Short Communication

The Neurofibromatosis Type 2 Gene Is Mutated in Perineurial Cell Tumors

A Molecular Genetic Study of Eight Cases

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Perineurial cell tumors (PNTs) are rare neoplasms derived from or showing differentiation toward specialized lining cells of the nerve sheath, the perineurial cells. In this study, we have evaluated neurofibromatosis type 2 (NF2) gene alterations in eight PNTs using archival formaldehyde-fixed, paraffin-embedded tissue. Two conventional soft-tissue PNTs from the upper back and chest wall, one retiform soft tissue variant from the scapular region, and five sclerosing PNTs from the fingers and palm were studied. All cases showed histological features of PNTs, and the neoplastic cells were positive for epithelial membrane antigen and negative for S100 protein. The coding sequences (exons 1 to 15) of the NF2 gene were polymerase chain reaction (PCR) amplified and evaluated for mutations by direct sequencing of the PCR products. Five NF2 point mutations, two in the 5'-untranslated region (UTR) and three in exons 3, 6, and 8, were identified in four of eight cases (50%) studied. Exon mutations resulted in changes of predicted amino acids sequences: Asp→Asn at codon 83, Glu→Asp at codon 182, and Leu→Val at codon 241. In two cases (one with a missense mutation in codon 241), the same point mutation in the 5'-UTR at the nucleotide position 8958 was identified. A loss of heterozygosity (LOH) study was performed in three cases. LOH at the NF2 locus was found in one case with a mutation in the 5'-UTR. However, in another case with exon 8 and 5'-UTR mutations, deletion of one allele of the NF2 gene was previously documented by fluorescence *in situ* hybridization. The coexistence

of NF2 gene mutations and LOH at the NF2 locus indicates that the NF2 tumor suppressor gene is altered in PNTs by the two-hit mechanism. (*Am J Pathol* 2001, 158:1223-1229)

The perineurium is a specialized component of the peripheral nerve sheath that forms a protective barrier around nerve fascicles and has a direct continuity with the pia-arachnoid membrane of the central nervous system. Perineurial cells have characteristic ultrastructural features, and they are typically immunoreactive for epithelial membrane antigen and vimentin, and negative for S100 protein.¹⁻⁴ These cells usually produce an external lamina that is immunoreactive for collagen IV and laminin.³⁻⁵

Although reports of tumors derived from perineurial cells date back to the 1970s, this concept was slow to gain acceptance because of the rarity of cases and an inability to confidently diagnose perineurial cell tumors (PNTs) without the aid of special studies.^{3,6} Although ultrastructural examination was once considered the only reliable means to diagnose these tumors, morphological assessment in conjunction with immunohistochemistry now seems sufficient to define the majority of these tumors.^{4,6,7}

PNTs are often categorized as intraneural or soft tissue in type. The former group encompasses lesions previously classified as localized hypertrophic neuropathy,

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hypertrophic mononeuropathy, localized hypertrophic neurofibrosis, intraneural neurofibroma, and hypertrophic interstitial neuritis.⁸ A sclerosing variant of PNT that typically presents as a superficial soft tissue mass in the fingers and palm of young adults,⁴ and a soft-tissue PNT variant with retiform growth⁹ have recently been described. The vast majority of PNTs pursue a benign clinical course. However, there is some evidence that malignant PNTs may also exist.¹⁰

There is only scant cytogenetic and molecular genetic data on PNTs. Previously published studies, based on small numbers of cases, indicated a loss of chromosome 22 or the deletion of genetic material from 22q in PNTs.^{11,12} More recently, a sclerosing PNT from the finger was reported to have cryptic deletions involving the 5'-BCR and neurofibromatosis type 2 (NF2) loci.¹³

