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Mutations of the neurofibromatosis type 2 gene and lack of the gene product in vestibular schwannomas

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Schwannomas are common tumors of the nervous system and are frequently found in patients with neurofibromatosis (NF) 2. Although loss of heterozygosity in NF2 tumors suggests that the NF2 gene functions as a tumor suppressor gene, the NF2 gene shows amino acid sequence homology to structural proteins in one of which dominantly acting mutations have been described. We performed a mutational analysis in 30 vestibular schwannomas and examined the effect of mutations on the NF2 protein. We detected 18 mutations in 30 vestibular schwannomas of which seven contained loss or mutation of both NF2 alleles. Most mutations were predicted to result in a truncated protein. Mutational hot spots were not identified. Immunocytochemical studies using antibodies to the NF2 protein showed complete absence of staining in tumor Schwann cells, whereas staining was observed in normal vestibular nerve. These data indicate that loss of NF2 protein function is a necessary step in schwannoma pathogenesis and that the NF2 gene functions as a recessive tumor suppressor gene.

INTRODUCTION

Vestibular schwannomas (VSs) are benign tumors of Schwann cell origin that occur commonly as single tumors on the vestibular branch of the eighth cranial nerve. Schwannomas account for 8% of intracranial tumors and 29% of intraspinal tumors (1). In patients with NF2, VSs are often bilateral and occur at a much earlier age than in patients with sporadic unilateral VSs. NF2 or bilateral acoustic neurofibromatosis is an autosomal dominant disorder, characterized by the presence of multiple tumors in the central nervous system (2). NF2 has been mapped to chromosome (CHR) 22q12 by genetic linkage analysis, and the NF2 gene was cloned recently (3,4).

The NF2 gene encodes a 595 amino acid protein with similarity to a family of proteins (moesin, radixin, ezrin) with the function to link the cell membrane and the cytoskeleton (5,6). Frequent loss of alleles in 22q12 in sporadic VSs suggested that the NF2 gene may act as a recessive tumor-suppressor gene (7–9) with the process of tumor formation following the two-hit model (10). However, the NF2 protein shows amino acid sequence homology to the red blood cell protein 4.1 which interacts with spectrin, the major cytoskeletal protein in the red blood cell, at the junction of spectrin tetramers. Mutation of one protein 4.1 allele is sufficient to cause altered red blood morphology, raising the possibility that dominant or dominant negative mutations may occur in VSs.

We undertook an extensive mutational analysis in 30 VSs to determine: (a) what proportion of VSs contain mutations of the NF2 gene and whether mutational 'hot-spots' can be identified; (b) whether specific types of mutation are found and what their predicted effect on the gene product is; and (c) whether mutations in both NF2 alleles and loss of the gene product can be demonstrated in VSs, thus supporting the function of the NF2 gene as a tumor suppressor gene.

RESULTS

Mutations in VS cDNAs

To detect mutations in the NF2 transcript we reverse transcribed total RNA from VSs and analyzed the cDNA by single-strand conformation polymorphism (SSCP). Eleven pairs of primers covering 100% of the NF2 coding sequence and part of the non-coding cDNA sequence were used this analysis (Table 1). Figure 1 shows examples of SSCP of cDNAs from VSs in which abnormal mobilities were detected. We characterized 18 mutations and one DNA variant affecting 16 tumors and 18 different alleles by DNA sequence analysis (Table 2). Sixteen of the mutations (89%) were 1–136 bp deletions and two were point mutations. Among the 16 deletion cases, 10 of them (62%) were of less than 9 bp. In two cases the deletion was accompanied by a DNA insertion, and in a third case by a single base pair change. Except in two cases, which were the only ones of deletion of a complete exon, all the deletions altered the reading frame creating new downstream stop codons and resulting in truncated proteins and/or proteins with a different protein product (Fig. 2). The two remaining abnormalities were point mutations; a missense mutation in codon 79 in tumor 27 (Lys to Glu) and a nonsense mutation in codon 57 in tumor 53 (Arg to STOP). Both were present in tumor DNA but not in lymphocyte DNA. The codon 57 mutation has been described previously (3). Since it creates a new *Dde*I site, a restriction enzyme analysis was used to detect any possible mutation that had escaped the sensitivity of the SSCP analysis. No additional mutations in codon 57 were identified.

