Short Communication

NF1 Deletions in S-100 Protein-Positive and Negative Cells of Sporadic and Neurofibromatosis 1 (NF1)-Associated Plexiform Neurofibromas and Malignant Peripheral Nerve Sheath Tumors

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Although plexiform neurofibroma (PN) is thought to represent a benign neoplasm with the potential for malignant transformation (malignant peripheral nerve sheath tumor; MPNST), its neoplastic nature has been difficult to prove due to cellular heterogeneity, which hampers standard molecular genetic analysis. Its mixed composition typically includes Schwann cells, fibroblasts, perineurial-like cells, and mast cells. Although *NF1* loss of heterozygosity has been reported in subsets of PNs, it remains uncertain which cell type(s) harbor these alterations. Using a dual-color fluorescence *in situ* hybridization and immunohistochemistry technique, we studied *NF1* gene status in S-100 protein-positive and -negative cell subpopulations in archival paraffin-embedded specimens from seven PNs, two atypical PNs, one cellular/atypical PN, and eight MPNSTs derived from 13 patients, seven of which had neurofibromatosis type 1 (NF1). *NF1* loss was detected in four of seven PNs and one atypical PN, with deletions entirely restricted to S-100 protein-immunoreactive Schwann cells. In contrast, all eight MPNSTs harbored *NF1* deletions, regardless of S-100 protein expression or NF1 clinical status. Our results suggest that the Schwann cell is the primary neoplastic component in PNs and that S-100 protein-negative cells in MPNST represent dedifferentiated Schwann cells, which harbor *NF1* deletions in both NF1-associated and sporadic tumors. (*Am J Pathol* 2001, 159:57–61)

Neurofibroma is defined as a benign nerve sheath tumor composed of a variable mixture of Schwann, perineurial-like, and fibroblastic cells, as well as ones with features

intermediate between these various cells.¹ Additional elements that may be encountered include mast cells, CD34-immunoreactive cells, melanocytic cells, heterologous epithelial elements, entrapped axons, ganglion cells or other native neural, dermal, or soft tissue components.^{1–3} This cellular heterogeneity has made it difficult to determine whether neurofibromas are neoplastic or hyperplastic in nature and, if the former, which cell type(s) are primarily neoplastic. Recognized variants of neurofibroma include localized cutaneous, diffuse cutaneous, localized intraneural, plexiform, and massive soft tissue forms.¹ Also, mitotically inactive examples with increased cellularity and/or pleomorphism are referred to as cellular and/or atypical neurofibromas or plexiform neurofibromas, and such cases may be difficult to distinguish from low-grade malignant peripheral nerve sheath tumor (MPNST). The plexiform neurofibroma (PN) is the only neurofibroma subtype with a significant rate of malignant transformation (~5%) into MPNST.¹ Because PN is encountered most commonly in the setting of neurofibromatosis type 1 (NF1), *NF1* is a logical candidate tumor suppressor gene for involvement in PN and MPNST tumorigenesis. Recent studies have demonstrated that ~63% of MPNSTs have *NF1* or 17q loss of heterozygosity (LOH); however, estimates of those genetic alterations in neurofibromas have ranged from 0 to 57% of cases^{4–12} (Table 1). Because most studies have not specified the growth patterns of their neurofibromas, these widely differing results likely reflect not only the complex cellular composition of individual tumors, but also varying subtypes of neurofibroma being analyzed. For example, those that have specified neurofibroma subtype have

Supported in part by Department of Defense grant DAMD 17-98-1-8611 (to A.P. and D.H.G.)

Accepted for publication April 11, 2001.

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Table 1. Repeated Loss of Heterozygosity Studies for *NF1* in Neurofibromas and Malignant Peripheral Nerve Sheath Tumors

Report (year)	All neurofibromas with <i>NF1</i> LOH	PNs with <i>NF1</i> LOH	MPNSTs with <i>NF1</i> LOH
Skuse GR ⁴ (1989)	0/11	NS	6/11 (55%)
Legius E ⁵ (1993)	ND	ND	1/1 (100%)
Lothe RA ⁶ (1993)	0/5	NS	1/1 (100%)
Lothe RA ⁷ (1995)	0/8	NS	4/6 (67%)
Colman SD ⁸ (1995)	5/22 (23%)	NS	ND
Däschner K ⁹ (1997)	1/38 (3%)	1/5 (20%)	ND
Serra E ¹⁰ (1997)	15/60 (25%)	NS	ND
Kluwe L ¹¹ (1999)	8/14 (57%)	8/14 (57%)	ND
Rasmussen SA ¹² (2000)	6/25 (24%)	4/10 (40%)	3/5 (60%)
Total	35/183 (19%)	13/29 (45%)	15/24 (63%)

