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Frequent *NF2* Gene Transcript Mutations in Sporadic Meningiomas and Vestibular Schwannomas

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Summary

The gene for the hereditary disorder neurofibromatosis type 2 (NF2), which predisposes for benign CNS tumors such as vestibular schwannomas and meningiomas, has been assigned to chromosome 22 and recently has been isolated. Mutations in the NF2 gene were found in both sporadic meningiomas and vestibular schwannomas. However, so far only 6 of the 16 exons of the gene have been analyzed. In order to extend the analysis of an involvement of the NF2 gene in the sporadic counterparts of these NF2-related tumors, we have used reverse transcriptase-PCR amplification followed by SSCP and DNA sequence analysis to screen for mutations in the coding region of the NF2 gene. Analysis of the NF2 gene transcript in 53 unrelated patients with meningiomas and vestibular schwannomas revealed mutations in 32% of the sporadic meningiomas (n = 44), in 50% of the sporadic vestibular schwannomas (n = 4), in 100% of the tumors found in NF2 patients (n = 2), and in one of three tumors from multiple-meningioma patients. Of the 18 tumors in which a mutation in the NF2 gene transcript was observed and the copy number of chromosome 22 could be established, 14 also showed loss of (parts of) chromosome 22. This suggests that in sporadic meningiomas and NF2-associated tumors the NF2 gene functions as a recessive tumor-suppressor gene. The mutations detected resulted mostly in frameshifts, predicting truncations starting within the N-terminal half of the putative protein.

Introduction

Neurofibromatosis type 2 (NF2) is a dominant hereditary disorder that predisposes patients to the development of a number of benign CNS tumors. These are typically (bilateral) vestibular schwannomas (tumors of the eighth cranial nerve), (multiple) meningiomas, schwannomas, and ependymomas. The incidence of NF2 is ~1:40,000, with >95% penetrance (Kanter et al. 1980; Evans et al. 1992). The tumors that occur in NF2 patients also occur sporadically. For instance, intracranial meningiomas represent ~13%-19% of all primary brain tumors that are treated by surgery (Russell and Rubinstein 1989). The actual incidence of meningiomas is probably higher, since they are observed in 33% of intracranial neoplasms found incidentally at necropsy (Wood et al. 1957).

The NF2 gene has been assigned to chromosome 22 by tumor deletion studies and linkage mapping (Seizinger et al. 1986, 1987*a*, 1987*b*; Rouleau et al. 1990) and recently has been isolated (Rouleau et al. 1993; Trofatter et al. 1993). The gene presumably encodes a 595-amino-acid protein called "merlin," or "schwannomin," of which the N-terminal 340 residues display a high homology to moesin, ezrin, and radixin. These proteins have been postulated to play a role in mediating interactions between the cell membrane and the cytoskeleton. In some NF2-related tumors the inacti-

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vating germ-line mutation was accompanied by loss of heterozygosity (LOH) of chromosome 22 (Rouleau et al. 1993; Trofatter et al. 1993), suggesting a classical tumor-suppressor–gene model analogous to that originally proposed by Knudson (1971).

In sporadic meningiomas and schwannomas, LOH of chromosome 22 is a frequent event, suggesting loss or inactivation of a tumor-suppressor gene(s) on chromosome 22 as the underlying mechanism for the development of these tumors (Zang 1982; Seizinger et al 1986, 1987*a;* Dumanski et al. 1990; Bijlsma et al. 1992). In a study by Rouleau et al. (1993), 30 meningiomas and 30 vestibular schwannomas from either NF2 or sporadic patients were analyzed for mutations by denaturing gradient gel electrophoresis analysis of 6 of the 16 exons of the gene. In total, six mutations were observed. Even when the limited region of the gene that so far has been investigated is considered, this raises the question about the extent in which the NF2 gene is involved—in particular, because there are indications of additional loci on chromosome 22 that may play a role in the development of these tumors (Dumanski et al. 1990; Lekanne Deprez et al. 1991; D. R. Cox, personal communication).

In the present paper we describe the analysis of mutations in the NF2 gene in 44 sporadic meningiomas, in 4 sporadic vestibular schwannomas, in 2 tumors from NF2 patients, and in 3 tumors from patients with multiple meningiomas. Mutations were detected in one-third (19/53) of the cases, and these were often (14/18) associated with loss of chromosome 22. This suggests that in a significant number of sporadic NF2-related tumors, the NF2 gene plays an important role in tumor development.

