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

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Abstract

Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive soft tissue tumors 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 tumor 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 downregulation 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 shows that p53 inactivation and subsequent loss of

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

expression of miR-34a may significantly contribute to the MPNST development. Collectively, our findings suggests that deregulation of miRNAs have a potential role in the malignant transformation process in peripheral nerve sheath tumors.

Keywords

peripheral nerve sheath tumors; malignant transformation; expression profiling; microRNA

Introduction

Malignant peripheral nerve sheath tumors (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 arises 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 that 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.].

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 signaling 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 mouse model of NF1, demonstrated that for neurofibroma formation, *Nf1* haploinsufficiency is required in the non-neoplastic cells of the tumor microenvironment and also implicated mast cells as critical mediators of neurofibroma initiation. Earlier studies have shown differences in 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 downregulation 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 noncoding 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 tumor suppressor function of the transcription factor p53 involves up-regulation of a network of microRNAs that includes miR-34a [19.]. Expression of miR-34a in turn regulates a large number of genes associated with cell cycle and proliferation [20.]. In order to understand the potential role of miRNAs in the malignant transformation process, we analyzed the global mRNA and miRNA

expression profiles of peripheral nerve sheath tumors using gene microarrays and used *in vitro* approaches to study the possible role of miR-34a in malignant transformation in MPNSTs.

Materials and Methods, (see Supplementary Methods Section for details)

Tumor samples

Ninety seven fresh frozen tumor samples (20 MPNSTs, 37 neurofibromas, 27 schwannomas and 13 synovial sarcomas (SS)) were obtained and centrally reviewed (CDMF). Clinicopathologic features of the tumor samples are shown in Table 1 and 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 Supplementary 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 Supplementary 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 tumor with p53 mutation (codon 254 deletion) and deletion of other p53 allele. See Supplementary methods section, Supplementary table 2 and Supplementary figure 1 for details.

Results

Global gene expression modules of peripheral nerve sheath tumors

Gene expression profiling was performed on 20 MPNSTs, 26 neurofibromas, 21 schwannomas and 13 SS cases. Using the gene filtering described in Materials and Methods, 5229 genes passed the criteria and were used for analysis. Unsupervised hybrid clustering separated the 80 tumors into two main groups. The majority of the benign tumors (neurofibromas and schwannomas) clustered in branch A of the dendrogram, leaving the majority of the malignant tumors (MPNSTs and SS) in branch B (Figure 1). Within branch B the MPNSTs and SS formed discrete subgroups. Ten of the 13 SS clustered together on a small sub-branch with 2 other SS on an adjacent sub-branch, a finding consistent with the relatively homogeneous gene expression profile reported for this tumor [21.]. One SS (STT3994) was distinct from the other 12 SS and had an unusual *SYT-SSX* fusion transcript (see below). The 20 MPNSTs show a more complex clustering pattern with 9/20 cases on a small sub-branch of branch B and 6 MPNSTs clustering along with the 12 SS. The

remaining 5 MPNST cases were distributed amongst the neurofibromas and schwannomas in branch A (Figure 1). The 3 MPNSTs (STT 3920, 4627 and 3990) that clustered with schwannomas in branch A showed diffuse S100 reactivity as well as histologic features indicative of schwannian differentiation. Of the 2 MPNSTs (STT4532 and 4541) that clustered with neurofibromas, one case (STT4541) was histologically a low grade nerve sheath tumor. Six neurofibromas clustered with MPNSTs in branch B however, clustering of MPNSTs and neurofibromas was not associated with known NF1 status of the tumor and there were no histologic or molecular features detected that distinguished these 6 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 4 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 supplementary table 3.

SAM analysis of gene expression in MPNST vs. neurofibroma

SAM analysis was performed to identify genes that are differentially expressed between MPNST samples and benign peripheral nerve sheath tumors. *CRABP1, BEX1, CPA3, DLK1, HIST1H2BD, CYTL1, LOC388152* and *IGFBP2* were the 8 genes that were highly expressed in all MPNSTs relative to the benign nerve sheath tumors (FDR 1%). Large numbers of genes (∼2200) were expressed at lower levels in MPNSTs when compared to benign peripheral nerve sheath tumors. The genes with low levels of expression in MPNSTs include *L1CAM, CDH1, ERBB3, MAL, SOX10, CRYAB, CD44, S100B* and *PMP22* (Figure 2). These genes have significant SAM negative scores. Genes such as *L1CAM, CRYAB, SOX10* and *S100B* were previously reported to be downregulated in MPNSTs [16.], and MPNST often show only focal reactivity for S100B in immunohistochemistry. The complete list of genes differentially expressed in MPNSTs relative to neurofibromas is given in 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 tumors, we considered the 15 MPNSTs in branch B as a group distinct from the 5 cases that clustered with the benign peripheral nerve sheath tumors in branch A. We compared the gene expression levels of these 15 ‘core’ MPNSTs to ‘core’ neurofibromas (neurofibromas clustered in branch A of figure 1). This SAM analysis identified over 300 genes that are significantly upregulated 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 upregulated 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 supplementary table 5 and 6 respectively.

