Otology & Neurotology
34:1355–1361 © 2013, Otology & Neurotology, Inc.

NF2 Genetic Alterations in Sporadic Vestibular Schwannomas: Clinical Implications

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Hypothesis: NF2 gene alterations may have a clinical impact in non-NF2 vestibular schwannomas (VSs).
Background: It has been suggested that *NF2* mutations might correlate with clinical expression of VS in NF2 patients. The aim of this study was to analyze the impact of genetic alterations in the *NF2* gene on epidemiologic, clinical, and radiologic features of patients with sporadic VS. The association between cigarette consumption and the molecular genetic findings was also studied.
Methods: The study group consisted of 51 patients who underwent surgery for removal of vestibular schwannoma in our institution between January 2006 and December 2010. Five highly polymorphic microsatellite DNA markers were used to observe the frequency of loss of heterozygosity (LOH) in chromosome 22. The NF2 gene mutations were detected using polymerase chain reaction amplification and denaturing high-performance liquid chromatography analysis (PCR/dHPLC), and direct sequencing of NF2. Multiplex ligation-dependent probe amplification (MLPA) of the NF2 gene was also performed.
Results: An *NF2* mutation was identified in 49%, 22q LOH in 57%, and MLPA alterations in 13.7% of the cases. One mutational hit was present in 27%, and 2 hits were present in 45% of the tumors. No association was found between the type of *NF2* mutation and relevant clinical parameters. The presence of *NF2* mutations detected by PCR/dHPLC was associated with no complaint of hearing loss at the time of diagnosis ($p = 0.023$), with subjective aural fullness ($p = 0.022$) and with an absence of tumor involvement of the internal auditory canal ($p = 0.029$). Patients with *NF2* mutations had lower mean corrected PTA thresholds compared with those with no NF2 mutation ($p = 0.037$). Inactivation of the *NF2* gene by mutation, MLPA, or LOH was more frequent in smokers when compared with never smokers ($p = 0.048$).
Conclusion: NF2 mutations may play a role in the pathophysiology of hearing loss as well as in the pattern of growth of VS. Cigarette smoking in patients with VS seems to play a role in both the risk of developing the tumor and also in its genetic profile. More studies are needed to corroborate these results and, more broadly, to establish links between molecular and clinical data. **Key Words:** Clinical implications—Hearing loss—*NF2* gene—NF2 mutation—Vestibular schwannoma.
Otol Neurotol **34:**1355–1361, 2013.

Vestibular schwannomas (VSs) are benign tumors arising from the sheath of the cochleovestibular nerve. These tumors generally grow slowly, causing minimal symptoms at the time of diagnosis. Hearing loss and tinnitus are the main features of VS, whereas vertigo, facial paralysis, or other incapacitating symptoms are less common initial symptoms. The growth rate of VS is known to be extremely variable, with most tumors growing slowly for many years. A peculiar aspect of these tumors is that their evolution in size and hearing level remains unpredictable. There is no correlation between tumor size and hearing loss (1).

Inactivation of Merlin, the product of the *NF2* tumor suppressor gene, is responsible for VS development, in both sporadic and bilateral cases. To date, the loss of 22q harboring the *NF2* gene has been the only constant genetic alteration reported in schwannomas (2). Inactivation of the *NF2* gene occurs in more than 60% of schwannoma cases, either by mutation of both alleles or by loss of one allele and mutation of the other (3). However, as Merlin protein seems to be absent in most, if not all VS (4), other mechanisms may lead to inactivation of this protein.

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Support for this work was provided by Grants 07/0577, 08/1849, and 10/1972 from Fondo de Investigación Sanitaria, Ministerio de Sanidad.
The authors disclose no conflicts of interest.

Osteopontin has been described as responsible for Merlin degradation in breast cancer (5), and recently, this gene has also been reported to be upregulated in VS (6). Thus, osteopontin may inhibit Merlin in tumors with no *NF2* DNA alterations.

