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Clinical Study

Molecular genetics alterations and tumor behavior of sporadic vestibular schwannoma from the People's Republic of China

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Key words: loss of heterozygosity, NF2 gene mutation, Schwannomin/merlin expression, Vestibular Schwannoma

Summary

Objectives: To analyze the molecular genetic alteration of sporadic vestibular schwannomas from the People's Republic of China and to correlate these alterations with the tumor behaviors. *Methods*: Four 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 by Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and DNA sequencing. The schwannomin/merlin (S/M) expression was examined using anti-NF2 (A-19) IgG under immunohistochemistry and western blot. The proliferative index (LI) of vestibular schwannoma was evaluated by proliferative cell nuclear antigen investigation. *Results*: Sixteen vestibular schwannomas (44.4%) showed allele loss. We found 22 mutations in 36 schwannomas. The LI and the growth rate of schwannomas with LOH or mutation were significantly higher than those without LOH or mutation. All of these vestibular schwannomas showed no immunoreaction to anti-NF2(A-19) IgG by immunohistochemistry. By immunoblotting technique, reduced expression of S/M was found in 31 cases (86%). The growth index of schwannomas with severely reduced expression of S/M was significantly higher than those with moderately reduced or normal expression. *Conclusion*: The molecular genetic changes in sporadic vestibular schwannomas from Chinese patients were similar to the previous reports. We demonstrate the relationship between tumor behaviors and genetic alteration (including LOH and mutation of NF2 gene). We propose that inactivation of S/M, may be an important step in tumorigenesis of sporadic vestibular schwannoma.

Introduction

Schwannomas are the tumors arising from schwann cells and account for 8–10% of all primary intracranial neoplasms [1]. Although these tumors do develop on other nerve roots, the most common type of schwannoma is tumors that originate from the vestibular branch of the 8th cranial nerve. The majority of vestibular schwannomas are sporadic, but they also occur with an increased frequency in individuals with the autosomal dominant disorder, neurofibromatosis type 2 (NF2), also known as central or bilateral acoustic neurofibromatosis. Approximately 90% of individuals with NF2 develop bilateral vestibular schwannomas [1].

The NF2 gene is located on chromosome 22. Loss of heterozygosity (LOH) in chromosome 22 has been observed in vestibular schwannoma carrying NF2 mutation, and thus is consistent with the two-hit model of tumor-suppressor genes [2–4]. However, few reports of these molecular genetic changes involved Chinese subjects of patients with sporadic vestibular schwannomas. We report our analysis of LOH in chromosome 22, NF2 gene mutation and S/M expression occurring in Chinese patients with sporadic vestibular schwannomas. This study also aims to investigate the molecular pathogenesis of sporadic vestibular schwannoma and to correlate these genetic alterations with tumor behaviors.

Materials and methods

Tumors from 36 unrelated patients without clinical NF1 or NF2, as determined by family histories, MRI of brain, as well as physical and ophthalmologic evaluations, were analyzed.

Clinical growth index of vestibular schwannoma

To obtain the data of tumor behavior, a growth index was reviewed based on tumor size and duration of symptom. The tumor growth index was calculated by dividing the tumor size (maximum diameter, centimeters) by the duration of presenting symptom in years (years).

Human tissue samples

Tumor specimens were obtained from 36 unrelated patients (15 men, 21 women, mean age 45.8 years, range 20–77 years). Tumor samples had been frozen in liquid nitrogen immediately after removal and were stored at −70 °C for up to 18 months before DNA extraction, protein extraction and immunohistochemistry were performed. Control study of S/M expression in sacral nerve tissue was examined, these tissues were obtained from unrelated patients with severe spasticity of lower extremities who underwent selective posterior rhizotomy.