The loss of one copy of chromosome 22 has been shown to be the most common genetic aberration in meningiomas and schwannomas.¹⁴ The NF2 tumor suppressor gene has been previously shown to be involved in tumorigenesis of sporadic meningiomas¹⁵⁻¹⁷ and schwannomas.¹⁸ In these tumors, one allele of the NF2 gene is lost, whereas the other allele is inactivated by a somatic mutation according to the two-hit hypothesis of inactivation of a recessive tumor suppressor gene.¹⁹ Protein studies have revealed reduced or absent expression of merlin/schwannomin (the protein product of the NF2 gene) in most sporadic meningiomas and schwannomas.²⁰⁻²²

The purpose of this study was to test the hypothesis that the NF2 tumor suppressor gene is altered in PNTs. A presence of mutations in the NF2 gene and loss of heterozygosity (LOH) at the NF2 locus would strongly suggest that PNTs share molecular genetic similarities with meningiomas and schwannomas.

Materials and Methods

Tissue Material

PNTs were obtained from the files of the Soft Tissue Registry of the Armed Forces Institute of Pathology, Washington, DC (cases 2, and 4 to 8) and the Department of Pathology, Katholieke Universiteit of Leuven, Leuven, Belgium (cases 1 and 3).

Immunohistochemistry

The tumors were immunohistochemically evaluated with a panel of antibodies, anti-epithelial membrane antigen, anti-vimentin, anti-collagen type IV, anti-laminin, anti-S100 protein and anti-glial fibrillary acidic protein, using the ABC-immunoperoxidase technique as previously described.⁴

Molecular Genetic Analysis of the NF2 Gene

DNA was extracted as previously described²³ using tumor and normal tissue obtained from unstained slides or paraffin blocks. Normal tissue was available in three

cases. The coding sequences (exons 1 to 15) of the NF2 gene were PCR amplified. The primer sequences and annealing temperatures for each PCR are shown in Table 1. The PCR reaction conditions were the standard ones recommended by Perkin Elmer (Norwalk, CT). To prevent PCR contamination, previously described precautions²⁴ were undertaken. The PCR products were size fractionated on 2.5% agarose gels, purified from the gels (Qiagen Inc., Chatsworth, CA), and sequenced directly on a 373 DNA-sequencer (Applied Biosystems, Foster City, CA) using forward and reverse primers. Computer analysis of the DNA sequences was performed using the Lasergene software (DNASTAR, Madison, WI) in connection with the data of the GenBank 110/EMBL 57 database (January 1999 edition) and NF2 Mutation Database (<http://neuro-trials1.mgh.harvard.edu/nf2/>).

LOH Studies

In three cases sampling of both normal and neoplastic tissue was possible. All were evaluated for LOH using a set of PCR-based markers mapped to the 22q12: D22S429, D22S310, D22S929 (first intron of the NF2 gene), D22S268, and D22S273. Primer sequences were obtained from the Genome Data Base (<http://www.gdb.org>). Nonradioactive fluorescent end-labeled primers were purchased from PE Applied Biosystems (Foster City, CA). PCR amplification was performed using AmpliTaq Gold DNA polymerase and the GeneAmp PCR System 9600 (Perkin-Elmer Corp.) with the following cycling profile. The initial pre-PCR heat step of 12 minutes at 95°C was followed by 10 cycles each of 94°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, and next by 25 cycles each of 89°C for 15 seconds, 60°C for 15 seconds, and 72°C for 30 seconds, and ended with a final 30-minute elongation at 72°C. Analysis of PCR products was performed using ABI Prism 310 and Genescan software according to the recommended procedure (PE Applied Biosystems). The LOH values were calculated as recommended by PE Applied Biosystems. LOH was defined in this study as a value less than 0.60.

Results

Clinical Features

The demographic and clinical data of all tumors analyzed in this study are summarized in Table 2. Of the eight patients, three were male and five were female. The age of the patients ranged from 13 to 56 years (median, 23 years; mean, 29 years). Sites of involvement were the finger ($n = 4$), palm ($n = 1$), upper back ($n = 1$), chest wall ($n = 1$), and scapular area ($n = 1$). None of the patients were diagnosed with NF2 syndrome.