Material and Methods

Tumor Samples and RNA Isolation

Fifty-three tumor samples were obtained from patients during surgery. The tumors were classified according to the World Health Organization histological typing of tumors of the CNS (Zülch 1979). The tumors were called "sporadic" when there was no family history for NF2 and when they were solitary cases. Patients were diagnosed as having NF2 when they fulfilled the criteria for NF2 according to the National Institute of Health Consensus Conference Statement on Neurofibromatosis (1987). When more than two meningiomas that did not fulfill the criteria for NF2 were found in one patient, this patient was considered as having multiple meningiomas. The tissue samples were kept frozen in liquid nitrogen until needed. The percentage of tumor cells in the tumor specimen was established by microscopic examination of a frozen tissue section. Most specimens contain >90% tumor cells. These samples and one cultured meningioma were used for total RNA isolation using the guanidinium thiocyanate method (Chirgwin et al. 1979).

RNA PCR Amplification

First-strand cDNA synthesis was carried out by denaturing 1.5 μg of total RNA at 65°C for 10 min in 12 μl of diethylpyrocarbonate–treated water with 30 pmol of each of the primers A3/3′ and B3/3′. The denatured RNA was chilled on ice for 2 min and incubated for 10 min at room temperature and then for 60 min at 42°C in a final volume of 20 μl containing 4 μl 5 × reverse-transcriptase buffer (Promega), 10 mM DTT, 0.5 mM each dNTP, and 200 units of reverse transcriptase (catalog M530; Promega). The reaction was terminated by heating to 95°C for 5 min and cooling on ice. A first amplification by PCR, resulting in the amplification products A and B, was performed by combining the following reagents in a 50-μl reaction: 2 μl of the reverse-transcribed product, 5 μl 10 × PCR buffer (Boehringer-Mannheim), 200 μM each dNTP, 1.5 units of *Taq* DNA polymerase (Perkin Elmer/Roche), 20 pmol of each primer (product A, A1/5′ and A3/3′; and product B, B1/5′ and B3/3′). Nested PCR amplifications A1 (A1/5′ and A1/3′), A2 (A2/5′ and A2/3′), and A3 (A3/5′ and A3/3′) were performed using 1 μl of amplification product A. Nested PCR amplifications B1 (B1/5′ and B1/3′), B2 (B2/5′ and B2/3′), and B3 (B3/5′ and B3/3′) were performed with 1 μl of amplification product B. The amplified products were separated on a 3% agarose gel (NuSieve 3:1; FMC). The conditions for nested PCR were identical to those for the amplification of fragments A and B. Amplification was carried out in a GeneAmp 9600 machine (Perkin Elmer), with the following parameters: initial denaturation for 5 min at 94°C, 32 three-step cycles (denaturation at 94°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 1 min 15 s), and 3 min at 72°C. The oligonucleotide primers used in reverse transcription and PCR amplification were as depicted in table 1.

SSCP Analysis

For SSCP analysis, identical nested PCR reactions were performed as described above, except that 20 μM dCTP and 0.1 μl of [α-^{32}P] dCTP (3,000 Ci/mmol; Du Pont–New England Nuclear) were added. After amplification, PCR products were diluted (1:6) with 0.1%

Table 1

Oligonucleotides for PCR and Sequence Analysis

Name	Sequence (5'→3')	Position[a]	Expected Size (bp)
A1/5'.....	CATGGCCGGGCCATCGCTTCC	−1/21	335
A1/3'.....	CCTGAACCAGCTCCTCTTCAGC	313/334	
A2/5'.....	TCAAAGGAAGAACCAGTCACC	259/279	353
A2/3'.....	TCAGCTTCATCCCTGGCTCG	592/611	
A3/5'.....	GGAGAGAATTACTGCTTGGTAC	555/576	370
A3/3'.....	CATAAATAGATCATGGTTCCCGAT	901/924	
B1/5'.....	CCTCAAAGCTTCGTGTTAATAAGC	860/883	365
B1/3'.....	TTCCTGCTCAGCCTCTGCGGC	1204/1224	
B2/5'.....	GGAGGCAAAACTTCTGGCCCAG	1179/1200	339
B2/3'.....	GACAGGCTGTCACCAATGAGG	1497/1517	
B3/5'.....	CAATTCCAGCACCGTTGCCTCC	1457/1478	350
B3/3'.....	GGGTGGCTGGGTCACCTGCT	1787/1806	

[a] Relative to the initiation codon (Rouleau et al. 1993).