Proliferative index (PCNA immunohistochemistry)

Tumor specimens were cut at 6.0 μm interval and immunostained for proliferative cell nuclear antigen (PCNA) with a mouse monoclonal antibody to PCNA, diluted to 1:100. This process was performed using avidin–biotin peroxidase. Subsequently the sections were counterstained with hematoxylin. A cell considered positive when all or part of the nucleus was stained.

On the PCNA-investigated slice, cells locating in three 40 × fields were counted to yield at least 1000 cells. Then positively staining cells were documented and divided by total cells (calculation in percentage).

Molecular genetic analysis

High molecular weight DNA was extracted from all 36 tumor samples by conventional methods. Corresponding blood DNA was also obtained from all of the 36 patients.

LOH

The analysis was performed using a previously reported method [5]. Four highly polymorphic linkage to NF2 gene microsatellite DNA markers: D22S264 [6], D22S268 [7], D22S280 [8], CRYB2 [5,6] were used. The most likely order for the chromosome 22q markers is cen-D22S264-CRYB2-D22S268-D22S280-tel [5–8]. These oligonucleotides were amplified by polymerase chain reaction (PCR), subsequently the PCR products were denatured and separated onto 6% polyacrylamide gels containing 7 mol/l of urea. Following electrophoresis, the gels were stained with silver [5].

PCR-SSCP (single-strand conformation polymorphism) and sequencing

PCR-SSCP analysis was performed on all 17 exons and flanking intronic sequence of the NF2 gene pursuing the techniques of previous reports [9]. PCR products were analyzed by SSCP (6% polyacrylamide gels, acrylamide: Bis ratio, 49:1 without glycerol) [9]. The gels were stained with silver nitrate [5]. Gel pieces containing abnormal bands were excised and DNA was extracted at 4 °C overnight and reamplified by PCR. After agarose gel electrophoresis, the PCR products were isolated from agarose following the manufacture's protocol (Qiagene, Qiaquick Gel Extraction Kit) and cloned into the vector pGEM-T (Promega) and TOPO TA (Invitrogen). Plasmid DNA from overnight LB cultures (75 μg/ml ampicillin) was isolated using Wizard plus Minipreps DNA purification system Kit (Promega). The plasmid DNA were directly sequenced using an automated sequencer (PRISM Dye Terminator Sequencing Kit and ABI PRISM, ABI 100, Model 377 DNA Sequencer, Applied Biosystems).

Schwannomin/merlin analysis

Antibodies. The commercial polyclonal antibodies raised in rabbits against S/M peptide were diluted in blocking buffer to the final concentration of 0.25 μg/ml (1:300). The anti-NF2 (A-19) immunoglobulin G (IgG) recognizes an epitope localized at the amino-terminus of the human NF2 protein (amino acids residues 2–21) (Santa Cruz. Co). *Anti-β*-actin monoclonal antibody AC-15 (Sigma, St Louis, MS) was used for the detection of β-actin.

Immunohistochemistry. Specimens were reacted with the primary NF2 antibody using a Vectastain ABC-DAB Kit (Vector Laboratories, Burlingame, CA). Only cytoplasmic immunostaining was documented as the positive cell.

Protein Analysis. Protein analysis was performed by western immunoblotting. Tumor specimens were homogenized in RIPA buffer [10]. Insoluble material was removed by 14,000 rpm of microfuge centrifugation at 4 °C and the resulting supernatant was saved for protein analysis. Afterwards the protein was electrophoretically separated by 10% SDS–PAGE, and was transferred onto nitrocellulose membrane for western blot (each time using the same amount of 2 mg protein). Then the membrane was incubated with the primary antibody for 4 h at room temperature. The secondary anti-rabbit horseradish peroxidase conjugated antibody (BRL) was diluted to 1:2000 in blocking buffer and incubated with the blots for 90 min at a room temperature. The amount of each sample loaded was adjusted to obtain the equivalent intensity for β-actin. Subsequently, the protein was detected by western blot chemiluminescence reagent (Amersham). Finally, scanning densitometry (using NTH-image 1.55 software) compared the density values of S/M 66-kD bands with the correspondent value of nerve tissue sample.

