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Immunohistochemical Detection of Schwannomin and Neurofibromin in Vestibular Schwannomas, Ependymomas and Meningiomas

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Abstract. In addition to schwannomas, patients with neurofibromatosis type 2 (NF2) frequently develop meningiomas and occasionally, ependymomas. Using DNA and protein analyses, we have shown NF2 gene mutations and lack of the gene product schwannomin in 29 schwannomas, 10 meningiomas, and in 7 ependymomas. We have raised antibodies (ABs) to peptides from the C-terminal (5990-AB) and N-terminal (5991-AB) domains of schwannomin. The ABs specifically detected a 65 kDa protein in a Schwann cell line and recognized schwannomin in the cytoplasm of Schwann cells (SCH), perineurial cells, and vestibular ganglion neurons. None of the 29 schwannomas were stained by the 5990-AB. Only 4 schwannomas were stained by the 5991-AB, indicating that most truncated schwannomins were unstable or not expressed in schwannomas. Seven of 10 meningiomas, including 3 tumors from NF2 patients, were not stained by either 5990-AB or 5991-AB. Only 2 of 7 ependymomas lacked schwannomin. Complete lack of schwannomin in these tumors supports a tumor suppressor function for schwannomin in some meningiomas and ependymomas. All tumors showed staining with an antibody to a C-terminal peptide of neurofibromin, confirming that full-length neurofibromin is present in these vestibular schwannomas, meningiomas, and ependymomas. The presence of schwannomin in some meningiomas and in the majority of ependymomas indicates that additional genes are likely to play a role in tumorigenesis of these tumors.

Key Words: Ependymoma; Meningioma; Merlin; Neurofibromin; Neurofibromatosis 2; Schwannoma; Schwannomin.

INTRODUCTION

The neurofibromatoses exist in 2 clinically and genetically distinct forms, neurofibromatosis type 1 (NF1) and neurofibromatosis type 2 (NF2). NF2 is an autosomal dominant inherited disorder that occurs with an incidence of approximately 1/30,000 to 1/40,000 live births, with approximately 50% of the cases representing new mutations (1, 2). Mutations in the NF2 gene predispose patients to a variety of brain and peripheral nerve tumors. Bilateral vestibular schwannomas are the most common tumors and are found in > 90% of NF2 patients (1). Spinal schwannomas and meningiomas occur frequently as well, whereas ependymomas, neurofibromas and gliomas are found less commonly (3–8).

The presence of these tumor types in NF2 patients suggested that mutations in the NF2 gene may cause the sporadic forms of these tumors as well. Analysis of NF2 mutations in sporadic schwannomas detected mutations in 60 to 70% (9–16), whereas mutations in sporadic meningiomas were found in smaller numbers (27%) (16–18). NF2 gene mutations appear to be relatively rare in ependymomas (7, 19, 20).

However, these studies were limited by the relatively low sensitivity of mutation detection as determined by the approximately 30% detection of germline mutations in lymphocyte DNA from NF2 patients (21–22). In order to assess the frequency of NF2 mutations in schwannomas and to determine if the NF2 gene is indeed a tumor suppressor gene with loss of function in both alleles, we examined vestibular schwannomas using antibodies (ABs) raised against the NF2 gene product (9). All schwannomas had lost schwannomin immunoreactivity (IR), indicating that NF2 gene mutations are the predominant molecular event in schwannoma pathogenesis and that inactivating mutations affect both NF2 alleles. Also in this report, we showed the expression of schwannomin in NF2-associated and sporadic meningiomas and ependymomas. Although lack of schwannomin IR was found in many tumors, the presence of schwannomin in several tumors suggested the existence of other, yet-unidentified meningioma/ependymoma genes.

MATERIALS AND METHODS

Antibody Production

We raised rabbit ABs against C-terminal and N-terminal NF2 peptides predicted from the NF2 cDNA sequence (23, Fig. 1). Peptide 5990 is located at amino acid (aa) residues 527–541 (EYMEKSKHLQEQLNE), and peptide 5991 is located at aa residues 10–23 (SFSSLKRKQPKTFT). Each peptide was conjugated to keyhole limpet hemocyanin and injected into 2 rabbits. Collected antisera were affinity purified and used for immunohistochemistry.

