

Oncogene Mutation Survey in MPNST Cell Lines Enhances the Dominant Role of Hyperactive Ras in NF1 Associated Pro-Survival and Malignancy

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Abstract: Malignant peripheral nerve sheath tumors (MPNST) are a type of soft tissue sarcoma that can be associated with germline mutations in Neurofibromatosis type 1 (*NF1*) or may occur sporadically. Although the etiology of MPNST is poorly understood, it is clear that a loss of function of the *NF1* gene, encoding a Ras-GAP, is an important factor in the tumorigenesis of the inherited form of MPNST. Tumor latency in NF1 patients suggests that additional mutational events are probably required for malignancy. In order to define oncogene mutations associated with 5 MPNST cell lines, we assayed the 238 most frequent mutations in 19 commonly activated oncogenes using mass spectroscopy-based analysis. All 238 mutation sites in the assayed oncogenes were determined to harbor only wild-type sequences. These data suggest that hyperactive Ras resulting from the loss function of neurofibromin may be sufficient to set up the direction of malignant transformation of Schwann cells to MPNST.

Keywords: MPNST, Ras-GAP, Neurofibromatosis, oncogene mutation

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Introduction

Malignant peripheral nerve sheath tumor (MPNST) is a type of soft tissue sarcoma that develops from the peripheral nerves, preexisting benign neurofibromas, or Schwann cells. Approximately 50% of MPNST occurs in the setting of NF1 genetic disease (OMIM ID #162200) with the remainder occurring sporadically. The lifetime risk for NF1 patients to develop a MPNST is 8%–13%, and the five-year survival rate is 20%–50%. NF1 patients are most frequently diagnosed with MPNST in the third and fourth decades of life whereas the sporadic form of MPNST is most frequently diagnosed in the sixth and seventh decades of life.¹

MPNST is thought to arise from the accumulation of additional genetic events in precursor Schwann cells. Neurofibromas are benign peripheral nerve sheath tumors and may or may not be associated with the *NF1* gene. Plexiform neurofibromas are almost exclusively *NF1*-associated and easily progress to MPNST. Mouse model studies have indicated a heterozygous *NF1* state was required for the Schwann cell derived plexiform neurofibroma formation and tumor microenvironment.² Localized cutaneous neurofibroma and diffuse cutaneous neurofibroma are 90% sporadic and have lower malignant potential.³ Whether the status of the *NF1* gene is the only requirement for malignancy remains questionable, however.

In *NF1*-associated neurofibroma, Ras signaling is hyperactive because of the loss of function of neurofibromin (encoded by *NF1* gene) which normally acts as a Ras GTPase activating protein (GAP). Whereas other cell types exhibit oncogene-induced senescence after activation of Ras due to missense mutations at amino acids 12,13, and 61, inactivation of *NF1* in neurofibromas typically results in a transitory growth arrest. Eventually escaping this growth limitation gives rise to MPNSTs.⁴ The mechanism behind this escape from oncogene-induced senescence it is not completely understood. A genetic study investigating neurofibroma occurrence in 175 patients from 48 pedigrees (including 6 monozygotic twins) suggested that there was highest correlation of tumor occurrence in monozygotic twins, which indicated that shared “modifier genes” rather than *NF1* mutations *per se* also played an important role on neurofibroma occurrence.⁵

In sporadic neurofibroma or Schwannoma, even less is known about the malignant transformation process. Mutations leading to the loss of function of *TP53*, *RBI* and deletions of the *CDKN2A* gene have been reported in both *NF1*-associated and sporadic MPNSTs suggesting that a series of mutations of tumor suppressor genes contributes to tumorigenesis and subsequent malignant transformation.^{6–8} In addition to tumor suppressor gene mutations, oncogenic mutations in platelet-derived growth factor receptor alpha (*PDGFR- α*), as well as gene amplification of epidermal growth factor receptor (*EGFR*) and hepatocyte growth factor (*HGF*) have been reported in MPNSTs, although these mutations are found at very low frequency.^{7,9,10} Taken together, these studies suggest that there is significant genetic heterogeneity in the initiation and progression of MPNSTs.