NS, not specified; ND, not done.

reported high rates of LOH in PNs, with only rare LOH in cutaneous examples.^{9,11,12} In contrast to neurofibromas, MPNSTs are obviously neoplastic and often demonstrate some degree of Schwann cell differentiation. Given that some MPNSTs arise from PNs, the Schwann cell is thought to represent the most likely neoplastic component in PNs as well. However, a small minority of MPNSTs demonstrate perineurial differentiation¹³ suggesting that other cell types may be occasionally implicated. Interestingly, none of the perineurial MPNSTs reported thus far have been associated with either an underlying neurofibroma or the NF1 syndrome.¹³ Most recently, Schwann cells have been further implicated in studies finding cytogenetic alterations¹⁴ and lack of neurofibromin expression¹⁵ in cultured Schwann cells from neurofibromas, with no detectable alterations from cultured fibroblasts obtained from the same specimens. However, it is not clear from these *in vitro* experiments what selection biases were introduced by expansion of these cell populations in culture. In this study, we have performed the first *in situ* evaluation of *NF1* deletions within intact PNs and MPNSTs.

Materials and Methods

Eighteen cases of PN, atypical PN, and MPNST were retrieved from the archives of the Lauren V. Ackerman Surgical Pathology Laboratory at the Washington University Medical Center in St. Louis. All available slides were reviewed, and diagnoses confirmed using current criteria.¹ Atypical PNs were defined by the presence of nuclear atypia in the absence of significant mitotic activity, whereas, cellular PNs were defined by hypercellularity in the absence of significant mitotic activity. A representative formalin-fixed paraffin-embedded block was selected per case for further study with dual-color immunohistochemistry/fluorescence *in situ* hybridization (FISH). Sporadic schwannomas were used as disomic (ie, normal 2 copies) *NF1* controls because they contain S-100 protein-positive Schwann cells of similar size and shape to those typically encountered in neurofibromas and would not be expected to harbor *NF1* deletions. Clinical records were reviewed, and the diagnosis of neurofibromatosis 1 (NF1) was rendered in patients fulfilling National Institute of Health (NIH) guidelines.¹⁶ Most of these

patients have been carefully examined and followed in the Neurofibromatosis Clinic at Washington University.

Unstained 5- μ m thick sections were cut onto superfrost/plus, precleaned glass slides from each paraffin block. The sections were deparaffinized in CitriSolv (Fisher, Pittsburgh, PA) and rehydrated in isopropanol and water. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in phosphate-buffered saline (PBS; 10 mmol/L; pH = 7.2) for 5 minutes. Non-specific antibody binding was inhibited by incubation in PBS-blocking buffer (PBS with 1% BSA, 0.2% powdered milk and 0.3% Triton X-100) for 20 minutes at room temperature and polyclonal rabbit anti-S-100 protein antiserum (Z311, Dako, Carpinteria, CA; 1:50,000 in PBS blocking buffer) was added to the sections overnight at 4°C. Sections were then washed in 1× PBS (3 × 5 minutes each) and incubated with horseradish peroxidase conjugated donkey anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA; diluted 1:1000 in PBS-blocking buffer) for one hour at room temperature. Antigen-antibody complexes were subsequently detected by direct tyramide signal amplification (Perkin Elmer Life Sciences, Boston, MA) using cyanine-3 conjugated tyramide (tyramide signal amplification plus cyanine 3) for 20 minutes at room temperature according to the manufacturer's instructions. Slides were washed in PBS and 2× SSC for 5 minutes each.

Subsequent FISH was performed on the S-100 protein immunolabeled slides using our previously published protocol¹⁷ and a fluorescein isothiocyanate (FITC)-labeled P1 artificial chromosome DNA probe targeting the exon 28 to 3' region of the *NF1* gene on chromosome 17q11.2 (donated by Dr. Eric Legius, Belgium). The probe was diluted 1:50 in DenHyb buffer (Insitus, Albuquerque, NM) and 10 microliters was directly applied to each tissue section. Probe and target DNA were co-denatured at 90°C for 13 minutes, followed by overnight hybridization at 37°C in a humidified oven. The slides were then washed for 5 minutes with 50% formamide in 1× SSC followed by two more washes of 2× SSC for 5 minutes each. The nuclei were counterstained with DAPI/ Antifade (Insitus). Fluorescent signals were enumerated under an Olympus B ×60 fluorescent microscope with appropriate filters. Because cytoplasmic borders were

