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DNA Methylation of Multiple Genes in Vestibular Schwannoma: Relationship With Clinical and Radiological Findings

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Hypothesis: The purpose of this study was to examine the DNA methylation profile of several genes in a series of vestibular schwannomas, and to analyze its relationship with clinical and radiological features.

Background: Aberrant methylation of promoter regions is a major mechanism for silencing of tumor suppressor genes in several tumors. There is limited information about methylation status in vestibular schwannoma, with no clinical or radiological implications described to date.

Methods: The methylation status of 16 tumor-related genes including *RASSF1A*, *RAR-B*, *VHL*, *PTEN*, *HMLH1*, *RB1*, *TP16*, *CASP8*, *ER*, *TIMP3*, *MGMT*, *DAPK*, *TP73*, *GSTP1*, *TP14*, and *THBS1* was examined in a series of 22 vestibular schwannomas. The bisulfite modification of genomic DNA was performed. Clinical and radiological features were compared with the methylation results.

Results: Methylation values from 9% to 27% were found in 12 of 16 genes tested, including *RASSF1A, VHL, PTEN*, *TP16*, *CASP8*, *TIMP3*, *MGMT, DAPK, THBS1, HMLH1*, *TP73*, and *GSTP1*. A significant association was found between *CASP8* and *RASSF1A* methylation. Methylation of *CASP8* was associated with the patient's age and the tumor size. Methylation of *TP73* was associated with hearing loss. *RASSF1A* methylation was inversely correlated with the clinical growth index.

Conclusion: Aberrant methylation of tumor-related genes may play a role in the development of vestibular schwannomas. Our results may provide useful clues to the development of prognostic assays for these tumors. **Key Words:** Vestibular schwannoma—DNA methylation—Tumor suppressor gene.

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Vestibular schwannomas (VSs) are common benign tumors accounting for 8–10% of intracranial tumors and about 80% of cerebellopontine tumors. Histologically, VSs arise from the sheath of the eight nerve. These tumors generally grow slowly, causing minimal symptoms at the time of diagnosis. The growth rate of VS is known to be extremely variable, with most tumors showing slow growth for many years (1). Complete information must be given to the patient, who must decide between wait and scan, surgery or radiotherapy. However, progressive and rapidly growing tumors may lead to brainstem compression and severe complications, and these cases may require prompt surgical treatment. Unfortunately, to date it is impossible to predict the expected behavior of VS based on information available at diagnosis (2).

The molecular changes involved in the pathogenesis of VS have been studied in the past years. Most studies have focused on the molecular genetic analysis of the *NF2* gene. Inactivation of merlin, the product of the *NF2* tumor suppressor gene, is responsible of VS development, both in sporadic and bilateral cases (3). Recently, the importance of DNA methylation in human tumors has been stressed. Hypermethylation of the promoter-associated CpG islands leading to transcriptional silencing is emerging as the major mechanism of epigenetic inactivation of tumor suppressor genes in cancer development. Methylation of specific subsets of CpG islands has been proposed to be associated with specific tumor types (4).

In VS, there is very limited information regarding abnormal methylation of tumor-related genes. Horiguchi

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et al. (5) studied the methylation status of the promoter region of *RASSF1A* in a series of malignant and benign brain tumors. Methylated alleles of the *RASSF1A* gene were detected in 10% (1/10) of the schwannoma cases. The authors suggested that methylated *RASSF1A* is not common in benign intracranial tumors. Kino et al. (6) characterized the promoter region of the human *NF2* gene and found methylation of 3 CpG islands in 14 of 23 VSs, suggesting this mechanism as an alternative mechanism for inactivation of the *NF2* gene. In a recent study, Gonzalez-Gomez et al. (7) studied the DNA methylation profile of 12 tumor-related genes in a series of 44 sporadic or NF-2 associated schwannomas. They found *THBS1*, *TP73*, *MGMT*, *NF2*, and *TIMP3* to be the most frequent methylated genes, suggesting that aberrant hypermethylation of *NF2* seems to be an early step in schwannoma development, whereas hypermethylation of the other tumor-related genes might represent events secondary to the inactivation of the *NF2* gene.

The aim of this study was to study the relationship between patient and tumor features that are available to the clinician at presentation, and the methylation profile in a series of VSs. To the best of our knowledge, no attempt to correlate clinical and radiological findings with the methylation profile of VSs has been performed to date.

