Table 2. Statistical Analysis of 12 Computer Prediction Software Programs and the Average z-Score of 12 Prediction Programs.[a]

Prediction Program	Group 1	Average	Group 2	Average	Difference in Each Group	SE	*P* Value[b]	95% Confidence interval	
								Lower	Upper
SIFT	Novel[c]	0.73	AD-Pathogenic	0.86	−0.13	0.06	1.2E-01	−0.28	0.03
			AR-Pathogenic	0.88	−0.14	0.04	2.6E-03	−0.24	−0.04
PP2-HVID	Novel	0.60	AD-Pathogenic	0.83	−0.23	0.09	1.9E-02	−0.44	−0.03
			AR-Pathogenic	0.87	−0.27	0.06	5.5E-06	−0.41	−0.14
PP2-HVAR	Novel	0.49	AD-Pathogenic	0.77	−0.27	0.09	3.7E-03	−0.47	−0.07
			AR-Pathogenic	0.79	−0.30	0.06	2.8E-07	−0.43	−0.17
LRT	Novel	0.71	AD-Pathogenic	1.00	−0.29	0.08	2.1E-03	−0.48	−0.09
			AR-Pathogenic	0.93	−0.22	0.06	2.2E-04	−0.35	−0.09
Mut Taster	Novel	0.69	AD-Pathogenic	0.91	−0.22	0.09	3.1E-02	−0.43	−0.02
			AR-Pathogenic	0.95	−0.26	0.06	2.1E-05	−0.39	−0.12
Mut Assesor	Novel	0.59	AD-Pathogenic	0.67	−0.08	0.03	2.9E-02	−0.16	−0.01
			AR-Pathogenic	0.69	−0.10	0.02	4.9E-06	−0.15	−0.05
FATHMM	Novel	0.41	AD-Pathogenic	0.50	−0.09	0.03	1.1E-03	−0.15	−0.03
			AR-Pathogenic	0.44	−0.04	0.02	9.1E-02	−0.08	0.00
RadialSVM	Novel	0.40	AD-Pathogenic	0.56	−0.16	0.03	1.0E-06	−0.23	−0.08
			AR-Pathogenic	0.56	−0.16	0.02	5.1E-09	−0.21	−0.11
LR	Novel	0.39	AD-Pathogenic	0.70	−0.31	0.06	1.8E-06	−0.45	−0.16
			AR-Pathogenic	0.71	−0.32	0.04	5.1E-09	−0.42	−0.23
GERP++	Novel	3.21	AD-Pathogenic	5.01	−1.79	0.60	7.6E-03	−3.19	−0.39
			AR-Pathogenic	4.52	−1.30	0.39	2.6E-03	−2.22	−0.38
PhyloP	Novel	1.41	AD-Pathogenic	2.38	−0.97	0.23	9.7E-05	−1.52	−0.42
			AR-Pathogenic	1.99	−0.59	0.15	3.9E-04	−0.95	−0.23
29-way LogOdds	Novel	11.72	AD-Pathogenic	15.94	−4.22	1.04	1.4E-04	−6.65	−1.79
			AR-Pathogenic	15.18	−3.47	0.68	1.2E-06	−5.07	−1.87
z Pred Ave	Novel	−0.03	AD-Pathogenic	0.66	−0.69	0.13	4.2E-07	−0.99	−0.38
			AR-Pathogenic	0.60	−0.62	0.09	5.1E-09	−0.82	−0.42

Abbreviations: SE, standard error; PP2, PolyPhen2; Mut Taster, Mutation Taster; Mut Assesor, Mutation Assesor; z Pred Ave, averaged z score of 12 prediction programs; AR, autosomal recessive; AD, autosomal dominant.
[a]All prediction programs listed above were including in the ANNOVAR software.[21, 22]
[b]Tukey HSD test.
[c]Novel, novel identified variants in this study (might include pathogenic variants and rare polymorphisms).

Molecular Epidemiology of Japanese Patients With Hearing Loss

In spite of the successful identification of deafness genes, no comprehensive etiological data on a genetic basis have been available. It is noteworthy that MPS-based studies can be made available for studying the molecular epidemiology of deafness. Recently, we first applied genetic epidemiology to determine the impact of each gene on hearing loss by using MPS analyses of 216 patients with hearing loss.[12] On the basis of our recent results, the gene having the greatest impact on the etiology of deafness was *GJB2*, mutations in which were found in exceptionally high numbers, followed by mutations in *SLC26A4, USH2A, GPR98, MYO15A, COL4A5*, and *CDH23*.[12] The present study, which was based on a different platform and used a larger cohort, generally corroborated our previous results. In this study, among the variants identified, the number of *GJB2* mutations was exceptionally high, followed by those in *CDH23, SLC26A4, MYO15A, COL11A2, MYO7A*, and *OTOF* (Figure 3). Nonsense, splicing, and frame shift deletion mutations were identified more frequently in autosomal recessive hearing loss genes, such as *GJB2, SLC26A4, MYO15A, COL11A2*, and *OTOF*, than in others. In contrast, most of the variants in autosomal dominant hearing loss genes, such as *MYO7A, TECTA, MYH14*, and *WFS1*, were missense variants, and only a limited number were nonsense, spliced junction, or frame shift insertions/deletions. These results were understandable because most autosomal recessive hearing loss is caused by loss-of-function mutations, whereas most autosomal dominant hearing loss is caused by dominant-negative or gain-of-function mutations, and a small portion are caused by haplo-insufficiency mutations. Notably, only a limited number of nonsense, splice junction, and deletion/