Currently, several MPNST cell line-based gene expression analyses have been used to identify gene expression signatures characteristic of MPNST,^{11,12} but the knowledge of the genetic background of these cell lines is quite limited. A detailed characterization of the genetic alterations in such cell lines will contribute to the understanding of MPNST tumorigenesis and decipher the gene expression alterations derived from microarray gene expression data. Moreover, the determination of actionable oncogenic mutations¹³ in MPNST cell lines may provide insight into rational MPNST therapeutics and drug discovery for the clinic. We therefore set out to determine whether the most frequent activating oncogene mutations found in human cancers were present in these MPNST cells. Mutational analysis of neurofibroma was carried out to investigate whether additional genetic alterations in any known oncogenes led to the development of MPNST. Here, we present the first report of oncogene mutation analysis of the most frequent 238 mutations in 19 oncogenes using mass spectroscopy based analysis among 5 MPNST cell lines.

Materials and Methods

Cell lines and tissue culture

Human MPNST ST88-14 cells (a generous gift from T. Glover, University of Michigan, Ann Arbor, MI, USA) and STS26T cells (a generous gift from D. Scoles, Cedars-Sinai Medical Center, Los Angeles, CA, USA) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with



5% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA). T265 cells (a generous gift from G. De Vries, Hines VA Hospital, Hines, Illinois USA) were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum. sNF96.2 and sNF02.2 cell lines were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% fetal bovine serum. Normal human Schwann cell line HSC361 (a kind gift from Patrick M. Wood, University of Miami Miller School of Medicine, Miami, FL, USA) was cultured in DMEM supplemented with 10% FBS, 2 μ M forskolin, and 10 nM heregulin. Cell lines were checked periodically for myco-plasma with Venor GeM Mycoplasma Detection Kit (Sigma, St. Louis, MO, USA). Cultures were propagated for no more than 3 months.

Western blot analysis

Cultures at 30% to 80% confluence were washed 3 times with ice-cold PBS, scraped and pelleted at 1,000 rpm for 5 min. Cell pellets were lysed with RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-Cl, pH 8.0) supplemented with 2% protease inhibitor cocktail, 1% PMSF, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, and 1 mM NaF. Secondary antibodies were conjugated to IRdye infrared dyes (Rockland Inc, Gilbertsville, PA, USA). Signal was detected and the bands were quantified using the Odyssey infrared imaging system and software (Licor Biosciences, Lincoln, NE, USA). Antibodies used in these experiments were rabbit poly-clonal anti-neurofibromin (#sc-67, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), mouse monoclonal anti-phospho-ERK1,2 (#9106, Cell Signaling), and mouse monoclonal anti-a-tubulin (#T5168, Sigma-Aldrich, St. Louis, MO, USA). Neurofibromin in cell lysates was detected by 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylated Erk1/2 was detected on 10% SDS-PAGE. 80 μ g protein lysate was loaded in each well, and PDVF membrane was used for protein transfer.

Oncogenomic profiles based on point mutation analysis

DNA was extracted from MPNST cell lines grown in culture using the DNeasy blood and tissue kit (Qiagen, catalog 69504). Point mutation analysis was performed

using the mass-spectroscopy based MassArray system developed by Sequenom (San Diego, CA, USA). In this study, we employed the Sequenom OncoCarta Panel v1.0. This mutational panel interrogates 238 mutations in 19 oncogenes (Table 1). These mutations include small indels and single base pair changes that result in non-synonymous coding mutations previously reported in human cancers.¹³ The assays are multiplexed into 24 reactions, each of which requires 20 ng of input DNA. Briefly, an initial PCR reaction is performed to amplify a small region of DNA (between ~80 to 120 base pairs) which includes the potential point mutation site. Next, a specific oligonucleotide primer with at least 10 bases of complementary DNA binds immediately upstream of the mutation site and is extended by one base into the potential mutation site. The oligonucleotide primers are subsequently separated on a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer which is able to quantitatively discern the specific nucleotide that was extended. This method can reproducibly detect mutations at a frequency as low as 10% within a given sample.