Expression profiles in MPNST associated with known function

MPNSTs showed lower levels of expression of the *NF1* 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 tumors, 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, MN1, GAS1, CCNB2, CDC2, CDC6, CDC7, CDC45L, PBK, PTN, TTK, BUB1* and *MCM6*) were upregulated in the majority of MPNSTs compared to benign nerve sheath tumors. Whitfield *et al* [23.] described a 'core set' of genes (*MCM4, MCM6, BUB1, PLK1, CCNB2, TOP2A, FOXM1* and *MK167/MIB1*) as a signature for cell proliferation in proliferating cells. Evaluation of expression profiles for these 'core sets' of genes in MPNSTs and other tumors revealed that the cell proliferation signature was a characteristic feature of MPNSTs and SS but not neurofibromas or schwannomas.

Loss of cell adhesion—Several cell adhesion genes, such as *MCAM, CDH2, L1CAM, VCAM1, CD44, CD58, ALCAM, and ITGAs* (integrins), were expressed at lower levels in MPNSTs relative to neurofibromas. E-cadherin (*CDH1*) was downregulated in both neurofibroma and MPNSTs compared to schwannomas and SS, whereas *CDH2*, a neuronal cell adhesion molecule, was downregulated 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 to the benign PNST, most MPNSTs and SS showed elevated expression of *TWIST1* and *UBEC2*, while increased expression of *HMMR* was more frequently seen in MPNSTs alone. In MPNSTs, there was a significant downregulation of thrombospondin (*THBS1* and *THBS2*, inhibitors of angiogenesis). The repression of thrombospondin leads directly to angiogenesis and tumor formation in mammary epithelial cells and kidney cells [26.].

Signaling pathways affected in MPNST

Gene set enrichment analysis [27.] was used to identify signaling pathways affected in MPNSTs. By comparing 'core' MPNSTs and 'core' neurofibromas, several signaling pathways were identified that appear associated with malignant transformation. Genes associated with signaling pathways (such as the extracellular matrix (ECM) pathway), cancer related genes involved in immune function, and genes involved in cell death were significantly downregulated 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 is shown in supplementary figure 2. The *TGFB* pathway related genes, such as *TGFBR2*, and *STAT1*, are downregulated in MPNSTs (Figure 2). We noticed relative low expression of several tumor 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 tumors and SS 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 MPNST 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 1 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 tumors and synovial sarcoma are shown in Figure 3C–H.

Loss of p53 function

Loss of p53 function has been associated with increased cell proliferation. We analyzed 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 upregulated in MPNSTs (Figure 4). Though most MPNSTs included in the analysis showed the signature for p53 inactivation, 4 MPNST tumor cases (STT4734, STT4737, STT4541 and STT3920) did not show the signature for p53 inactivation. Of these 4 cases, 2 (cases STT3920 and STT4541) clustered with the benign tumors 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 downregulated due to inactivation of p53 (*PDE5A, TAGLN, CUL4B* and *UGCG*) showed elevated expression in the majority of neurofibromas compared to 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 tumors (6 MPNSTs, 11 neurofibromas and 6 schwannomas). The 6 MPNSTs had also been used for mRNA profiling (Figure 1). Distinct miRNA expression profiles clearly distinguished MPNSTs from the benign tumors in unsupervised clustering using the 64 miRNAs that met the filtering criteria. Among the benign tumors, neurofibroma 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 tumor class. In comparison with benign tumors,

five miRNAs (miR-214, miR-377, miR409-3p, miR-487b and miR-99b) were relatively upregulated and miR-517, miR-34a, miR-29a, miR-30e-5p and miR-27a were downregulated in MPNSTs (FDR 1%). The gene expression profiling data showed the gene signature for p53 inactivation in 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 patient's tumor samples validated the microRNA array data (Figure 5b).

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

We determined the expression levels of 10 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 expression levels of these miRNA in MPNST-14 and MPNST-724 cells with HEK-293 cells (p53 positive) shows that both these MPNST cell lines have at least 2–10 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 the MPNST cell lines by transfection with wt-p53-GFP containing plasmid. We found a significant increase in the levels of miR-34a and 9 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 to its transfection efficiency with the miR-34a construct (about 40%, Supplementary figure 3). Further, the presence of transfected miRs were verified in these cells using qRT-PCR (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 (Supplementary figure 4).

Transfection experiments were also carried out with the second MPNST cell line, MPNST-724, a cell line derived from non-NF1 individual (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 qRT-PCR. These mRNA targets are known to be influenced by miR-34a [30, 31.]. We found that expressions of these mRNAs were decreased (by 38–40%) upon miR-34a transfection (Figure 6d).

