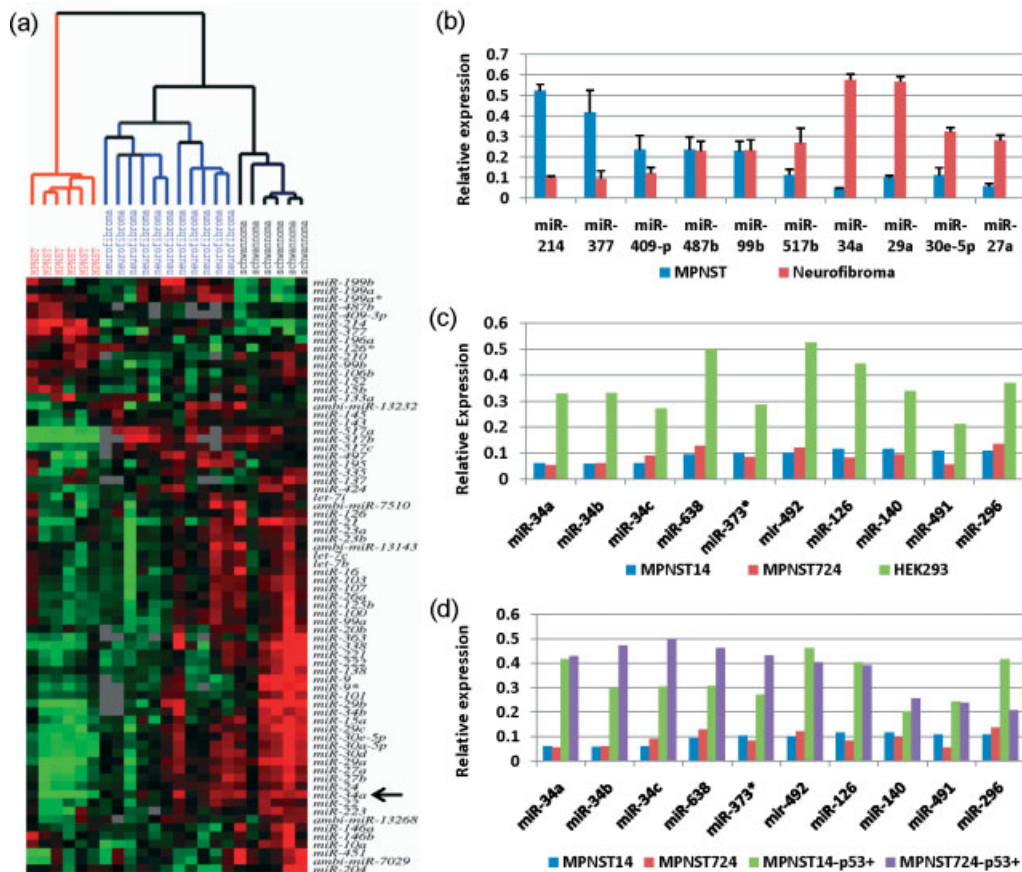


Figure 5. (a) MicroRNA expression profiling and unsupervised hierarchical clustering analysis of 23 peripheral nerve sheath tumours. Each row represents the relative levels of expression for a single miRNA and each column shows the expression levels for a single sample. The red or green colour indicates relatively high or low expression, respectively, while grey indicates absent data points. (b) qRT-PCR analysis of differentially expressed miRNAs in five MPNST and five neurofibroma samples. Relative values for each miRNA from five different neurofibroma and MPNST patients were averaged and plotted. Data represent mean \pm SD. (c) Expression levels of p53-dependent miRNAs (miR-34a, miR-34b, miR-34c, miR-638, miR-373*, miR-492, miR-126, miR-140, miR-491, and miR-296) in MPNST-14 and MPNST-724 cells compared with HEK-293 cells. Expression levels are normalized to U6 small RNA expression. (d) qRT-PCR analysis of p53-dependent miRNAs in MPNST-14 and MPNST-724 cells after transfection with wild-type p53. Relative expression of miR-34a, miR-34b, miR-34c, miR-638, miR-373*, miR-492, miR-126, miR-140, miR-491, and miR-296 was normalized to U6