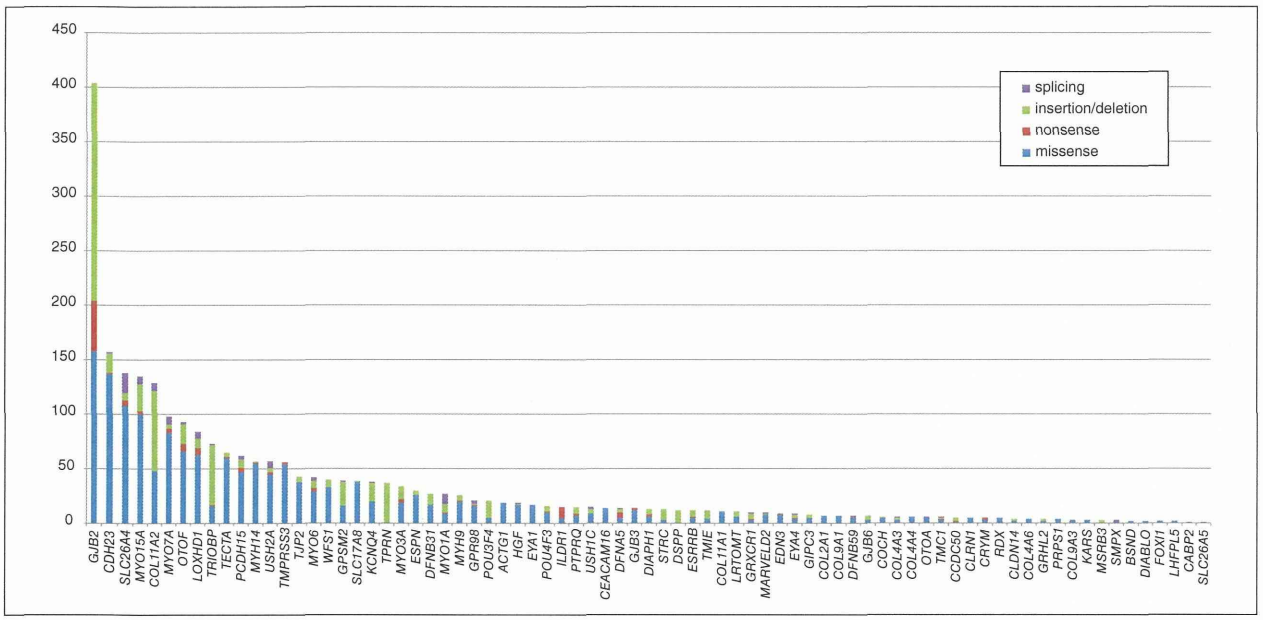

Figure 3. The number of previously reported pathogenic and candidate variants selected using the allele frequencies of a large number of controls and results of 12 computer prediction programs. The number of variants indicates that the majority of responsible gene variants accumulated in particular major causative genes.

insertion mutations were observed in *CDH23*. *CDH23* is reported to have a clear phenotype-genotype correlation, and a *CDH23* truncation mutation causes Usher syndrome,[30,31] which is why we observed a low number of nonsense, splice junction, and deletion/insertion mutations in the *CDH23* mutations.

This result indicates that 30% to 40% of patients are deaf due to recurrent mutations in particular genes, such as *GJB2, CDH23*, and *SLC26A4*. *GJB2* is the most common gene responsible for deafness worldwide, and 14% to 16% of Japanese patients with hearing loss have a *GJB2* mutation.[12,27,28] Mutations in *CDH23* and *SLC26A4* are also frequent and important causes of deafness in Japanese.[27,31,32] The majority of the responsible gene mutations are limited to a few genes due to recurrent mutations; however, the remainder consist of rare genes or mutations. MPS is a powerful tool with which to identify such rare genes or mutations. At the same time, we should keep in mind the limitations of MPS technologies. Careful interpretation is needed to analyze results for genes with identical sequences or pseudogenes, such as *ESPN* and *STRC*. Most current MPS technologies used are based on short reads; therefore, it is difficult to distinguish "true genes" and "pseudogenes." The Ion PGM system yields read lengths longer than those obtained by the Illumina system, and better results should be expected, particularly for such pseudogenes. However, it is impossible to distinguish "true genes" and "pseudogenes" completely, so further investigation is required for these genes.

In conclusion, we performed MPS analyses and confirmed the genetic background of hearing loss in Japanese patients. This data set will be a powerful tool with which to discover rare causative genes mutations in a highly heterogeneous monogenic disease and reveal the genetic epidemiology of deafness. We are currently performing segregation analysis for the newly identified candidates.

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Mutational Spectrum and Clinical Features of Patients With *ACTG1* Mutations Identified by Massively Parallel DNA Sequencing

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Abstract

Objectives: *ACTG1* has been reported to be a causative gene for autosomal dominant sensorineural hearing loss, DFNA20/26. In this study we sought to clarify the detailed mutational spectrum, clinical features, and genotype-phenotype correlations.

Methods: Massively parallel DNA sequencing (MPS) of 63 target candidate genes was used to screen 1120 Japanese hearing loss patients.

Results: MPS screening successfully identified 4 *ACTG1* mutations in 5 families. The majority of patients showed high frequency–involved progressive hearing loss, with the age of onset mostly in the first or second decade. One patient received electric acoustic stimulation (EAS), which showed a good outcome.

Conclusions: Target exon-sequencing using MPS was proven to be a powerful new clinical diagnostic tool for the identification of rare causative genes such as *ACTG1*. The present clinical findings not only confirmed those previous reports but also provided important new clinical information.