MATERIALS AND METHODS

The study group consisted of 22 patients who underwent surgery for removal of unilateral VS in our institution between February 2002 and January 2004. Age, sex, tumor side, main clinical complaint, duration of symptoms (i.e., the delay between the appearance of symptoms and surgical treatment), and the presence of each symptom (hearing loss, tinnitus, dizziness, vertigo, and facial alterations) were studied. Facial nerve function was reported using the House-Brackmann grading system (8). Audiovestibular investigations included pure tone audiometry, speech discrimination score (SDS), and videonystagmography. Pure tone threshold was calculated for 500, 1000 and 2000 Hz. To avoid the effect of presbycusis, the hearing loss of the contralateral ear was substracted from the hearing loss of the affected ear (corrected threshold) as previously described (9). Tumor size was measured in terms of the largest diameter in the axial plane of MRI. In addition, the tumor volume was also measured from MRI, using the formula for an ellipsoid, as previously described (10). Involvement of the fundus of the internal auditory canal, and brainstem compression were recorded. To assess the tumor growth, the clinical growth index (CGI) was used. The CGI is calculated by estimating the tumor size as the maximal tumor diameter and the period of growth as the length of the patient's symptoms (2).

There were 15 women and 7 men with a mean age at surgery of 49 years (range 16 to 71 years). The tumor was on the left side in 14 cases (64%). The main complaints at the time of diagnosis were hearing loss (45%), tinnitus (23%), dizziness (18%), and other symptoms (14%). When specifically asked about each symptom, the percentage of patients having each symptom was: hearing loss (82%), tinnitus (68%), dizziness (59%), vertigo (9%), and facial alterations (13%). The duration of symptoms varied from 1 to 240 months. The preoperative facial function was grade I in 91% of patients, and grade II in 9% of patients. Mean pure tone threshold for the tumor ear and the unaffected ear were 42 dB and 23 dB, respectively. The mean corrected threshold was 16 dB. Data about videonystagmography were available in 16 patients, 9 of them showing different kind of anomalies. Maximum SDS ranged from 0% to 100%, with a mean of 70 %. Tumor size ranged from 13 mm to 55 mm (mean, 28 mm). Mean tumor volume at presentation was 935 mm^3, ranging from 25 to 5363 mm^3. Involvement of the fundus of the internal auditory canal and brainstem compression were present in 15 and 16 cases, respectively. Mean CGI was 21 mm/year ranging from 1.3 to 84 mm/year (median, 12.8 mm).

The methylation profile of 22 VSs was studied. All tumors arose from the vestibular nerve. One patient had NF2 and the other 21 patients had no evidence of this disease. In addition to the tumor samples, two samples of nonneoplastic peripheral nerve sheath tissue and two nonneoplastic brain tissue samples (all four obtained by autopsy) were used as controls. Genomic DNA was isolated from frozen tissues by standard methods, and the histological examination before DNA extraction estimated tumor cell content of the tumor tissue samples to be 75–90%. We analyzed the status of 16 genes frequently showing promoter methylation in other neoplasms. The genes studied were *RASSF1A*, *RARB*, *VHL*, *PTEN*, *HMLH1*, *RB1*, *TP16*, *CASP8*, *ER*, *TIMP3*, *MGMT*, *DAPK*, *TP73*, *GSTP1*, *TP14*, and *THBS*. The genes were chosen for study on the basis of their critical tumor-related function, as they are known to be frequently hypermethylated and silenced in other neoplasms, or on the basis of their localization al genomic regions involved in chromosome deletions in schwannomas. Bisulfite modification of genomic DNA was performed as reported previously (11).

Statistical analyses for differences between groups were performed using the Fisher's exact tests and the nonparametric Mann-Whitney *t* test.

RESULTS

Methylation values >10% were obtained for 9 genes: *RASSF1A* (23%), *VHL* (27%), *PTEN* (18%), *TP16* (14%), *CASP8* (18%), *TIMP3* (14%), *MGMT* (18%), *DAPK* (18%), and *THBS1* (18%) (Table 1). Three genes displayed methylation frequencies <10%, *HMLH1*, *TP73*, and *GSTP1* (9% each), whereas 4 genes *RARB*, *RB1*, *ER*, and *TP14* showed no methylation at all. Methylation did not occur in the autopsy samples of nonneoplastic tissue. All but 3 tumors displayed methylation of one or more genes (19 of 22 tumors: 86%). The frequency of methylation of multiple genes in a tumor was determined using the methylation index (MI) defined as the number of loci methylated/the number of loci tested. MI ranged from 0 to 0.31, with a mean of 0.126. Three of 5 VSs with *RASSF1A* methylation also demonstrated *CASP8* methylation but only one of 17 VSs without *RASSF1A* methylation demonstrated *CASP8* methylation ($p = 0.02$).