Discussion

Our gene microarray analysis identified expression signatures for the 4 classes of tumors (MPNST, neurofibroma, schwannoma and synovial sarcoma) included in the study. Unsupervised hybrid clustering separated the majority of the benign tumors from the malignant tumors. There were a few outliers in each class of tumor, which may reflect alterations of gene expression seen in different stages of tumor progression. For example, 6 of 26 neurofibroma cases clustered in the branch containing most of the MPNSTs. Though these neurofibroma cases show the 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 resulted in a gene expression profile more similar to MPNSTs than with typical neurofibromas. For 5 of these 6 neurofibromas, the NF1 status was known and 4 were resected from NF1 patients. In contrast, of the 20 neurofibromas clustering on branch A, the NF1 status of 16 cases were known, 8 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 to neurofibromas, 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 tumors, 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 have been found in plexiform neurofibromas [34.].

A search for genes that are uniquely overexpressed in all 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 analyzing 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 were found. Genes such as *TOP2A* and *TWIST1* that were previously implicated in the progression of MPNSTs [7, 16.] were also found to be over expressed in our gene array analysis. Likewise, *CRABP1* was one of the top 5 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 SS from other mesenchymal tumors. Loss of expression of genes such as E-cadherin and over-expression of the zinc-finger transcriptional repressor gene ‘snail’ (*SNAI2*) is associated with tumor invasion [36, 37.]. This interactive mechanism of downregulation of E-cadherin and upregulation of *SNAI2* was observed in most MPNSTs in our mRNA expression study.

Similar to earlier reports [13, 38.], our TMA analysis revealed an elevated expression of EGFR in a subset of MPNSTs and neurofibromas. EGFR expression was not associated with the mutational status of *NF1*. Gene and tissue microarray 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.]. Further, a majority of the synovial sarcomas stained for EGFR, which was 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 SS as compared to MPNSTs (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 297bp (the usual fusion product seen in other cases of SS is 585bp). Sequencing identified a novel, previously unreported fusion of exon 8 *SYT* gene to exon 7 *SSX1* (Supplementary figure 5). Interestingly, this SS case had relatively lower levels of TLE1 expression compared to other SS samples (Figure 1).

Two noticeable miRNA-mRNA associations became apparent in our study, (i) microRNA expression analysis indentified 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 majority of MPNSTs and thus may be involved in miR-214 expression in MPNSTs as well.

(ii) Several miRNAs were downregulated in MPNST compared to the benign nerve sheath tumors, including miR-34a. Initial studies on primary and tumor derived cell lines shown that p53 induces the expression of miR-34a [19, 29, 42.]. Subsequent studies have demonstrated miR-34a expression induces apoptosis in colon cancer and neuroblastomas [30, 43.], however, no sarcomas samples were included in these studies. To better understand 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 to HEK-293 cells. Relative downregulation of miR-34a in these cell lines is consistent with the lower level of miRNA seen in MPNST tumor samples and with our observation the majority of MPNSTs show a gene expression signature that is associated with p53 inactivation. Transfection of p53 in MPNST cells induced 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 majority of MPNSTs, p53 is inactivated either by mutation or through other unknown mechanisms. In our study, miR-29a was significantly downregulated in MPNSTs, miR-29 family miRs can activate p53 by blocking CDC42 and p85α, the negative regulators of p53[44.]. Further, in our functional assay only miR-34a showed apoptotic mediated cell death in the MPNST cells whereas mR-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 determinator for mRNA target specificity [45.]. However, a search in miRgen for consensus target prediction showed only a partial overlap in the targets for these 2 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 downregulated 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 proapoptotic 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 tumor cell proliferation and survival through cell cycle arrest and apoptosis [43, 46.].