Cytogenetic and LOH (loss of heterozygosity) studies have shown monosomy or partial loss of the chromosome 22 in approximately 40% to 50% schwannomas (7–9). Other molecular alterations have been proposed to explain the molecular biology of VS. DNA copy number analysis has revealed recurrent chromosomal gains of 9q34 and 17q and losses of 1p and chromosome Y in a few cases (10,11). The DNA methylation process, which consists of the methylation of cytosine at position C5 in CpG dinucleotides, has been described in VS involving the *NF2* gene (12–15) and other tumor-related genes (16); nevertheless, controversial findings have been reported, as *NF2* frequencies of methylation in those reports ranged from 0% to 50%. Clinical implications of this feature have been analyzed in patients with VS (17). Recent studies on microarray gene expression profiling have provided data allowing the identification of gene expression patterns linked to differentially expressed genes and pathways that play key roles in schwannoma development (18–22). Cyclin D1 is a cell cycle regulatory protein for the mammalian G1-S phase transition and is implicated in cell proliferation and differentiation. Lassaletta et al. (23) found cyclin D1 immunohistochemical expression in 52% of cases of VS. Recently, immunogenic factors (24), cytokines, and growth factors (25) have also been proposed to play a role in the molecular pathophysiology of VS. Whereas a clear progression in mutation type distribution by severity has been described in NF2 patients (26), the clinical significance of molecular alterations in sporadic VS is still unknown.

Little is known about the cause of VS. Risk factors including loud noise, mobile phone use, and some occupational hazards have been investigated with inconclusive results. Cigarette consumption has recently been suggested to have an effect on VS risk (27).

The aim of this study was to analyze the impact of genetic alterations of the *NF2* gene on epidemiologic, clinical, and radiologic features of patients with sporadic VS. A possible association between the smoking status of the patients and the genetic findings was also studied.

MATERIALS AND METHODS

Patients

The study group consisted of 51 patients who underwent surgery for removal of vestibular schwannoma in our institution between January 2006 and December 2010. Patients with a history of previous irradiation, previous VS surgery, known NF2 disease, or origin different from the vestibular nerve were excluded. The study was approved by the hospital's ethics committee.

Epidemiologic, Clinical, and Radiologic Data

The population included 24 women and 27 men with a mean age at surgery of 48 years (range, 14–75 yr). The tumor was on the left side in 23 cases (45%). The primary clinical complaint at the time of diagnosis was hearing loss (50%), tinnitus (20%), dizziness (11%), and vertigo (4%). Other symptoms accounted for 14% of cases. When specifically asked about particular complaints, the percentage of patients having each symptom was hearing loss (90%), tinnitus (72%), dizziness (47%), vertigo (20%), ear fullness (10%), and facial alterations (29%). Preoperative facial function was House-Brackmann Grade 1 in 96% and Grade 2 in 4% of patients. Mean pure-tone threshold for the tumor ear and the contralateral ear were 53 and 30 dB, respectively. To avoid the effect of presbycusis, the hearing loss of the contralateral ear was subtracted from the hearing loss of the affected ear (corrected threshold) as previously described (28). The mean corrected threshold was 30 dB. Mean speech reception threshold was 50 dB (range, 0–85 dB). Maximum speech discrimination score (SDS) ranged from 0% to 100%, with a mean of 58%. Tumor size measured as the largest diameter in the axial plane of MRI ranged from 5 to 52 mm (mean, 25 mm). In addition, the tumor volume was also measured using MRI, using the formula for an ellipsoid, as previously described (29). Mean tumor volume at presentation was 6.7 cm^3, ranging from 0.16 to 37.483 cm^3. Size was evaluated as Stage 1 (intracanalicular) in 2 cases (4%), Stage 2 (15 mm in its greatest diameter in the CPA) in 12 cases (23%), Stage 3 (16–30 mm in the CPA) in 29 cases (57%), and Stage 4 (>30 mm in the CPA) in 8 cases (16%). Tumor appearance was homogeneous (53%), heterogeneous (29%), and cystic (18%). Tumors in the CPA were centered in the internal auditory canal (IAC) in 82%, posterior in 12%, and anterior in 4% of the cases. Involvement of the fundus of the IAC was assessed on T2-weighted MRI as absence of the normal high cerebrospinal fluid signal intensity between the fundus and the distal end of the tumor. The fundus of the IAC was involved in 68% of the cases. Twenty-six patients (52%) were never smokers, whereas 14 (28%) were current smokers, and 10 (20%) were past smokers. Therefore, the number of never and ever smokers was 26 (52%) and 24 (48%), respectively.

