Genetic variants of cancer-associated genes analyzed using next-generation sequencing in small sporadic vestibular schwannomas

TAKESHI FUJITA[1], KAZUKO SAKAI[2], NATSUMI UEHARA[1], YUJIRO HOSHI[3], ANJIN MORI[4], HAJIME KOYAMA[5], MITSUO SATO[6], KAZUYA SAITO[7], YASUHIRO OSAKI[6], KAZUTO NISHIO[2] and KATSUMI DOI[6]

[1]Department of Otolaryngology Head and Neck Surgery, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017; [2]Department of Genome Biology, Kindai University Faculty of Medicine, Osaka-Sayama, Osaka 589-8511; [3]Department of Otorhinolaryngology, Mitsui Memorial Hospital, Tokyo 101-8643; [4]Department of Otorhinolaryngology - Head and Neck Surgery, NTT Medical Center Tokyo, Tokyo 141-8625; [5]Department of Otolaryngology and Head and Neck Surgery, Graduate School of Medicine, The University of Tokyo Hospital, Tokyo 113-8655; [6]Department of Otolaryngology, Kindai University Faculty of Medicine, Osaka-Sayama, Osaka 589-8511; [7]Department of Otorhinolaryngology, Izumi City General Hospital, Izumi, Osaka 594-0073, Japan

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Abstract. Vestibular schwannoma (VS) is the most common tumor of the cerebellopontine angle. Despite the increasing diagnosis of sporadic VS over the past decade, the use of traditional microsurgeries to treat VS has decreased. This is likely a result of the adoption of serial imaging as the most common initial evaluation and treatment strategy, especially for small-sized VS. However, the pathobiology of VSs remains unclear, and elucidating the genetic information of tumor tissue may reveal novel insights. The present study performed a comprehensive genomic analysis of all exons in the key tumor suppressor and oncogenes from 10 small (<15 mm) sporadic VS samples. The evaluations identified *NF2*, *SYNE1*, *IRS2*, *APC*, *CIC*, *SDHC*, *BRAF*, *NUMA1*, *EXT2*, *HRAS*, *BCL11B*, *MAGI1*, *RNF123*, *NLRP1*, *ASXL1*, *ADAMTS20*, *TAF1L*, *XPC*, *DDB2* and *ETS1* as mutated genes. The current study could not draw any new conclusions about the relationship between VS-related hearing loss and gene mutations; however, it did reveal that *NF2* was the most frequently mutated gene in small sporadic VS.

Correspondence to: Dr Takeshi Fujita, Department of Otolaryngology Head and Neck Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunokicho, Chuoku, Kobe, Hyogo 650-0017, Japan
E-mail: fujitake@med.kobe-u.ac.jp

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Introduction

Vestibular schwannoma (VS) is the most common tumor in the cerebellopontine angle (1). These tumors arise from the myelin-producing Schwann cells within the vestibular branch of the eighth (VIII) cranial nerve (2). Although these tumors are histologically benign, VSs most commonly present with hearing loss and tinnitus and can also cause dizziness, facial paralysis, other cranial neuropathies, and even death from brainstem compression. Most of VS cases are sporadic and unilateral, while bilateral tumors are associated with Neurofibromatosis type 2 (NF2).

The number of sporadic VS cases diagnosed has increased dramatically over the last decade, mainly due to the widespread adoption of, and easy access to magnetic resonance imaging (MRI). The prevalence of sporadic VS is estimated to be 1 in 2,000 adults and 1 in every 500 persons aged 70 years or older (3). The prevalence of NF2 patients is estimated to be 1 in 56,161 (4). Despite the increasing number of VS patients, the traditional microsurgery treatment for VS via the retrosigmoid, middle cranial fossa, or translabyrinthine approach has decreased (5). Serial imaging is now the most common initial strategy for most patients with small- or medium-sized VS tumors (6).

The *NF2* gene located on chromosome 22 at 22q12.2, which encodes for the tumor suppressor protein Merlin, is considered to be the most important gene for understanding VS pathobiology in both sporadic VSs and familial (NF2 related) VSs (7-9). Various pathways, including those involving the *NF2* gene, have been investigated for the treatment of VSs (10). However, *NF2* alone cannot fully explain all tumor development and growth. Therefore, it is essential to analyze genetic information obtained from tumor tissue to gain novel insights into the pathobiology of VSs and devise effective treatment

strategies. In this study, we focused on key tumor suppressor genes and oncogenes.