In conclusion, molecular profiling of nerve sheath tumors reveal MPNSTs to be a heterogeneous group of tumors. The major trend in malignant transformation towards MPNST is the downregulation of large number of genes. MPNSTs are characterized by hallmark molecular events such as upregulation of cell cycle genes, loss of cell adhesion, gene signature for inactivation of p53, and elevated expression of genes that regulates tumor metastasis. Based on 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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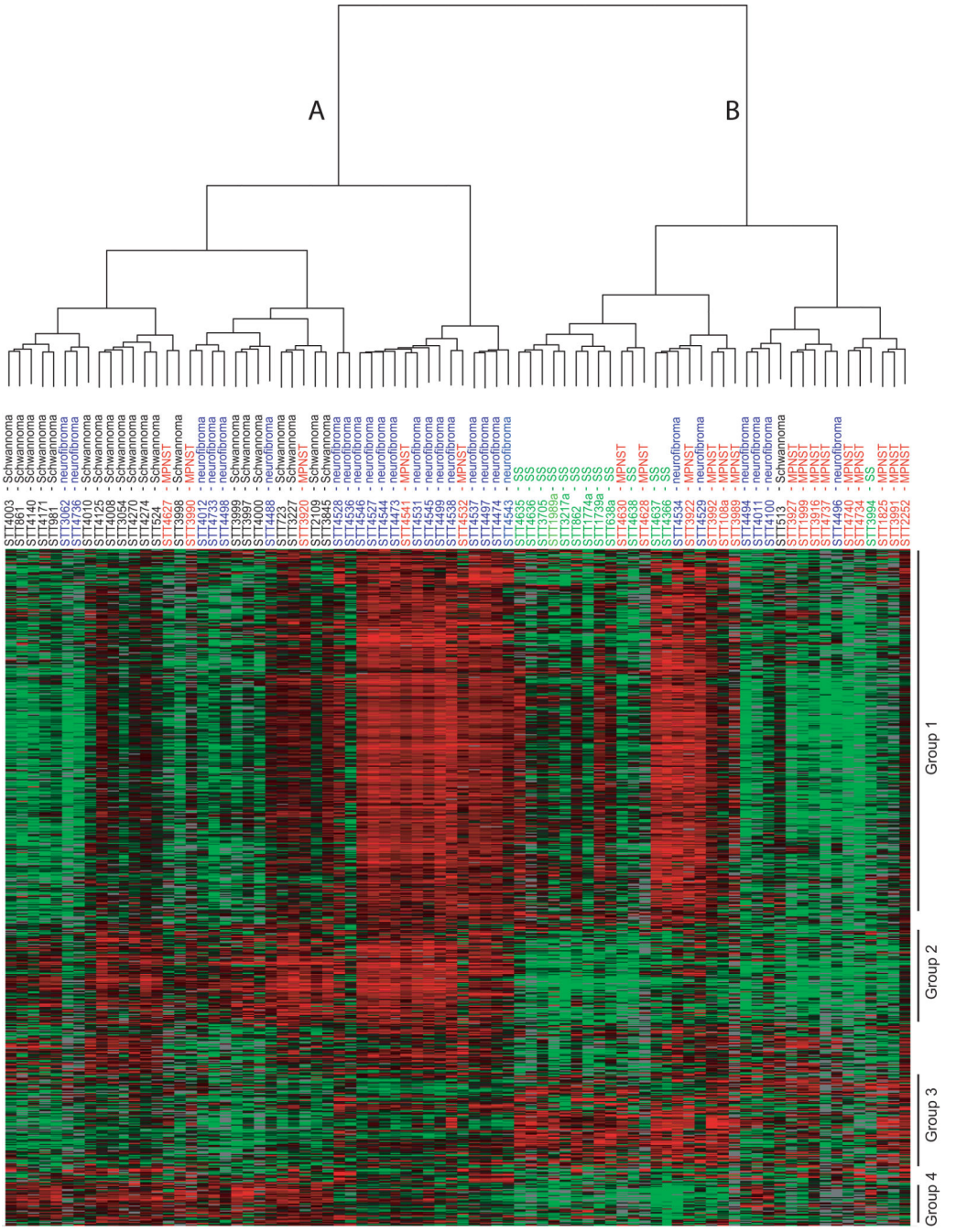

Figure 1.

Unsupervised hierarchical cluster analysis of gene expression profiles of 67 nerve sheath tumors 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 colors indicate high and low expression, respectively. Grey indicates missing data. The two main branches of the dendrogram labeled A and B predominantly separate the benign tumors (neurofibroma and schwannoma) from the malignant tumors (MPNSTs and SS).