Statistical analysis

Statistical analysis of tumor growth rate and proliferative index of tumor which harboring different expression of S/M and genetic alteration (LOH and mutation) was performed using Student *t*-test.

Results

LOH

All tumors were investigated for LOH. Sixteen (44.4%) vestibular schwannomas demonstrated LOH [D22S268 (11 cases), D22S280 (7 cases), D22S264 (8 cases), CRYB2 (6 cases)] (Figure 1, see Table 1), but 20 tumors retained the constitutional genotype.

NF2 gene mutation

Results of the SSCP analysis and DNA sequencing are shown in Figures 2 and 3. From all specimens, 22 inactivating mutations of the NF2 gene were found (Table 1). The identified types of mutation included 13 deletions or insertions, which resulted in frameshift, 2 nonsense mutations, 3 missense mutations, and 4 splice site mutations, which affected acceptor or donor splicing sites. The mutational site involved 11 exons: most of them were in exon 2, 4, 6 ($n = 4, 3, 4$). The detected NF2 gene mutations were summarized in Table 2. The

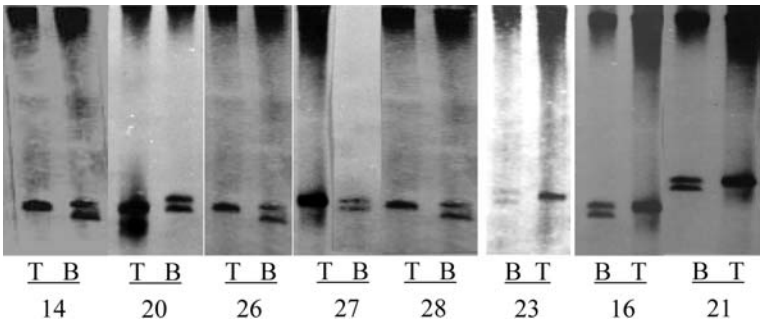

Figure 1. Microsatellite assay for D22S268 of constitutional DNA (B) and vestibular schwannoma DNA (T) reveals various patterns of Loss of heterozygocity (LOH). Comparing to corresponding blood DNA, the DNA marker that is present in blood DNA, but not in tumor DNA, is demonstrating allele loss (LOH) in the tumor tissue (Case 14, 16, 21, 23, 26, 27, 28 reveal loss of the allele 1, whereas Case 20 shows loss of the allele 2).

Table 1. The clinic data, growth index, proliferative index, loss of heterozygosity, mutation and S/M expression in vestibular schwannoma

No.	Age (yr)	Sex	Size (cm)	GR (cm/yr)	LI (%)	D22S268	D22S280	D22S264	CRYB2	Mutation	S/M expression
1	49	M	4	6.21	4.2		+	+		+	0.04
2	32	M	3.8	1.6	1.88						0.52
3	56	F	3.2	6.82	1.92	+	+				0.39
4	47	M	2.8	6.4	2.3					+	0.22
5	62	F	5	4.58	3.4			+		+	0.18
6	45	M	3.4	5.16	2.4					+	0.32
7	34	F	6	4.3	2.2			+	+	+	0.42
8	36	M	3	5.4	3.7	+	+	+		+	0.38
9	60	F	5.6	2.8	3.05						0.48
10	57	F	4	6.94	2.1					+	0.37
11	61	M	4	6.25	3.3					+	0.54
12	46	M	3.2	6.82	2.04						0.24
13	49	F	2.6	0.61	2.1						0.7
14	48	M	4.7	6.94	2.5	+	+	+	+	+	0.33
15	68	F	4	6.3	4.5		+		+	+	0.47
16	60	F	4.6	7.25	2.9	+	+	+	+	+	0.01
17	48	M	4	5.31	2.7	+			+	+	0.49
18	43	F	6	6.8	3.2					+	0.43
19	49	M	4.6	6.13	2.3					+	0.41
20	36	F	6.2	10.63	3.69	+					0.29
21	57	F	5	5.8	2.2	+				+	0.55
22	22	F	5	6.63	2.7				+	+	0.21
23	31	M	3	4.85	1.7	+				+	0.04
24	46	F	4	7.49	2.6					+	0.28
25	20	F	4.8	9.94	2.02						0.81
26	56	F	3	6.97	2.2	+		+		+	0.12
27	35	F	5	6.58	2.7	+				+	0.25
28	47	M	4	6.3	1.2	+	+	+		+	0.45
29	25	M	2.8	2.16	2.34						0.72
30	77	M	3.9	4.97	1.56						0.81
31	43	M	2.6	0.61	2.41						0.66
32	48	F	4	5.35	2.3					+	0.02
33	30	F	3.2	6.82	2.62						0.69
34	60	F	3.1	4.97	2.17						0.34
35	56	F	3.6	0.61	2.52						0.81
36	47	M	3.7	1.94	1.65						0.28

