CpG Island Methylation in Sporadic and Neurofibromatis Type 2-Associated Schwannomas

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ABSTRACT

Purpose: **The purpose of this research was to examine the DNA methylation profile of schwannomas.**

Experimental Design: **We examined the DNA methylation status of 12 tumor-related genes (*NF2*, *RB1*, *p14^{ARF}*, *p16^{INK4a}*, *p73*, *TIMP-3*, *MGMT*, *DAPK*, *THBS1*, *caspase-8*, *TP53*, and *GSTP1*) in 44 sporadic and/or NF2-associated schwannomas using methylation-specific PCR.**

Results: **The most frequently methylated genes were *THBS1* (36%), *p73* (27%), *MGMT* (20%), *NF2* (18%), and *TIMP-3* (18%). The *RB1/p16INK4a* gene pair displayed aberrant methylayed alleles in 15% of cases, whereas methylation was relatively rare in the other genes (<5%). Methylation was tumor specific because it was absent in two nonneoplastic nerve sheath samples and two nonneoplastic brain samples studied as controls.**

Conclusions: **Our findings indicate that aberrant methylation seems to be a mechanism for *NF2* gene inactivation, considered an early step in schwannoma tumorigenesis, and as well, aberrant hypermethylation of other tumor-related genes might represent secondary events that also contribute to the development of these tumors.**

INTRODUCTION

Schwannomas are usually encapsulated with WHO histological grade I tumors that arise from the schwann cells surrounding peripheral nerves (1). These neoplasms account for ~8% of intracranial and 29% of spinal tumors, although cutaneous lesions have also been described previously (1). The clinical presentation can be as solitary sporadic tumors or as multiple lesions, primarily involving the vestibular branch of the eighth cranial nerve. Taken together with meningiomas, schwannomas represent the hallmark of NF2[8] disease (2), a severe autosomal dominant genetic disorder affecting ~1 in 40,000 live births (3). The *NF2* gene was isolated in 1993 after linkage studies had assigned the gene to chromosome 22, and a large number of mutations accompanied by the loss of heterozygosity at 22q have been reported in both sporadic and NF2-associated schwannomas (4, 5).

Aberrant methylation of normally unmethylated CpG islands located in the promoter regions has been associated with transcriptional inactivation of tumor-related genes in human cancers (6). On the basis of these findings, aberrant promoter methylation is suspected of participating in the pathogenesis and progression of neoplastic diseases. Information on the methylation status in a variety of malignant tumors is available, and this suggests each tumor type may have a distinct methylation profile (7). Especially regarding nervous system tumors, the available data primarily focus on malignant gliomas (8–15), with little information on schwannomas; this data indicates that suppressed expression by aberrant promoter methylation would be an alternative mechanism for inactivation of the *NF2* gene in ~50% of schwannoma cases (16). Additional information was reported on four cases, two of which displayed aberrant *p16^{INK4a}* promoter methylation (17). Nevertheless, a methylation profile for schwannomas using a panel of genes has yet to be published.

In this study, we used MSP to analyze a series of sporadic and NF2-associated schwannomas for methylation of 12 genes. The genes studied were chosen on the basis of their critical cancer-related function because they are known to frequently be hypermethylated and silenced in other neoplasms. The target genes include tumor suppressor genes, angiogenesis and invasion inhibitors, DNA repair genes, and a detoxification gene.

MATERIALS AND METHODS

Tissue Samples and DNA Extraction. We studied the methylation profile of 44 schwannomas arising in 37 patients

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[8] The abbreviations used are: NF2, neurofibromatosis type 2; MSP, methylation-specific PCR; MGMT, O^6-methylguanine DNA-methyl transferase; DAPK, death-associated protein kinase; *THBS1*, thrombospondin-1; GSTP1, glutathione *S*-transferase P1; MI, methylation index.

(28 women; 9 men) ranging from 19 to 74 years of age (mean, 55.9). Thirteen tumors presented in 8 patients with NF2, and the other 29 patients had no evidence of this disease. Three patients had two tumors each, and 2 patients had three tumors each. Tumor localization was the eighth cranial nerve in 20 cases, spine in 14 cases, cervical spine in 4 cases, and skin/other in 6 cases. In addition to tumors, blood samples from the patients, two samples of nonneoplastic peripheral nerve sheath tissue and two nonneoplastic brain tissue samples (all four obtained by autopsy) were studied 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%.