A sequence variant was found in codon 351 in tumor DNA of sample 17, a sporadic unilateral VS in a 65-year-old otherwise healthy man; instead of the normal CGC codon a CAC was present resulting in a conservative amino acid change (Arg to His). This change very likely represents a polymorphism, since

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Table 1. Oligonucleotide pair of primer sequences, written 5′–3′

NP1A:	CTTCAACCTCATTGGTGACAG	(1709)
NP1B:	TGGTATTGTGCTTGCTGCTG	(1947)
NP2A:	AGGTACTGGATCATGATGTTTC	(457)
NP2B:	TTTGGAAGCAATTCCTCTTGG	(730)
NP3A:	CGGGCCATCGCTTCCCGCAT	(225)
NP3B:	AAATTTGGCCAAGAAGTGAA	(518)
NP4A:	GACTACGACCCCAGTGTTCA	(672)
NP4B:	AGCTCTGTGCCCTTTTTATT	(913)
NP5A:	TGTACGGTGTGAACTACTTT	(865)
NP5B:	CCGATACATAGCTGGAGAAT	(1123)
NP6A:	CTCCTCAAAGCTTCGTGTTA	(1076)
NP6B:	CTCCTCAGACCGCATCAGTG	(1355)
NP7A:	AAAGAAGAAGCAACAATGGCC	(1308)
NP7B:	TGGCCCTCCTCTCTGACTCC	(1563)
NP8A:	GCCGAGGTGCTGGCACTGAAGA	(1515)
NP8B:	CCATGGAAAGCCGCTTCATGTCA	(1776)
NP9A:	TCTGGATATTCTGCACAATGAGA	(1889)
NP9B:	CTACAGGGTCGTAGTTCAAGGC	(2194)
NP10A:	ACCGTCCCCCAACTCCCCTT	(17)
NP10B:	CGTCTTGGGTTGCTTCCTCTTG	(281)
NP11A:	TTCCCGCATGAGCTTCAGCT	(236)
NP11B:	GGCCAAGAAGTGAAAGGTGACTG	(512)

The position of the 5′ base according to the published sequence (4) is indicated in the numbers between brackets. Upper primers are indicated with an A, and lower primers are indicated with a B.

the same change was found in the patient's lymphocyte DNA. In addition, the NF2 allele containing the polymorphism also contained a 1 bp deletion predicted to result in a truncated protein.

Two hit mutations in tumors

Seven (23%) tumors contained alterations or loss of both NF2 alleles (Table 3). We detected loss of heterozygosity (LOH) in eight tumors, seven of which had been reported in a previous study (9), using microsatellite markers flanking the NF2 gene (see Materials and methods). In 12 (40%) tumors only one genetic abnormality was detected, and no alteration was detected in 11 (37%) tumors. Of the seven tumors with two abnormal or missing alleles, in five tumors one allele was lost and the other contained a second mutation. The second mutation was a 1 or 2 bp deletion that resulted in a truncated protein in four of them (tumors 15, 17, 19 and 48) and a missense mutation in one (tumor 27); tumor 27 contained a missense mutation that resulted in a non-conservative amino acid change (Lys to Glu). Two more tumors contained mutations in both alleles, all of them resulting in truncated proteins. Tumor 10 contained different deletions in the same region of the sequence, thus in different alleles. Tumor 37 harbored two mutations: one allele contained a 59 bp insertion and 2 bp deletion, the second allele a 136 bp deletion. Since primers NP5A and NP1B flanked the region containing both mutations, we could easily determine that the mutations had occurred in separate alleles. If the mutations had been in the same allele two products of 1083 (normal allele) and 1004 bp would have been detected. However, we detected products of approximately 1140 (allele with insertion) and 950 bp (allele with deletion).