SDS and 10 mM EDTA. A 5-μl sample of the diluted reaction was then mixed with 5 μl of stop solution (U.S. Biochemical Corporation). After denaturing at 94°C for 2 min and chilling on ice, 3 μl of the sample was loaded onto a 0.5 × Mutation Detection Enhancement gel (J. T. Baker) and electrophoresed at 8 W constant power for 14–16 h at room temperature, with 0.6 × TBE (Tris-borate EDTA) buffer. Gels were transferred to 3 MM Whatman paper, dried, and exposed to Kodak XAR-5 film.

Subcloning and Sequencing

Individual bands were carefully excised from dried SSCP gels and were placed in 100 μl of deionized water for 3 h at 37°C, with gentle shaking to elute the DNA from the gel. Five microliters of eluted DNA was reamplified using the appropriate primers as described above. Amplified products were subcloned into the plasmid vector pCM^{TM}II (Invitrogen). A mixture of four independent plasmids from one PCR amplification were sequenced with the appropriate NF2 primers from both orientations by using double-stranded recombinant plasmids as template for the dideoxy chain-termination method (U.S. Biochemical).

In Situ Hybridization, Cytogenetic and RFLP Analysis of Chromosome 22

The copy number of chromosome 22 was determined using three different techniques, depending on the material available. Karyotyping was performed after 3–50 d of culture. Cosmids from the q arm of chromosome 22 and a chromosome 22–specific centromere probe were used for in situ hybridization on both metaphase spreads and interphase nuclei of cultured and fresh tumor tissue. Chromosome 22–specific cosmid and centromere probes were used along the centromere and the long arm of chromosome 22. RFLP analysis was done by using 10 polymorphic probes for loci on chromosome 22. Only tumor samples with >80% tumor cells were used for this analysis. No constitutional DNA was available for most of the tumor DNA samples. Therefore, LOH for a specific marker was only scored when a heterozygous DNA sample showed considerable reduction of intensity of one of the two alleles. Further details of this study and information about the probes will be published elsewhere.

Results

RNA samples isolated from a total of 48 meningiomas (1 NF2 associated, 3 from patients with multiple meningiomas, and 44 sporadic tumors) and 5 vestibular schwannomas (1 NF2 associated and 4 sporadic tumors) were investigated for mutations in the *NF2* gene transcript. Reverse-transcriptase PCR was used to amplify the whole coding region of the NF2 mRNA in six different overlapping fragments of ~350 bp. All PCR-amplified products were first analyzed by agarose gel electrophoresis to search for large deletions and insertions in *NF2* gene transcripts. Some tumors revealed fragments of altered size. After this preliminary study, SSCP analysis was carried out. All cases showing alterations on agarose gels also detected aberrant fragments by SSCP techniques. Aberrantly migrating fragments