Previous studies showed that both ABs detected a 65 kDa protein in protein extracts of a Schwann-like cell line, STS26T,

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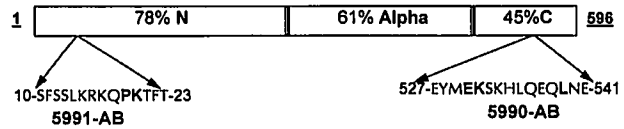

Fig. 1. Location of peptides 5990 and 5991 in schwannomin. Peptide 5990 is a 15 aa peptide located in the C-terminal domain at aa residues 527 to 541. Peptide 5991 is a 14 aa peptide located in the N-terminal domain at aa residues 10 to 23. Bold capitalized letters indicate conserved aa residues among schwannomin, ezrin, moesin, and radixin. Percentage indicates the average percent of identity in the N-terminal, alpha-helical, and C-terminal domains of schwannomin, ezrin, moesin, and radixin.

and that incubation of this cell line with NF2 antisense oligonucleotides blocked the synthesis of schwannomin (24). In addition, both ABs also detected the 65 kDa protein in normal human sciatic nerve (9). Pre-immune sera collected prior to immunization did not precipitate the 65 kDa band (24). Furthermore, both 5990-AB and 5991-AB preabsorbed with the respective peptides did not detect the 65 kDa protein in Western blots of protein extracts from a normal human sciatic nerve (9).

The NF1C antibody detected a 290 kDa protein in a human neuroblastoma cell line, SK-N-M, and was described previously (25–26).

Immunohistochemistry

Tumors and normal vestibular nerves were briefly washed in 100 mM PBS and processed by the Tissue-Tek VIP (Miles Scientific, Indiana) in 10% formalin fixative. Tissues were embedded in paraffin. Seven-micron sections were cut and mounted onto microscopic slides. The sections were dehydrated by rinsing twice in xylene, 100% ethanol, 95% ethanol, and 70% ethanol. Prior to AB treatment, rehydrated sections were treated with a protease cocktail (Autozyme from Biomeda, CA) and nonspecific sites were blocked as previously described (26). Sections were then incubated with a 1/200 dilution (20 μg/ml) of affinity-purified NF2 ABs (5990-AB and 5991-AB), or a 1/500 dilution (20 μg/ml) of affinity-purified NF1C AB (25–26). After incubation for 2 hours (h) at 37°C, the primary ABs were detected using the Vector ABC elite Peroxidase kit (Vector, CA), enhanced by DAB enhancer and visualized with diaminobenzidine (DAB) (Biomeda, CA). Sections were counterstained with aqueous hematoxylin (Xymed, CA). Absorption controls were performed with NF2 ABs preabsorbed with their respective peptides at 100 μM each for 2 h at room temperature and overnight at 4°C.

RESULTS

To investigate the presence of schwannomin in vestibular schwannomas, ependymomas, and meningiomas, we used ABs to a C-terminal peptide (5990-AB) and an N-terminal peptide (5991-AB) of schwannomin to stain normal human vestibular nerves, 23 sporadic vestibular schwannomas, 6 NF2 derived vestibular schwannomas, 10 meningiomas, and 7 ependymomas. Positive and negative immunoreactivity was determined by comparing the relative level of staining intensity between tumors and normal 8th nerve, or between schwannomin-positive and schwannomin-negative tumors.

Both NF2 ABs showed an identical pattern of immunoreactivity in normal vestibular nerve (Fig. 2A, C). Cytoplasmic staining was observed in vestibular sensory neurons (SN), satellite cells (Sa), and Schwann cells (SCH) (Fig. 2A, C). All staining was absorbed out with the addition of 100 (M of the respective peptide (Fig. 2B, D). For additional controls, we used an antibody to neurofibromin, NF1C. Neurofibromin was detected abundantly in SCH and SN (Fig. 2E). Low levels of neurofibromin were detected in Sa (Fig. 2E). NF1C-AB preabsorbed with 100 μM was immunonegative (Fig. 2F).

None of the 29 schwannomas were stained with the 5990-AB (Table 1, Fig. 3A, D, G, J). Only 4 tumors showed immunoreactivity with the N-terminal antibody 5991-AB (Table 1, Fig. 3E). In Figure 3H, only occasional cells showed weak staining. All tumors were immunoreactive (IR) with the S100 (Fig. 3I) and the NF1C (Fig. 3C, F, L) ABs. Interestingly, in some tumors, endothelial cells were stained by both NF1 and NF2 ABs (Fig. 3A–C). In a majority of tumors, however, endothelial cells lining vessels within the tumor mass were not stained by NF2 ABs (Fig. 4B), but with NF1 antibody (Fig. 4C). In contrast, endothelial cells lining blood vessels residing outside of the tumor were commonly immunoreactive with NF2 antibody (data not shown).