Western blot analysis of VSs

In order to further define the effect of NF2 mutations on the NF2 protein we produced antibodies against a C-terminal peptide (antibody 5990) and an N- terminal peptide (antibody 5991). Both antibodies detected a protein of 65 kDa in normal sciatic nerve

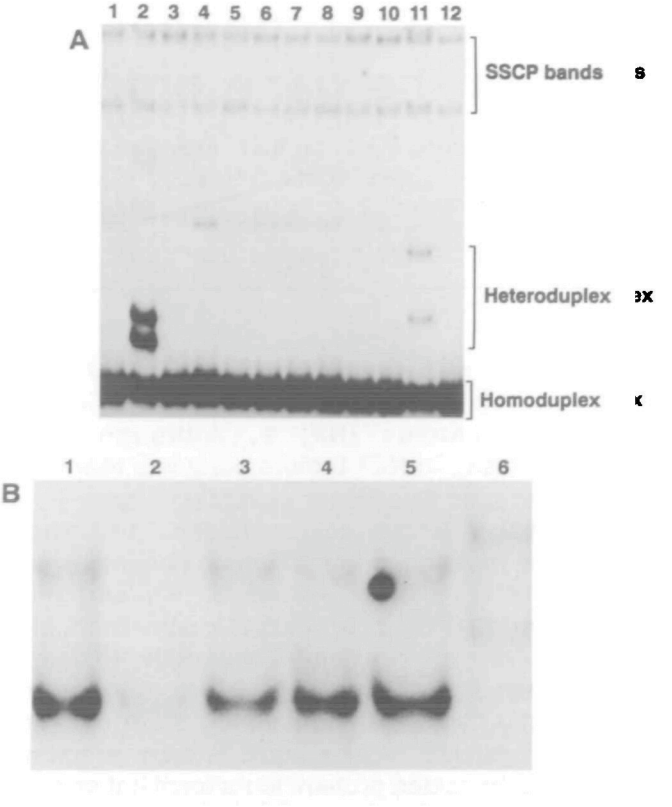

Figure 1. PCR–SSCP conformers specific to the NF2 gene from VSs. (A) PCR products in lanes 2 and 11 (tumors 15 and 29 respectively) show abnormal conformers in single strand DNA (SSCP bands) and in heteroduplex DNA; the abnormalities detected were a 2 bp deletion in tumor 15 and a 4 bp deletion in tumor 29. (B) A SSCP abnormal conformer is present in lane 6 (tumor 53). The abnormal migration is caused by a nonsense mutation.

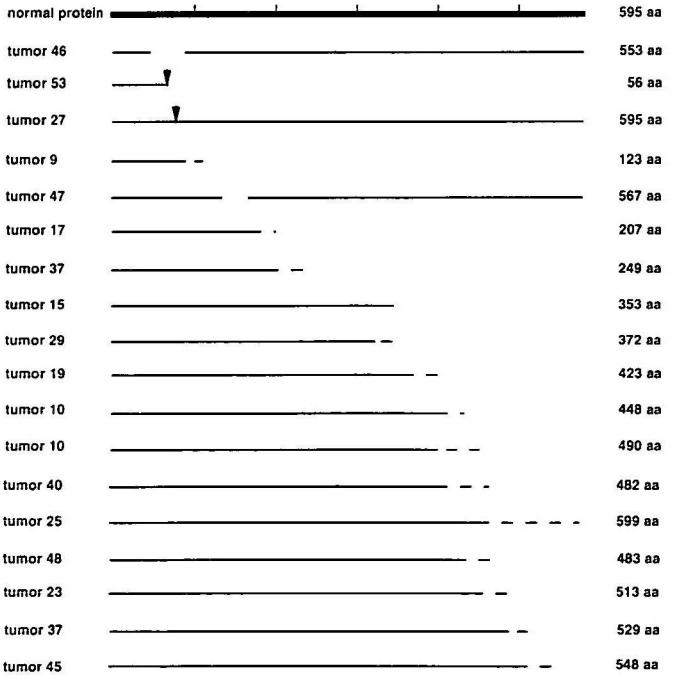

Figure 2. Predicted protein product of the NF2 gene in 18 mutated alleles in VSs. Fifteen of the predicted products result in a truncated protein and two (tumors 46 and 47) in proteins missing a complete exon. Arrows represent point mutations; gaps, deleted exons; dashed lines, altered amino acid sequence due to frameshift.