Table 2. Summary of Clinical Cases and FISH Results

Case no.	Age/sex	NF1 status	Diagnosis	Tumor location	NF1 in S-100 ⁺ cells	NF1 in S-100 ⁻ cells
681	3 M	Yes	PN	Eye	Deleted	Normal
957-A	16 F	Yes	PN	Buttock	Deleted	Normal
957-B	16 F	Yes	PN	Flank	Normal	Normal
957-C	16 F	Yes	PN	Scalp	Normal	Normal
017-A	2 F	No	PN	Neck	Deleted	Normal
017-B	5 F	No	PN	Neck	Normal	Normal
482	8 F	No	PN	Ulnar nerve	Deleted	Normal
795	3 F	Yes	At-PN	Perineum	Deleted	Normal
882-A	24 M	Yes	At-PN	Scalp	Gains	Normal
147-A	11 F	Yes	Cell-At-PN	Thigh	Gains	Normal
501	13F	Yes	MPNST	Paraspinal	Insufficient cells*	Deleted
882-B	24 M	Yes	MPNST	Neck	Deleted	Deleted
147-B	13 F	Yes	MPNST	Thigh	Deleted, gains	Deleted
215	33 M	Yes	MPNST	Retroperitoneum	Insufficient cells*	Deleted
459	62 F	No	MPNST	Brachial plexus	Deleted	Deleted
566	36 F	No	MPNST	Brachial plexus	Deleted	Deleted
286	42 F	No	MPNST	Brachial plexus	Insufficient cells*	Deleted
883	47 M	No	MPNST	Mediastinum	Insufficient cells*	Deleted

At-PN, atypical plexiform neurofibroma.

* MPNST with too few S-100⁺ cells to enumerate.

often indistinct under fluorescence microscopy, only cells with immunopositive nuclei (ie, some red fluorescence over the nucleus) were scored for *NF1* signals in the evaluation of S-100 protein-positive cellular subsets. However, because cytoplasmic staining was also frequently observed, only immunonegative (ie, blue) nuclei with no surrounding red fluorescence were scored for *NF1* signals in the evaluation of S-100 protein-negative cellular populations. Because the S-100 protein staining sometimes obscured the underlying *NF1* signals when the colors were viewed simultaneously, signal enumeration required the consecutive viewing of individual nuclei under each single-pass filter (ie, blue, red, and green).

Given the truncation artifact (ie, fewer signals in sectioned nuclei with incomplete DNA complement) associated with thin tissue FISH, cutoffs for genetic alterations were based on results from four control hybridizations (see above). The cutoff for *NF1* gene deletion was based on the mean percentage of nuclei with one signal in controls plus two standard deviations. Because nuclei with >2 signals were never seen in these controls, *NF1* (17q) polysomy (gain) was arbitrarily defined as >5% nuclei with three or more FISH signals.

Results

The clinical features, tumor diagnoses, and FISH results are summarized in Table 2. There were 18 tumors obtained from 13 patients, 7 of which had diagnostic features of NF1. The seven PNs, two atypical PNs, and one cellular/atypical PN came from five female and two male patients ranging in age from 2 to 24 years of age (median 8 years). All but two (017 and 482) fulfilled criteria for NF1. Given the young ages of these two patients, however, it seems likely that they either represent mosaic forms of NF1 or as of yet undiagnosed NF1 in young individuals with insufficient clinical criteria to warrant a

definitive diagnosis. The NF1-associated MPNST patients consisted of two males and two females ranging in age from 13 to 33 years (median 18.5 years). The sporadic MPNSTs were derived from one male and three female patients ranging in age from 36 to 62 years (median 44.5 years). One of the NF1-associated and one of the sporadic MPNSTs had rhabdomyoblasts (ie, Triton tumor). Three of the sporadic MPNSTs were probably radiation-induced sarcomas based on clinical history (radiation for prior breast cancer or Hodgkin's disease).

Representative examples of dual immunohistochemistry/FISH results are illustrated in Figure 1. Control sections demonstrated 1 *NF1* signal in 27 to 40% of nuclei. Based on the mean (35%) plus 2 standard deviations (12%), a cutoff of >47% nuclei with one signal was established for *NF1* deletion. These results are similar to those obtained using other DNA FISH probes in our laboratory on thin paraffin sections from non-neoplastic controls (data not shown). The fraction of cells with one *NF1* signal ranged from 50 to 93% (median 67%) in populations interpreted as deleted versus 14 to 39% (median 27%) in populations interpreted as nondeleted. *NF1* deletion was detected within the S-100 protein-positive cellular populations of four (57%) PN and one (33%) atypical PN (Table 2). The S-100 protein-negative populations from these same tumors were disomic (normal 2 copies). Four of the MPNSTs had too few S-100 protein-positive cells to determine *NF1* status within this subset of tumor cells. However, *NF1* deletion was found in the S-100 protein-negative cells of these same cases (Table 2). The remaining four MPNSTs demonstrated *NF1* deletion in both the S-100 protein-positive and -negative components. Polysomies (gains with 3 to 4 signals per nucleus) of *NF1* were identified in subpopulations of S-100 protein-positive cells of one atypical PN, one cellular/atypical PN and one MPNST. These cells likely represent polyploid or aneuploid clones within these tumors.