Abbreviations: GR: tumor growth index; No: number; LI: proliferative index; S/M: Schwannomin/merlin; yr: year.

mutations that detected in tumor specimens were not noticed in constitutional DNA.

LOH mutation and tumor behavior

The size of tumor, growth rate as well as proliferative index of tumor in patients with LOH (4.27 cm, 6.30 cm/year, 2.77%) were significantly higher than those in patients without LOH (3.40 cm, 4.59 cm/year, 2.55%, $P = 0.08$, 0.013, 0.024 respectively). Between tumors with and without NF2 gene mutation, there were also significant differences of these three indexes (4.1 cm vs. 3.59 cm, 6.09 cm/year vs. 4.38 cm/year, 2.69 % vs. 2.28 %; $P =$ 0.044, 0.044, 0.039 respectively). Tumors with

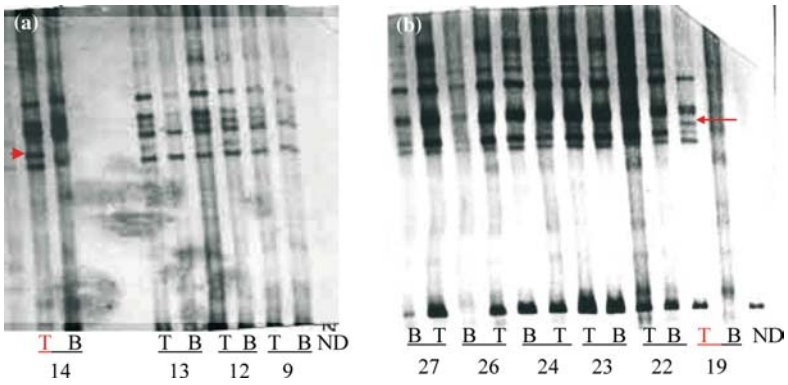

Figure 2. Single-strand conformation polymorphism (SSCP) analysis of exon 2 (a) and exon 6 (b). For each assay, a lane of PCR product is run with non-denaturated (ND) DNA to identify fully reannealed, double-stranded DNA (first lane from right). The other bands in the test lanes represent various conformation of the single-stranded DNAs of the PCR products. If a band with abnormal migration appears in the tumor specimen, comparing to the corresponding bands in the blood, it represents the presence of a somatic mutation in the tumor tissue (Case 14 and 19, see Figure 3a and b).