MSP and Direct Sequencing. We analyzed the status of 12 genes frequently showing promoter methylation in other neoplasms. The genes studied were *NF2*, *RB1*, *p14^{ARF}*, *p16^{INK4a}*, *p73*, *TIMP-3* (the tissue inhibition of metalloproteinase 3 gene), *MGMT*, *DAPK*, *THBS1*, *caspase-8*, *TP53*, and *GSTP1*. Bisulfite modification of genomic DNA was performed as reported previously (18). Briefly, 2 μg of genomic DNA were denatured with 2 M NaOH and modified with sodium bisulfite treatment. After purification with the DNA clean-up kit (Promega, Madison, WI), the DNA was treated with NaOH, precipitated with ethanol, and resuspended in water. PCR was conducted with primers specific for either the methylated or the unmethylated alleles in standard conditions with variable annealing temperatures (55°C–66°C), using the Biotools DNA polymerase kit (Madrid, Spain). We used the primer sets described previously (16, 19–25). Water was substituted for DNA as a negative control, and as a positive control for methylated alleles, we treated DNA (from lymphocytes of healthy volunteers) with *Sss*I methyl-transferase (New England Biolabs, Beverly, MA) and then subjected it to bisulfite treatment. PCR products were loaded onto 6% polyacrylamide gels or 2–3% agarose gels, stained with ethidium bromide, and visualized under UV. Samples giving signals approximately equivalent to the positive control were designated as methylated. To verify the identity of PCR products, they were purified and sequenced using the Abiprism Byg-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on AB model 310 or 377 DNA sequencers.

RESULTS

Methylation values > 10% were obtained for 6 genes: *THBS1* (36%); *p73* (27%); *MGMT* (20%); *NF2* (18%); *TIMP-3* (18%); and *p16^{INK4a}* (11.3%) (Fig. 1; Table 1). Three genes displayed methylation frequencies < 10%, *RB1* and *DAPK* (4.5% each) and *caspase-8* (2.2%), whereas *p14^{ARF}*, *TP53*, and *GSTP1* showed no methylation at all. Methylation did not occur in the autopsy samples of nonneoplastic tissue. All but eight tumors displayed methylation of 1 or more genes (36 of 44 tumors: 81.8%), and the PCR products were sequenced and showed the changes to be expected from bisulfite treatment (Fig. 2). The frequency of methylation of multiple genes in a tumor was determined using the MI defined as the number of loci methylated/the number of loci tested. MI ranged from 0 to 0.33, with a mean of 0.119. There were no significant differences when the overall MI values from the NF2-associated tumors (MI: 0.102; $n = 13$) were compared with the MI values from the sporadic schwannomas (MI: 0.126; $n = 31$). Aberrant *NF2* promoter methylation was detected in 2 of 13 (15.3%) NF2-associated tumors and in 6 of 31 (19.3%) sporadic schwannomas. There was no significant association between overall MI and other clinicopathological data (Table 2). In regard to gender distribution, the frequency of methylation for the *THBS1* gene was greater in tumors arising in female patients (12 of 29, 41.3%) than in tumors arising in male patients (4 of 15, 26.6%). Other sex-associated