To date, obtaining small tumor samples has become increasingly difficult as nonsurgical management options have been shown to produce good results in slow-growing and smaller VS tumors, especially those with a total length of <2 cm (11). Tumor size does not correlate with degree of hearing loss (12-14), as small tumors limited to the auditory canal can still result in hearing loss (15). Patients in the small tumor (<1.0 cm) group presented more frequently with tinnitus and sudden hearing loss than those in the large tumor (>4.0 cm) group, suggesting differences in the biological characteristics of large and small tumors (16).

Thus, it is important to investigate the genetic profile of small tumors to understand the pathobiology of VS. To elucidate the genetic landscape of small VSs, especially the genes related to tumor genesis and tumor growth, this study involved a comprehensive genomic analysis of all the exons from the key tumor suppressor genes and oncogenes obtained from 10 sporadic, small VS samples collected through surgical intervention.

Materials and methods

Patients. We enrolled a total of 15 patients who underwent surgical resection for small unilateral VS (<2 cm total length) between October 2012 and March 2017 at the Kindai University Hospital. Ten out of 15 samples yielded genomic DNA of adequate quality for library preparation. Five samples were excluded from this study because of the inadequate quality of genomic DNA. These tumors were classified using the Koos acoustic neuroma grading system (17) and 10 of these samples were then analyzed using next generation sequencing (NGS) in this study. Our study was approved by the ethics committee at the Kindai University Hospital (approval no. 29-015).

DNA extraction and library preparation. Genomic DNA was extracted from the FFPE tumor tissues using the QIAamp DNA FFPE Tissue Kit (Qiagen). This was then used as the template in four independent multiplex polymerase chain reactions (PCRs) which amplified 15,992 regions across 409 cancer-related genes (Table SI). These amplifications were completed using an Ion AmpliSeq Library Kit 2.0 with Comprehensive Cancer Panel (Thermo Fisher Scientific). After multiplex PCR, Ion Xpress Barcode Adapters (Thermo Fisher Scientific) were ligated to the PCR products, which were then purified using Agencourt AMPure XP beads (Beckman Coulter). Purified libraries were pooled and sequenced on an Ion Torrent Proton instrument using an Ion Proton Hi-Q Sequencing Kit, and an Ion PI v3 Chip (all from Thermo Fisher Scientific). DNA sequencing data were then accessed using the Torrent Suite v.5.0 program (Thermo Fisher Scientific).

Sequence analysis. DNA sequencing data were accessed using the Torrent Suite ver. 5.2 software program and the reads were aligned against the hg19 human reference genome. Variants were called using the variant caller ver. 5.2. and the VCF files were annotated using CLC Genomics Workbench ver. 9.0.1 (Qiagen). Variants were filtered based on quality (quality score >100, read depth >50 and allele frequency >5%) and were manually checked using the integrative genomics viewer (IGV; Broad Institute). Germline mutations were excluded using the use of the Human Genetic Variation Database (http://www.genome.med.kyoto-u.ac.jp/SnpDB) and the Exome Aggregation Consortium (ExAC). The pathogenicity of the mutations was predicted using SIFT (18), PolyPhen2 (19), FATHMM (20), and CScape (21).

Results

Clinical characteristics of the patients. The clinical characteristics of the 10 patients profiled in this study are summarized in Table I. This cohort was comprised of five men and five women, with a mean age of 61.4 years (median 62.5, range 40-73). Tumor size ranged from those limited to the internal auditory canal to a maximum diameter of 15 mm. A total of 6 patients presented with Koos grade I (intracanalicular) tumors while the other 4 had Koos grade II (small tumor with protrusion into the CPA; no contact with the brainstem) tumors. The middle cranial fossa approach was used in five patients, and translabyrinthine approach was used in the other five patients. Complete resection of the tumor was reported for nine patients, and a 99% resection was performed in one patient.

Genetic landscape of our small vestibular schwannoma samples. *NF*2 (40%) was the most frequently mutated cancer-related gene in these VS samples (Fig. 1) and was followed by *SYNE1* (20%), *IRS*2 (10%), *APC* (10%), *CIC* (10%), *SDHC* (10%), *BRAF* (10%), *NUMA1* (10%), *EXT2* (105), *HRAS* (10%), *BCL11B* (10%), *MAGI1* (10%), *RNF123* (10%), *NLRP*1 (10%), *ASXL*1 (10%), *ADAMTS20* (10%), *TAF1L* (10%), *XPC* (10%), *DDB2 (*10%), and *ETS1* (10%). Most of these mutations were predicted to be pathogenic (Table II). Closer evaluation of the cases harboring mutations in the *NF2* revealed that one case harbored a missense mutation in exon 4 (case no. 3) and its pathogenicity was predicted as 'damaging' by SIFT, 'probably damaging' by Polyphen2, 'pathogenic' by FATHMM-XF, and 'oncogenic with high confidence' by CScape. Two cases harbored a nonsense mutation in exon 2 (case no. 7 and 10) and its pathogenicity was predicted as 'benign' by FATHMM-XF, and 'oncogenic with high confidence' by CScape. One case harbored a nonsense mutation in exon 6 (case no. 6) and its pathogenicity was predicted as 'pathogenic' by FATHMM-XF, and 'oncogenic with high confidence' by CScape.