Molecular Genetic Analysis

DNA Extraction

DNA was isolated from frozen vestibular schwannoma samples, using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). DNA from the corresponding patients' peripheral blood was also extracted.

Loss of Heterozygosity Studies

The allelic status of 5 microsatellite markers at the D22S275, D22S264, D22S929, D22S268, and D22S280 loci (22q11-q12.3) was determined by labeling 5' primers with fluorescent markers (6-FAM/HEX and ROX as a size standard; Applied Biosystems, Foster City, CA, USA). Allelic ratios were defined according to previously described criteria: T2xN1/T1xN2 in which LOH < 0.6–1.67 > LOH (30).

PCR/dHPLC Analysis and Direct Sequencing of NF2

For *NF2* mutation screening, PCR/dHPLC analysis and direct sequencing of *NF2* were performed. Genomic DNA amplification was performed using standard PCR methods (total volume of 20 μl). A set of 15 primer pairs was used as described (31). Mutational screening was carried out using dHPLC following the manufacturer's protocols (Transgenomic WAVE dHPLC Systems). Samples with abnormal patterns were sequenced bidirectionally (ABI 3100 Avant; Applied Biosystems), using the

Big Dye sequencing kit (Applied Biosystems), to determine the position and nature of the alteration. For name mutations, sequence NM_000268.3 was used when the alteration appeared within mature mRNA, and NC_000022.10 when the mutation was inside of the intron sequence. Mutations were named according to den Dunnen and Antonarakis (32).

Multiplex Ligation-Dependent Probe Amplification of the NF2 Gene

We used a commercial MLPA kit for *NF2* gene analysis (SALSA P044 NF2). Information on the probe sequences and ligation sites can be found at http://www.mlpa.com. The MLPA protocol was performed as described by the manufacturers and according to the method we described previously (33), using 100 ng of DNA from control and tumor samples. The data analysis was performed with MRC-Coffalyser software (MRC-Holland) and Microsoft Excel. Values less than 0.1 were considered as background.

As MLPA and LOH by microsatellites give us overlapping information regarding 22q status, only microsatellite markers were taken into account for hits in *NF2*. MLPA was only considered for individual exon-probe deletion, in agreement with Hadfield et al. (9).

Statistical Analyses

Categorical variables were described by frequency (percentage) and continuous variables as mean (standard deviation). Groups of patients were compared using the Mann-Whitney test for continuous variables, and χ^2 and Fisher's exact tests for categorical values. Because of the limited sample size, only univariate (unadjusted) associations were generated. All analyses were performed using SPSS 13.0 for Windows. Differences were considered significant at a level of $p < 0.05$.

RESULTS

Table 1 shows the main genetic findings.

LOH Analysis

Using 5 microsatellites markers, LOH of 22q11-q12.3 occurred in 29 (57%) of 51 tumors.