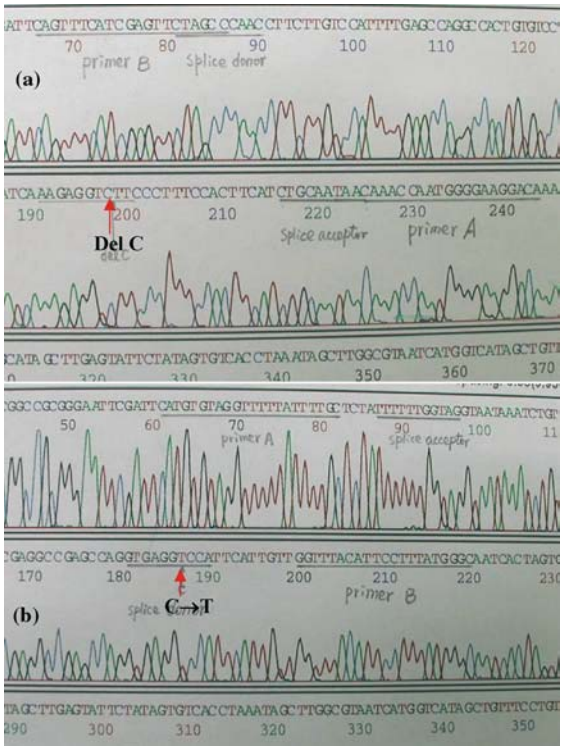

Figure 3. (a) and (b) demonstrate a frameshift and splice site donor mutation in Case 14 and 19, respectively. In case 14, comparison to wild-type E2 sequence, there was deletion of a base C, corresponding the position 126 of E2, or condon 42 (resulting in frameshift mutation). In case 19, there was splice donor mutation, the base C changed to base T (B, Control blood DNA; T, tumor DNA; ND, a non-denatured sample).
(a) *DNA sequence results*: AAA GAG GT CTT CCC TTT,
Wild-type E2 sequence:
61 ttattgcag *atg aag tgg* $\frac{\textit{aaa ggg aag gac ctc ttt}}{117\ 120\ 123\ 126\ 129\ 132}$ *gat ttg gtg tgc cgg act ctg ggg ctc cga ttc ttt*.
(b) *DNA sequence results*: splice donor GTGAGGTCCA,
Wild-type E6 sequence:
121 ·····*att act gct tgg tac gca gag cac cga ggc cga gcc* $\frac{\textit{agg}\ \text{tga ggccca}}{600\ \text{splice donor}}$
ttca ttgttggttt.

frameshift and missense mutation had more rapid growth rate than those tumors with splice mutation (6.36 and 6.44 cm/year vs. 5.32 cm/year, $P = 0.017$, 0.032 respectively).

S/M expression

None of the tumor cells were positively stained with anti-NF2 (A-19) IgG by immunohistochemistry (Figure 4a). However, the positively staining cells might be neurocyte, monocyte or endothelial cells lining in blood vessels of the tumor (Figure 4b and c).

Immunoblotting with anti-NF2 (A-19) IgG was performed in order to investigate the level of S/M expression in 36 sporadic vestibular schwannomas (Figure 5). The density value of S/M expression at 66-kD band was evaluated from the exposed films. Then, the density value of S/M expression from tumor sample was divided by the correspondent value of normal nerve tissue (Number N1, N2 and N3 in Figure 5a, b and c respectively) using scanning densitometry (see also Table 1).

In five tumors (Group I: Case 13, 25, 29, 30, 35 see also Table 1), the 66-kD band was detected at the similar level to those observed in control group (S/M indicator ratio ≥0.70). The levels of S/M were, however, decreasing in the remaining 31 tumors (Groups II and III). In Group II (Case 2, 3, 7–11, 15, 17–19, 21, 28, 31, 33), which consisted of 15 tumors, the ratio of S/M density was moderatly reduced (S/M density ratio from 0.36 to 0.70). Whereas the S/M 66-kD band was severely reduced (S/M density ratio ≤0.35) in Group III ($n = 16$; Case 1, 4–6, 12, 14, 16, 20, 22–24, 26, 27, 32, 34, 36) (Table 1). The growth rate with severely reduced expression of S/M was faster than the remaining tumors (6.17 cm/year vs. 4.82 cm/year, $P = 0.018$). There was significant difference of S/M expression between tumors with LOH or mutation and tumors without LOH or mutation (0.29 vs. 0.48 and 0.30 vs. 0.55, respectively; $P < 0.01$). Besides, the 66-kD S/M band, bands harboring molecular weight standards near 110-kD were also detected (Figure 5).