Keywords

ACTG1, DFNA20/26, hearing loss, massively parallel DNA sequencing, next generation sequencing, EAS

Introduction

Hereditary hearing loss is the most common sensorineural deficit and shows the highest degree of genetic heterogeneity. Autosomal dominant sensorineural hearing loss (ADSNHL) accounts for 20% of hereditary hearing loss, and more than 30 genes have been reported to be associated with ADSNHL.

ACTG1 has been reported as one of the causative genes for ADSNHL and is linked to the DFNA20/26 locus (OMIM #604717) on chromosome 17q25.3.[1-3] *ACTG1* encodes γ-actin, the predominant actin isoform in auditory hair cells and, more specifically, in the cuticular plate, adherens junctions, and stereocilia.[4,5]

Based on previous reports, most patients develop hearing loss during the first or second decades, particularly for the frequencies from 6 to 8 kHz, and this hearing loss is slowly progressive, with threshold shifts observed at all frequencies. However, only 18 cases of this mutation have previously been reported,[2,3,6-14] so the detailed clinical features remain unknown.

Recent advances in targeted exon sequencing of selected genes using massively parallel DNA sequencing (MPS) technology have allowed the successful identification of causative mutations in relatively rare genes such as *ACTG1*. In this study, we further examined the detailed clinical characteristics of patients with *ACTG1* mutations and discussed the appropriate intervention.

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Subjects and Methods

Subjects

A total of 1120 Japanese hearing loss (HL) patients with bilateral sensorineural hearing loss (ADSNHL, 266; autosomal recessive sensorineural hearing loss, 600; unknown, 254) from 53 otolaryngology departments nationwide participated in this study. Written informed consent was obtained from all subjects (or from their next of kin, caretaker, or guardian in the case of minors/children) prior to enrollment in the project. This study was approved by the Shinshu University Ethical Committee as well as the respective ethical committees of the other participating institutions.

Amplicon Library Preparation

Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, California, USA) for 63 genes reported to cause nonsyndromic hearing loss according to the manufacturer's instructions. The detailed protocol was described elsewhere.[12] After preparation, the amplicon libraries were diluted to 20 pM, and equal amounts of 6 libraries for 6 patients were pooled for 1 sequence reaction.

Emulsion Polymerase Chain Reaction and Sequencing

Emulsion polymerase chain reaction (PCR) and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere.[12] MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using an Ion PGM 200 Sequencing Kit and an Ion 318 Chip (Life Technologies).

Base Call and Data Analysis

The sequence data were mapped against the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program. After sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. After variant detection, their effects were analyzed using ANNOVAR software.[15,16] The missense, nonsense, insertion/deletion, and splicing variants were selected from among the identified variants. Variants were further selected as less than 1% of (1) the 1000 genome database,[17] (2) the 6500 exome variants,[18] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[19] and (4) the 269 in-house Japanese normal hearing controls.

To predict the pathogenicity of missense variants, the following functional prediction software was used: PhyloP,[20] Sorting Intolerant from Tolerant (SIFT),[21] Polymorphism Phenotyping (PolyPhen2),[22] LRT,[23] MutationTaster,[24] and GERP++.[25]

Candidate mutations were confirmed by Sanger sequencing, and the responsible mutations were identified by segregation analysis using samples from among the patients' family members.

Results

Detected Mutations

MPS screening and subsequent Sanger sequencing identified a total of 4 missense mutations in *ACTG1* (c.142G>C [p.G48R], c.353A>T [p.K118M], c.721G>A [p.E241K], and c.895C>G [p.L299V]) in 5 families among the 1120 probands (Table 1). The pedigrees for all 5 families were compatible with autosomal dominant inherited hearing loss, and segregation analysis confirmed that the mutations were plausible disease-causing mutations. All detected mutations were predicted to be pathologic by several software programs. The 3 mutations, excluding c.142G>C, had already been reported as causative mutations for deafness.[2,8,12]

Clinical Findings

Figures 1, 2, 3, 4, and 5 and Table 2 show the clinical features for the 5 families. All pedigrees exhibited a typical autosomal dominant inheritance pattern, and all affected patients displayed progressive, symmetrical sensorineural hearing loss beginning in the high frequencies.

Family 1 (Figure 1: 2973, 2974, SNS5888, SNS5889)

Patient 2973, a 21-year-old male, had a heterozygous c.142G>C (p.G48R) mutation, with hearing loss detected by school physical examination at 6 years of age. His pedigree was compatible with autosomal dominant inherited hearing loss. His mother (patient 2974), who had noticed her hearing loss at 11 years of age, carried the same mutation. She experienced progressive hearing loss and tinnitus. The patient's older sister and youngest sister carried the same mutation, although the audiogram of the youngest sister (11 years) appeared to be normal.

Family 2 (Figure 2: JHLB964)

Patient JHLB964 is a 33-year-old female, with a history of hearing loss from 26 years of age. Due to the rapid progression of her hearing loss, the patient began using hearing aids at 28 years of age. She demonstrated associated vertigo. Her

Table 1. *ACTG1* Variants in Autosomal Dominant Sensorineural Hearing Loss (ADSNHL).