Mutational Analysis by PCR/dHPLC

Mutational analysis by PCR/dHPLC showed 25 (49%) of 51 of the samples with at least 1 alteration at the *NF2* gene. Three of 25 altered tumors presented 2 mutations (Samples 35, 41, and 48), with a total of 28 mutations detected. By analyzing the changes in the nucleotide sequence, small deletions of 1 to 15 pairs of bases were the most common events with 13 cases (46%) followed by 12 point mutations (43%). Small insertions occurred in 2 cases. Seven mutations (25%) affected the intronic region. Patient 32 had both intron and exon affected regions. Nonsense mutation c.169C > T was the only one repeated in our series. Most changes of sequence occurred at exon 4 (8 cases) followed by exon 2 (4 cases). Exons 6 and 9 were not affected by any mutation. The first half of the *NF2* gene (exons 2–8) showed 71% of the total mutations. Two samples (11 and 48) presented the *NF2* gene mutated in both tumor DNA and peripheral blood. These 2 cases had no features of NF2 preoperatively or postoperatively, probably representing mosaic cases.

MLPA Analysis of the *NF2* Gene

In agreement with LOH findings, MLPA analysis of the *NF2* gene (SALSA P044) showed results compatible with the total loss of an *NF2* allele in 19 cases (37%). Additionally, 7 (13.7%) of 51 cases displayed deletion of at least 1 exon. In 4 tumors with a MLPA deletion, no sequence alterations were found by PCR/dHPLC. However, 3 of these cases were concomitant with LOH of 22q. Multiple deletions were also present in 4 cases, that is, tumor 15, which showed a deletion from exons 14 to 17. By MLPA, exons 4 and 14 were the most commonly altered in the series with 3 cases each.

Summary of NF2 Analyses

Because of the biallelic gene constitution of the cell, at least 2 hits (by mutation, 22q LOH, etc) are theoretically needed to inactivate the tumor suppressor *NF2* gene (26). As shown in Table 1, two or more mutational hits were found in 23 (45%) tumors. Two were exclusively due to 2 mutations at the *NF2* sequence (Samples 41 and 48); 3 tumors had 2 hits because of a MLPA alteration adding LOH of 22q with no PCR/dHPLC alterations; the rest presented this pattern because of a combination of several LOH and sequence mutations found by MLPA and/or PCR/dHPLC. A single mutational event was established in 14 (27%) cases: 8 with LOH of 22q, 5 with a mutation detected by PCR/dHPLC, and 1 with a deletion found by MLPA. In 14 (27%) of 51 tumors, neither molecular alteration of the *NF2* gene nor 22q LOH / MLPA alteration were found.

Clinical Associations

In our study, the presence of *NF2* mutations by PCR/dHPLC was associated with no complaint of hearing loss at the time of diagnosis, subjective aural fullness, and no tumor involvement of the IAC (Table 2). *NF2* mutations detected by PCR/dHPLC were also associated with hearing level at the time of diagnosis. Patients with *NF2* mutations had lower mean corrected PTA thresholds compared with those with no *NF2* mutation (Table 2). No association was found between the mutation of the *NF2* gene and other epidemiologic, clinical, and radiologic relevant parameters.

An association was found between *NF2* gene status and smoking habits. Inactivation of the *NF2* gene by mutation, MLPA or LOH was more frequent in ever smokers when compared with never smokers (Table 3). No association was found between the status of the *NF2* gene and other relevant epidemiologic, clinical, and radiologic parameters.

DISCUSSION

VS arise from inactivation of the *NF2* tumor suppressor gene, which regulates Schwann cell growth. Mutations of the *NF2* gene have been found in both NF2 and unilateral sporadic schwannoma patients (34). More than