Table 2. NF2 mutations in VSs

Tumor	Exon	Codon	Nucleotide change	Effect of mutation	Type of mutation	LOH
46	2	39	(126 bp)GTA→GTA	126 bp deletion	Exon deletion	
53	2	57	CGA→TGA	Arg→Stop	Nonsense	
27	2	79	AAG→GAG	Lys→Glu	Missense	Yes
9	4	122–150	G(83 bp)TA→GTA	83 bp deletion	Frameshift	
47	4	122–150	(84 bp)TAT→TAT	84 bp deletion	Exon deletion	
17	7	202	GAAG→GAG	A deletion	Frameshift	Yes
37	7–8	225–270	CG(136 bp)T→CGT	136 bp deletion	Frameshift	
15	11	353–354	AGGGA→AGA	GG deletion	Frameshift	Yes
29	11	364–365	AAAGAAG→AAG	AGAA deletion	Frameshift	
19	12	396–397	AAACTTC→AAC	ACTT deletion	Frameshift	Yes
10	13	447–449	AGGGCCAAAGA→AGA	GGGCCAAA deletion	Frameshift	
10	13	447–482	A(106 bp)GC→AGC	106 bp deletion	Frameshift	
40	13	459–470	CAG(32 bp)→CCG	Gln→Pro, 32 bp deletion	Frameshift	
25	13	480–482	ACGTACCCG→A(20 bp)G	CGTACCC deletion, 20 bp insertion	Frameshift	
48	13	480	ACGT→CGT	A deletion	Frameshift	Yes
23	15	490	CCGT→CGT	C deletion	Frameshift	
37	15	525	AAA→A(59 bp)	AA deletion, 59 bp insertion	Frameshift	
45(N)	15	538	CAGC→CAC	G deletion	Frameshift	

(N) Represents tumor derived from an NF2 patient.
LOH represents loss of heterozygosity.
The codon numbers are according to the published sequence (3).
The inserted sequence in tumor 37 is: TGCTGTGGGC AGCTGTGAAC TAGACTGAGT GATTGGGGCC TTGGGAAGCT GGGGCAGAG
The inserted sequence in tumor 25 is: CTGGGGCTGC CTTAGTCCTG

(Fig. 3A, lanes 1 and 3). Staining of this protein was absorbed out by addition of the respective peptide (Fig. 3A, lanes 2 and 4). In addition, antibody 5991 detected two proteins at 97 and 45 kDa; however, only the 45 and 65 kDa proteins were absorbed out (Fig. 3A, lane 4). A similar reaction with presera did not detect the 65 kDa protein (not shown). Antibody 5990 also detected a single protein at 65 kDa in protein extracts from several VSs (Fig. 3B, lanes 1–4) which was not detected after preabsorption (lanes 5–8) or with pre-immune sera (data not shown). Although equal amounts of total protein were loaded, the relative amounts of NF2 protein in different tumor samples varied greatly.

Localization of NF2 protein in normal vestibular nerve and NF2 tumors

To elucidate the presence of NF2 protein in VSs we stained paraffin sections of normal vestibular nerve and 27 of our 30 VSs (tissue from tumors 7, 47 and 53 was not available) with antibody 5990. Staining was seen in nerve fascicles (Fig. 4A) and the epi- and perineurium (not shown). In the nerve fascicle, cytoplasmic and perinuclear immunoreactivity (IR) was observed in Schwann cells, but not in axons (Fig. 4A insert). In addition, ganglion cell bodies and satellite cells in the eighth nerve and epineural fibroblasts showed strong cytoplasmic and perinuclear staining (not shown). All IR was absorbed out by addition of peptide 5990 (Fig. 4B).

In contrast, in all tumors Schwann cells were devoid of IR (Fig. 4C). Schwannomas are known to form specific morphologic patterns referred to as Antoni A regions for cell dense areas and Antoni B regions for areas of low cellularity. Despite their different morphology and positive staining for S100 protein, Schwann cells in both regions lacked NF2 protein immunoreactivity. Several controls indicated that lack of staining was not due to technical problems: (a) normal vestibular nerve processed under identical conditions showed strong immunoreactivity; (b) tumor cells stained strongly with S100 antibody (Fig. 4D); (c) remnants of normal nerve and vestibular ganglion cells could be identified in some tumors and showed normal staining (data not shown); (d) some tumors contained regions rich in fat-laden phagocytes which were stained; however, adjacent S100 positive Schwann cells did not stain (Fig. 4E, F).