To determine the stability of truncated schwannomins or schwannomins containing missense mutations, we correlated NF2 mutations identified by SSCP analysis with staining results (Table 1). Tumor 27, which had a missense mutation at codon 79, showed no IR with either the C-terminal antibody 5990-AB (Fig. 3J) or the N-terminal antibody 5991-AB (Fig. 3K), but it was strongly immunoreactive with an antibody to a peptide of the C-terminal region of neurofibromin, the NF1C-AB (Fig. 3L). In addition, tumor 81, another vestibular schwannoma from an NF2 patient, had a missense mutation at codon 62 (13) and was also devoid of staining with both NF2 ABs (Table 1).

To investigate the expression of schwannomin in ependymomas, we examined 7 sporadic ependymomas for the presence of schwannomin. One ependymoma showed light staining, and the other 4 ependymomas showed moderate-to-strong schwannomin IR (Fig. 4A). Two ependymomas showed no IR for both 5990 and 5991 ABs (Fig. 4B). In these tumors, blood elements were stained with the NF2 antibody but endothelial cells were not stained (Fig. 4B). All ependymomas were strongly stained with the NF1C antibody (Fig. 4C).

Since NF2 patients develop meningiomas, we also examined 10 meningiomas, including 3 meningiomas from NF2 patients using 5990, 5991, and NF1C ABs. Of the 7 sporadic meningiomas, 3 tumors showed IR. Two of these were meningothelial meningiomas (Fig. 4D) and