TABLE 1. *Mutational analysis of NF2 gene*

Sample	LOH	MLPA	Tumor mutation NM_000268.3	Codon	Consequence	Blood status	Hits in NF2
1	N	−/−	-	-		-	0
2	N	−/−	c.359_360del2	p.Leu20Serfs*	Frame shift	-	1
3	+	−/+	-	-	-	-	1
4	N	−/−	-	-	-	-	0
5	+	−/+	NC_000022.10:g.71279A > C (IVS13-2A > C)	-	Splice acceptor	-	2
6	N	−/−	-	-	-	-	0
7	N	−/−	-	-	-	-	0
8	+	−/+	c.351T > A	p.Leu120Stop	Non-sense	-	2
9	+	−/+	-	-		-	1
10	+	−/−	c.459C > G	p.Tyr153Stop	Non-sense	-	2
11	+	−/+	c.431_432insAA	p.Tyr144Stop	Non-sense	Mutated	2
12	+	−/11	-	-		-	2
13	N	−/−	-	-		-	0
14	+	−/+	c.447G > A	p.=	Silent	-	2
15	+	+/14-17	-	-		-	2
16	N	−/−	-	-		-	0
17	+	−/+	c.625A > T	p.Lys209Stop	Non-sense	-	2
18	N	−/−	-	-	-	-	0
19	+	−/+	NC_000022.10:g.54588_54622dup35 IVS6	-	Silent	-	2
20	+	−/+	c.1592delA	p.Lys531Argfs*	Frame shift	-	2
21	+	−/+	c.663C > G	p.Tyr221Stop	Non-sense	-	2
22	+	−/+	NC_000022.10:g.64892G > A (IVS10+1G > A)	-	Splice acceptor	-	2
23	+	+/4,14	c.386_398del13	p.Glu129Alafs*	Frame shift	-	3
24	N	−/−	-	-	-	-	0
25	N	−/−	c.169C > T	p.Arg57Stop	Non-sense	-	1
26	+	−/+	c.737delC	p.Pro246Leufs*	Frame shift	-	2
27	+	−/+	-	-	-	-	1
28	+	+/11,13	-	-	-	-	2
29	N	−/−	-	-	-	-	0
30	+	+/4	c.401delC	p.Pro134Leufs*	Frame shift	-	3
31	+	−/+	NC_000022.10:g.38646G > A (IVS4-1 G > A)	-	Splice acceptor	-	2
32	+	−/+	NC_000022.10:g.71379_71405del	p.Thr480Serfs*	Frame shift	-	2
33	+	−/−	c.436_443del8	p.Val146Glnfs*	Frame shift	-	2
34	+	−/+	c.1076insT	p.R359MfsX*	Frame shift	-	2
35	+	+/5,14	c.467G > A	p.Ser156Asn	Missense	-	4
			c.465_474del10	p.P155QfsX*	Frame shift	-	
36	N	−/−	-	-		-	0
37	N	−/−	-	-		-	0
38	+	−/−	-	-		-	1
39	N	−/4	-	-		-	1
40	+	−/+	-	-		-	1
41	N	−/−	c.169C > T	p.Arg57Stop	Non-sense	-	2
			NC_000022.10:g.74615_74636del(IVS14-26del)	-	Silent	-	
42	N	−/−	-	-	-	-	0
43	N	−/−	c.1230_1243del14	p.Gln410Hisfs*	Frame shift	-	1
44	N	−/−	-	-	-	-	0
45	+	−/+	-	-	-	-	1
46	N	−/−	c.206delA	p.Lys69Argfs*	Frame shift	-	1
47	N	−/−	-	-	-	-	0
48	N	−/−	c.414delT	p.Val139Cysfs*	Frame shift	-	2
			c.1600C > T	p.His534Tyr	Missense	Mutated	
49	+	−/+	-	-		-	1
50	+	−/−	-	-		-	1
51	N	−/−	NC_000022.10:g.38750delA (IVS4+20delA)	-	Silent	-	1

In chromosome 22q analysis, N is normal and + is positive for LOH. To name mutations, *del* corresponds to deletion, *ins* to insertion, > to change of a single nucleotide, and *dup* to duplication. When mutations occurred within introns, (IVS), the number of the affected is shown. Changes in codons and consequence of mutations in proteins are predicted from those changes obtained in DNA.

LOH indicates loss of heterozygosity; −/− indicates no losses; −/+ indicates complete deletion of one NF2 allele; −/"number" indicates partial deletion of the stated exons.