Results

Five different MPNST cell lines were used to detect oncogene mutations according to the OncoCarta Panel V1.0. ST88-14, T265, sNF96.2 and sNF02.2 are *NF1*-associated MPNSTs with either undetectable or extremely low neurofibromin protein levels as shown by western blot analysis (Fig. 1). STS26T cells were derived from a sporadic MPNST and have functional neurofibromin as did normal human Schwann cells (HSC361) which were used as a positive control. Cytogenetic characterization of ST88-14 cells has shown that the *NF1* locus has been deleted from one allele on chromosome 17 while the gene on the remaining allele exhibits greatly reduced transcriptional activity.¹⁴ A heterozygous nonsense mutation in *NF1* (C910T) in codon 304 (R304X) of exon 7 was also reported in ST88-14.¹⁵ In the T265 cell line, no *NF1* mutation has been reported, and the neurofibromin was hardly detectable (Fig. 1). The sNF96.2 cell line was derived from a recurrent mass associated with nerve tissue and diagnosed as MPNST in an *NF1* patient. This line has an abnormal karyotype and complete loss of heterozygosity with no detectable DNA from the wt-*NF1* allele.¹⁶

Table 1. Mutations assayed for each of the 19 Genes in the Oncocarta v1.0 Mutation Panel. A total of 238 mutations were assayed.

Gene name	Mutation sites checked
<i>ABL-1</i>	G250E, Q252H, Y253H, Y253F, E255K, E255V, D276G, F311L, T315I, F317L, M351T, E355G, F359V, H396R
<i>AKT-1</i>	V461L, P388T, L357T, E319G, V167A, Q43X, E17del
<i>AKT-2</i>	S302G, R371H
<i>BRAF</i>	G464R, G464V/E, G466R, F468C, G469S, G469E, G469A, G469V, G469R, G469R, D594V/G, F595L, G596R, L597S, L597R, L597Q, L597V, T599I, V600E, V600K, V600R, V600L, K601N, K601E
<i>CDK-4</i>	R24C, R24H
<i>EGFR</i>	R108K, T263P, A289V, G598V, E709K/H, E709A/G/V, G719S/C, G719A, M766_A767insAI, S768I, V769_D770insASV, V769_D770insCV, D770_N771>AGG/V769_D770insASV/V769_D770insASV, D770_N771insG, N771_P772>SVDNR, P772_H773insV, H773>NPY, H773_V774insNPH/PH/H, V774_C775insHV, T790M, L858R, L861Q, E746_T751del, E746_A750del, E746_T751del, E746_T751del, S752D, L747_E749del, L747_T750del, L747_S752del, L747_T751del, L747_S752del, P753S, A750P, T751A, T751P, T751I, S752I/F, S752_I759del, L747_Q ins, E746_T751del, I ins (combined), E746_A750del, T751A (combined), L747_E749del, A750P (combined), L747_T750del, P ins (combined), L747_S752del, Q ins (combined)
<i>ERBB2</i>	L755P, G776S/LC, G776V/CVC, A775_G776insYVMA, P780_Y781insGSP, P780_Y781insGSP, S779_P780insVGS
<i>FGFR-1</i>	S125L, P252T
<i>FGFR-3</i>	G370C, Y373C, A391E, K650Q/E, K650T/M
<i>FLT-3</i>	I836del, D835H/Y
<i>JAK-2</i>	V617F
<i>KIT</i>	D52N, Y503_F504insAY, W557R/R/G, V559D/A/G, V559I, V560D/G, K550_K558del, K558_V560del, K558_E562del, V559del, V559_V560del, V560del, Y570_L576del, E561K, L576P, P585P, D579del, K642E, D816V, D816H/Y, V825A, E839K, M552L, Y568D, F584S, P551_V555del, Y553_Q556del
<i>MET</i>	R970C, T992I, Y1230C, Y1235D, M1250T
<i>PDGFRa</i>	V561D, T674I, F808L, D846Y, N870S, D1071N, D842_H845del, I843_D846del, S566_E571>K, I843_S847>T, D842V
<i>PIK3CA</i>	R88Q, N345K, C420R, P539R, E542K, E545K, Q546K, H701P, H1047R/L, H1047Y, R38H, C901F, M1043I
<i>H-RAS</i>	G12V/D, G13C/R/S, Q61H/H, Q61L/R/P, Q61K
<i>K-RAS</i>	G12C, G12R, G12S, G12V, G12D, G12A, G12F, G13V/D, A59T, Q61E/K, Q61L/R/P, Q61H/H
<i>N-RAS</i>	G12V/A/D, G12C/R/S, G13V/A/D, G13C/R/S, A18T, Q61L/R/P, Q61H, Q61E/K
<i>RET</i>	C634R, C634W, C634Y, E632_L633del, M918T, A664D