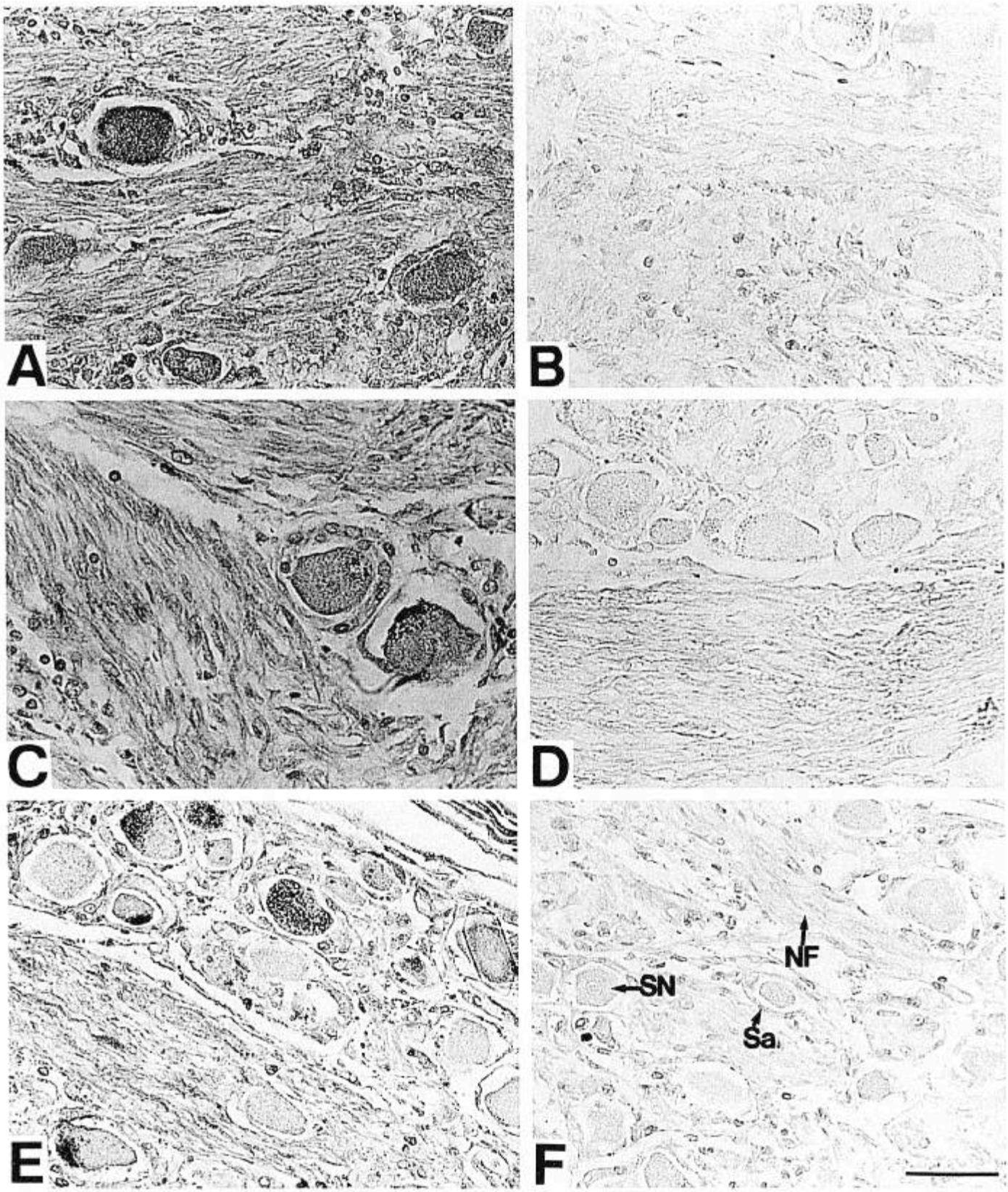

Fig. 2. Detection of schwannomin and neurofibromin in a normal human vestibular ganglion. Sections of a normal human vestibular ganglion stained with the 5990 Ab (A), 5991 AB (C), and neurofibromin antibody NF1C (E). Adjacent sections stained

TABLE 1
NF2 Gene Mutations and Expression in Vestibular Schwannomas

Tumor	Codon	Exon	Effect of mutation	Mutation type	LOH	5990	5991	S100	NF1C
1	UNK		UNK	UNK	UNK	−	−	+	+
2 (NF2)[a]	262	8	Arg → Stop	Nonsense	UNK	−	−	+	+
4 (NF2)[a]	UNK		UNK	UNK	UNK	−	−	+	+
9	122–150	4	83 bp deletion	Frameshift	UNK	−	−	+	+
10	447–449	13	8 bp deletion	Frameshift	UNK	−	+	+	+
	447–482	13	106 bp deletion	Frameshift	UNK				
11	UNK		UNK	UNK	Yes	−	−	+	+
12	UNK		UNK	UNK	UNK	−	−	+	+
14	UNK		UNK	UNK	Yes	−	−	+	+
15	353–354	11	GG deletion	Frameshift	Yes	−	−	+	+
17	202	7	A deletion	Frameshift	Yes	−	−	+	+
18 (NF2)[a]	341	10	Arg → Stop	Nonsense	Yes	−	−	+	+
19	396–397	12	ACTT deletion	Frameshift	Yes	−	−	+	+
20	UNK		UNK	UNK	UNK	−	−	+	+
22	UNK		UNK	UNK	UNK	−	−	+	+
23	490	13	C deletion	Frameshift	UNK	−	−	+	+
25	480–482	13	20 bp deletion	Frameshift	UNK	−	−	+	+
27	79	2	Lys → Glu	Missense	Yes	−	−	+	+
29	364–365	11	AGAA deletion	Frameshift	UNK	−	−	+	+
32 (NF2)[a]	UNK		UNK	UNK	UNK	−	−	+	+
34	UNK		UNK	UNK	UNK	−	+	+	+
37	525	15	59 bp deletion	Frameshift	UNK	−	−	+	+
39	UNK		UNK	UNK	UNK	−	−	+	+
40	459–470	13	32 bp deletion	Frameshift	UNK	−	+	+	+
43	UNK		UNK	UNK	UNK	−	−	+	+
45 (NF2)[a]	538	15	G deletion	Frameshift	UNK	−	−	+	+
46	39	1	126 deletion	Exon deletion	UNK	−	−	+	+
48	480	13	A deletion	Frameshift	Yes	−	−	+	+
51	466	13	Arg → Stop	Nonsense	UNK	−	+	+	+
81 (NF2)[a]	62	2	Phe → Ser	Missense	UNK	−	−	+	+

This table combines mutation data of 29 vestibular schwannomas we previously published (9–13) with immunohistochemical data obtained with ABs to the C-terminal domain (5990) and N-terminal domain (5991) of schwannomin. For controls, the tumors were stained with S100 antibody and an antibody to the C-terminal region of neurofibromin, NF1C (25, 26). LOH, loss of heterozygosity; UNK, unknown; [a]tumor obtained from known NF2 patients.

one was a fibroblastic meningioma. Four sporadic meningiomas showed no schwannomin immunoreactivity (Fig. 4E). All meningiomas derived from NF2 patients lacked schwannomin IR. All meningiomas showed strong IR for neurofibromin (Fig. 4F).