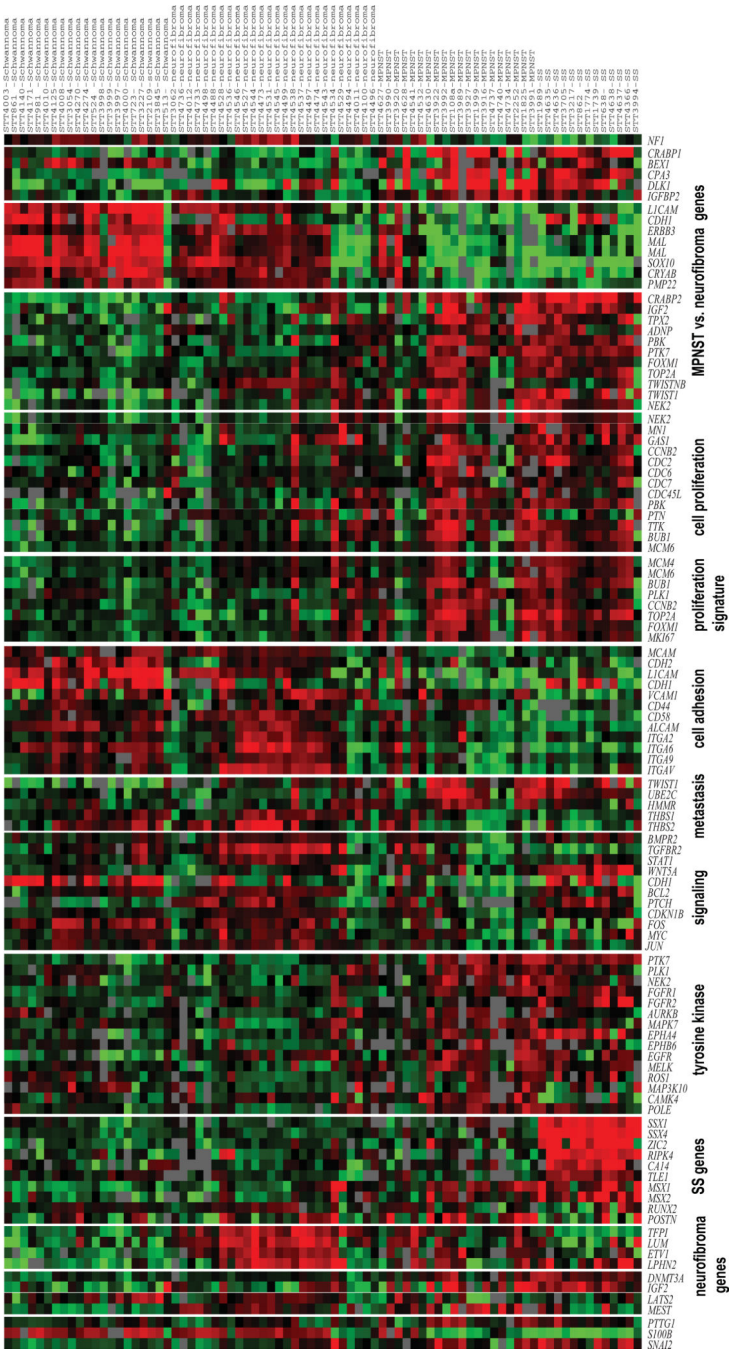

Figure 2.

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.