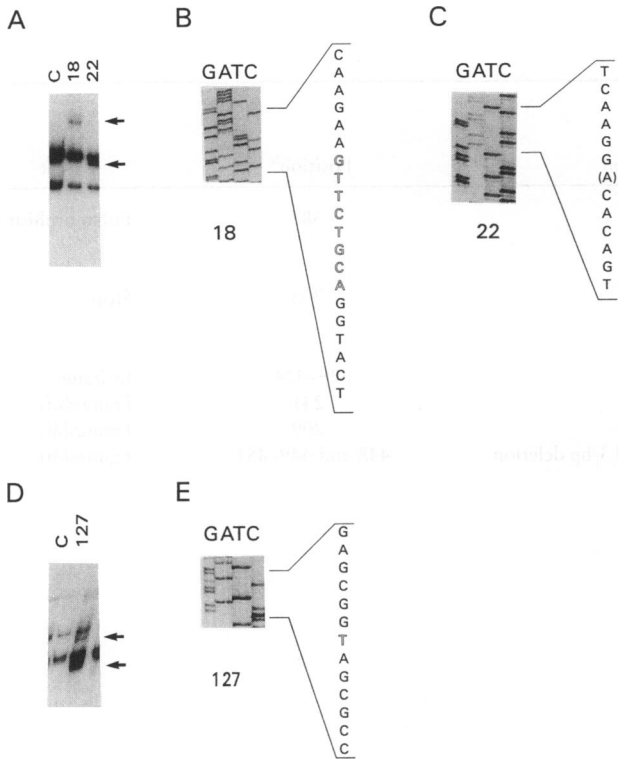

Figure 1 *NF2* gene–transcript mutation analysis. *A* and *D*, SSCP PAGE of reverse-transcribed and PCR-amplified RNA from tumors 18, 22, and 127. The aberrantly migrating SSCP fragments are indicated by arrows. Lanes C, Control RNA samples. *B*, *C*, and *E*, DNA sequence analysis of the area surrounding the mutations in tumors 18, 22, and 127. In *B* the 8-bp insertion in tumor 18 is indicated by unblackened letters; in *C* the 1-bp deletion in tumor 22 is indicated by parentheses; and in *E* the C→T transition in tumor 127 is indicated by the unblackened letter. The 5′→3′ orientation is from top to bottom.

were isolated from the SSCP gels. These fragments were amplified once more and were subcloned into a plasmid. In each case, four separate clones were combined for double-stranded sequence analysis in both orientations. Sequence analysis demonstrated the presence of deletions, insertions, and point mutations in all but one sample, which displayed an altered migration on agarose and/or SSCP gels. In figure 1, examples of all three types of alterations are depicted. Figure 1*A* and *D* shows the SSCP analysis of RNAs from tumors 18, 22, and 127, and figure 1*B*, *C*, and *E* shows the corresponding sequences that identify an 8-bp insertion in tumor 18, a 1-bp deletion in tumor 22, and a point mutation in tumor 127.

Table 2 shows a compilation of the results of the *NF2* gene–transcript mutation analysis in the 44 sporadic meningiomas, 1 meningioma from an NF2 patient, and 3 meningiomas from patients with multiple meningiomas. A summary of the chromosome 22 status of these tumors is also included. The copy number of chromosome 22 in the meningiomas and vestibular schwannomas was studied by cytogenetic analysis and by FISH and RFLP analysis, with a variety of chromosome 22–specific probes. Thirteen meningiomas revealed both mutations in the *NF2* gene and loss of (parts of) chromosome 22, one of which represented meningioma from a multiple-meningioma patient (tumor 22) and one of which represented meningioma from a NF2 patient (tumor 121). In 14 (32%) of the 44 sporadic meningiomas, mutations in *NF2* gene transcripts were observed. In tumor 128 we detected two different mutations. It remains to be established whether these represent different mutations in each of the two alleles of the *NF2* gene. Two meningiomas showed SSCP variations due to point mutations not resulting in amino acid substitutions (table 2, tumors 1 and 94). In the meningiomas analyzed here, we found no correlation between the presence of *NF2* mutations and age or sex of the patients (data not shown). A slight overrepresentation of mutations was found in meningiomas with a (partial) fibroblastic histology. However, this was not statistically significant (two-tail Fisher's exact test; $P = .075$).

Table 3 summarizes the results obtained with RNA samples from vestibular schwannomas. Two of four sporadic vestibular schwannomas showed mutations in the *NF2* gene transcript; none showed loss of (parts of) chromosome 22. We also performed mutation analysis on RNA from a meningioma (tumor 121) and a vestibular schwannoma (tumor 106), both of which were derived from NF2 patients. Both cases showed mutations in the *NF2* gene: in tumor 121, an in-frame insertion of 105 bp was detected (table 2), and in tumor 106 a 21-bp deletion was observed, creating a stop codon at the junction (table 3). Both tumors also showed loss of (parts of) chromosome 22.