DISCUSSION

Only 30% to 50% of NF2 germline mutations are detected when the coding region of the NF2 gene was examined by SSCP and DGGE analyses (9–16, 21–23). This suggests that many mutations involve promoter, intronic or noncoding expressed sequences. Due to the presence of 2 hits in NF2 and sporadic schwannomas, approximately twice the percentage of mutations are detected in tumors (when corrected for allelic loss) (9–12). Extrapolating the sensitivity of mutation detection to analysis of schwannoma DNAs, it is likely that most if not all schwannomas harbor mutations in the NF2 gene. We analyzed whether analysis of the NF2 gene product schwannomin might be more sensitive in detecting NF2 gene mutations than DNA analysis. Indeed, 29 out of 29 schwannomas had lost immunoreactivity for schwannomin when a C-terminal AB was used.

The ABs used for these investigations were highly specific and well characterized. The two NF2 ABs recognize a 65 kDa protein (9, 24) that is close to the calculated

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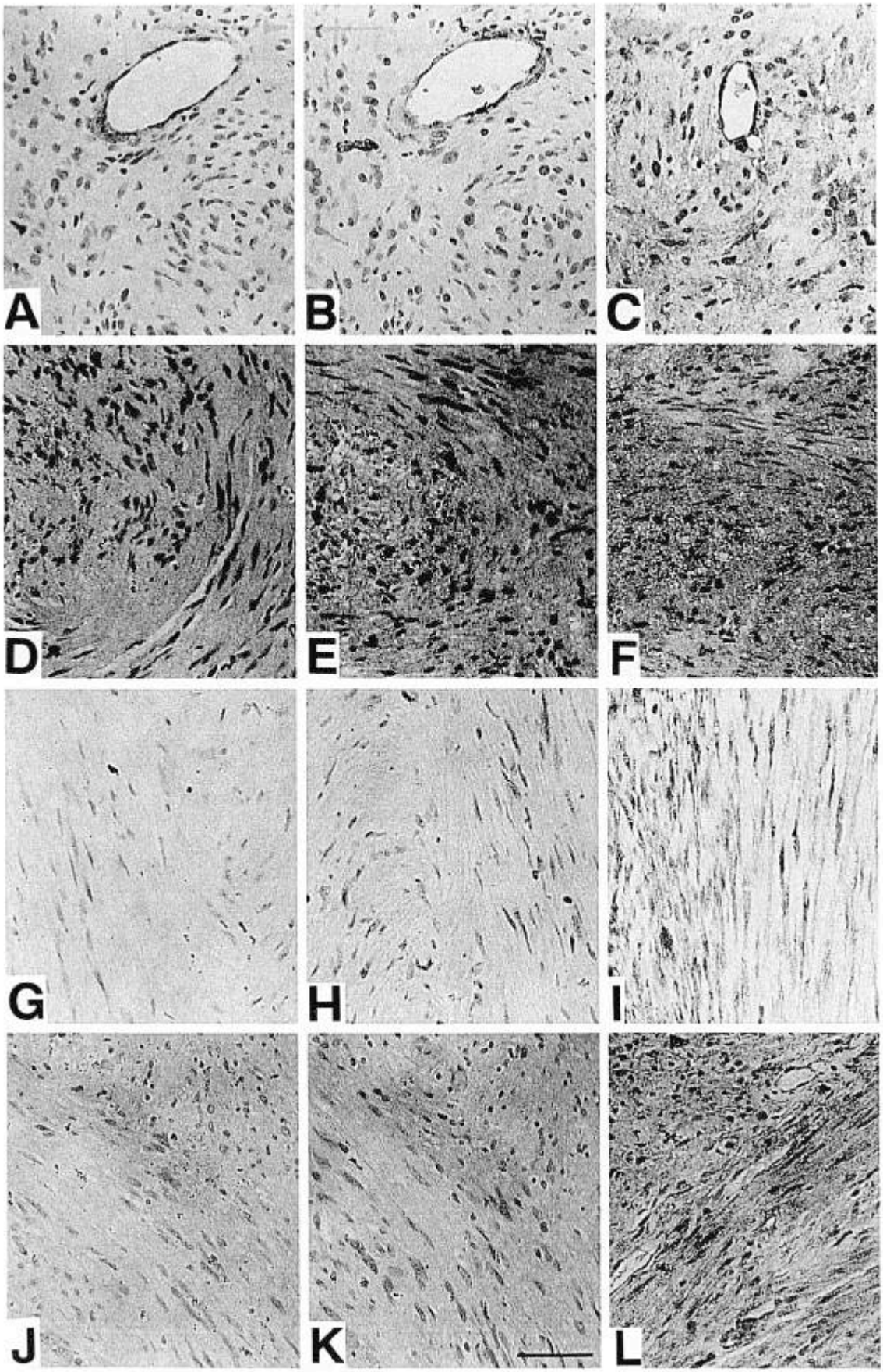
with preabsorbed 5990 AB (B), 5991 (D), and NF1C (F). Each antibody was preabsorbed with 100 μM of its antigenic peptide overnight at 4°C. Sensory neurons and Schwann cells (SCH) were strongly stained by all 3 ABs. NF1C stained Sa weakly. All sections were lightly counterstained with Methylene Green. Bar indicates 100 μm.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molecular weight of 69 kDa for schwannomin (27). Several lines of evidence establish that the 65 kDa protein is indeed schwannomin: First, ABs raised against peptides derived from different regions of schwannomin recognized the same protein in both immunoprecipitation and Western blots (9, 28). Second, pre-immune sera did not detect the 65 kDa protein in either Western blot or immunoprecipitation of a cell line derived from a sporadic malignant schwannoma (24). Third, ABs preabsorbed with 100 μM of the corresponding peptide did not detect the 65 kDa protein (9). Fourth, the 65 kDa protein was not synthesized when cells were incubated with NF2 antisense oligonucleotides (24). And fifth, both ABs showed the same pattern of staining in a variety of human tissues including normal vestibular nerve and mouse embryonic tissues (9, 28, Fig. 2A and C). Interestingly, schwannomin expression in vestibular nerve was not limited to SCH, but strong immunoreactivity was also observed in vestibular ganglion cells and Sa (Fig. 2A, C).