Discussion

LOH

Our finding that 44.4% of vestibular schwannomas showing LOH on chromosome 22 was similar to those

Table 2. NF2 gene mutations in sporadic vestibular schwannomas

No.	Exon	DNA sequence alteration	Codon change	Consequence	LOH
1	5	Del 4 bp AAT C	167–168	Frameshift	+
4	11	Del T CTC(Leu)→CC	331	Frameshift	
5	4	Del C		Splice donor site	+
6	10	C→G		Splice acceptor site	
7	13	CAG(Gln) →TAG(stop codon)	456	Nonsense	+
8	6	C→T		Splice donor site	+
10	7	Del 14 bp (GCTGAAATGGAATA)	195–199	Frameshift	
11	4	Del AAG (Lys)	123	Frameshift	
14	2	AAA (Lys) →CCA (Pro)	42	Missense	+
15	4	ATT (Ile) →TAG (Stop Codon)	127	Nonsense	+
16	2	Del C	44	Frameshift	+
17	6	CAG(Gln) →CAAG	178	Frameshift	+
18	6	CCG (Pro) →CG	181	Frameshift	+
19	6	C→T		Splice acceptor site	
21	15	ATC(Ile) →ATG(Met)	537	Missense	+
22	10	Del G GGG (Gly) →GG	294	Frameshift	+
23	13	CCC (Pro) →CC	479	Frameshift	+
24	8	Del C CTG (Leu) →TG	224	Frameshift	
26	8	Del 8 bp (CTTGGAGT)	226–228	Frameshift	+
27	2	TTT (Phe) →TTA (Leu)	47	Missense	+
28	12	Ins C TCT (Ser) →TCCT	369	Frameshift	+
32	2	GTG (Val)→GG	72	Frameshift	

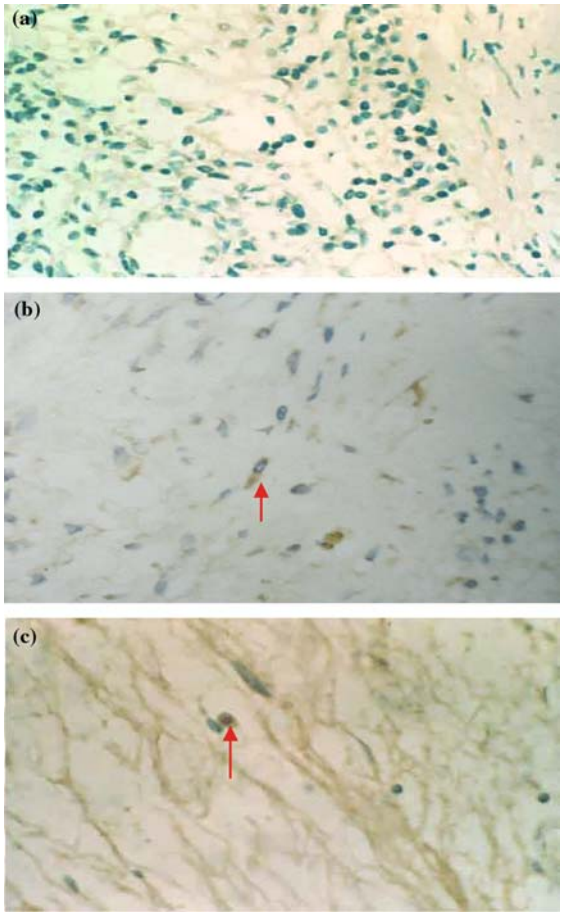

Figure 4. Immunohistochemical study of S/M with antibody NF2 (A-19) IgG (dilution 1:300). A cell is considered positive when all or part of plasma is stained. A: There is no immunohistochemical reaction on tumor cells. (b) and (c) reveal the positively staining cells which are not tumor cells, these cells might be neutrocyte or monocyte or normal tissue cells (400×).