to its transfection efficiency with the miR-34a construct (about 40%; Supporting information, Supplementary Figure 3). Furthermore, the presence of transfected miRs was verified in these cells using qRT-PCR (Supporting information, Supplementary Figure 3b). In contrast, transfection of MPNST-14 cells with scrambled sequence vectors showed only 5.19% dead cells. In addition, transfection with miR-34b and miR-34c had little or no effect on cell survival, with 5.16% and 4.46% cell death, respectively.

We performed FACS analysis using transfected cells and could show that the dead cells contained fragmented DNA, a characteristic feature of apoptosis (Figure 6b). We examined the effect of exogenous expression of wild-type p53 in MPNST-14 cells. Transfection of wild-type p53 resulted in 40% dead cells (Figure 6a, iv), similar to that of miR-34a transfection. Transfection with empty vector controls yielded far fewer dead cells (2.73%).

The transfection efficiency of MPNST cells is only about 40%. In order to determine the actual percentage of dead cells amongst successfully transfected cells,

miR-34a and p53-transfected cells were enriched by FACS based on GFP fluorescence. The transfection-enriched cell populations were next subjected to FACS analysis using PI staining. The miR-34a transfection-enriched population and p53 transfection-enriched population had over 90% dead cells (Supporting information, Supplementary Figure 4).

Transfection experiments were also carried out with the second MPNST cell line, MPNST-724, a cell line derived from a non-NF1 individual (Supporting information, Supplementary Table 7). With miR-34a transfection, the apoptotic response in MPNST-724 cells was 38.79%, similar to that seen in MPNST-14 (NF1 mutant) cells (36.23%). We also found a similar percentage of apoptosis (40.43%) with p53 transfection of MPNST-724 cells. Transfection with the scrambled sequences and empty vector controls had 5.11% and 2.19% dead cells, respectively (Figure 6c).

In order to understand the possible mechanism by which miR-34a induces apoptosis in these MPNST cells, we determined the levels of *MYCN*, *E2F*, and *CDK4* in miR-34a transfected MPNST cells by