The observation of neurofibromin staining in endothelial cells of human blood vessels and weak staining of Sa in the vestibular ganglion is inconsistent with observations by Daston et al (29) and Nordlund et al (30). Using paraformaldehyde-fixed, frozen human tissues and rat DRG, neurofibromin was not detected in endothelial and Sa (29, 30). The discrepancy is probably due to differences in techniques and the sensitivity of the staining methods. Our staining method is probably more sensitive due to the use of a protease cocktail (26) to unmask cross-linked proteins and a peroxidase enhancer (Biomeda, CA) to enhance the peroxidase reaction, especially for endothelial and satellite cells.

Most NF2 germline and somatic mutations are predicted to result in truncated proteins that leave the N-terminus of the protein intact. Therefore, one would have predicted that most schwannomas would be immunoreactive for the N-terminal AB provided that mutated schwannomins are stable. However, only 4 tumors were reactive with the N-terminal 5991 antibody. In 3 tumors, the mutations were identified at the DNA level in exon 13. In tumor 10, mutations in both NF2 alleles are known, both resulting in a frameshift mutation in exon 13. Tumor 40 had a frameshift mutation at codons 459–470 and tumor 51 had a nonsense mutation at aa residue 466. It is not clear why these tumors had retained IR, since other tumors with truncations distal to exon 13 had lost IR. These results suggest that most mutated transcripts are not effectively translated or that truncated schwannomins have a short half-life or are not efficiently integrated into the cytoskeleton. This hypothesis received further support when we analyzed 2 tumors known to contain missense mutations. Two tumors (Table 1 and Fig. 3G–H), which contain missense mutations at codons 62 and 79, respectively, showed no IR with either the C-terminal (5990-AB) or the N-terminal (5991-AB) ABs (Fig. 3G–H), although the antigenic sites used for AB generation were not affected by these mutations.