Exon	Subdomain	Nucleotide Change	Amino Acid Change	NM No.	Audiogram Configuration	Progression	Family Origin	Onset Age	Reference
3	2	c.142G>C	p.G48R	NM_001614	High frequency involved	Progressive	Japanese	First decade	This study
3	2	c.151G>A	p.D51N	NM_001614	High frequency involved	Progressive	Dutch	First decade	9
3	1	c.266C>T	p.T89I	NM_001614	High frequency involved	Progressive	American	Third decade	2
3	1	c.353A>T	p.K118M	NM_001614	High frequency involved	Progressive	American, Japanese	First, second, or third decade	2, this study
3	1	c.354G>C	p.K118N	NM_001614	High frequency involved	Progressive	Spanish	Childhood	8
4	1	c.364A>G	p.I122V	NM_001614	High frequency involved, profound	Progressive	Chinese	First decade	7
4	4	c.559G>C	p.D187H	NM_001614	Ascending to sloping to flat	Progressive	South Korean	At birth	10
4	4	c.721G>A	p.E241K	NM_001614	High frequency involved	Progressive	Spanish, Japanese	First decade	8, this study
4	4	c.791C>T	p.P264L	NM_001614	High frequency involved	Progressive	American	First or second decade	2
4	4	c.802G>C	p.G268S	NM_001199954	Normal to moderate	Unknown	Japanese	First or fourth decade	13
5	3	c.833C>T	p.T278I	NM_001614	High frequency involved, profound	Progressive	Dutch	First or second decade	3
5	3	c.895C>G	p.L299V	NM_001614	High frequency involved	Progressive	Japanese	Second decade	12, this study
5	3	c.914T>C	p.M305T	NM_001614	Severe to profound	Progressive	Korean	Fourth decade	11
5	3	c.974T>A	p.M325K	NA	High frequency involved	Unknown	German	At birth	14
6	3	c.994C>G	p.P332A	NM_001614	High frequency involved	Progressive	American	Second decade	2
6	C-terminal	c.1109T>C	p.V370A	NM_001614	High frequency involved	Progressive	Norwegian	First or second decade	6

father and brother also displayed progressive sensorineural hearing loss, and her father received cochlear implantation at the age of 45. Due to a lack of samples, segregation analysis could not be performed.

Family 3 (Figure 3: 3070, 3121)

Patient 3070, a 37-year-old male, had developed hearing loss at the age of 17. His father (patient 3121) also noticed the onset of hearing loss at high frequencies around 17 years of age and eventually developed profound hearing loss. The c.353A>T mutations were detected in both patients.

Family 4 (Figure 4: JHLB1181, GNM5164, GNM5089)

Patient JHLB1181, a 7-year-old boy, his younger sister (GNM5164), and his mother (GNM5089) all had heterozygous c.721G>A (p.E241K) mutations. He had passed newborn hearing screening but demonstrated a delay in language development. Auditory brainstem response (ABR) evaluation at 4 years, 11 months showed about 80 dB hearing loss in the high frequencies. He started using hearing aids and some language development was seen. Developmental disorders including speech development were found in this patient. According to the Wechsler Preschool and Primary Scale of Intelligence (WPPSI), an intelligence test designed for children aged 2 years, 6 months to 7 years, 3 months,[26] his results (VIQ 45, PIQ 74, and total IQ 50) at 4 years and 11 months were compatible with the existence of a developmental disorder. His younger sister (GNM5164) carrying the same mutation has mild high frequency–involved hearing loss, first evaluated at age 3 years of age. His mother (GNM5089) also has the same mutation and a similar type of hearing loss. The mother's hearing loss was identified by school physical examination at 14 years of age, and she began using a hearing aid at age 18. She demonstrated involuntary movement at ages 10 and 24 and was eventually diagnosed with Moyamoya disease (occlusion of the circle of Willis).

Family 5 (Figure 5: 3837, 3839, JHLB0109, JHLB0111)

Patient 3837, a 41-year-old male, had a heterozygous *ACTG1* mutation, c.895C>G (p.L299V).[12] He was first diagnosed with hearing loss in the high frequencies during a primary school physical examination at the age of 12. He became aware of progressive hearing loss and episodes of tinnitus at around age 20. He started wearing a hearing aid at age 33. One year later, he made his first visit to a clinic. Audiometric examination confirmed high frequency–involved SNHL, and distortion product otoacoustic