Table 3. Genetic alterations at the NF2 locus in 30 VSs

Tumor	Genetic Alteration		Number of alterations
10	T	T	2
15[a]	T	LOH	2
17[a]	T	LOH	2
19[a]	T	LOH	2
27	M	LOH	2
37	T	T	2
48[a]	T	LOH	2
9[b]	T		1
11[a]	LOH		1
14[a]	LOH		1
18(N)[a]	LOH		1
23[b]	T		1
25[b]	T		1
29	T		1
40[b]	T		1
45(N)	T		1
46	E		1
47	E		1
53	T		1
1			0
2(N)			0
7			0
12			0
20[b]			0
22[b]			0
32(N)[b]			0
34[b]			0
39			0
43			0
51			0

(N) represents tumors derived from NF2 patients.
T indicates a truncation of the NF2 product caused by nonsense mutation, deletion or insertion; E, deleted exon; M, missense mutation.
LOH represents loss of heterozygosity; [a]LOH detected in a previous study (9); [b]No data for LOH.

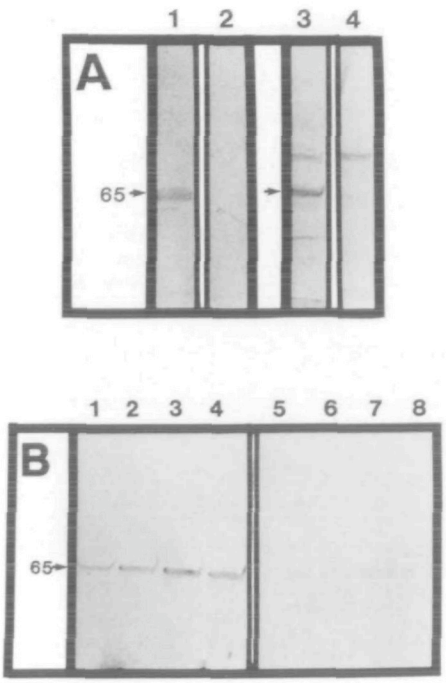

Figure 3. Immunoblots of normal human sciatic nerve and VS. (**A**) Western blots of protein extract from normal sciatic with NF2 antibodies. Antibody 5990 detected a single 65 kDa protein (lane 1), while no bands were detected after preabsorption with 100 μM peptide 5990 (lane 2). Antibody 5991 detected the 65 kDa protein, but also two additional bands at 95 kDa and 45 kDa (lane 3). Only the 45 kDa and 65 kDa bands were absorpted out (lane 4). (**B**) Immunoblots of protein extracts from four different VSs with the 5990 antibody (lanes 1–4) and antibody 5990 preabsorbed with 100 μM peptide 5990 (lanes 5–8). A 65 kDa band was specifically detected by the 5990 antibody.

DISCUSSION

We have detected mutations of the NF2 gene in a large number of VSs most of which are predicted to result in a truncated NF2 protein. Seventeen (94%) of the mutations led to an incomplete product of the NF2 gene due to premature termination, a different amino acid sequence or an absence of a complete exon. Two of the deletions (tumors 46 and 47) involved a complete exon. A missense mutation was detected in the only allele remaining in tumor 27. Given that it is not present in the patient's lymphocyte DNA this missense change likely represents a tumorigenic mutation and not a polymorphism.

In tumor 53 a C to T transition in codon 57 results in a missense mutation also described in a previous study (3) and reported in abstract form (Strachan *et al.*, personal communication). Correcting for varying denominators due to methodologic differences, this mutation accounts for approximately 7% of the described mutations. These results are consistent with the high frequency of C to T transitions reported in CpG dinucleotide. Such mutations are frequently observed in the mammalian genome and in tumor suppressor genes as the p53 gene (11). Because a new *Dde*I restriction site is created, this mutation can be rapidly detected by restriction enzyme analysis of polymerase chain reaction (PCR) products. Our studies probably underestimate the true prevalence of NF2 mutations in VSs. First, the current analysis did not include the promoter region or intronic portions of the NF2 gene. Mutations in these regions could also result in loss or reduction of NF2 function. Secondly, the SSCP analysis could not detect a complete loss of NF2 transcript. Thirdly, the sensitivity of SSCP analysis is very likely lower than 100%.