200 different mutations have been identified to date, including single-base substitutions, insertions, missense, and deletions (35). *NF2* gene inactivation is necessary for VS to grow. According to Knudsen's 2-hit hypothesis, in NF2 patients, the germline *NF2* allele is inactivated and tumors occur when a wild-type allele is inactivated by allelic loss, silencing, or mutation. On the other hand, sporadic unilateral VS formation is explained by somatic biallelic *NF2* inactivation as a result of the same molecular mechanisms (allelic loss, gene silencing, or mutation) (36).

In this study of 51 samples of unilateral VS, LOH alterations were found in 57% of cases, *NF2* mutations

TABLE 2. *Association between NF2 mutation and clinical and radiologic variables*

		No *NF2* mutation	*NF2* mutation	
Hearing loss	No	0 (0%)	5 (100%)	Fisher's exact test $p = 0.023$
	Yes	26 (56%)	20 (43%)	
Aural fullness	No	25 (57%)	19 (43%)	Fisher's exact test $p = 0.022$
	Yes	0 (0%)	5 (100%)	
Involvement of the IAC	No	5 (29%)	12 (71%)	Fisher's exact test $p = 0.029$
	Yes	21 (62%)	13 (38%)	
Corrected PTA threshold (500, 1,000, and 2,000 Hz)	Mean	36 dB (SD 15)	23 dB (SD 25)	Mann-Whitney test $p = 0.037$

were found in 49%, and MLPA alterations (other than those compatible with the total loss of chromosome 22) occurred in 13.7% of cases. Therefore, one mutational hit was present in 27%, and 2 hits were present in 45% of the tumors. High sensitivity in detection of DNA sequence variations is essential for mutation analysis in genetic diseases. In this study, we performed 3 different techniques to cover all the *NF2* mutational screening: dHPLC for small sequence mutations, MLPA for large scale rearrangements, and LOH of 22q for detecting the absence of an entire copy of the gene. Even using these 3 methodologies, the possibility of missing a small subset of *NF2* mutations cannot completely be discarded, that is, an insertion of a foreign gene (37). However, our results are similar to those reported by other authors, which suggests that the combination of DHPLC, MLPA, and LOH of 22q may be enough sensitive to detect most *NF2* gene mutations in patients with VS. Martinez-Glez et al. (21) studied 23 frozen samples of schwannomas and found *NF2* mutations in 34.8% and LOH alterations in 30.4% of the cases. Hadfield et al. (9) identified at least 1 point mutation in the *NF2* gene in 65 (66%) of 98 sporadic VSs, with a higher incidence of frameshift (34%) than nonsense mutations (17%) in comparison with NF2-related tumors. In this study including 104 patients with unilateral sporadic VS, loss of heterozygosity (LOH) was found in 56% of the cases (9).

Previous studies have attempted to correlate the clinical expression of tumors with *NF2* mutations, especially in NF2 patients (38); however, scarce information is available on this subject in terms of patients with sporadic VS. Deletion mutations that cause truncation of the NF2 protein have been reported to cause a more severe phenotype in NF2 patients, whereas missense mutations or small in-frame insertions have been reported to be associated with a mild phenotype (38). In the present study performed on 51 unilateral VS, no association was found between the type of *NF2* mutation and relevant clinical parameters. However, a negative association was found between mutation of the *NF2* gene and hearing loss, as well as between mutation of the *NF2* gene and involvement of the IAC fundus. Patients with *NF2* mutations had better corrected hearing thresholds, less frequent involvement of the IAC fundus (which is usually associated with poorer hearing), and complained less frequently about hearing loss when compared with those without *NF2* mutations.