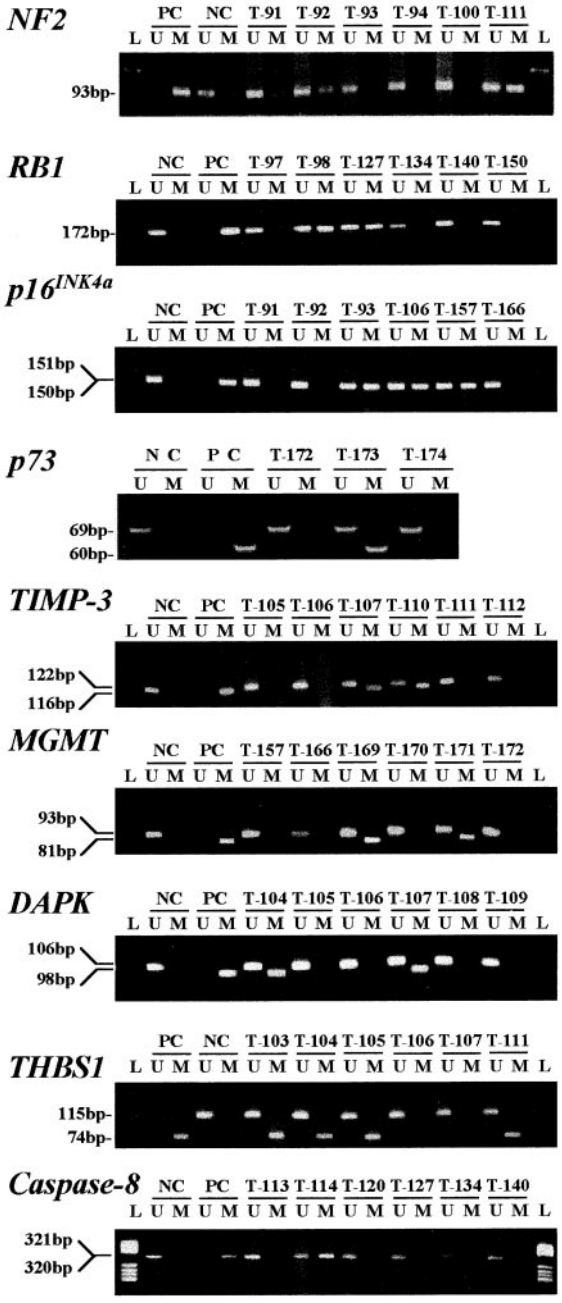

Fig. 1 Methylation analysis in schwannomas. The gene studied is given at the left of each panel. *Lane U:* amplified product with primers recognizing unmethylated sequence; *Lane M:* amplified product with primers recognizing methylated sequence. NC, normal control; PC, positive control for methylation. The PCR product sizes of all of the genes are shown to the left. L: ladder.

Table 1 Summary of methylation of all 12 genes in Schwannomas

+, methylated loci; −, unmethylated loci.

	NF2	*RB1*	*p14^{ARF}*	*p16^{INK4a}*	*p73*	*TIMP-3*	*MGMT*	*DAPK*	*THBS1*	*Caspase-8*	*TP53*	*GSTP1*	*MI*
T-89	−	−	−	−	−	−	−	−	+	−	−	−	0.08
T-90	−	−	−	−	−	+	−	−	−	−	−	−	0.08
T-91	+	−	−	−	−	−	−	−	+	−	−	−	0.17
T-92 (e)[a]	+	−	−	−	−	−	−	−	+	−	−	−	0.17
T-93 (b)[b]	−	−	−	+	−	−		−	−	−	−	−	0.08
T-94 (b)[b]	−	−	−	−	−	−	+	−	+	−	−	−	0.17
T-95	−	−	−	−	−	−	−	−	+	−	−	−	0.08
T-97	−	−	−	−	+	−	−	−	+	−	−	−	0.17
T-98	−	+	−	−	−	−	−	−	−	−	−	−	0.08
T-99	−	−	−	−	−	−	−	−	−	−	−	−	0
T-100 (d)	−	−	−	−	−	−	−	−	−	−	−	−	0
T-101 (e)	−	−	−	−	+	−	+	−	−	−	−	−	0.17
T-102 (c)[b]	−	−	−	−	−	−	−	−	−	−	−	−	0
T-103 (d)	−	−	−	−	−	+	−	−	+	−	−	−	0.17
T-104	−	−	−	−	+	−	−	+	+	−	−	−	0.25
T-105[b]	−	−	−	−	−	−	−	−	+	−	−	−	0.08
T-106 (c)[b]	−	−	−	+	−	−	−	−	−	−	−	−	0.08
T-107	−	−	−	−	+	+	−	+	−	−	−	−	0.25
T-108	−	−	−	−	−	−	−	−	−	−	−	−	0
T-109	−	−	−	−	−	−	−	−	−	−	−	−	0
T-110	−	−	−	−	+	+	−	−	−	−	−	−	0.17
T-111	+	−	−	−	−	−	+	−	+	−	−	−	0.25
T-112	+	−	−	−	−	−		−	+	−	−	−	0.17
T-113	−	−	−	−	−	−	+	−	−	−	−	−	0.08
T-114	+	−	−	−	−	−	−	−	+	+	−	−	0.25
T-120	−	−	−	−	−	+	−	−	+	−	−	−	0.17
T-127	−	+	−	−	−	−	−	−	−	−	−	−	0.08
T-134	−	−	−	−	−	−	+	−	−	−	−	−	0.08
T-140	−	−	−	−	−	−	−	−	−	−	−	−	0
T-150[b]	−	−	−	−	−	−	−	−	+	−	−	−	0.08
T-152 (a)[b]	−	−	−	−	+	+	−	−	−	−	−	−	0.17
T-153 (a)[b]	+	−	−	−	+	−	−	−	−	−	−	−	0.17
T-155[b]	−	−	−	−	−	−	−	−	−	−	−	−	0
T-157[b]	−	−	−	+	+	−	−	−	−	−	−	−	0.17
T-166 (b)[b]	+	−	−	−	−	−	−	−	−	−	−	−	0.08
T-169 (a)[b]	−	−	−	−	−	+	+	−	−	−	−	−	0.17
T-170[b]	−	−	−	−	+	−	−	−	−	−	−	−	0.08
T-171	−	−	−	−	−	−	+	−	−	−	−	−	0.08
T-172	−	−	−	−	−	−	−	−	−	−	−	−	0
T-173	+	−	−	+	+	−	−	−	+	−	−	−	0.33
T-174	−	−	−	−	−	−	+	−	+	−	−	−	0.17
T-175	−	−	−	−	+	−	+	−	−	−	−	−	0.17
T-176	−	−	−	+	+	−	−	−	−	−	−	−	0.17
T-177	−	−	−	−	−	+	−	−	−	−	−	−	0.08
Totals	8/44	2/44	0/44	5/44	12/44	8/44	9/44	2/44	16/44	1/44	0/44	0/44	0.119
	18%	4,5%	0%	11,3%	27%	18%	20%	4,5%	36%	2,2%	0%	0%	