We compared the relationships between aberrant methylation of the 16 genes and clinical and radiological features in VS (Tables 2 and 3). Interestingly, some statistically significant correlations between VS features and gene methylation status of specific genes were established. Methylation of the *CASP8* gene was associated with the patient's

TABLE 1. *Summary of methylation of all 16 genes in vestibular schwannomas*

	RASSF1	*RARB*	*VHL*	*PTEN*	*HMLH1*	*RB1*	*P16*	*CASP8*	*ER*	*TIMP3*	*MGMT*	*DAPK*	*P73*	*GSTP1*	*P14*	*THBS1*	*MI*
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
3	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	0.12
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	0.06
5	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0.06
6	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	0.12
7	−	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	0.25
8	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−	−	0.12
9	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	0.06
10	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	0.19
11	+	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	0.19
12	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	0.06
13	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	0.19
14	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	0.12
15	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	0.06
16	+	−	+	−	−	−	+	+	−	−	−	−	−	−	−	+	0.31
17	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	0.06
18	−	−	+	−	−	−	−	−	−	−	+	−		+	−	−	0.19
19	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	0.06
20	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	0
21	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	0.19
22	+	−	+	−	−	−	−	+	−	−	+	−	−	−	−	−	0.25
	5 (23%)	0	6 (27%)	4 (18%)	2 (9%)	0	3 (14%)	4 (18%)	0	3 (14%)	4 (18%)	4 (18%)	2 (9%)	2 (9%)	0	4 (18%)	

MI, methylation index.

TABLE 2. *Methylation status of tumor-related genes and qualitative clinico-radiological findings*

	RASSF1A	*VHL*	*PTEN*	*HMLH1*	*P16*	*CASP8*	*TIMP3*	*MGMT*	*DAPK*	*P73*	*GSTP1*	*THBS1*
Sex												
M (n = 7)	2(29%)	2(29%)	0	1(14%)	1(14%)	1(14%)	1(14%)	1(14%)	0	0	1(14%)	2(29%)
F (n = 15)	3(20%)	4(27%)	4(27%)	1(7%)	2(13%)	3(20%)	2(13%)	3(20%)	4(27%)	2(13%)	1(7%)	2(13%)
Side												
R (n = 8)	0 (0%)	2(25%)	2(25%)	1(12%)	1(12%)	1(12%)	1(12%)	2(25%)	1(12%)	0	1(12%)	1(12%)
L (n = 14)	5(36%)	4(29%)	2(14%)	1(7%)	3(21%)	3(21%)	2(14%)	2(14%)	3(21%)	2(14%)	1(7%)	3(21%)
Symptom												
Hearing loss (n = 18)	5(28%)	5(28%)	2(11%)	2(11%)	3(14%)	3(14%)	3(14%)	3(14%)	4(22%)	2(11%)	2(11%)	4(22%)
Tinnitus (n = 15)	3(20%)	5(33%)	2(13%)	2(13%)	3(20%)	3(20%)	3(20%)	2(13%)	3(20%)	2(13%)	2(13%)	4(27%)
Dizziness (n = 13)	3(23%)	3(23%)	2(15%)	1(7%)	3(23%)	3(23%)	2(15%)	2(15%)	4(30%)	1(7%)	0	2(15%)
Vertigo (n = 2)	1(50%)	0	0	0	0	0	0	1(50%)	0	0	0	0
Facial (n = 3)	0	0	0	0	0	0	1(33%)	0	1(33%)	1(33%)	0	1(33%)
Preoperative facial function												
I (n = 20)	5(25%)	6(30%)	4(20%)	2(10%)	3(15%)	4(20%)	2(10%)	4(20%)	3(15%)	1(5%)	2(10%)	3(15%)
II (n = 2)	0 (0%)	0(0%)	0	0	0	0	1(50%)	0	1(50%)	1(50%)	0	1(50%)
IAC involvement												
No (n = 7)	1(14%)	3(43%)	2(29%)	0	2(29%)	1(14%)	0	2(29%)	2(29%)	0	1(14%)	1(14%)
Yes (n = 15)	2(27%)	3(20%)	2(13%)	2(13%)	1(7%)	3(20%)	3(20%)	2(13%)	2(13%)	2(13%)	1(7%)	3(20%)
Brainstem compression												
No (n = 6)	1(17%)	0	0	1(17%)	0	1(17%)	2(33%)	0	1(17%)	0	1(17%)	0
Yes (n = 16)	4(25%)	6(37%)	4(25%)	1(6%)	3(19%)	3(19%)	1(6%)	4(25%)	3(19%)	2(12%)	1(6%)	4(25%)
VNG												
Normal (n = 9)	1(14%)	3(43%)	0	0	0	1(14%)	1(14%)	2(29%)	1(14%)	1(14%)	1(14%)	2(29%)
Altered (n = 7)	3(33%)	3(33%)	1(11%)	1(11%)	0	3(33%)	1(11%)	1(11%)	2(22%)	1(11%)	0	1(11%)