However, the sensitivity of our mutation analysis is within the range observed for the extensively studied APC gene. Using a variety of methods germline mutations were detected in 16% (12), 29% (13), 30% (14) and 67% (15) of the samples. As expected, a greater percentage of mutations was found in colorectal carcinomas and adenomas, and ranged from 60% (16) to 80% (17). Absent or truncated APC proteins were detected in 57% of lymphoblastoid cell lines from APC patients and 75% of colon carcinoma and adenoma cell lines by direct analysis of the APC protein (18). These numbers agree well with our sensitivity of 35% per chromosome analyzed and a detection of either LOH or mutation of the NF2 gene in 63% of tumors.

It is thought that tumorigenesis in NF2 tumors and in sporadic schwannomas follows a two-hit model. This model predicts the existence of tumors in which inactivation of both NF2 alleles can be detected. We found seven tumors that are predicted to lack any functional NF2 protein. In five tumors, one 'hit' was loss of CHR 22, the second 'hit' was a truncating mutation in four, and a missense mutation in one. Two tumors had truncating mutations in each allele. Since the abnormalities were close enough to be amplified by the same set of PCR primers, we could exclude the possibility that the mutations occurred on the same allele.

Based on mutational analysis we could not exclude the possibility that mutation in one NF2 allele may confer a growth advantage possibly in conjunction with mutations in other genes or that some mutations have a dominant negative effect. There are several other candidate tumor suppressor genes on CHR 22 (19) including oncostatin (20), LIF (21) and one or more genes involved in the formation of meningiomas (22). If proteins that show homology to the NF2 protein serve as a model, one may predict a cellular phenotype associated with certain mutations of one allele. Protein 4.1 interacts with spectrin, the major cytoskeletal protein in the red blood cell, at the junction of spectrin tetrameres. Mutation of one allele may cause an altered shape of red blood cells, but mutations in both alleles may cause severe hemolysis.

To demonstrate that mutations in both NF2 alleles are a necessary step in VS tumorigenesis we examined all tumor samples for loss of the gene product. All tumors had lost immunoreactivity for the NF2 protein. However, strong staining was seen in Schwann cells in normal vestibular nerve. Since our tumor samples had been removed by the trans-labyrinthine approach which removes tumor plus normal structures *in toto*, our tumor samples contained additional cellular elements. As in samples of normal eighth nerve, cells showing staining included vestibular ganglion cells, fat-laden phagocytes, peri-, and epineural cells. The varying admixture of these cells to tumor samples likely explains the presence of faint normal alleles in LOH studies (7,9) and the presence of the gene product in Western blots of VSs. Most importantly, it may mask complete