TABLE 3. *Association between NF2 gene inactivation and smoking status*

		NF2 normal or one mutational hit	*NF2* inactivation (2 hits)	
Smoker	Never	19 (73%)	7 (27%)	Chi-squared
	Ever	11 (46%)	13 (54%)	test $p = 0.048$

Involvement of the IAC fundus is an important feature for the VS surgeon. Absence of fluid between the lateral end of the tumor and the IAC fundus on MRI seems to have a negative influence both on hearing outcome (39) and on facial function outcome (40) after VS surgery. The exact origin of VS in the VIIIth nerve remains uncertain. According to a recent study, VS may arise anywhere along the course of the axons of the cochleovestibular nerve, from the glial-Schwann cell junction up until terminations within the auditory and vestibular end organs (41). Moreover, its pattern of growth, spreading laterally toward the IAC fundus or medially from the IAC into the CPA angle is also unpredictable. The reason why tumors with NF2 mutations had less frequent involvement of the IAC fundus in our study may be related to these possible growth patterns and may be useful for predicting facial function or hearing outcome.

Hearing loss is the most common symptom in patients with VS. In the past, compressive mechanisms caused by the tumoral mass and its growth have been regarded as the most likely causes of hearing loss associated with VS. In recent years, molecular mechanisms have been proposed to explain this symptom (42). Lassaletta et al. (17) explored the methylation status of 16 tumor-related genes in 22 unilateral VS. DNA methylation values of 9% to 27% were found in 12 of the genes tested. A significant association was found between *TP73* aberrant methylation and hearing loss. In another study using immunohistochemistry, cyclin D1 expression was observed in 52% of VS. Patients with negative cyclin D1 expression had a longer duration of deafness and higher 2,000-Hz hearing thresholds than cyclin-positive patients (23). Using microarray gene expression technology, tumor samples surgically removed from 11 patients with unilateral VSs were analyzed (43); the expression of platelet-derived growth factor A (PDGFA) was inversely correlated with hearing loss. In the present study, patients with *NF2* mutation had better corrected hearing thresholds than those with no mutation. According to all these data, either *NF2* mutation, other non-random genetic events, or

deregulated expression of other genes also contribute to VS patients' hearing status.

In our study, inactivation of the *NF2* gene by mutation, MLPA or LOH was more frequent in smokers when compared with never smokers. To our knowledge, in contrast to several malignant tumors in which genetic alterations have been clearly related to cigarette smoking, no association between smoking and genetic alterations was previously reported in VS. The relationship between cigarette smoking and VS etiology has been studied in recent years. The results of a multicenter case-control study showed a significantly lower risk of VS in current smokers than in never smokers (44). Benson et al. (45) in a large prospective study confirmed a decreased incidence of VS in current smokers, and no clear association was found between smoking and the incidence of other tumors of the CNS, including glioma or meningiomas. Recently, Palmisano et al. (27) studied 451 VS cases and 710 controls. They confirmed that the risk of VS was greatly reduced in male current smokers and moderately reduced in female current smokers. The effect of cigarette smoking on hormonal status or the effects of hypoxia and reoxygenation on Schwann cells because of cigarette combustion products are possible mechanisms to explain the association between smoking and the risk of *NF2* inactivation. Furthermore, polyphenols present in tobacco may mimic the results obtained by Angelo et al. (46), whose experiments showed that, after applying curcumin (a polyphenol) to a schwannoma cell line, growth was inhibited in the tumor cells. Thus, smoking and VS may be related in 3 different ways. First, population studies show a lower rate of VS in smokers than in never-smokers; second, a component of tobacco (polyphenol) may exert growth inhibition in VS cells based on schwannoma cell lines studies (46); an third, our results seem to be contradictory with the previous points and show an increased *NF2* mutation rate in smokers with VS than nonsmokers with VS. Therefore, further studies are warranted to explain these phenomena. As new tobacco regulations are leading to a significant reduction in smoking in our country (47), it will be interesting to see if both the incidence of VS and the rate of *NF2* mutation in patients with VS is affected by this decrease.

In conclusion, there is growing evidence to explain hearing loss associated with VS patients using molecular data. *NF2* mutations may play a role in the pathophysiology of this symptom as well as in the growth pattern of these tumors. The role of cigarette smoking in patients with VS seems to play a role in both the risk of developing the tumor and also in its genetic profile. More studies are needed to corroborate these results and, more broadly, to establish links between the molecular and clinical data.

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