The sNF02.2 cell line was derived from a lung metastasis MPNST in an NF1 patient. A very weak full length NF1 band was observed in these two cell lines (Fig. 1). The STS26T cell line was derived from a sporadic malignant Schwannoma, a type of soft tissue sarcoma.¹⁷ This cell line has a wild type neurofibromin but p53 expression was completely absent.¹²

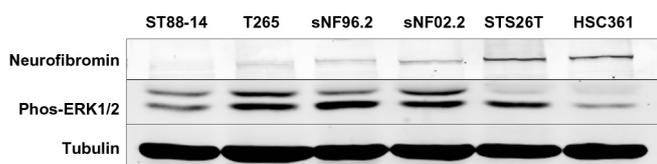


Figure 1. Neurofibromin expression and phosphorylated Erk1/2 status in the cell lines.

Increased MAPK activity has been reported in NF1 patients.¹⁸ We confirmed this increased MAPK activity by testing the phosphorylation state of Erk1/2 in all the MPNST cell lines in our study. All the MPNST cell lines exhibited more phosphorylated Erk 1/2 than the normal HSC361 cell line. However, the relative intensity of Erk1 and Erk2 varied among the different cell lines. Notably, even with functional neurofibromin, the phosphorylated Erk1/2 in STS26T was significantly higher than the normal HSC361 cells which emphasizes the indispensable role of MAPK pathway activation in NF1-related MPNSTs.

The mass-spectroscopy based MassArray system provides a high-throughput method to characterize the oncogenic alterations in tumors with high accuracy



and acceptable cost. In this study, we assayed the 238 most frequent mutations in 19 commonly activated oncogenes. This panel covered 90%~99% of the mutation prevalence reported thus far in the 19 oncogenes.¹³ All 238 mutation sites in the assayed oncogenes were determined to harbor only wild-type sequences in all 4 *NF1*-defective MPNST cell lines or STS26T (*NF1*-wild type) using the OncoCarta Panel V1.0 (Table 1).