results reported by others (22–80%) [14–17]. Two markers (D22S268, D22S280) are telomeric and the others (D22S264, CRYB2) are centromeric to the NF2 gene. Of these markers, location of D22S268 is nearest to NF2 gene. Eleven of thirty-six (31%) vestibular schwannoma have lost D22S268, which noticed to be higher than other loci. It may suggest that D22S268 marker can represent a specific marker for detecting of LOH in vestibular schwannoma.

Our data demonstrate that (1) allele loss of chromosome 22 is a frequent event in sporadic vestibular schwannoma, (2) the region of LOH includes the NF2 gene, (3) patients with chromosome 22 LOH tend to have a larger tumor size, higher tumor growth index and more rapid proliferative index than those without LOH. Mechanism leading to tumor formation in the 55.6% of our tumor series, which absence of LOH, remains unknown. Recently, Warren et al. [18] hypothesized that other allele loss in schwannomas might exist but not locate on chromosome 22, or other 22q-located genes, or these tumors harbored small deletions or point mutations leading to loss of NF2 gene function. Hence, future schwannoma-related projects should extensive study to better define additional, putative 22q-located loci and analyze the entire chromosome 22 for schwannoma development [18,19].

Mutations

In this study, we analyzed 36 sporadic vestibular schwannomas for mutations in the exons of NF2 gene. Mutations are found in 61% of our samples ($n = 22$) and consist of deletions, insertions, and point mutations (see Table 2). Most of these mutations produce frameshift, which leading to a truncated and abnormal protein [15].

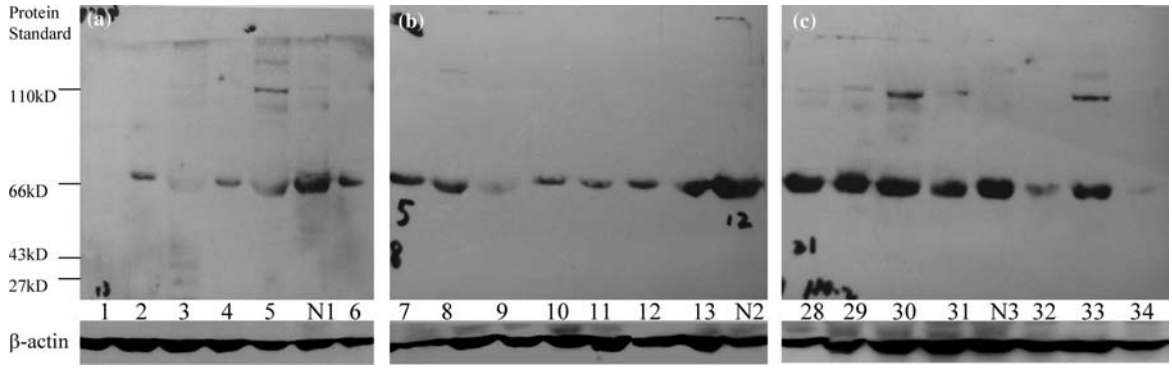

Figure 5. S/M expression in sporadic vestibular schwannomas: two milligrams protein from each specimen are separated by 10% SDS–PAGE. After incubation with the primary antibody (dilution 1:300) is performed for 4 h at room temperature, the secondary anti-rabbit horseradish peroxidase conjugated antibody (diluted to 1:2000) is incubated with blots for 1.5 h at room temperature. The protein is detected by chemiluminescence reagent. β-actin was used as loading control. Number N1, N2 and N3 in (a), (b) and (c) represent normal nerve tissue respectively. Absent or reduced S/M expression is observed in all tumors, except number 13, 29 and 30, when comparing to normal tissue.