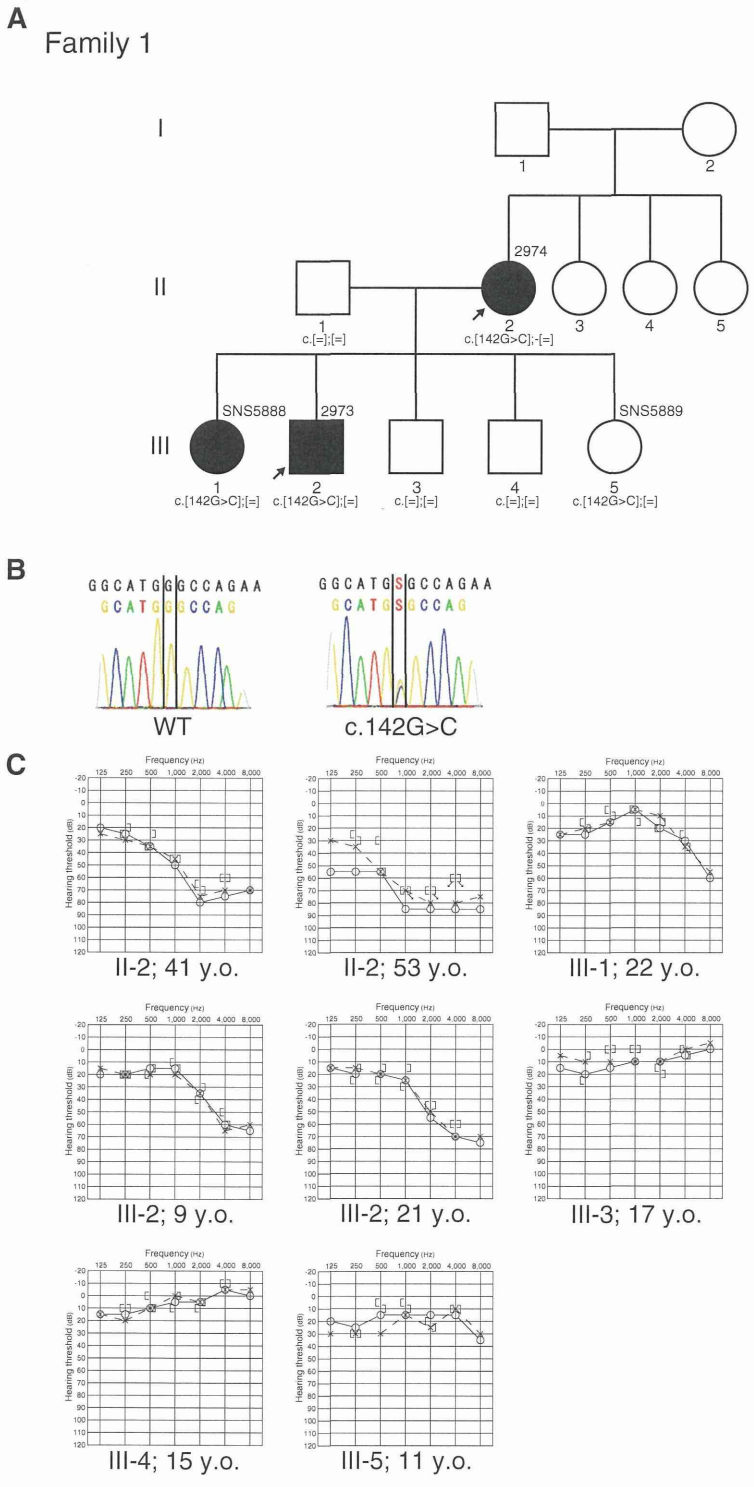

Figure 1. (A) Patient 2973 (a 21-year-old male), his mother (2974; 53 years), his older sister (22 years), and his young sister (11 years) had heterozygous c.142G>C (p.G48R) mutations. (B) The results of Sanger sequencing. (C) Audiograms of family members, showing high frequency–involved progressive hearing loss. WT, wild type.

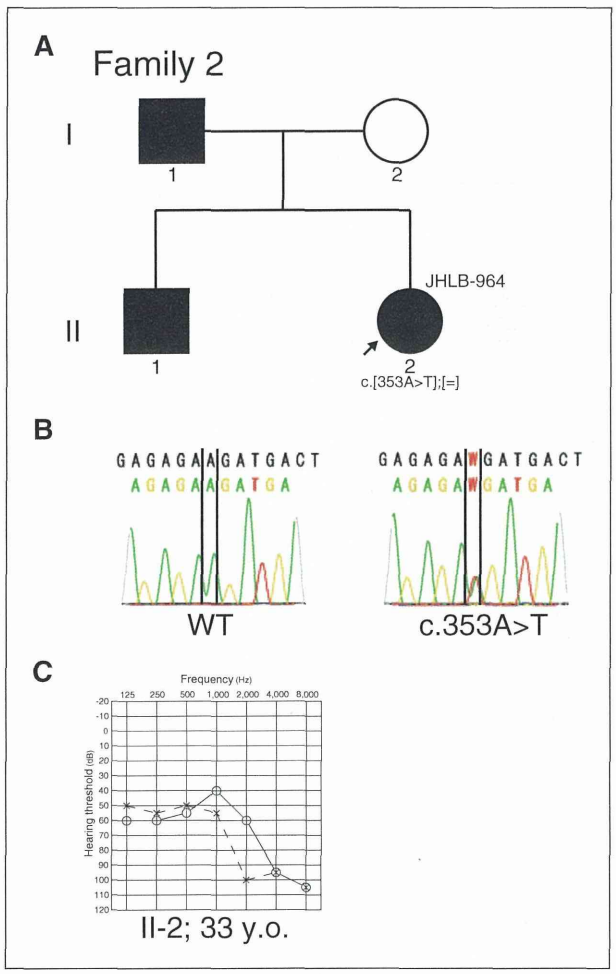

Figure 2. (A) The family tree of patient JHLB964 (a 33-year-old female). (B) The results of Sanger sequencing. (C) Audiogram of JHLB964. WT, wild type.