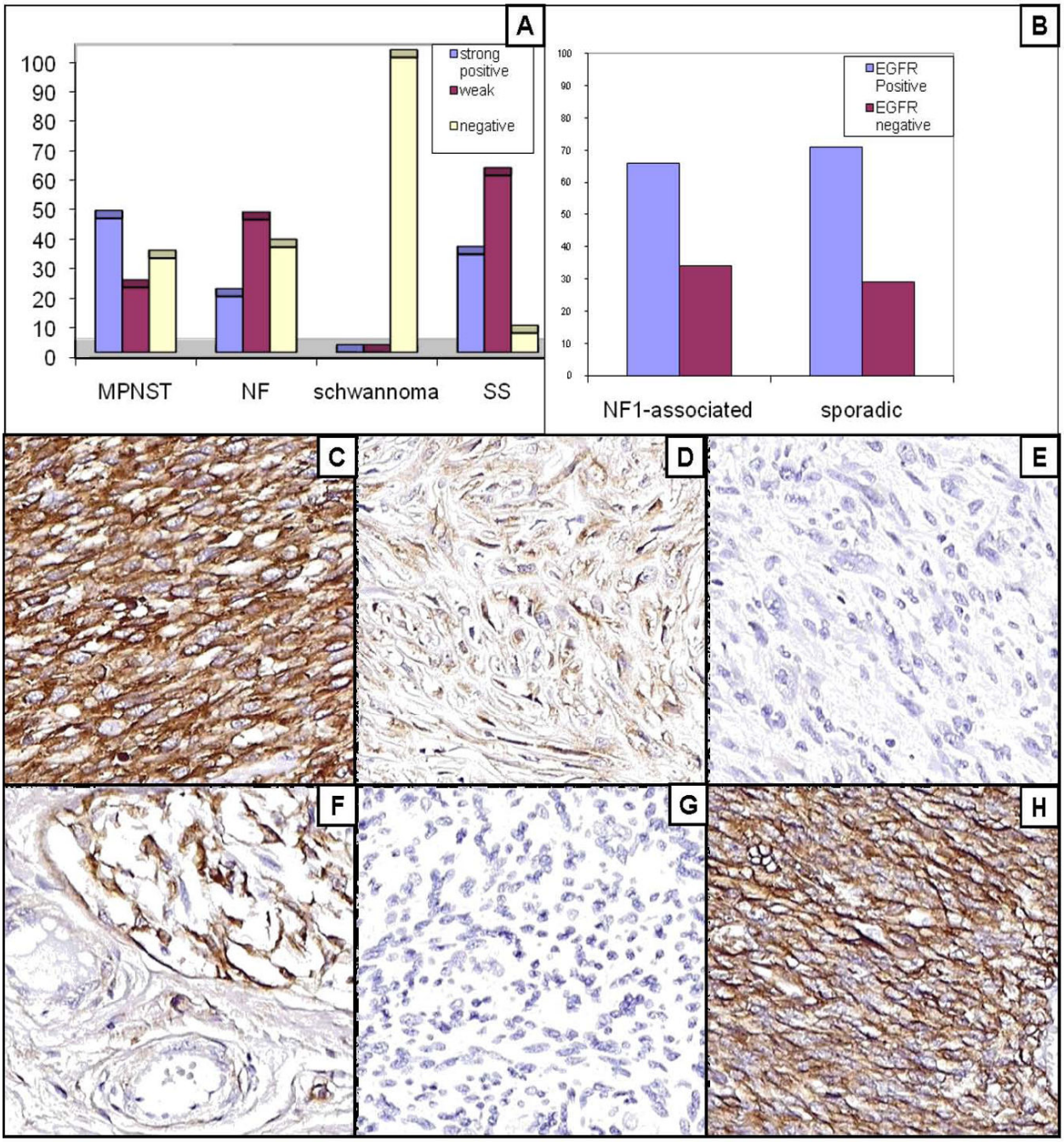

Figure 3.

Graphical representation of the percentage of positive IHC for EGFR staining in: a) PNSTs and synovial sarcomas; 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 f) strong expression in a synovial sarcoma.

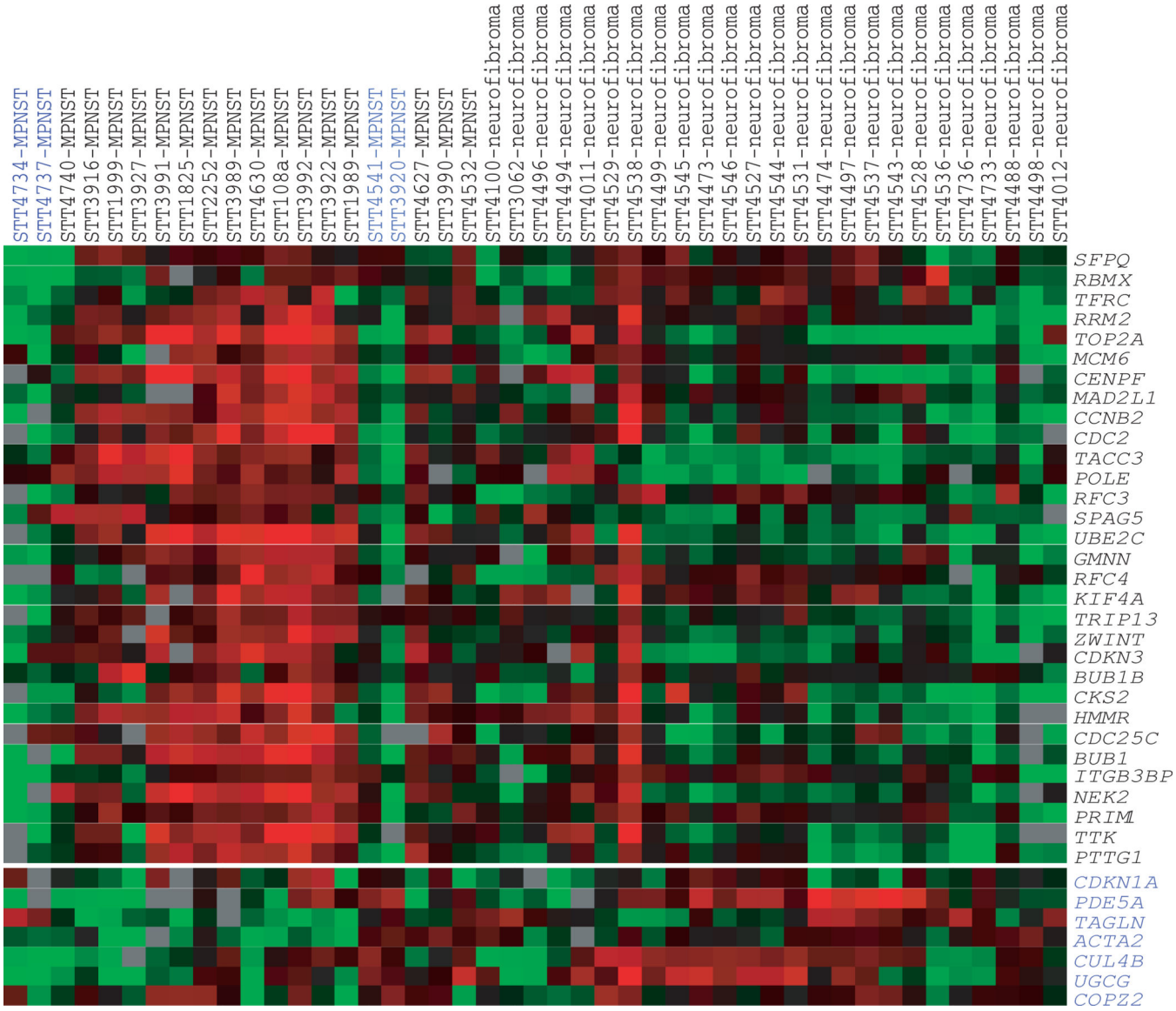

Figure 4.