[a] a, b, c, d, e: five patients with several tumors.
[b] Tumors from patients with *NF2*.

methylation frequency variations were obtained for *NF2*, 2 of 15 tumors arising in male (13%) *versus* 6 of 29 in female patients (21%), and for *TIMP-3*, 4 of 15 tumors in male (26.6%) *versus* 4 of 29 tumors in female patients (13.7%). Nevertheless, given the sample size these differences were not significant.

DISCUSSION

This study is the first to analyze the methylation status of multiple genes in schwannomas. Results indicate that gene methylation is a common event in this neoplasm because 81.8% of cases had at least one methylated gene and 47.7% of samples had two or more methylated genes.

Nine of the 12 genes studied were found aberrantly hypermethylated in at least one tumor. *RB1* and *p16^{INK4a}* have closely related functions in cell cycle regulation (26). Both genes were alternatively hypermethylated in a total of 7 cases; this suggests that this cell cycle regulation pathway might be altered through epigenetic changes in a subset of schwannomas and that this finding could be related to the RB1-CDK pathway deregulation recently identified in vestibular schwannomas (27).

THBS1 is a known angiogenesis inhibitor, which, when its expression is altered, has been associated with neovascularization in human cancers, including malignant gliomas (13, 15, 28), gastric carcinoma (21), and several carcinoma cell lines (28). We have recently reported significant rates of *THBS1* promoter methylation in neurofibromas (11 of 21 samples: 52%; Ref. 29),