age and the tumor size. The frequency of methylation for the *CASP8* gene was greater in smaller tumors and in older patients. The mean age of patients with methylated *CASP8* was 61 years whereas the mean age of patients with the unmethylated gene was 46 years ($p = 0.03$). Mean tumor size was 30 mm and 20 mm in patients with the unmethylated and methylated *CASP8* gene respectively ($p = 0.04$). Methylation of the *TP73* gene was associated with hearing loss. The corrected hearing thresholds were 43 dB and 17 dB for patients with the methylated and unmethylated *TP73* gene respectively ($p = 0.04$), 1000 Hz being the most affected frequency. *RASSF1A* methylation was associated with the duration of symptoms, being 74 months and 20 months for the methylated and unmethylated cases,

TABLE 3. *Methylation status of tumor-related genes and quantitative clinico-radiological findings*

	Methylation status	Age (yr)	Duration of symptoms (mo)	PTA threshold (dB)	Corrected PTA (dB)	SDS (%)	Tumor size (mm)	Tumor volume (mm^3)	CGI (mm/yr)
RASSF1A	−	49	20	41	20	72	29	938	25
	+	50	74	47	18	64	26	926	9
VHL	−	46	33	44	22	62	30	1086	23
	+	56	29	37	14	92	25	535	17
PTEN	−	51	24	44	20	69	27	764	21
	+	40	67	31	16	72	34	1706	18
HMLH1	−	48	31	42	20	70	29	1009	23
	+	55	42	49	14	69	20	203	6
P16	−	48	32	44	20	65	28	957	22
	+	56	32	32	15	100	28	797	13
CASP8	−	46	33	44	23	70	30	1076	24
	+	61	30	34	4	71	20	303	11
TIMP3	−	49	32	43	19	74	30	1026	23
	+	47	30	39	20	46	21	362	11
MGMT	−	49	36	41	17	72	28	968	20
	+	50	13	49	29	62	29	788	26
DAPK	−	50	34	42	21	78	27	714	23
	+	45	26	46	10	34	34	1932	11
P73	−	48	33	40	17	72	28	955	19
	+	56	21	62	43	47	30	735	35
GSTP1	−	50	34	44	21	67	29	1011	20
	+	38	15	25	8	100	19	181	28
THBS1	−	50	23	43	19	69	29	1010	19
	+	44	74	40	21	72	27	598	29

CGI, clinical growth index.

respectively ($p = 0.039$). As the CGI is related to the length of symptoms, a significant association was also found between CGI and methylation of the *RASSF1A*. The mean CGI of patients with methylated *RASSF1A* was 9 mm per year whereas the mean CGI of patients with the unmethylated gene was 25 mm per year ($p = 0.03$). No correlation was found between methylation status of each gene and other clinical and radiological relevant parameters. The MI for each patient was neither correlated with clinical and radiological features.

DISCUSSION

Recently, great strides have been made concerning the role of aberrant DNA methylation in the genesis and development of several nervous system tumors (5,12,13). This study is the first to assess the clinical and radiological implications of the methylation status of multiple genes in VS. We found different methylation values in 12 of 16 genes tested, including *RASSF1A, VHL, PTEN*, *TP16*, *CASP8*, *TIMP3*, *MGMT, DAPK, THBS1, HMLH1*, *TP73*, and *GSTP1*. Moreover, some correlations between the methylation status and certain clinical and radiological factors were found.