Histological and Immunohistochemical Features

There were five sclerosing PNTs, two conventional soft-tissue PNTs, and one retiform PNT. The sclerosing PNTs

Table 1. Sequences of the Oligonucleotide Primers Used in this Study

NF2 gene	Primer	Sequence	Annealing temperature [°C]
Exon 1	NF2.1F1	5' GCT-AAA-GGG-CTC-AGA-GTG-CA 3'	60
	NF2.1R2	5' AGC-TTC-CAC-CTC-GAC-TGT-CA 3'	
Exon 2	NF2.2F1	5' AGA-GTG-GAG-AGT-GCA-GAG-AA 3'	50
	NF2.2R2	5' CTC-TAC-TAT-ACA-GCT-ACA-GCG-C 3'	
Exon 3	NF2.3F1	5' TAG-CAC-AGG-AGG-AAG-TGC-CAA-T 3'	50
	NF2.3R1	5' CAA-GTT-CTC-TCA-GAA-CTG-GG 3'	
Exon 4	NF2.4F1	5' ATG-TCT-CCC-TTG-TTG-CTC-CT 3'	57
	NF2.4R1	5' GAG-TGA-TCC-CAT-GAC-CCA-AA 3'	
Exon 5	NF2.5F1	5' AAT-CTC-AAT-CGC-CTG-CTC-TC 3'	55
	NF2.5R1	5' CCA-CAT-ATC-TGC-TAT-GTC-TTC-CTG 3'	
Exon 6	NF2.6F1	5' TGA-CTA-TCT-CCC-TGG-GTG-TA 3'	50
	NF2.6R1	5' AAA-CCA-ACA-ATG-AAT-GGG-CC 3'	
Exon 7	NF2.7F1	5' TCC-AAT-GAC-AGT-GTC-TTC-CG 3'	50
	NF2.7R1	5' TAC-ACA-AGG-AGC-TCA-GAG-AG 3'	
Exon 8	NF2.8F1	5' TGT-TGG-GAC-CTG-CTG-AAA-CT 3'	50
	NF2.8R1	5' CCC-ATC-TGC-AGT-ACA-CAC-AT 3'	
Exon 9*	NF2.9F3	5' GTT-CTG-CTT-CAT-TCT-TCC 3'	45
	NF2.9R2	5' GTA-ATG-AAA-ACC-AGG-ATC 3'	
Exon 10*	NF2.10F2	5' CCT-TTT-TGT-CTG-CTT-CTG 3'	45
	NF2.10R2	5' TCA-GTT-AAA-ACA-AGG-TTG 3'	
Exon 11	NF2.11F1	5' TCT-CGA-GCC-CTG-TGA-TTC-AA 3'	50
	NF2.11R2	5' AAG-TCC-CCA-AGT-AGC-CTC-CTG-GAA 3'	
Exon 12	NF2.12F1	5' CTT-CAG-CTA-AGA-GCA-CTG-TG 3'	50
	NF2.12R3	5' AGG-ACA-ACT-GCT-GTA-GAG-CT 3'	
Exon 13	NF2.13F1	5' GCT-GAC-ATC-TCA-TCC-TTT-CC 3'	55
	NF2.13R1	5' AAC-ATC-ACC-AGG-ACT-AAG-GC 3'	
Exon 14	NF2.14F1	5' GTC-CTT-CTG-TGC-TTG-TAT-GA 3'	55
	NF2.14R1	5' GTT-CAC-AGC-TGC-CCA-CAG-CA 3'	
Exon 15	NF2.15F1	5' TGA-GCC-GTG-TCT-CAC-TGT-CT 3'	55
	NF2.15R1	5' TAA-TGA-TGG-TCC-TGA-TCA-GC 3'	

F, forward; R, reverse.