Figure 1. Representative examples of dual S-100 protein immunohistochemistry and *NF1* FISH hybridization. **A:** Low-power image from a control schwannoma, demonstrating relatively diffuse S-100 protein immunoreactivity (red). As is typical of this antibody, some of the staining is cytoplasmic and some is nuclear. **B:** At higher magnification, two S-100 protein-positive cells demonstrate the normal disomic state, with two copies of *NF1* (green signals) per nucleus. **C:** Two adjacent nuclei from a representative plexiform neurofibroma (case 957-A) are shown. The nucleus on the right demonstrates S-100 protein immunoreactivity and only a single *NF1* signal, whereas the nucleus on the left is S-100 protein-negative with the normal disomic *NF1* dosage. Hybridization counts from this case revealed one *NF1* signal in 67% of S-100 protein-positive *versus* 27% of S-100 protein-negative nuclei. This is consistent with a gene deletion that is restricted to the S-100 protein-positive population of cells. **D:** This S-100 protein-negative region of an MPNST (case 566) demonstrated one *NF1* signal in 93% of nuclei, consistent with deletion. S-100 protein-positive regions of the same tumor similarly showed evidence of deletion (not illustrated).

Discussion

Using a dual-color FISH-immunohistochemical method, we have demonstrated, for the first time, *NF1* gene copy numbers in S-100 protein-positive *versus* -negative cellular populations in PNs and MPNSTs. One of the primary advantages of this technique is that it is applied *in situ* with preserved tissue morphology. In this fashion, some entrapped native tissue elements, such as uninvolved nerve fascicles and infiltrated fat, skeletal muscle, sweat glands, etc can easily be excluded from genetic analysis. Our results provide the most conclusive evidence thus far that the S-100 protein-positive Schwann cell is the primary target for *NF1* deletions in PNs, both typical and atypical subsets. Furthermore, it adds support to the growing body of literature suggesting that most, if not all PNs are neoplastic, rather than hyperplastic in nature^{9,11,12,14,15}. Because some of our cases, and many of those in the literature, harbor no detectable genetic alterations, however, we cannot exclude the possibility that a subset of PNs, and perhaps most cutaneous neurofibromas, are in fact, hyperplastic or hamartomatous. Alternatively, these cases may harbor inactivating mutations beyond the resolution of FISH or LOH, involve alterations of other genes besides *NF1*, or consist of tumors with minute neoplastic clones that induce an overshadowing

reactive process including non-neoplastic fibroblasts, perineurial-like cells, native intraneural Schwann cells, etc. Further resolution of these issues will likely require sophisticated screening techniques capable of detecting genetic alterations within individual cells.

Another interesting finding in our study was the prevalence of *NF1* deletion in MPNSTs, regardless of S-100 protein expression or *NF1* status. The simplest interpretation is that S-100 protein-negative tumor cells within MPNSTs represent dedifferentiated Schwann cells that still harbor *NF1* deletion. In other words, the loss of *NF1* represents an early tumorigenic event that is still detectable in high-grade neoplastic clones no longer manifesting immunohistochemical evidence of Schwann cell differentiation. The finding of divergent epithelial and/or mesenchymal differentiation in some MPNSTs (eg, Triton tumors) and complete lack of S-100 protein expression in others would further support this dedifferentiation hypothesis. In any case, only a few MPNSTs have been genetically characterized in terms of *NF1*. Reported LOH studies have been largely limited to examples from *NF1* patients (Table 1),^{4-7,12} where *NF1* loss has been common. In a small cytogenetic study by Rao and colleagues,¹⁷ monosomy was identified in one of four sporadic MPNSTs, suggesting that *NF1* may be implicated in

some of these cases as well.¹⁸ Gómez and colleagues found no mutations in nine sporadic MPNSTs within the GAP-related domain by polymerase chain reaction/single-strand conformational polymorphism.¹⁹ However, this was a fairly limited screening and additional studies are obviously needed with larger numbers of both sporadic and NF1-associated examples.

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