Discussion

We investigated the genomic landscape of the small VSs using a comprehensive genomic analysis of all the exons from key tumor suppressor genes and oncogenes in 10 small sporadic VS tumors. These evaluations identified *NF2, SYNE1, IRS2, APC, CIC, SDHC, BRAF, NUMA1, EXT2, HRAS, BCL11B, MAGI1, RNF123, NLRP1, ASXL1, ADAMTS20, TAF1L, XPC, DDB2*, and *ETS1* as mutated genes. Five mutations were identified in a single tumor

Table I. Clinical characteristics of ten patients with sporadic vestibular schwannoma.

No.	Sex	Age, years	Side	Tumor size, mm	Koos grade	HL affected side, dB HL	HL intact side, dB HL	Surgery	Resection mode
1	M	58	L	15	II	63	16	MCF	Total
2	M	65	L	IAC (6.0)[a]	I	48	25	TL	Total
3	M	60	L	6.8	II	101	10	TL	Total
4	F	72	L	7.7	II	89	48	TL	Total
5	F	48	L	7.2	II	38	26	MCF	Total
6	F	40	R	6.2	II	6	9	MCF	Total
7	F	69	L	8.3	II	56	30	MCF	Total
8	M	73	R	IAC (6.6)[a]	I	36	36	MCF	99%
9	F	58	R	IAC (6.0)[a]	I	s.o	14	TL	Total
10	M	71	R	IAC (5.9)[a]	I	86	13	TL	Total

[a]The size of the tumor localized to the IAC is provided for reference. M, male; F, female; L, left; R, right; HL, hearing level; IAC, internal auditory canal; s.o., scaled out in pure tone audiometry; MCF, middle cranial fossa approach; TL, translabyrinthine approach.

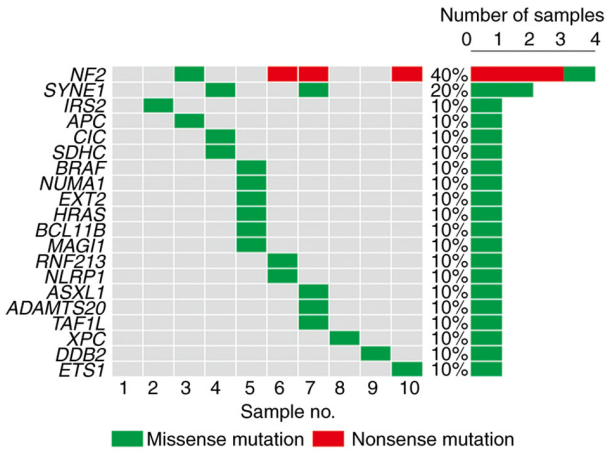

Figure 1. The mutations in various cancer related genes across the ten vestibular schwannomas.

sample (sample no. 5), but did not indicate high tumor mutation burden (TMB) (22).

To date, the genetic profile of sporadic VS is not completely understood. The only consistent genetic alteration in these tumors appears to be the inactivation of the *NF2*. Merlin, a cytoskeletal protein encoded by the *NF2* gene, suppresses tumorigenesis by interacting with integrins and receptor tyrosine kinases. Mutational analysis of the *NF2* gene in sporadic VSs has been reported to be identified in 49 to 100% of analyzed samples (23-35). In this study, we identified mutations in NF2 in four of ten cases (40%), and revealed that even in small sporadic VSs, genetic mutations were most frequently found in the *NF2*.

The second most frequent mutation was found in *SYNE1*, and appeared in 20% of the samples. Synaptic nuclear envelope protein 1 (SYNE1) encodes a series of spectrin structural proteins that play important roles in cytoskeletal, nuclear, and vesicular anchoring (36), and mutations in this gene are associated with a form of cerebellar ataxia (37). Recently, it has also been suggested that altered expression, somatic mutations, and single nucleotide polymorphisms in the *SYNE1* are associated with the development and progression of lung cancer (38), oral cancer (39), and hepatocellular carcinoma (40). There have been no reports of these mutations in schwannomas. The amino acid changes 'p.Ala977Thr' and 'p.Lys4121Ser' in the *SYNE1* gene identified in this study have not been reported in any of the germline mutation databases. Therefore, we considered these mutations to be somatic.