*Primer sequences previously published.¹⁵

were composed of cords of small epithelioid cells often arranged in a concentric manner in a moderately dense collagenous background. Two of the sclerosing PNTs (cases 1 and 2) have been previously reported.^{4,13} The two conventional soft-tissue PNTs were composed of uniform spindle cells with predominantly storiform and whorled growth patterns. The retiform soft-tissue PNT had a marked predominance of loosely arranged spindled cells with a basket-weave or net-like growth pattern. All tumors were positive for epithelial membrane antigen and negative for S100-protein and glial fibrillary acidic protein. Positivity for vimentin was documented in two cases.

Pericellular reactivity for collagen IV and laminin was documented in six and two cases, respectively. Representative histological and immunohistochemical images are shown in Figure 1.

Evaluation of NF2 Gene for Mutation

Coding sequences of the NF2 gene were evaluated for the presence of mutations by PCR amplification and direct sequencing of PCR products. Exons 1 to 15 were analyzed in all cases. Because of the lack of a DNA

Table 2. Summary of Demographic, Clinical, Pathological, and Genetic Data

Case	Age/ sex	Location	Tumor size	Diagnosis	LOH value at D22S929	DNA sequence alteration	Predicted amino acid change
1	15/F	Finger	0.5 cm	Sclerosing PNT	0.99	G to A at n.p. 8958 C to G at n.p. 65734 A to T at n.p. 60108	5'-UTR Codon 241, Leu→Val Codon 182, Glu→Asp
2	24/M	Ring finger	0.7 cm	Sclerosing PNT		G to A at n.p. 43583	Codon 83, Asp→Asn
3	52/F	Middle finger	0.8 cm	Sclerosing PNT		Wild type	—
4	22/F	Middle finger	2.0 cm	Sclerosing PNT		Wild type	—
5	13/M	Palm	1.9 cm	Sclerosing PNT		G to A at n.p. 8958	5'-UTR
6	36/M	Upper back	1.4 cm	Soft-tissue PNT	0.57	Wild type	—
7	56/F	Chest wall (anterior)	4.0 cm	Soft-tissue PNT	0.83	Wild type	—
8	17/F	Scapular area	8.0 cm	Soft-tissue PNT retiform variant		Wild type	—

n.p., nucleotide position based on the human NF2 gene sequence obtained from EMBL/GenBank/DDBJ databases (EMBL: HSAY 1800).

Figure 1. PNT variants: histological and immunohistochemical findings. Case 1, a sclerosing PNT (**A**); case 8, soft-tissue PNT with retiform histology (**B**); and case 6, soft-tissue PNT with conventional histology (**C**) are depicted. All PNT variants showed strong positivity for epithelial membrane antigen (**D**), laminin (**E**), and collagen IV (**F**). The illustrated immunohistochemical results are from soft-tissue PNT in **C**. 5'-UTR sequences of the NF2 gene obtained from the tumor and corresponding normal tissue in case 1 (**G**), red **arrow** indicates homozygous mutation.

template, case 2 could not be analyzed for exon 14 and cases 1 to 4 for exon 15. Five somatic mutations were identified. In case 1, there were two somatic mutations, a G to A mutation at nucleotide position 8,958 [5'-untranslated region (UTR)] and a C to G mutation at nucleotide position 65734 (exon 8). In case 2, there was an A to T mutation at nucleotide position 60,108 (exon 6). Case 3 had a G to A mutation at nucleotide position 43,583 (exon 3). In case 6, a somatic G to A mutation at nucleotide position 8,958 (5'-UTR), similar to that observed in case 1, was found. However, the mutation in the 5'-UTR in

case 1 was homozygous, and in case 6, it was heterozygous. All other mutations were heterozygous. In cases 1 and 6, PCR products amplified from normal tissue revealed a wild-type sequence for the NF2 gene. The exon mutations resulted in changes of predicted amino acid sequences: Asp→Asn at codon 83, Glu→Asp at codon 182, and Leu→Val at codon 241. The presence of a G to A mutation in cases 1 and 6 and an A to T mutation in case 2 was confirmed by sequencing of the PCR products obtained from two different PCR reactions. Homozygous mutation in the 5'-UTR in case 1 is shown in Figure 1.