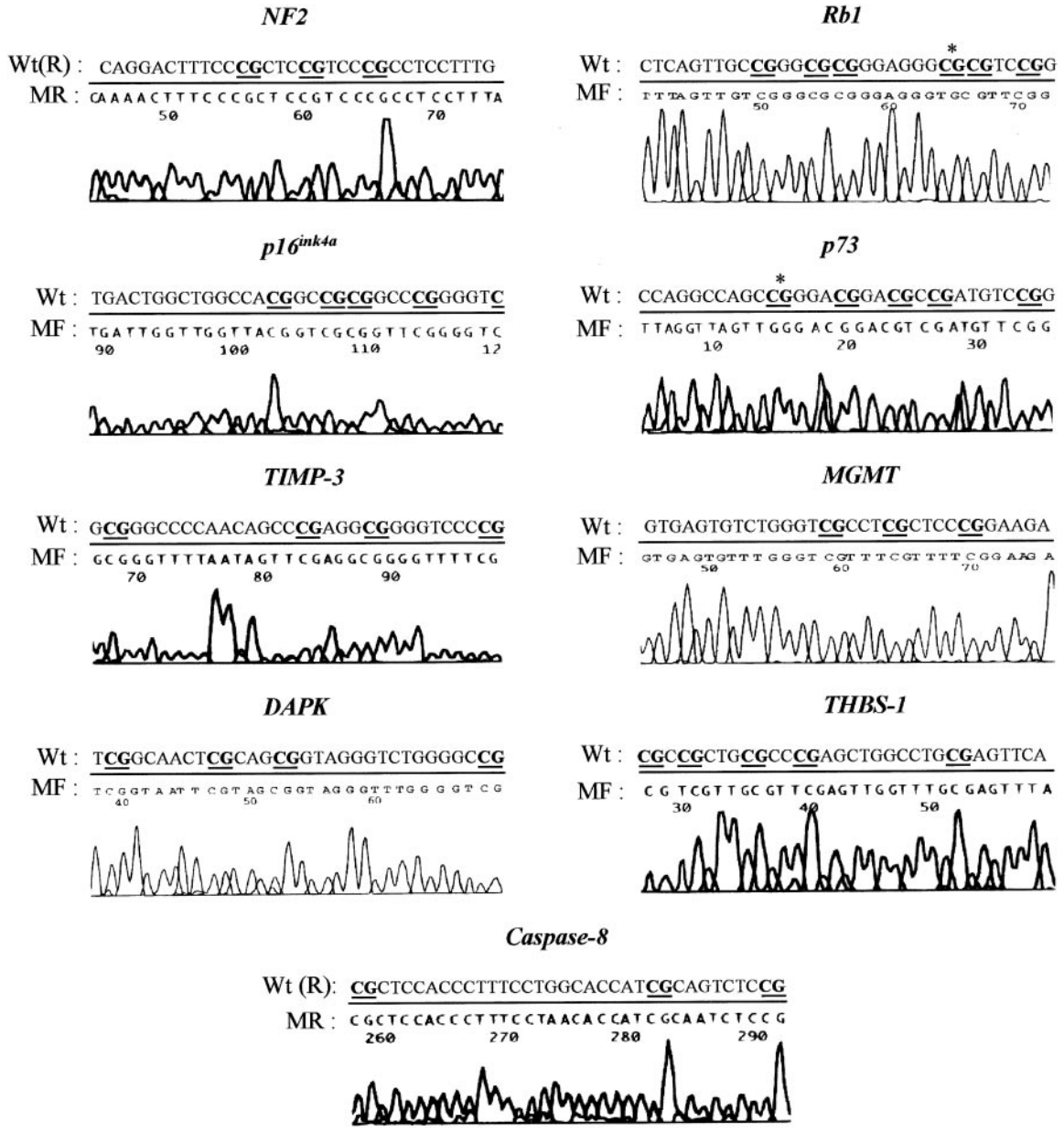

Fig. 2 Sequences of the methylated MSP products from representative cases of all 9 genes with positive results in schwannomas. Sequences are aligned against the wild-type (Wt) sequences showing change of C to T (in the F strand) or G to A (in the R strand) by bisulfite treatment. C in CpG islands that were methylated remains unaffected. *Asterisks* show two CpG islands not methylated.

and a significant rate of *THBS1* methylation characterized the series of schwannomas reported here. Both types of neurogenic neoplasms, *i.e.*, neurofibromas and schwannomas are characterized by a schwannian component, and thus *THBS1* hypermethylation in these neoplasms might reflect some of this relationship.

Our study also demonstrated significant methylation frequencies for *MGMT* (20%) and *TIMP-3* (18%). These genes are respectively known to encode for a DNA repair protein and an extracellular matrix binding protein (30, 31). DNA repair and matrix binding are key functions in tumor development and progression and could play important roles in the formation of a subset of schwannomas.