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* (37) 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 Figure 4 (shown in blue) are a group of 8 genes that are downregulated due to p53 inactivation in the study of Milyavsky (37) and are expressed at low levels in MPNSTs. A few MPNST cases (4 cases total) did not show the p53 inactivation signature and are highlighted in blue; 2 of these (STT 4541 and −3920) clustered with the benign PNSTs in branch A of Figure 1.

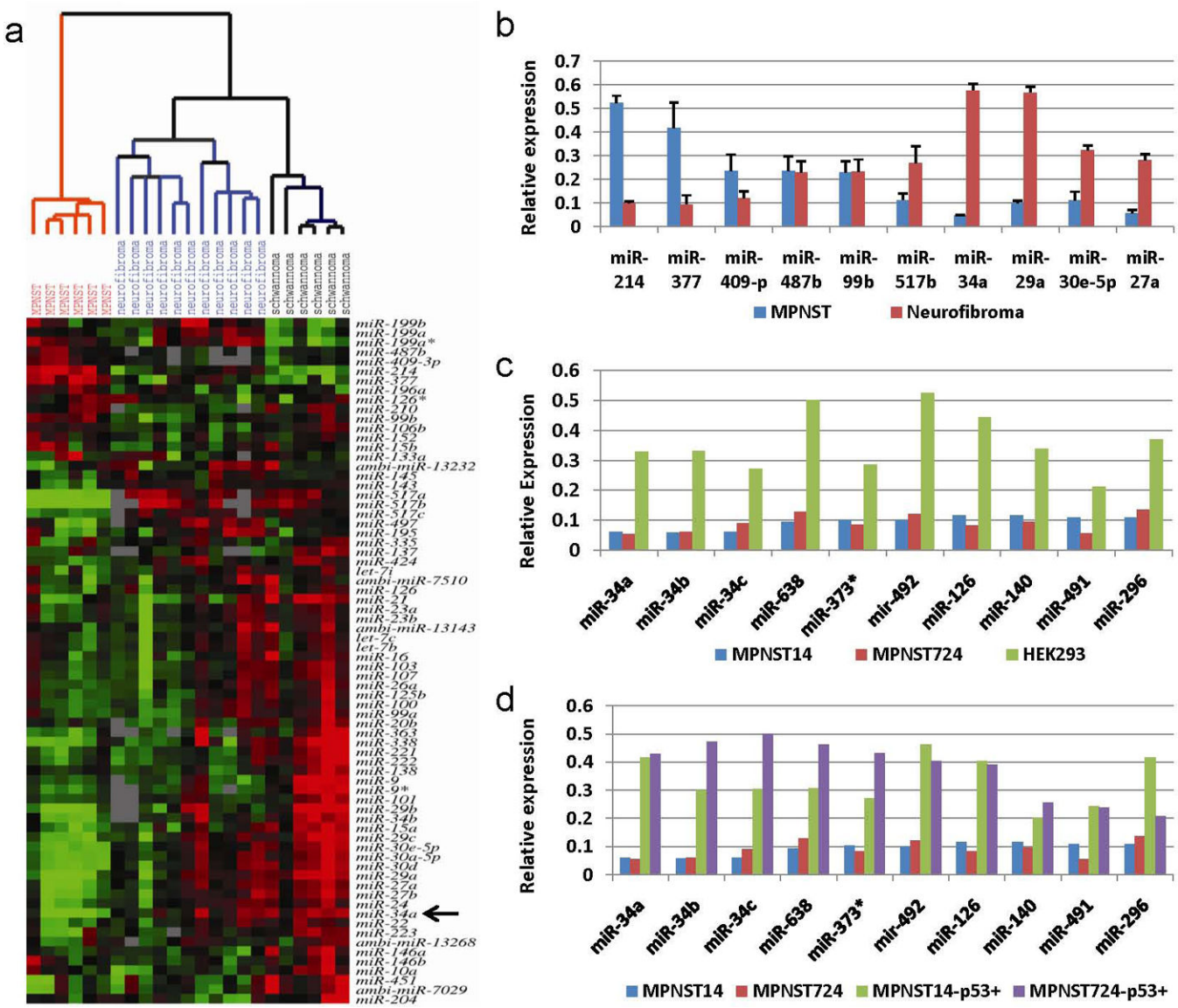

Figure 5.

a) MicroRNA expression profiling and unsupervised hierarchical clustering analysis of 23 peripheral nerve sheath tumors. 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 color indicates relatively high or low expression, respectively, while gray indicates absent data points

b) qRT-PCR analysis of differentially expressed miRNAs in 5 MPNST and 5 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, miR34b, 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 to 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 were normalized to U6.