CASP8 is an initiator caspase (cysteine–aspartyl–protease) involved in death receptor–mediated apoptosis and apoptosis triggered by other stimuli such as chemotherapeutic agents (14). The *CASP8* gene is located at the chromosome locus 2q33, a region of loss of heterozygosity in several cancers. Loss of *CASP8* expression has been correlated with tumor severity in malignant lesions (15). However, partial methylation of *CASP8* has been also described in normal cerebellar samples (16), questioning the relationship between gene methylation and increased aggressiveness. In our study, methylation of the *CASP8* gene was associated with older patients. A similar association has been described in neuroblastoma patients, with no other clinicopathological implications (17). As methylation of several CpG islands has been described in an age–related manner in non-malignant tissues (18,19), the latter could be simply explained as an aging event.

In our series of VSs we detected *CASP8* methylation in 60% of schwannomas with *RASSF1A* methylation and demonstrated a significant correlation between *RASSF1A* and *CASP8* methylation ($p = 0.02$). Although this finding may reflect frequent targets in tumors showing a high MI, it also suggests that a subset of VSs might have a CpG island methylator phenotype (CIMP). The CIMP was described in colorectal cancer by Toyota et al. (18) to identify some tumors that are prone to transcriptional silencing linked to promoter methylation, with the potential to inactivate multiple tumor suppressor genes simultaneously. A high concordance rate between methylation of *CASP8* and *RASSF1A* has been stated in neuroblastomas (20), as well as in certain pediatric tumors (17), suggesting that concordant methylation of both genes may contribute to the pathogenesis of several types of tumors. To our knowledge, CIMP has been never described in schwannomas.

The *TP73* gene is a *TP53* homologue localized at 1p36.3 that induces apoptosis and inhibits cell growth. Although both genes share an extensive degree of homology, a typical tumor suppressor gene role for the *TP73* has been excluded (21). Unlike *TP53*, the *TP73* gene is not inactivated by mutation in a large number of cancers, *TP73* null–mice show specific developmental defects but no spontaneous tumors, and *TP73* gives rise to multiple protein isoforms with opposite biological properties (22). In our study, an association was found between methylation of the *TP73* gene and hearing loss. In contrast, methylation of this gene was not related to age, CGI or tumor size. It is well known that hearing is affected by VS in several ways. Compression of the cochlear nerve, changes in the vascular supply of the cochlea, biochemical alterations in the inner fluids, hair cell degeneration, and dysfunction of the stria vascularis have been proposed as possible pathogenetic factors, which are thought to vary independently to tumor size (23). As it has been recently suggested that *TP73* plays a main role in cellular differentiation and apoptosis in neuronal tissues, its function being required for neuron survival (24), methylation of *TP73* could be implicated in the complex pathogenesis of hearing loss in VS. This mechanism would be independent from tumor size.

The *RASSF1A* gene is a candidate tumor suppressor gene that was isolated at 3p21.3 in lung and breast cancers (25). This gene acts at downstream of the Ras mediated apoptotic pathway and is capable of binding to Ras in a GTP dependent manner (26). Although, theoretically *RASSF1A* methylation should imply unfavorable prognosis in patients with malignant tumors, correlations with outcome have shown inconsistent results (27,28). The occurrence of *RASSF1A* methylation in benign tumors has been reported rarely with various results. Horiguchi et al. (5) described a 16.7% *RASSF1A* methylation in 12 meningiomas. In a recent study, no *RASSF1A* methylation was found in 13 benign ganglioneuroma (29). Astuti et al. (20) reported a 22% *RASSF1A* methylation in 23 pheochromocytomas. In our study, the hypermethylated state of the *RASSF1A* promoter CpG island was found in 23% of VSs, being correlated with the CGI. Tumor growth was inversely correlated to the methylation status. This apparently conflicting finding could be explained by the nature of VS, a benign tumor with variable growth patterns, not comparable with the expected growth of malignant tumors. Although the relation between the CGI and the real VS growth rate is uncertain (30), our results suggest that methylation of the *RASSF1A* promoter region may correspond to an early event in the progression of VS. During surgery of VS it would be sometimes useful to have a parameter of prediction of further tumor growth, especially in tumors that are very adherent to the facial nerve and complete tumor resection may lead to severe facial impairment.

In conclusion, the results of this study show some significant correlations between the CpG methylation status and clinical and radiological features in VS. Due to the small size of our series and the relatively high number of genes tested, further studies in large prospective cohorts

are now required to confirm these findings, and to fully establish any further clinical significance of these tumor-specific methylation events.

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