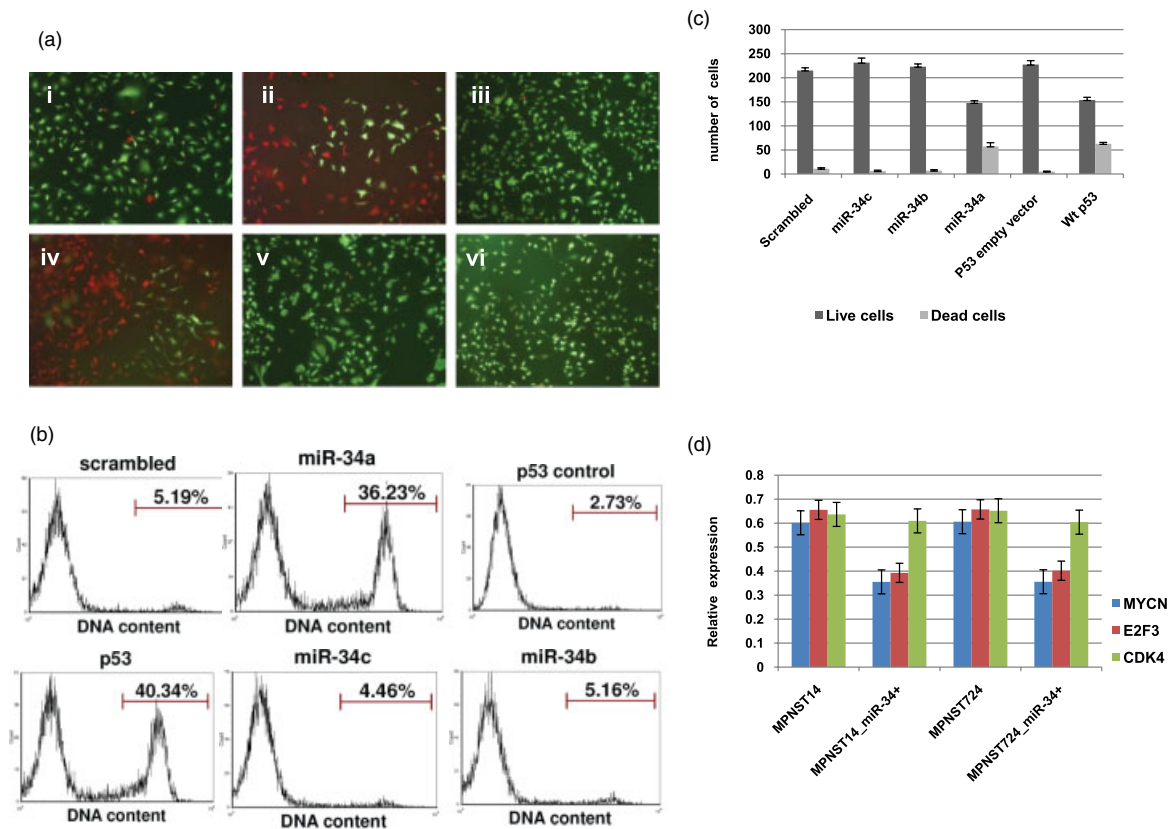


Figure 6. (a) Live dead assay of transfected MPNST cells. Cells were transfected with scramble sequence control (i), miR-34a (ii), empty vector control (iii), and wild-type p53 (iv), and subjected to cell viability assay 48 h after transfection. (b) FACS analysis of miR-34 or p53 transfected cells. Panels correspond to the transfection experiments shown in a. (c) Pro-apoptotic effect of miR-34a and p53 is independent of NF1-mutation status. MPNST-724 cells (non-NF1 mutant) transfected with constructs containing miR-34a and p53 were subjected to live/dead assay. Viability assays were carried out in duplicate and values indicate the mean of the total numbers of live and dead cells counted in three fields per sample. The number of dead cells was equal to the transfection efficiency and was comparable to the cell death ratio of MPNST-14 cells (NF1 mutant). (d) Apoptotic activity of miR-34a in MPNSTs is mediated by possibly targeting MYCN, E2F3, and CDK4. MPNST-14 and MPNST-724 cells were transfected with miR-34a construct and mRNA isolated from these cells was subjected to qRT-PCR analysis. miR-34a transfection reduces these transcripts by 38–40%. Data are normalized to GAPDH expression levels and values are mean \pm SD

qRT-PCR. These mRNA targets are known to be influenced by miR-34a [30,31]. We found that expression of these mRNAs was decreased (by 38–40%) upon miR-34a transfection (Figure 6d).

Discussion

Our gene microarray analysis identified expression signatures for the four classes of tumours (MPNST, neurofibroma, Schwannoma, and synovial sarcoma) included in the study. Unsupervised hybrid clustering separated the majority of the benign tumours from the malignant tumours. There were a few outliers in each class of tumour, which may reflect alterations of gene expression seen in different stages of tumour progression. For example, six of 26 neurofibroma cases clustered in the branch containing most of the MPNSTs. Though these neurofibroma cases show morphological features diagnostic of a typical neurofibroma, the possibility cannot be excluded that early pre-malignant disturbances at the molecular level have occurred in these cases and have resulted in a gene

expression profile more similar to MPNSTs than to typical neurofibromas. For five of these six neurofibromas, the NF1 status was known and four were resected from NF1 patients. In contrast, of the 20 neurofibromas clustering on branch A, the NF1 status of 16 cases were known, eight of which were resected from NF1 patients. In our study, we were unable to distinguish NF1-associated and sporadic MPNSTs based on gene expression profiles. This finding is in agreement with previous studies [13,15].