Discussion

MPNST is a rare malignant tumor with poor prognosis, accounting for 3%~10% of all soft tissue sarcomas.¹⁹ Microarray studies failed to distinguish the *NF1*-associated and sporadic form of MPNST²⁰ which indicates accumulated aberrant gene regulation is common in this kind of tumor. It is generally believed that MPNST probably comprises multiple distinct tumors sharing similar histological manifestations and therefore may arise from heterogeneous mechanisms of tumorigenic transformation. It is a consensus that *NF1*-associated neurofibroma is derived from *NF1*^{-/-} Schwann cells and that the *NF1*^{+/-} haplo-deficient cells within the tumor microenvironment contribute to malignant transformation. However, the key biological events occurring during MPNST development are not clear. Schwannoma is also a benign peripheral nerve sheath tumor and 1% can undergo malignant conversion such as the STS26T cell line. Although *TP53* mutations appear to be more associated with the sporadic form, it is not known whether other genetic alterations contribute to the malignant transformation of these benign tumors. Indeed, four of the cell lines in this study are known to be *TP53* wild-type and these are the cell lines derived from *NF1*-associated MPNSTs (ST88-14, T265, sNF96.2 and sNF02.2).^{21,22} Only the sporadic MPNST-derived cell line STS26T is *TP53* null.²² By analyzing potential oncogene mutations, we sought to identify whether such genetic alterations play a role in the evolution of MPNSTs.

Among the 19 oncogenes we investigated for activating mutations, only 4 genes have been reported to be mutated in MPNST tumors. Specifically, activating point mutations have been found at a very low frequency²³ in *BRAF* (1/83 tumors tested)²⁴⁻²⁶ *KRAS* (1/98),^{27,28} *NRAS* (1/33),^{25,28} and *PDGFRA* (2/61)^{10,25,29} (COSMIC database). Furthermore, gene

amplification of *EGFR*,³⁰ *ERBB2*³¹ and *c-KIT*¹⁰ have also been reported previously. In addition, among the 19 oncogenes we assayed, no activating point mutations were found in the following: *EGFR* (81 tumor samples analyzed), *ERBB2* (40), *HRAS* (33), *KIT* (38), *PIK3CA* (23) and *RET* (4) (COSMIC Database).

The results presented here suggest that oncogene mutation related effects were not necessary in the formation of MPNSTs, especially in the context of hyperactive Ras due to *NF1* loss. In the *NF1*-associated MPNSTs, the constitutively active Ras pathway provides strong pro-survival signals through its downstream Raf-MEK-MAPK and PI3K-AKT-mTOR signaling pathway. These activated pathways may abrogate the selective pressures against mutations in oncogenes such as *AKT1*, *AKT2*, *BRAF*, and *PI3K* during tumorigenesis. This may explain why the common oncogene mutations in cancer were not observed in this study. On the other hand, the absence of oncogene mutations also suggests that elevated Ras activation due to *NF1* deficiency in an *NF1* haplo-insufficient microenvironment may be sufficient to initiate the cells and microenvironment to facilitate the pro-survival of the *NF1* mutation-bearing Schwann cells to circumvent the anti-cancer mechanism and finally develop malignancy. For the sporadic MPNST, limited availability of tissue has made it more difficult to study than its *NF1*-associated counterpart. Here, we first explored possible oncogene mutations in the STS26T cell line, and the results indicated the mechanism giving rise to the sporadic MPNST is more complicated than the known changes in pathways downstream of Ras or other oncogenes.

It is possible that potential mutations were not known or covered by our panel of 238 assays in this study. However, based on the COSMIC database and our study, extra oncogene activation is rarely reported in *NF1* patients and MPNST cell lines. This study cannot rule out other regulatory mechanisms such as epigenetic modification of the expression of other key cellular regulatory pathways, however. In the *NF1*-associated MPNSTs, our data suggests that hyperactive Ras due to *NF1* loss of function provides a sufficient impetus to malignancy. The *NF1* haplo-deficient cells and the microenvironment are involved in the final selection process of tumor



formation, which partially explains the latency of MPNST in NF1 patients.

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Disclosures

Author(s) have provided signed confirmations to the publisher of their compliance with all applicable legal and ethical obligations in respect to declaration of conflicts of interest, funding, authorship and contributorship, and compliance with ethical requirements in respect to treatment of human and animal test subjects. If this article contains identifiable human subject(s) author(s) were required to supply signed patient consent prior to publication. Author(s) have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have consent to reproduce any copyrighted material. The peer reviewers declared no conflicts of interest.

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