It has been suggested that missense mutations and mutations resulting in minimally truncated schwannomins are associated with a milder NF2 phenotype. The failure to detect these less severely altered schwannomins in the majority of vestibular schwannomas suggests that the proteins are not stable in vivo. Thus, it is unlikely that mutated schwannomins exert a partial function that may result in a mild phenotype. The lack of genotype/phenotype correlation has also been supported by the study of recurrent missense mutations, deletions, and by analysis of identical twins (13, 31, 32).

The immunohistochemical analysis of meningiomas and ependymomas confirmed that the NF2 gene is important for the pathogenesis of these tumor types, but that other genes likely play a role as well. No schwannomin IR was detected in 7 of 10 meningiomas, including all 3 meningiomas from NF2 patients, and 2 of 7 ependymomas. We demonstrated that absence of schwannomin IR was not artifactual. First, IR was detected in non-tumor elements that were part of the tumor specimen such as parts of normal meningeal tissue or in blood vessels residing outside of the tumor mass. Second, both NF2-ABs gave identical results. And finally, NF1 ABs stained tumor cells in all ependymomas and meningiomas. The last finding also underscores that NF1 mutations do not play a major role in the genesis of meningiomas and ependymomas.

The immunocytochemical observations in ependymomas and meningiomas extend the results obtained by mutational analysis of tumor DNA (7, 16–19). These studies suggested that only some meningiomas were caused by

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Fig. 3. Staining of vestibular schwannomas with NF2 and NF1 ABs. A, B, and C show sections of an NF2-associated vestibular schwannoma (tumor 18) containing a truncated mutation at codon 341 stained with 5990 AB, 5991 AB, and NF1C-AB, respectively. Both the schwannomin C-terminal (5990 AB) and the N-terminal (5991 AB) ABs did not detect any immunoreactivity on tumor cells. D, E, and F show sections of a sporadic vestibular schwannoma (tumor 10) containing frameshift deletions between codons 447–482 stained with 5990 AB, 5991 AB, and NF1C-AB, respectively. The N-terminal Ab 5991 and NF1C-AB showed staining. G, H, and I show sections of an NF2 associated vestibular schwannoma (tumor 4), in which mutation was not detected by SSCP and DGGE methods, stained with 5990-AB, 5991-AB, and S100. J, K, and L show sections of a sporadic vestibular schwannoma (tumor 27) with a missense mutation at codon 79 stained with 5990-AB, 5991-AB, and NF1C-Ab. Only the NF1C-Ab was immunoreactive. All sections were counterstained with aqueous hematoxylin. Bar indicates 70 μm.