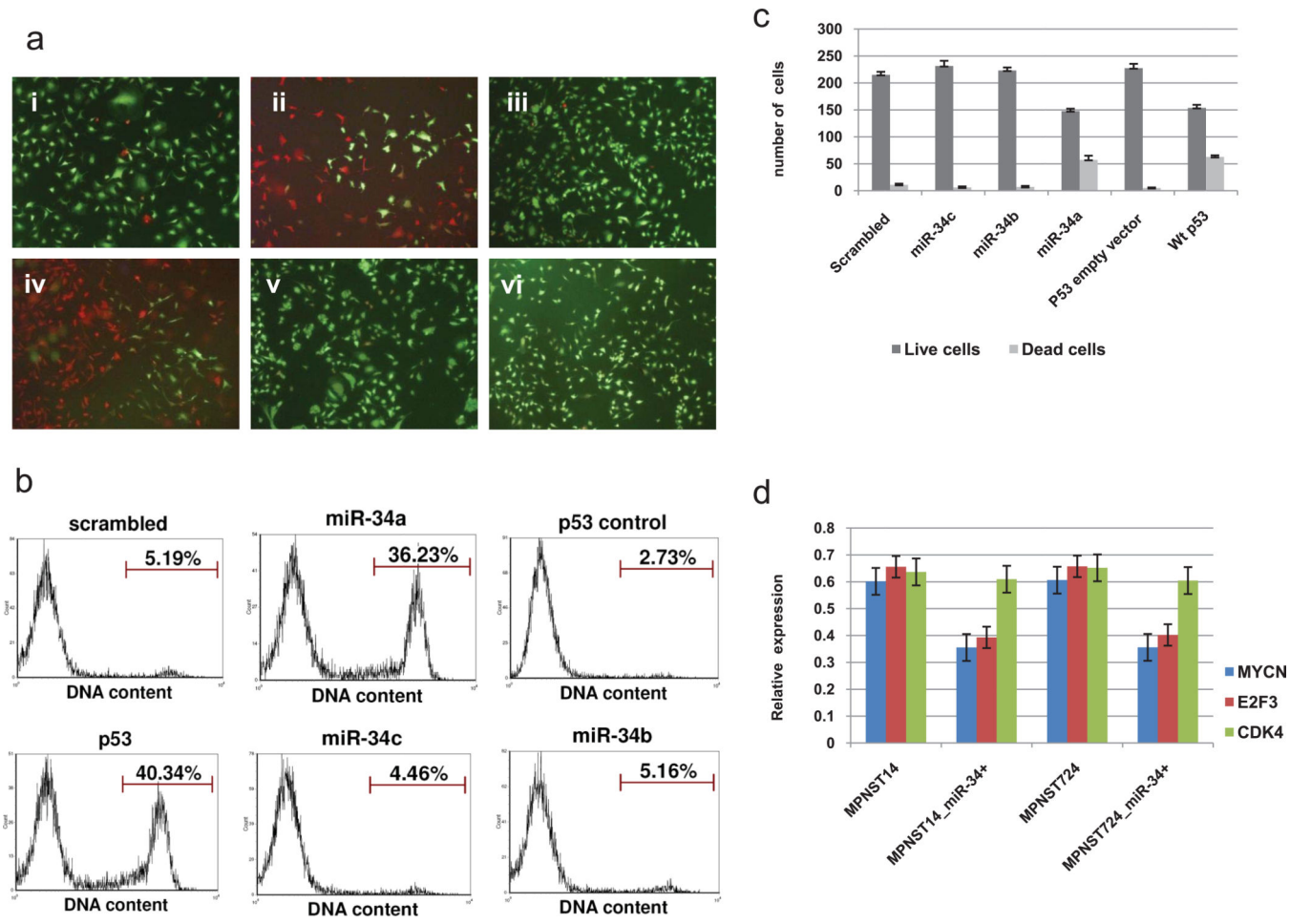

Figure 6.

a) Live dead assay of transfected MPNST cells. Cells were transfected with scramble sequence control (i) with miR-34a (ii) empty vector control (iii) and with wt-p53 (iv) and subjected to cell viability assay, 48 hrs after transfection.

b) FACS analysis of miR-34 or p53 transfected cells. Panels correspond to the transfection experiments shown in figure 6a.

c) Pro-apoptotic effect of miR-34a and p53 is independent of NF1-mutations 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 duplicates 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 were subjected to qRT-PCR analysis. miR-34a transfection reduces these transcripts by 38–40%. Data is normalized to GAPDH expression levels and values are mean $_{\pm}$SD.

Table 1

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

Clinical Fearure	MPNST	neurofibroma	schwannoma	synovial sarcoma
Sample size (n)	20	37	27	13
Male gender, (%)	55	35	28	42
Median age (yr)	33	34	49	33
Median tumor size (cm)	9	6	4.1	21
NF1 (%)	55	67	nd	nd
SYT-SSX fusion (%)	0	nd	nd	100

nd, not done

Table 2

Gene ontology terms associated with each group of gene 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

Table 3

Gene set enrichment analysis of core neurofibromas and MPNSTs

Gene Sets	Size	ES	NES	NOM p value
PIP3 signaling in B lymphocytes	37	0.6385	1.8572	0.0000
Extra cellular 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 p val, normalized p value