On the other hand, we found 4 splice site mutations. Alterations at splice sites are most likely causing skipping exon or premature codon, which resulting in a stop codon [15]. Although there were no hot-spot mutation, most mutations were in the first seven exons (59%, 13/22, see also Table 2). These findings are in consistent with other previous studies [17,20].

According to a 'two-hit' mutation model, tumorigenesis in sporadic vestibular schwannoma complies with two mechanisms of genetic alteration [17]. In our study, we can demonstrate that 42% (15/36) tumor specimens harboring both LOH and mutations (see also Table 1). Failure to identify mutation in all of our cases is likely due to several causes [19]. Moreover, the sensitivity of SSCP analysis has been reported to be related to the size of the PCR amplified DNA fragment. For example, 97% of single base substitutions were detected in DNA fragments of 155 base pairs (bp). Whereas fragments of 135 and 175 bp, containing the same mutations, resulted in a decrease of sensitivity to 80% [20–23].

LOH, mutation and tumor behavior

Although Irving et al. [12] found that there was no correlation between the nature of NF2 gene mutation and tumor behavior (proliferative index), our data demonstrated the significant difference of tumor behavior between tumor with and without NF2 gene mutation. We found that the proliferative activity of tumor cells might be associated with the NF2 gene inactivation, especially with the missense mutation of NF2 gene. According to the previous study in NF2-related vestibular schwannomas, nonsense mutations of the NF2 gene fail to produce stable merlin protein, while missense mutations result in generation defective merlin proteins and in negative growth regulation [24–26]. The same genetic mechanism might occur as well in sporadic vestibular schwannomas. Recently, Scoles et al. [27,28] proposed a possible role of missense mutation on schwannoma cell proliferation. Fraenzer et al. [29] found the overexpression of the NF2 gene inhibits schwannoma cell proliferation. Therefore, the role of missense mutation in sporadic vestibular schwannoma should be further studied.

S/M expression

In the present study, we found the positively S/M staining cells might be non-tumor cells and none of tumor cells have the immunoreaction to S/M antibody. These findings are similar to other previous reports [30–32]. Because from our data, only 61% tumors presented with NF-2 gene mutation, one might argue that in the immunohistochemical study of S/M, the remaining tumor specimens (without NF-2 gene mutation) should be immunoreactive to the anti-NF2 (A-19) IgG. The explanation of this finding is that those tumors might have harbored undetected mutations, which led to the suggestion that loss of merlin occurred without NF2 mutation [33].

Furthermore, the expression of S/M was significantly lower in tumors with mutation than without mutation tumors. The 66-kD bands of S/M in tumors identified by immunoblotting were reduced in 78% and severely reduced in 42% vestibular schwannomas. These results are also similar to other reports [21,24]. When we correlate our immunohistochemical result with the immunoblotting one, this 66-kD band may presumably originate from the non-tumorous cells such as macrophages, lymphocytes and cells of the blood vessels. This hypothesis is also previously suggested by Bianchi [34].

At the same time protein bands harboring molecular weight standards near 110-kD were also detected (Figure 5). The relationship of this protein to S/M is unknown. However, this protein is not likely to be isoforms of S/M, because only 66 kD is immunoprecipitated by both amino terminus form and carboxy specific antibodies [10].

Conclusion

This study suggested that the molecular genetic changes of sporadic vestibular schwannomas in Chinese patients were similar to the previous reports. NF2 gene acts as tumor suppressor gene in vestibular schwannoma and is inactivated by two-hit process. There are some degrees of relationship between tumor behaviors and genetic

alterations including LOH and mutation of NF2 gene. The proliferative activity of tumor cells might be associated with the NF2 gene inactivation, especially with the missense mutation of NF2 gene. We propose that inactivation of S/M may be an important step in tumorigenesis of sporadic vestibular schwannoma.

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