In contrast to the low frequency of *p73* inactivating mutations detected in malignant neoplasms (for a review see Ref. 32), *p73* inactivation associated to CpG island methylation has been identified in oligodendrogliomas and in some hematological malignancies (13, 33, 34). Our findings show that 14 of 44 (27%) of schwannomas presented methylation involving *p73*; we have also previously detected significant rates of methylation for this gene in astrocytic gliomas (19 of 88, 21%), neuroblastomas (11 of 44, 25%) and neurofibromas (4 of 21, 19%; Refs. 15, 29, 35). The *p73* gene maps to a region (1p36.33) that is frequently deleted in neuroblastomas (36) and oligodendrogliomas (37), and some degree of deletion at this regions has also been reported in astrocytic

Table 2 Summary of the overall MI findings related to clinicopathological data

	n^a	MI value
NF2-associated tumors	13	0.119 ± 0.062
Sporadic tumors	31	0.126 ± 0.089
Eighth cranial nerve tumors	20	0.129 ± 0.080
Other localizations	24	0.110 ± 0.084
Tumors arising in male patients	15	0.111 ± 0.076
Tumors arising in female patients	29	0.123 ± 0.086
Total tumors	44	0.119 ± 0.082

[a] *n*, number of cases examined.

gliomas (37, 38). Allelic loss at 1p36 has been reported in a few schwannomas (38, 39), a finding recently confirmed through comparative genomic hybridization analysis of sporadic cases (4 of 14, 28%; Ref. 40). Thus, inactivation of *p73* might occur through a pathway of allelic loss plus CpG island methylation in some neurogenic neoplasms, including schwannomas. However, except for oligodendrogliomas (33), more studies linking *p73* CpG island methylation and loss of gene expression in neurogenic neoplasms are needed before we can firmly establish the functional significance of this epigenetic change during tumorigenesis in these neoplasms.

We detected aberrant methylation of the *NF2* gene promoter elements in ~18% (8 of 44) of schwannomas in our series. This finding represents a lower frequency of methylation than previously identified (16). Schwannomin has been reported to be absent in virtually all schwannoma samples (41), although the rate of *NF2* coding region inactivating mutations described in this tumor type is usually lower (20–75%; Refs. 42, 43). In agreement with the data reported by Kino *et al.* (16), our findings demonstrate that CpG island methylation of the *NF2* promoter region is a relatively frequent event in schwannomas and could therefore represent an alternative mechanism that would contribute to *NF2* inactivation. In this respect, two NF2-associated tumors in our series (T-153 and T-166) displayed aberrant *NF2* promoter methylation. Both cases retained their constitutional heterozygosity when the chromosome 22 allelic constitution was studied, and no *NF2* mutations could be evidenced by PCR-single-strand conformational polymorphism analysis. Identical findings (no loss of heterozygosity at 22q and no *NF2* mutations) were detected in three of the sporadic schwannomas with aberrant *NF2* promoter methylation (T-91, T-92, and T-114); chromosome 22 allelic losses accompanied the epigenetic change in the other three sporadic tumors (T-111, T-112, and T-173; Refs. 39, 44 and unpublished data).

As reported in previous methylation studies that demonstrate the link between CpG island methylation and gene silencing in other neoplasms (45), our findings show that in those tumors displaying hypermethylation, this was accompanied by amplification in the unmethylated reaction, as well in all of the genes we studied. Tumoral heterogeneity and the fact that tumor specimens represent macroscopically isolated samples containing both tumor and a small fraction of nonmalignant tissues might explain this finding. The existence of cytosine hemimethylation, or of methylation occurring outside the critical sites for gene silencing, as previously shown with *GSTP1* in prostate cancer cells (46) or with *NF2* in schwannomas (16), could explain the presence of certain degree of gene expression. In these situations, CpG island methylation would have no functional implications at all or would only imply a partially reduced gene expression. However, additional anomalies (deletion, mutation, and so forth) would contribute to inactivate the methylation-targeted genes. In fact, abnormal methylation has been proposed to predispose to and precede gene deletion in some instances (47).

Our report is the first methylation profile of tumor-related genes in schwannomas. In addition to *NF2*, the *THBS1*, *MGMT*, *TIMP-3*, and *p73* genes are frequently found to be aberrantly hypermethylated. Additionally, the *RB1/p16^{INK4a}* cell cycle regulation pathway genes displayed methylation in ~15% of our cases. The significance of the epigenetic changes of the studied genes, changes that most probably represent events secondary to the inactivation of the *NF2* gene, should be further determined to identify its potential clinical and biological implications.

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