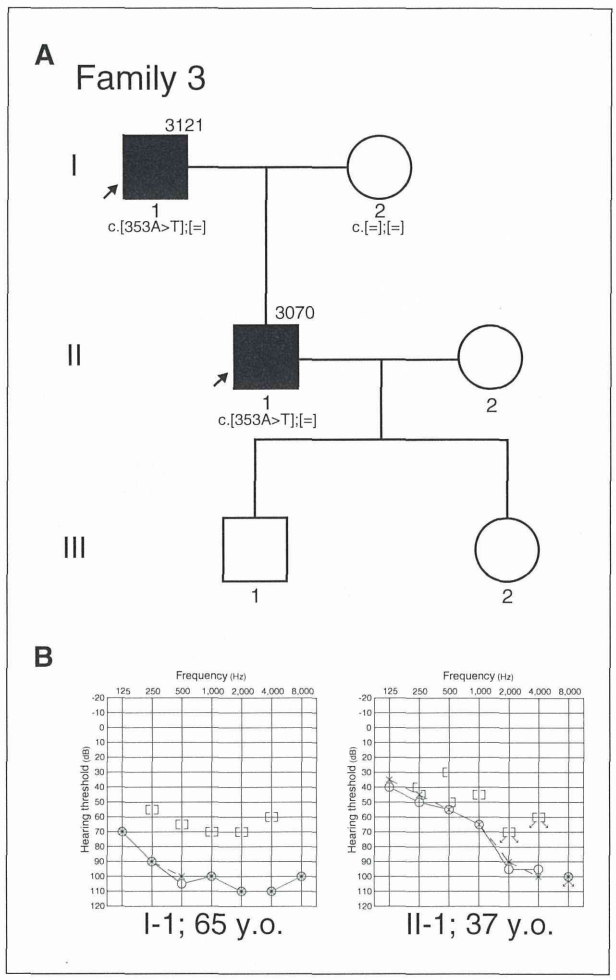

Figure 3. (A) The family tree of patient 3070 (a 37-year-old male) and his father (patient 3121). (B) Audiograms of affected members.

emissions (DPOAE) found no response in all frequencies, which confirmed that the hearing loss originated in the cochlea. He received electric acoustic stimulation (EAS) due to progressive hearing loss at the age 39: the effectiveness of EAS for this patient was previously reported.[12] His father, brother, and younger son carried the same mutation. His brother (JHLB0109) showed the same type of audiogram since he was around 15 years of age. The audiogram for the last 5 years showed slightly progressive hearing loss in the high frequencies, although hearing was preserved in the lower frequencies. His father (patient 3839) also displayed signs leading to profound hearing loss. The patient's younger son (JHLB0111) with the same causative mutation had slight hearing loss at 8 kHz and showed normal DPOAE.

None of the patients experienced vertigo, and the findings of vestibular testing (caloric test and cervical vestibular evoked myogenic potential [cVEMP]) for patient 3837 were normal.

Discussion

Targeted exon sequencing of selected genes using MPS technology successfully identified 4 *ACTG1* mutations in 5 families, indicating that this technology is a powerful tool for the identification of causative mutations in relatively rare genes. Previously, linkage analysis and mutation analysis have identified many responsible genes, and several *ACTG1* mutations have been identified. However, those classical approaches are sometimes difficult because of small family size and the fact that one-by-one gene screening is time-consuming. Screening based on MPS technology can resolve these issues, and in this study, we conducted genetic analysis of 63 deafness-causative genes using MPS-based genetic screening for Japanese patients with hearing loss. This screening identified *ACTG1* mutations in 0.4% (5/1120) of bilateral hearing loss probands