Discussion

In this report we have shown that mutations in *NF2* gene transcripts are a frequent event associated with sporadic meningiomas. RNA SSCP analysis of the coding region of the *NF2* gene in 53 unrelated patients with meningiomas and vestibular schwannomas revealed mutations in 32% of the sporadic meningiomas ($n = 44$), in 50% of the sporadic vestibular schwannomas

Table 2

***NF2* Gene–Transcript Mutations in Meningiomas**

Tumor	Loss of (parts of) Chromosome 22[a]	Mutation	Position[b]	Result
1	?	GAA→GAG	387	Polymorphism
3	−22			
7	−22			
9	−22	TAT→TAA	303	Stop
10	−22			
12	?			
15	Diploid	96-bp deletion	329–424	In frame
18	−22	8-bp insertion	241	Frameshift
22[c]	−22	1-bp deletion	209	Frameshift
25	−22	128-bp insertion and 3-bp deletion	448 and 449–451	Frameshift
32	−22			
35	−22			
41	−22			
48	?			
54	−22			
55[c]	−22			
82	−22			
85	Diploid			
88	Diploid			
90	Diploid			
92	−22			
93	−22	1-bp deletion	1107	Frameshift
94	−22	GCC→GCT	219	Polymorphism
99	−22	20-bp deletion	448–467	Frameshift
108	−22	CAA→CT	53–54	Frameshift
109	−22			
111	−22	1-bp deletion	76	Frameshift
116	−22			
118	Diploid			
119	−22			
121[d]	−22	105-bp insertion	517	In frame
125	−22			
127	−22	CAG→TAG	1009	Stop
128	Diploid	2-bp deletion	36–37	Frameshift
		82-bp deletion and 18-bp insertion	496–577	Frameshift
130	Not done			
133	Diploid			
135	−22			
136	Diploid			
140	−22	14-bp deletion	1223–1236	Frameshift
141	?	2-bp deletion	68–69	Frameshift
143	−22			
144	−22			
145	−22	119-bp insertion	364	Frameshift
147	−22			
149	−22			
150	−22	13-bp deletion	153–165	Frameshift
153	Diploid			
154[c]	Diploid			

[a] −22 = Complete or partial loss of chromosome 22; Diploid = no loss of chromosome 22; and ? = not conclusive.
[b] Relative to the initiation codon (Rouleau et al. 1993).
[c] Patient with multiple meningiomas.
[d] Meningioma in an NF2 patient.

Table 3

***NF2* Gene–Transcript Mutations in Vestibular Schwannomas**

Tumor	Loss of (parts of) Chromosome 22[a]	Mutation	Position[b]	Result
68	Not done			
106[c]	−22	21-bp deletion	38–58	Stop
110	Diploid	53-bp deletion	440–492	Frameshift
117	Diploid	22-bp deletion	1023–1044	Frameshift
151	Diploid			

[a] −22 = Complete or partial loss of chromosome 22; and Diploid = no loss of chromosome 22.
[b] Relative to the initiation codon (Rouleau et al. 1993).
[c] Vestibular schwannoma in an NF2 patient.

(n = 4), in 100% of the tumors found in NF2 patients (n = 2), and in one of three tumors from multiple-meningioma patients. The mutations found can be divided into three categories: point mutations, deletions, and insertions. Three tumors carried mutations creating a stop codon at the mutated site (table 2; tumors 9, 106, and 127). Most insertions and deletions introduced frameshifts leading to premature termination of the reading frame within 14–200 bp of the site of mutation. The insertions found in RNA from tumors 18, 25, and 121 occurred at the border of two exons, indicating that they could be the result of splice-junction mutations. The 20-bp deletion in RNA from tumor 99 is at the beginning of exon 5 and is probably caused by a mutation in the splice acceptor site of exon 5. In our series of samples, no deletions of complete exons were observed. In two cases, an in-frame deletion of 96 bp and a 105-bp in-frame insertion were observed in RNA from tumors 15 and 121, respectively (table 2). The mutations observed predict major alteration in the structure of the merlin protein, suggesting that these represent real loss-of-function mutations, leading to inactivation of the *NF2* tumor-suppressor gene.

Most of the NF2 mutations described in this paper map to the N-terminal region of the predicted merlin protein (fig. 2), leading predominantly to deletions in the C-terminal part of the protein. However, it is as yet unclear whether the N-terminal moesin-ezrin-radixin–homology region of the merlin protein is more frequently associated with mutations in meningiomas. Mutations in sporadic vestibular schwannomas also appear to cluster somewhat in the N-terminal region of the merlin protein. However, mutations also have been found scattered throughout the *NF2* gene coding region in multiple tumor types (Bianchi et al. 1994). More extensive studies will be required to satisfactorily address the question about the pattern of mutations in human tumors.