Chromosome 22 LOH Studies

In three cases, samples of tumor and normal tissue were microdissected from the paraffin blocks and subjected to DNA extraction. The LOH study was done using five polymorphic markers. In case 3, LOH (value, 0.57) was detected at D22S929, a marker mapped to the first intron of the NF2 gene. In cases 1 and 7, LOH values did not indicate loss of genetic material from 22q12.

Discussion

PNTs are a rare group of peripheral nerve sheath tumors that display phenotypic features of perineurial cells.⁸ In this study, we evaluated NF2 gene alterations in eight well-documented PNTs using archival formaldehyde-fixed, paraffin-embedded material.

The previously published cytogenetic and molecular genetic data on PNTs are based on an analysis of only five cases.^{7,11-13} Tsang and colleagues⁷ karyotyped an intraneural PNT and demonstrated monosomy of chromosome 22 and a structurally abnormal remaining chromosome 22 with loss of genetic material in the region 22q11.2-qter. Emory and colleagues¹¹ showed possible loss of chromosome 14 or 22 in another case of intraneural PNT. In two soft-tissue PNTs, Giannini and colleagues¹² found a loss of hybridization signal with a probe specific for 22q11.2 (BCR locus) indicating a specific deletion, partial loss, or entire loss of chromosome 22. More recently, Sciort and colleagues¹³ reported a case of sclerosing PNT with a cryptic deletion of the 5'-BCR and NF2 loci and suggested that the NF2 gene may be a target of genetic alteration in PNTs.

The NF2 gene is mapped to chromosome 22q12. It encodes for an intracellular cytoskeleton-associated protein of 595 amino acids, designated as merlin or schwannomin.^{18,25} Inactivating mutations of the NF2 gene have been observed in 34 to 66% of screened NF2 patients.²⁶⁻²⁸ The majority of the mutations are nonsense, frameshift, or spliced donor site mutations, all of which result in a nonfunctional, truncated merlin protein. More recently, it was found that large deletions in the NF2 gene may be present in NF2 patients, raising the frequency of detectable NF2 gene alterations in this population to 84%.²⁹

Inactivating NF2 mutations, similar to that seen in NF2 patients, have been found in 15 to 35% of sporadic meningiomas^{15-17,30} and 20 to 60% of sporadic schwannomas.^{31,32} In these tumors, one allele of the NF2 gene is lost, whereas the other is mutationally inactivated. The majority of mutations in meningiomas and schwannomas are clustered in NF2 exons, corresponding to the N-terminal half of the merlin protein.^{17,31} Protein studies have revealed reduced or absent expression of merlin in most sporadic meningiomas and schwannomas.²⁰⁻²²

We evaluated eight PNTs for the presence of mutations in the NF2 gene. In three cases, nucleotide substitutions were identified in exons 3, 6, or 8. These exons encode the N-terminal half of the merlin protein that is often mutated in meningiomas and schwannomas. The NF2

wild-type sequence of exon 8 was identified in normal tissue from case 1, confirming a somatic mutation in this tumor. In the two other cases, normal tissue was not available for testing. Because the NF2 gene has been extensively studied in hundreds of individuals and no polymorphism has been found in the exons we examined³³ the nucleotide substitutions that were detected in two of our PNTs at codon 83 and 182 most likely represent somatic mutations. It is noteworthy that a nonsense mutation and 1-bp deletion involving codon 182 have previously been reported in two NF2 patients (<http://neuro-trials1.mgh.harvard.edu/nf2/>).