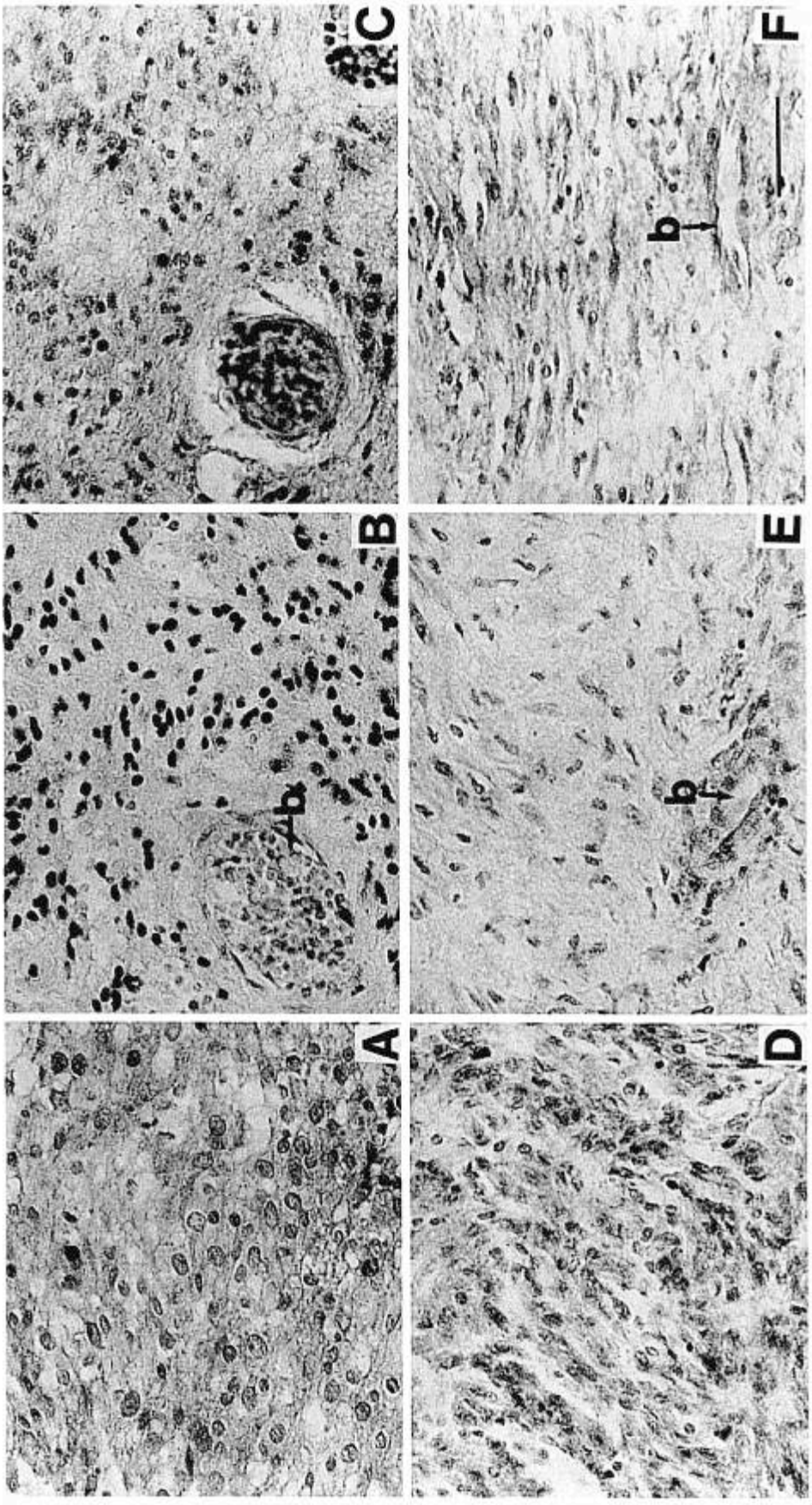

Fig. 4. Staining of meningiomas and ependymomas by NF2 and NF1 ABs. A. Presence of schwannomin in an ependymoma as shown by its immunoreactivity with 5990 AB. B. Negative staining of another ependymoma with 5990 AB. C. Same as B but stained with NF1C-AB. D. Detection of schwannomin in a sporadic meningioma with 5990 AB. E. Negative staining of a sporadic fibroblastic meningioma with 5990 AB. F. Same as E stained with NF1C-AB. Bar indicates 70 μm.

mutations in the NF2 gene (16–18). This conclusion was based on the observation that NF2 gene mutations were statistically associated with loss of heterozygosity for chromosome 22 markers in a subgroup of meningiomas. However, conclusions drawn from DNA mutation analysis are at the moment limited by the relatively low sensitivity of mutation detection, which is approximately 30% to 50% for various forms of mutation detection (22). Immunocytochemical analysis now provides direct evidence that loss of schwannomin underlies the formation of about half of the meningiomas.

Ependymomas arise from ependymal cells lining the CNS ventricles and subependymal glial cells and comprise approximately 5 to 6% of intracranial and 63% of spinal gliomas (20, 33). Approximately 3% to 4% of NF2 patients develop ependymomas (2, 20). Loss of chromosome 22 alleles has been reported in NF2-associated ependymomas (7). Recently, Rubio et al (19) found a single base deletion in exon 7 of the NF2 gene in an ependymoma as the only abnormality in 8 sporadic ependymomas screened for NF2 mutations. This study now confirms the low rate of NF2 gene mutation in ependymomas in that only 2 out of 7 sporadic ependymomas lacked schwannomin IR.

The presence of schwannomin IR in half of the meningiomas and the majority of ependymomas suggests the existence of as-yet-unidentified meningioma/ependymoma genes. Their existence is also supported by genetic linkage analysis. Although pedigrees with autosomal dominant meningioma and ependymoma are exceedingly rare, the analysis of one such a pedigree excluded the mutation from a large region of chromosome 22 including the NF2 locus (35). The excluded region also encompassed the recently identified MN1 gene that is adjacent to NF2 (34).

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