The alterations observed are in agreement with a recessive tumor-suppressor gene model, because, in 13 of 15 meningiomas and in 1 of 3 vestibular schwannomas, mutations in *NF2* gene transcripts were observed together with loss of (parts of) chromosome 22, which is in accordance with the two-hit hypothesis first described by Knudson (1971). In two tumors derived from NF2 patients, both mutations in the *NF2* gene and loss of (parts of) chromosome 22 were observed. Thus, these mutations most likely represent germ-line alteration responsible for the disease in these patients. Further analysis of somatic tissue should prove this. Patient 121 belongs to a family in which the patients suffer mostly from multiple meningiomas (e.g., see patient FIII,2 in Delleman et al. 1978). However, bilateral vestibular schwannomas, the hallmark for NF2, were

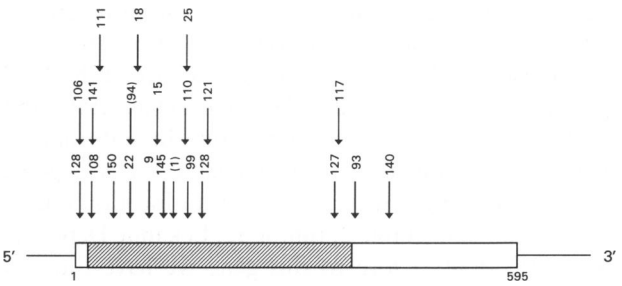

Figure 2 Outline of the position of the different mutations, with respect to the open reading frame of the NF2 protein. The region that displays homology to moesin, ezrin, and radixin is hatched. The two tumor samples showing silent mutations are in parentheses.

observed at autopsy in one member of the family in 1939. This, together with the presence of an *NF2* gene mutation in one of the tumors, suggests that this is indeed an NF2 family.

In most cases where we discovered aberrant fragments by agarose gel electrophoresis and/or SSCP, the intensity of the aberrant fragment was less than that of the wild-type fragment; this was also so in cases where, in addition to the mutation, loss of chromosome 22 was observed (fig. 1*A* and *D*). A possible explanation for the differences in intensity between wild-type and mutant RNA could be that a considerable number of normal endothelial cells may be present in the tumor. This is also obvious from RFLP studies, where complete loss of one allele is hardly ever observed (authors' unpublished results). An additional explanation is that the mutant RNA molecules, which in most cases can lead only to very short, truncated proteins, are less stable than their normal counterparts (Sachs 1993).

The frequent occurrence of *NF2* gene mutations in meningiomas and vestibular schwannomas suggests that this gene is important for the development of these types of tumors in both their sporadic form and their hereditary form. The actual frequency of *NF2* gene mutations may be even considerably higher, in light of the limits of techniques such as SSCP analysis, which depends on factors such as fragment size and position of the mutation (Sheffield et al. 1993). In the paper by Sheffield et al. (1993), the detection frequency of fragments of 300–400 bp is ~60%. Thus, when the presumed efficiency of the SSCP method is corrected for, the mutation frequency would increase to 50%. In addition, mutations in the promoter of the gene and very large deletions and insertions will not be detected by SSCP analysis. However, it is very much the question whether, when all these limitations might be solved, the percentage of mutations would reach 100. This is especially interesting because evidence exists for putative additional loci on chromosome 22. Aberrations of chromosome 22 that are not in the vicinity of the *NF2* gene have been described by Dumanski et al. (1990) and D. R. Cox (personal communication). In addition, we have recently cloned a gene proximal to the *NF2* gene, which is disrupted by a reciprocal translocation t(4;22) in a meningioma (table 2, tumor 32; Lekanne Deprez et al. 1991, 1994). Close to this gene we have found a germ-line deletion in a patient with multiple meningiomas (table 2, tumor 55; Lekanne Deprez et al. 1994). Neither case revealed mutations in NF2 transcripts by SSCP analysis of the coding region (present paper). This suggests that, besides the *NF2* gene, other genes on chromosome 22 may exist that play a role in the pathogenesis of meningiomas and vestibular schwannomas.

Acknowledgments

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