Compared with neurofibromas, the majority of the MPNSTs showed a decreased level of expression for a large number of genes. A possible explanation for this finding is that the majority of the 'group 1' genes that are down-regulated in MPNST are associated with nucleic acid-binding functions (Table 2). This may explain the down-regulation of many genes downstream from these transcription factors. Alternatively, epigenetic changes, such as DNA methylation, can also affect gene expression levels. For example, *DNMT3A* (an effector of DNA methylation) and genes known to be affected by DNA methylation (such as *CDH1*, *LATS2*, *IGF2*, and *MEST*) [32]

are differentially expressed in peripheral nerve sheath tumours, suggesting a possible role for DNA hypermethylation. In addition, a recent study showed that PTEN is hypermethylated in 29% of MPNSTs [33], and site-specific methylation involving transcription factor binding sites for AP2 and SP1 in the *NF1* gene has been found in plexiform neurofibromas [34].

A search for genes that are uniquely overexpressed in all the MPNSTs included in the study yielded only eight genes. This is consistent with the heterogeneous nature of MPNSTs, and this heterogeneity is also reflected in their variable histological appearance and their clustering pattern in our unsupervised cluster analysis. However, when analysing the 'core MPNSTs', those present in branch B of Figure 1, high expression of genes that are involved in cell proliferation and that are implicated in the pathogenesis of other cancer types was found. Genes such as *TOP2A* and *TWIST1*, which have been previously implicated in the progression of MPNSTs [7,16], were also found to be overexpressed in our gene array analysis. Likewise, *CRABP1* was one of the top five genes highly expressed in MPNSTs in our study. In a previous study, Henderson *et al* [35] noted that high expression of *CRABP1* distinguished MPNSTs and SSs from other mesenchymal tumours. Loss of expression of genes such as *E-cadherin* and overexpression of the zinc-finger transcriptional repressor gene 'snail' (*SNAI2*) are associated with tumour invasion [36,37]. This interactive mechanism of down-regulation of *E-cadherin* and up-regulation of *SNAI2* was observed in most MPNSTs in our mRNA expression study.

Similar to earlier reports [13,38], our TMA analysis revealed elevated expression of EGFR in a subset of MPNSTs and neurofibromas. EGFR expression was not associated with the mutational status of *NF1*. Gene and TMA analyses showed no EGFR expression in any of the Schwannomas included in this study. Earlier studies have shown that EGFR is expressed in a subset of *NF1*-mutant Schwann cells and not in normal Schwann cells [39]. Furthermore, a majority of the synovial sarcomas stained for EGFR, which is in agreement with previous findings from our laboratory [21].

Histological distinction between SS and MPNST often poses a diagnostic challenge. SAM analysis revealed *SSX*, *ZIC2*, *RIPK4*, *CA14*, and *TLE1* to be the top-ranking genes that are expressed at higher levels in SSs compared with MPNSTs (Supporting information, Supplementary Table 8). We have previously shown that an antibody against *TLE1* is a useful diagnostic tool for SS [40]. One of the SS cases, STT3994, clustered with the MPNSTs (Figure 1). RT-PCR for the diagnostic t(X;18) showed an unusually small fusion product of 297 bp (the usual fusion product seen in other cases of SS is 585 bp). Sequencing identified a novel, previously unreported fusion of exon 8 *SYT* gene to exon 7 *SSX1* (Supporting information, Supplementary Figure 5). Interestingly, this SS case had

relatively lower levels of *TLE1* expression compared with the other SS samples (Figure 1).

Two noticeable miRNA-mRNA associations became apparent in our study. Firstly, miRNA expression analysis identified miR-214 as the top overexpressed miRNA in MPNSTs. *TWIST1*, a master regulator of metastasis, has been shown in mouse neural cells to induce miR-214 expression [41]. It is notable that *TWIST1* is highly expressed in the majority of MPNSTs and thus may be involved in miR-214 expression in MPNSTs as well.