The genes whose mutations were predicted as 'damaging', 'pathogenic', or 'oncogenic' by at least two pathogenicity prediction models were *NF2*, *IRS2*, *CIC*, *EXT2*, *HRAS*, *BCL11B*, *TAF1L*, and *ETS1* (Table II). *IRS2* encodes the insulin receptor substrate 2, a cytoplasmic signaling molecule regulating the effects of insulin, insulin-like growth factor 1, and other cytokines (41). Alterations in *IRS2* have been reported in several cancers such as colorectal cancer, lung cancer, and breast cancer (42). Capicua transcriptional repressor (*CIC*), a part of the high mobility group (HMG)-box family, encodes a transcription repressor protein.

CIC mutations occur most frequently in oligodendroglioma (43). *EXT2* (exostosin glycosyltransferase 2) encodes glycosyltransferases responsible for heparan sulfate biosynthesis. Mutations in *EXT2* cause the type II form of hereditary multiple exostoses (44). *HRAS* (Harvey rat sarcoma viral oncogene homolog) encodes the GTPase HRas protein, one of the three human RAS proteins. *HRAS* mutations occur in various cancers such as head and neck squamous cell carcinoma and bladder urothelial carcinoma (45). *BCL11B* encodes B-cell leukemia/lymphoma 11B, a C2H2-type zinc finger protein that functions as a transcriptional repressor. *BCL11B* mutations occur in T-cell acute lymphoblastic leukemia (T-ALL) (46). TATA-Box Binding Protein Associated Factor 1 (TAF1) possesses intrinsic protein kinase, histone acetyl-transferase and ubiquitin-conjugating activities. *TAF1L* encodes TAF1 Like (TAF1L), a TAF1 homolog that shows 95% amino acid identity with TAF1. *TAF1L* mutations occur in oral squamous

Table II. The pathogenicity of the somatic mutations in sporadic vestibular schwannoma using SIFT, PolyPhen2, FATHMM and CScape.

Gene symbol	Effect	Sample	Genomeposition[a]	cDNA change	Amino acid change	VAF, %	SIFT	Polyphen2	FATHMM-XF	CScape
NF2	Missense	3	chr22:30038274	c.447G>T	p.Lys149Asn	33.2	D	Prob-D	P	O (HC)
NF2	Non-sense	6	chr22:30070927	c.1443C>G	p.Tyr481*	36.3	NA	NA	P	O (HC)
NF2	Non-sense	7,10	chr22:30032794	c.169C>T	p.Arg57*	46.5/61.2	NA	NA	B	O (HC)
SYNE1	Missense	4	chr6:152774819	c.2929G>A	p.Ala977Thr	45.5	T	B	B	O
SYNE1	Missense	7	chr6:152658141-152658142	c.12362_12363delAGinsGT	p.Lys4121Ser	47.7	NA	NA	B	O
IRS2	Missense	2	chr13:110434998	c.3403G>A	p.Val1135Ile	46.1	D	Prob-D	P	B
APC	Missense	3	chr5:112177052	c.5761G>A	p.Gly1921Ser	44.4	T	B	B	B
CIC	Missense	4	chr19:42796489	c.5773C>T	p.Pro1925Ser	50.5	T	Prob-D	B	O
SDHC	Missense	4	chr1:161298236	c.128A>G	p.Asn43Ser	50.2	T	B	B	B
BRAF	Missense	5	chr7:140500192	c.950C>T	p.Ser317Phe	52.4	T	B	B	O
NUMA1	Missense	5	chr11:71726642	c.1907C>T	p.Thr636Ile	51.3	T	B	B	B
EXT2	Missense	5	chr11:44129270	c.107C>T	p.Ala36Val	48.5	NA	B	P	O (HC)
HRAS	Missense	5	chr11:532688	c.518C>T	p.Pro173Leu	48.6	D	B	P	B
BCL11B	Missense	5	chr14:99697747	c.575C>T	p.Ser192Leu	47.1	T	Poss-D	P	O (HC)
MAGI1	Missense	5	chr3:65464432	c.592A>G	p.Ser198Gly	48.0	T	B	B	O
RNF213	Missense	6	chr17:78343382	c.12236A>C	p.Lys4079Thr	51.5	D	NA	B	B
NLRP1	Missense	6	chr17:5462461	c.1555G>A	p.Val519Met	42.8	D	B	B (HC)	B
ASXL1	Missense	7	chr20:31025035	c.4520C>T	p.Ala1507Val	55.7	T	B	B	B
ADAMTS20	Missense	7	chr12:43826171	c.3032G>A	p.Arg1011Gln	30.1	T	B	B	O
TAF1L	Missense	7	chr9:32634817	c.761G>T	p.Arg254Leu	46.7	D	Poss-D	B	O
XPC	Missense	8	chr3:14190071	c.2411C>G	p.Ser804Cys	48.0	T	B	B	O
DDB2	Missense	9	chr11:47256837	c.897T>C	p.Met240Thr	49.0	NA	NA	B	B
ETS1	Missense	10	chr11:128332425	c.1289A>T	p.Tyr430Phe	37.7	D	Prob-D	P	O (HC)