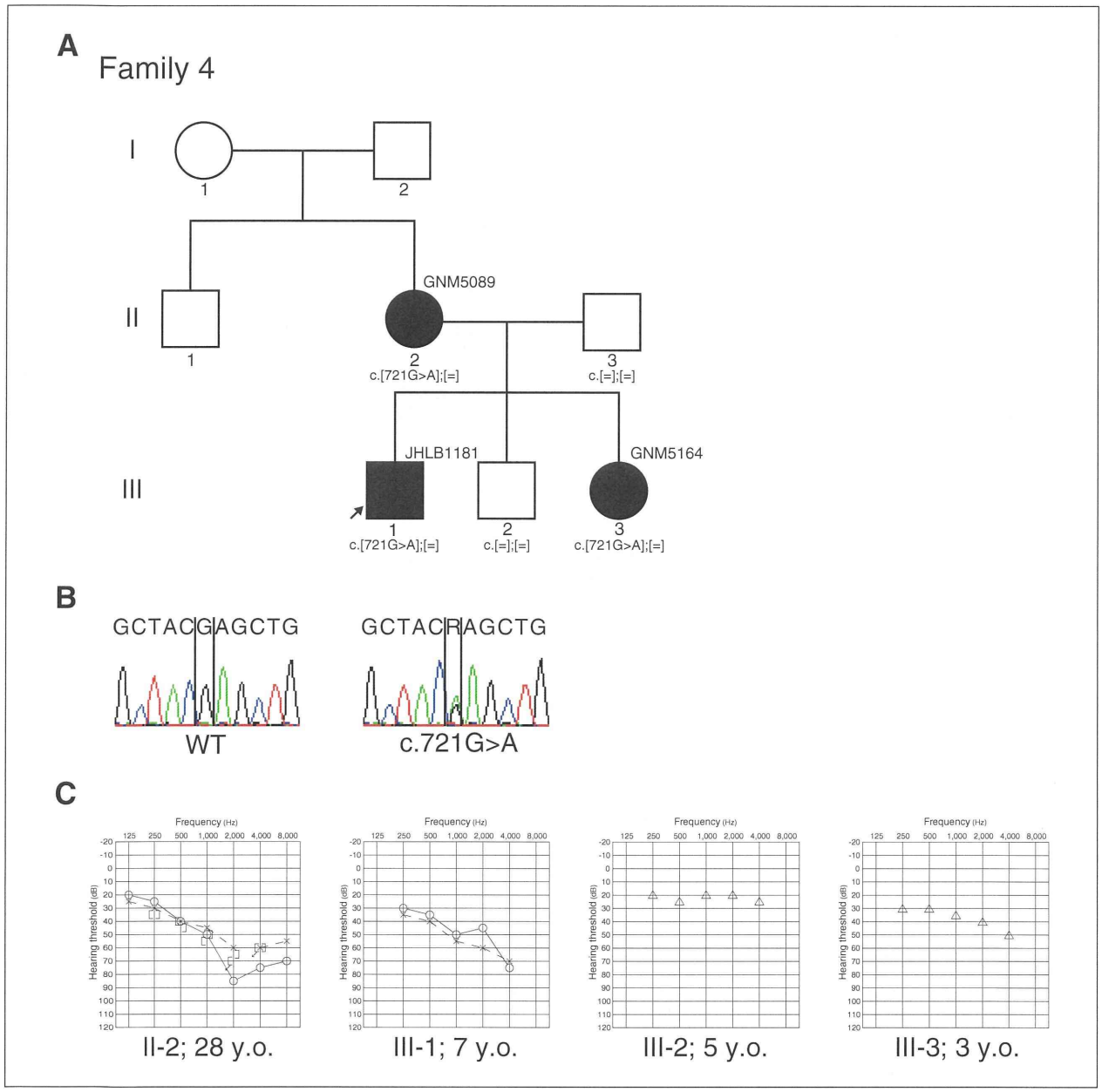

Figure 4. (A) The family tree of patient JHLB1181 (a 7-year-old boy). (B) The results of Sanger sequencing. (C) Audiograms of affected members. WT, wild type.

and in 1.9% (5/266) of patients with autosomal dominant hearing loss and not in any of the patients with congenital hearing loss.

Two (c.353A>T [p.K118M], c.721G>A [p.E241K]) out of 4 mutations had already been reported as causative mutations in the American and Spanish populations, suggesting the existence of mutational hot spots rather than ancestral mutations. Using haplotype analysis, we have previously demonstrated that a particular *KCNQ4* mutation occurred independently in different populations and, therefore, most likely represents a mutational hot spot.[27]

ACTG1 encodes γ-actin, the predominant actin isoform in auditory hair cells and, more specifically, in the cuticular plate, adherens junctions, and stereocilia.[4,5]

The γ-actin is 1 of 6 highly conserved actin proteins in humans. Four actin genes encode the isoforms responsible for contractile muscle movement, with the other 2 non-muscle actin genes, *ACTG1* and *ACTB*, encoding cytoskeleton

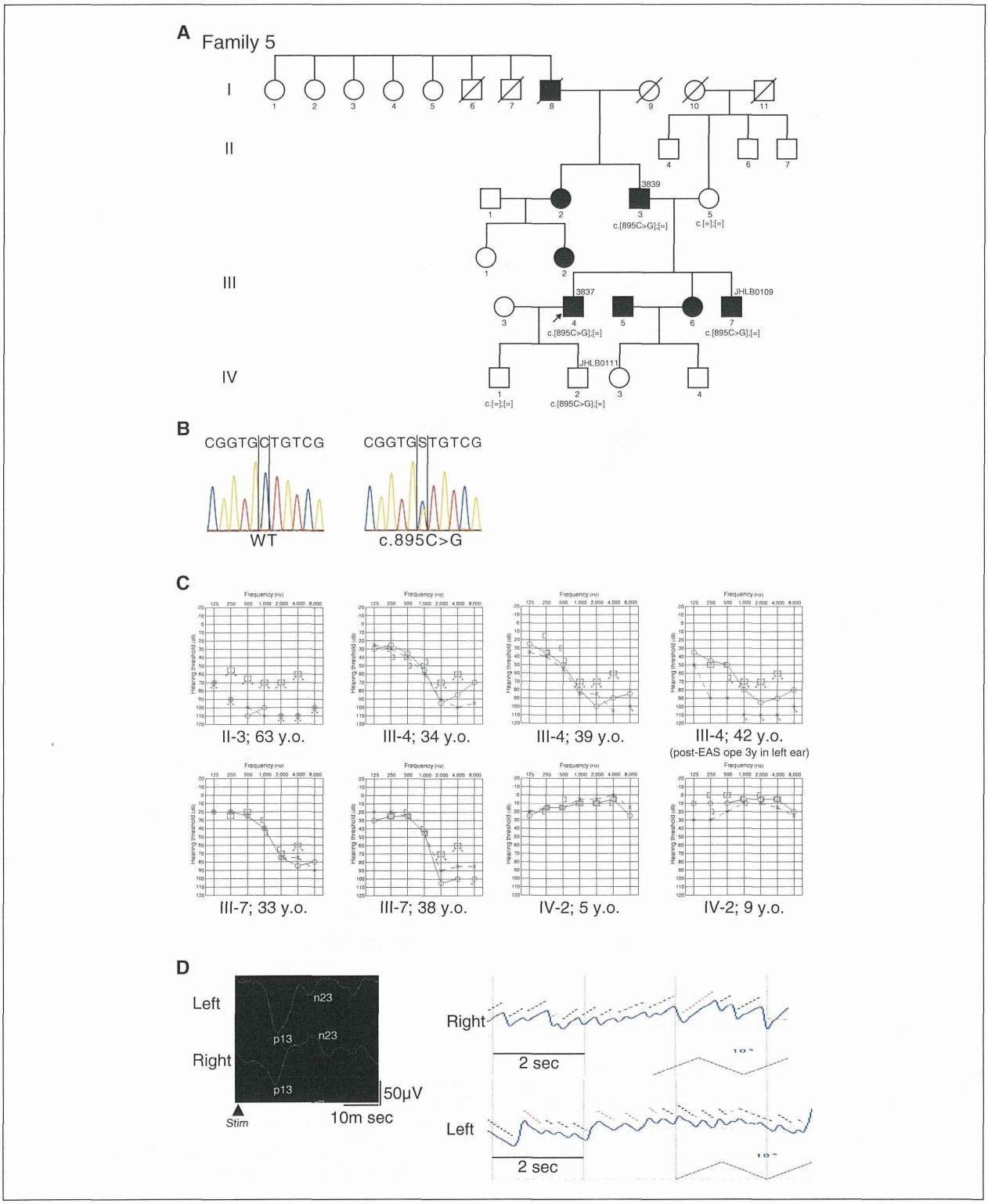

Figure 5. (A) The family tree of patient 3837 (a 42-year-old male). (B) The results of Sanger sequencing. (C) Audiograms of affected members showing progression. (D) Vestibular examination of patient 3837. His vestibular testing, including caloric testing and cervical vestibular evoked myogenic potential (cVEMP), showed normal findings. WT, wild type.