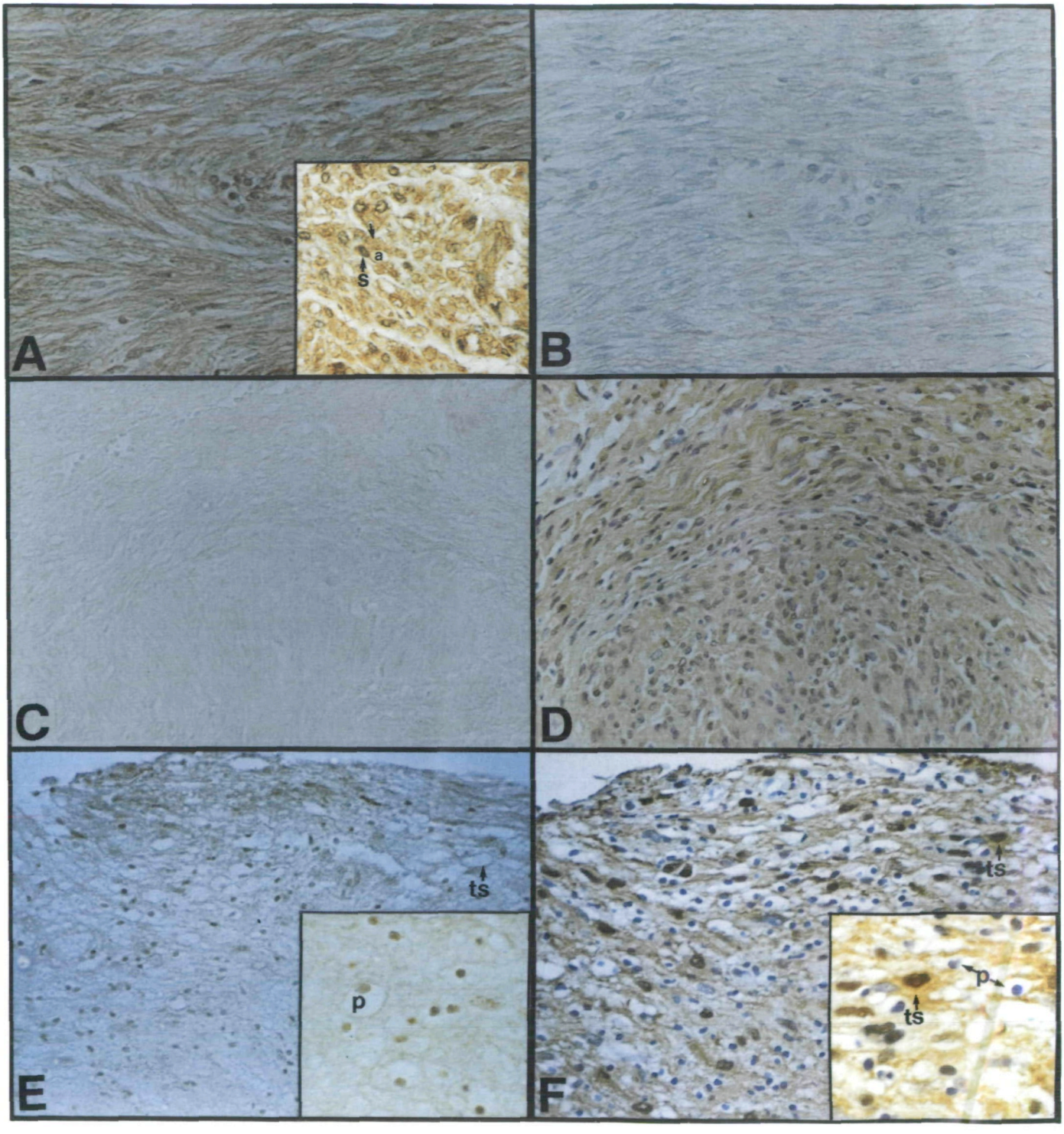

Figure 4. Photomicrographs of normal eighth nerve and VSs stained with antibody 5990. (**A**) Longitudinal section through a normal eighth nerve fascicle. Insert shows a normal eighth nerve cut in cross-section at higher magnification showing positive staining in Schwann cells (s) and lack of staining in axons (a). Arrow points to Schwann cell cytoplasm surrounding an axon. (**B**) Adjacent section of (A) stained with antibody 5990 preabsorbed with 100 μM peptide 5990. Both sections counterstained with methylene green. (**C**) VSs stained with antibody 5990 without counterstaining. (**D**) Adjacent section stained with S100 antibody and counterstained with Mayer's hematoxylin. (**E**) Staining of fat-laden phagocytes in an area of a VS. Insert shows higher magnification detail of stained phagocytes (p). No counterstain. (**F**) Section adjacent to E stained with S100 antibody and counterstained with Mayer's hematoxylin. Insert shows lack of staining of phagocytes (p), but nuclear and cytoplasmic staining of tumor Schwann cells (ts).

loss of NF2 transcript when an RT−PCR approach is used for mutational analysis.

In summary, we can conclude that: (a) the majority of VSs contain mutations in the NF2 gene, which frequently are deletions ranging from 1 to 136 bp; (b) a C to T transition in codon 57 may represent a mutational 'warm' spot; (c) inactivation of both alleles of the NF2 gene is likely required to develop VSs and is accompanied by loss of the normal gene product in all tumors examined; and (d) our results are consistent with a recessive tumor suppressor function of the NF2 gene.

MATERIALS AND METHODS

Human tissue samples and DNA and RNA extraction

Blood and tissue samples from VSs were obtained from unrelated patients. Standard procedures were used for DNA and RNA extraction (23).