[a]GRCh37. T, tolerant; D, damaging; Prob-D, probably damaging; Poss-D, possibly damaging; B, benign; NA, not available; P, pathogenic; O, oncogenic; HC, high confidence.

Table III. Summary of studies describing tumor size in the genetically analyzed sporadic VS samples.

First author, year	Sample size range, mm (average)	Number of VS samples	(Refs.)
This study	6.2-15 (8.3)	10	-
Aaron *et al*, 2020	13-35 (24.8)	12	(49)
Havik *et al*, 2018	16-56 (31.0)	46	(33)
Carlson *et al*, 2018	6-49 (24.8)	23	(35)
Chen *et al*, 2017	8-70 (26.8)	281	(34)

VS, vestibular schwannoma.

cell carcinoma (47), and gastric and colorectal cancers (48). ETS proto-oncogene 1 (*ETS1*) encodes a transcription factor and is mutated in cancers such as colon carcinoma (42).

Here, we focused on previously resected tumors, but our current strategy for evaluating and treating small VSs is to follow up with serial MRI scans and audiological tests. Serial imaging is the most common initial strategy for slow-growing, especially small-sized VS with a length of less than 2 cm (6,11). Previous reports describing tumor size in the genetically analyzed sporadic VS samples reported that the mean size of their surgically removed tumors ranged from 24.8 to 31.0 mm in length (33-35,49) (Table III). In this study, the average size of the sporadic VSs was 8.3 mm. In the present study, we did not identify any gene mutations that were specific to small tumors. However, we did find that one gene mutation in smaller-sized Koos grade I tumors (cases 2, 8, and 9). Only two mutations, those in *NF2* and *ETS1,* were found in case 10. The mean number of gene mutations detected in the four Koos grade I tumors was 1.25. In the six Koos grade II tumors, the mean number of gene mutations detected was 3.0. It is possible that tumors with larger sizes may harbor a greater number of mutated genes, but this will require further examination in a future study featuring a larger number of cases.

This study has several limitations. First, the small sample size limited the accuracy of the conclusions. Second, the samples lacked reference materials such as blood samples, so we need to rely on databases to determine whether the mutations were somatic or germline. Third, the pathogenicity of the mutant genes in schwannomas needs to be further investigated, as do the effects of these potentially pathogenic genes and their underlying mechanisms. The current study could not draw any new conclusions about the relationship between VS-related hearing loss and gene mutations. However, this study revealed that *NF2* was the most frequently mutated gene in small sporadic VSs. Moreover, the novel mutations in *SYNE1* were identified by comprehensively analyzing the genomic data obtained from small tumors. In the future, establishing diagnostic and prognostic biomarkers using blood samples may be a critical strategy to predict tumor growth and hearing loss, since tumor specimens are difficult to obtain from small VSs.

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Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to the research proposal approved by the ethics committee of our institution but are available from the corresponding author on reasonable request.

Authors' contributions

TF and KSak designed the study. KSak and KN conducted the genetic analyses. TF and KSak confirm the authenticity of all raw data. TF and NU substantially contributed to the manuscript drafting. NU, HK, YH, AM, MS, YO, KSai and KD significantly contributed to data analysis and interpretation. All authors critically reviewed and revised the manuscript draft, and read and approved the final manuscript.

Ethics approval and consent to participate

The study complied with the standards of the Declaration of Helsinki and was approved (approval no. 29-015) by the Institutional Review Board of Kindai University Hospital (Osaka, Japan). Opt-out informed consent from patients was obtained by exhibiting the research information on the official website of our hospital (Kindai University Hospital, Osaka, Japan). The authors guarantee the opportunity for refusal by document, call or e-mail whenever possible. Patients who rejected participation in this study were excluded.

Patient consent for publication

Opt-out informed consent from patients was obtained by exhibiting the research information on the official website of our hospital (Kindai University Hospital, Osaka, Japan). The authors guarantee the opportunity for refusal by document, call or e-mail whenever possible. Patients who rejected publication of their information in this study were excluded.

Competing interests

The authors declare that they have no competing interests.

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