Secondly, several miRNAs were down-regulated in MPNSTs compared with the benign nerve sheath tumours, including miR-34a. Initial studies on primary and tumour-derived cell lines have shown that p53 induces the expression of miR-34a [19,29,42]. Subsequent studies have demonstrated that miR-34a expression induces apoptosis in colon cancer and neuroblastomas [30,43]; however, no sarcoma samples were included in these studies. To understand better the mechanism of the association between p53 and miR-34a expression in MPNST, we used two MPNST cell lines: MPNST-14 and MPNST-724. Despite the presence of intact genomic loci for miR-34 family miRNAs (data not shown), both MPNST cell lines had lower levels of endogenous miR-34 levels compared with HEK-293 cells. Relative down-regulation of miR-34a in these cell lines is consistent with the lower level of miRNA seen in MPNST tumour samples and with our observation that the majority of MPNSTs show a gene expression signature that is associated with p53 inactivation. Transfection of p53 in MPNST cells induced the expression of miR-34a and other p53-dependent miRNAs. In addition, exogenous expression of either p53 or miR-34a in the MPNST cell lines induced apoptosis-mediated cell death in over 90% of the transfected MPNST cells.

In the majority of MPNSTs, p53 is inactivated either by mutation or through other unknown mechanisms. In our study, miR-29a was significantly down-regulated in MPNSTs; miR-29 family miRs can activate p53 by blocking CDC42 and p85 α , the negative regulators of p53 [44]. Furthermore, in our functional assay, only miR-34a showed apoptosis-mediated cell death in the MPNST cells, whereas miR-34b and miR-34c did not produce any significant effect in the transfected MPNST cell line. miR-34a and miR-34c have an identical seed sequence, an important determinant of mRNA target specificity [45]. However, a search in miRgen for consensus target prediction showed only a partial overlap in the targets for these two miRNAs (data not shown), indicating that other factors play a role in the determination of targets.

In the MPNST cell lines, *MYCN*, *E2F3*, and *CDK4* transcript levels were down-regulated upon miR-34a transfection. *MYCN* oncogene mRNA is a known target of miR-34a, and MYC protein levels are reduced up to 80–95% upon miR-34a expression [31]. E2F family members are pro-apoptotic and miR-34a is known to mediate apoptosis by significantly reducing

the levels of E2F3 in neuroblastoma cells [30]. Earlier studies have shown that expression of miR-34a has a drastic effect on tumour cell proliferation and survival through cell cycle arrest and apoptosis [43,46].

In conclusion, molecular profiling of nerve sheath tumours reveals MPNSTs to be a heterogeneous group of tumours. The major trend in malignant transformation towards MPNST is the down-regulation of a large number of genes. MPNSTs are characterized by hallmark molecular events such as up-regulation of cell cycle genes, loss of cell adhesion, gene signature for inactivation of p53, and elevated expression of genes that regulate tumour metastasis. On the basis of our observations, we conclude that loss of miR-34a expression in MPNSTs may be partly due to p53 inactivation, which may play a major role in the transformation process of MPNSTs. As a consequence, miR-34a could be further investigated as a miRNA-based therapeutic treatment of MPNST.

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Note: References 47–55 are cited in the Supporting information to this article.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary Materials and methods. Detailed experimental methods.

Figure S1. Plasmid constructs used in the study.

Figure S2. Genes enriched in extracellular matrix (ECM) pathway are down-regulated in MPNSTs.

Figure S3. Transfection of MPNST-14 and MPNST-724 cells.

Figure S4. FACS analysis of miR-34a or p53 transfection enriched cells.

Figure S5. Novel SYT–SSX1 fusion transcript in synovial sarcoma

Table S1. Clinical, histopathologic and molecular data for the PNST and SS cases used in gene expression profiling studies.

Table S2. Oligos used in the study

Table S3. Gene ontology associated with transcriptional gene groups

Table S4. Differentially expressed genes: all MPNSTs vs neurofibromas

Table S5. SAM ‘core MPNSTs’

Table S6. SAM neurofibromas

Table S7. Effect of exogenous expression of miR-34a, and p53 in MPNST-724 cells.

Table S8. SAM analysis: SS versus MPNST