RT−PCR

The coding region and approximately 350 bp of the 5′ and 3′ non-coding region of the NF2 gene were divided in 11 segments and each segment was separately amplified using PCR. The primers used are listed in Table 1. To analyze genomic DNA, primers previously described (3) were used. cDNA templates from the tumor RNA were obtained using the Reverse Transcription System (Promega). For a 20 μl reaction 1 μg RNA was used to synthesize cDNA. One microliter of the cDNA obtained was then used as a template for the PCR. The PCR was performed in 20 μl with 6 pmol of each primer, standard PCR buffer, 1.5 mM $MgCl_2$, 250 mM of each dATP, dGTP and dTTP, 60 mM dCTP, 2.5 μCi of [α^{32}P] dCTP and 2.5 units of *Taq* DNA polymerase. Reactions were heated to 94°C for 2 min and then cycled 40 times; each cycle consisted of 30 s at 94°C, 30 s at the appropriate annealing temperature, 1 min at 72°C for strand elongation. Annealing temperatures were 55°C for primers NP1 to NP7, 57°C for primers NP9, 60°C for primers NP8 and NP10 and 61°C for primers NP11. All the reactions were performed in a Perkin-Elmer or a Biometra (TRIO-thermoblock) DNA thermocyclers.

SSCP gel analysis

PCR products were diluted in 1 vol. of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol and denatured at 98°C for 2 min and chilled on ice until 2 μl were loaded onto polyacrylamide gels. Each set of reactions was analyzed using two gel conditions described as the most informative (24): (A) 6% acrylamide/bis (37.5:1), 0.5×TBE (0.045 M Tris-borate pH 8.0; 0.001 M EDTA) at 4°C; (B) same as in (A) with the addition of 5% glycerol and at room temperature. Electrophoresis was carried out in a 50 cm sequencing apparatus (Bio-Rad) at 300 V during 17−21 h at room temperature and 500 V during 17−24 h at 4°C. After electrophoresis gels were covered with Saran-wrap and exposed without drying at −70°C.

Direct PCR sequencing

SSCP bands with abnormal migration were cut with a razor blade from acrylamide gels and placed in 75 μl of distilled water. After 30 min incubation at room temperature or overnight at 4°C, 10 μl were used as a template for PCR using the same pair of primers used in the original PCR. Samples were purified with a Magic PCR prep DNA purification system (Promega) and then were PCR sequenced using the CircumVent DNA sequencing kit (New England Biolabs).

LOH analysis

The analysis was done using a previously reported method (9). Four microsatellite markers were used: D22S351 (25), CRYB2 (26) centromeric to NF2 and D22S430 (27), D22S268 (28) telomeric to NF2.

Protein extraction and Western blot

We raised rabbit antibodies against NF2 peptides. Peptide 5990 is located at amino acid (aa) residues 527−541 (EYMEKSKHLQEQLNE), and peptide 5991 is located at aa residues 10−22 (SFSSLKRKQPKTF) (3). Each peptide was conjugated to keyhole limpet hemocyanin, and injected into two rabbits. Collected antisera were affinity purified as previously described (29). Fresh tumors and sciatic nerve tissues were immediately frozen in liquid nitrogen. RNA, DNA, and protein were extracted sequentially from 50 to 200 mg tissue using the TRI-REAGENT™ kit (Molecular Research Center, Inc., Cincinnati, OH). Protein concentration was determined using the Bio-Rad Protein Assay Kit I, 100 μg of total protein from each sample was loaded on a 4−20% gradient SDS−PAGE (BIO-RAD), and Western blot was performed as previously described (30).

Immunohistochemistry

VSs and normal acoustic eighth nerves were briefly washed in 100 mM PBS and then processed by the Tissue-Tek VIP (Miles Scientific, Indiana) in 10% formalin fixative. Tissues were then embedded with paraffin. Seven micron sections were cut and mounted onto Fisherbrand Superfrost/Plus microscope slides. They were air-dried overnight at room temperature, then rehydrated by rinsing twice in xylene, 100% ethanol, 95% ethanol, and 70% ethanol. Prior to antibody incubation, rehydrated sections were treated as previous described (31) to block endogenous peroxidase and nonspecific antibody binding. Sections were then incubated with 1/500 dilution (7 μg/ml) of affinity purified NF2 antibody (5990 antibody) for 2 h or with 1/200 dilution of S100 antibody (DAKO, Z311) for 30 min at room temperature. The primary antibodies were detected using Vector ABC elite Peroxidase kit (Vector). Absorption controls were performed by incubating 7 μg/ml of NF2 antibody with 100 μM of the respective peptide (5990 or 5991 peptides) for 2 h at room temperature and then overnight at 4°C.

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