Missense mutations in NF2 have only been occasionally reported in NF2 patients.^{28,34} It has been suggested that these mutations may be associated with mild clinical phenotypes of NF2. In such cases, tumors are said to present later in adult life and to grow more slowly. Also, the number of separate tumors in the patients was reportedly smaller and the survivals were longer.^{28,34} Missense mutations have also been identified in a few cases (<5%) of sporadic meningiomas, two of them classified as World Health Organization grade I benign tumors.^{17,30,35}

A lack of nonsense, frameshift, or spliced donor site mutations, and the presence of missense mutations, that are very rare in meningiomas and schwannomas, may be characteristic for PNTs. Also, the identification of missense mutations exclusively in the sclerosing PNT subtype suggests a genotype/phenotype correlation between the type of mutation and the histological variant of PNT. However, more cases need be studied to confirm these observations.

In two PNTs, an identical point mutation was identified in the 5'-UTR of the NF2 gene. Mutations in this region have not been reported in familial or sporadic tumors that commonly show involvement of the NF2 gene. Structural alteration in the 5'-UTR may result in low levels of mRNA because of transcript instability or reduction in gene expression.¹⁵ The role of mutations in the 5'-UTR of the NF2 gene in PNTs should be studied further.

LOH at the NF2 locus was identified in only one of three PNTs analyzed. A polymorphic marker mapped to the first intron of the NF2 gene was lost in one of the soft-tissue PNTs (case 6) carrying an NF2 5'-UTR mutation. The other two tumors (cases 1 and 7) did not show LOH at the NF2 locus, however one of these (a sclerosing PNT, case 1) was previously demonstrated by fluorescence *in situ* hybridization¹³ to have a loss of one NF2 allele. The differences between the current LOH data and the previously published fluorescence *in situ* hybridization data are most likely related to the investigative methodology. In the current study, LOH status of 22q12 was evaluated with five polymorphic markers, one of them mapped to the first intron of the NF2 gene. In the previous study, pooled PCR fragments specific for different regions of the NF2 gene were used for fluorescence *in situ* hybridization,¹³ this allowed screening of the entire NF2 gene and detection of the deletions involving other than first intron region of the NF2 gene. The identification of a G to A homozygous mutation at nucleotide position 8958 in the 5'-UTR indicates that a sequence from one allele is present and confirms the previously published fluores-

cence *in situ* hybridization observation. However, the mis-sense mutation identified in exon 8 was heterozygous indicating that sequences from both alleles are present and confirms our LOH data.

These differences cannot be explained by a simple loss of one NF2 allele. In case 1, complex molecular changes including intragenic deletions coexisting with other rearrangements might be responsible for contradictory results obtained using different molecular techniques. Structural chromosomal alterations of the NF2 gene, deletions of variable size and localization, and chromosomal inversions have been previously identified in the NF2 patients.^{29,36} Cloning of both NF2 alleles is necessary to understand the type of structural changes in the NF2 gene. However, this will require high molecular weight DNA that is not currently available.

No NF2 mutations were identified in the remaining four PNTs. However, we cannot exclude the possibility that mutations were present but remained undetected because they occurred in exons that were not examined. Also, the possibility of NF2 inactivation by large deletions cannot be excluded. Such deletions cannot be detected by PCR analysis when using the partially degraded DNA obtained from formaldehyde-fixed, paraffin-embedded tissue.

Recently, increased proteolytic degradation of merlin by calpain was shown in schwannomas and meningiomas without detected NF2 mutations.³⁷ This alternative mechanism of merlin inactivation should be explored in perineuriomas. However, immunohistochemical evaluation for merlin is currently hampered by the lack of anti-merlin antibodies reactive in formaldehyde-fixed, paraffin-embedded tissues.

Because the NF2 gene seems unaffected in up to 40% of meningiomas and schwannomas other genetic mechanisms may also contribute to the development of these tumors.^{15-17,31,32} Recently, several interstitial deletions in 22q, outside of the NF2 locus, were identified in a group of schwannomas and may be involved in tumorigenesis.³⁸ Thus, the possibility of several different pathways leading to the development of PNTs should also be considered. A comprehensive molecular genetic study involving a large number of PNTs is needed to address this issue.

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