Table 2. Clinical Features for 14 Affected Patients in 5 Families.

Family No.	Patients	Nucleotide Change	Amino acid Change	Age	Onset Age	Hearing Level (dB)	Progression	Tinnitus	Intervention	Vertigo
1	2974	c.142G>C	p.G48R	53	11	56.3	Progressive	+	Hearing aids	Unknown
1	SNS5888	c.142G>C	p.G48R	22	N/A	16.3	Progressive	Unknown	None	Unknown
1	2973	c.142G>C	p.G48R	21	6	31.3	Progressive	–	None	—
1	SNS5889	c.142G>C	p.G48R	11	Precritical	15	Unknown	Unknown	None	
2	JHLB964	c.353A>T	p.K118M	33	26	62.5	Progressive	+	Hearing aids	+
3	3121	c.353A>T	p.K118M	65	17	105	Progressive	Unknown	Hearing aids	Unknown
3	3070	c.353A>T	p.K118M	37	17	77.5	Progressive	+	Hearing aids	—
4	JHLB1181	c.721G>A	p.E241K	7	3	51.3	Progressive	–	Hearing aids	—
4	GNM5164	c.721G>A	p.E241K	3	3	38.8	Unknown	Unknown	None	Unknown
4	GNM5089	c.721G>A	p.E241K	28	14	51.3	Progressive	–	Hearing aids	—
5	3837	c.895C>G	p.L299V	41	12	78.8	Progressive	+	EAS	—
5	3839	c.895C>G	p.L299V	63	46	107.5	Progressive	+	Hearing aids	—
5	JHLB0109	c.895C>G	p.L299V	38	15	61.2	Progressive	+	Hearing aids	—
5	JHLB0111	c.895C>G	p.L299V	9	Precritical	6.3	Progressive	–	None	—

Abbreviations: +, existing symptoms; –, without symptoms.

proteins found in all mammalian cells.[2,28] Notably, γ-actin is the predominant actin found in the auditory hair cells of the cochlea and intestinal epithelial cells.[29] The distinct expression pattern of γ-actin compared to the other actins is thought to account for the nonsyndromic hearing loss phenotype caused by mutations in this gene.[1] It is notable that actin structures appear to be structurally damaged as a consequence of noise exposure and aging.[30,31]

Based on previous reports, in most cases with hearing loss caused by *ACTG1* the mean age at onset of hearing loss is the first or second decade. Findings show that hearing loss first affects the high frequency portion before progressing to involve all frequencies. Audiograms show a sloping configuration with age. Audiological features of the affected patients found in this study showed a similar tendency. De Heer et al[9] performed a detailed analysis of progression and stated that the rate of deterioration varied across the different mutations from 2 dB to 6 dB/year. Among the present families, detailed time courses of deterioration could be obtained for patient 3837 and JHLB0109, with the rate of deterioration found to be 1.4 dB/year and 1.0 dB/year, respectively. Their father (63 years) had profound hearing loss, suggesting that further deterioration will occur in the future and proper intervention will be required due to the progressive nature of their hearing loss.

Tinnitus has been reported in only 1 family with a mutation in subdomain 2.[9] However, the patients in our cohort experienced tinnitus regardless of subdomain (the mutations identified in this study were located in the various subdomains, 2974; subdomain 2, JHLB964; subdomain 1, 3070; subdomain 1, 3837: subdomain 3), indicating that tinnitus is not a subdomain-specific symptom but is associated with the severity of hearing loss.

Functional studies have demonstrated that actin is involved in the relationship between the onset age and individual *ACTG1* mutations.[8]

In the present study, we also found that the age of onset in patients with c.721G>A (p.E241K) mutations was comparatively early. Since 2 affected patients passed newborn hearing screening, hearing loss is suggested to be early onset rather than congenital. In family 5, JHLB0111 did not show any hearing disorders either in pure-tone audiometry or in otoacoustic emissions (OAE) response.

Vertigo was previously reported in some cases, but no associated abnormalities were observed on vestibular testing.[9] In the present cases, vertigo was not a consistent symptom; ie, 1 patient with c.353A>T (p.K118M) had experienced vertigo, whereas the other subjects had not. It is not surprising that vertigo is associated with vestibular symptoms because the stereocilia of vestibular hair cells are also composed of γ-actin.[32] Vestibular symptoms are, however, often masked by vestibular compensation; therefore, detailed vestibular function testing is needed to evaluate their real function.

In this study, we succeeded in performing a detailed vestibular examination for patient 3837 carrying the c.895C>G (p.L299V) mutation. His vestibular testing, including caloric testing and cervical vestibular evoked myogenic potential (cVEMP), showed normal findings. As this patient had typical high frequency–involved hearing loss, this indicates the presence of discrepancies between auditory and vestibular function.

With regard to phenotypes other than hearing loss, *ACTG1* and *ACTB* have recently been reported as causative genes for Baraitser-Winter syndrome, being associated with a well-defined developmental disorder characterized by a

combination of congenital ptosis, high-arched eyebrows, hypertelorism, ocular colobomata, and brain malformation consisting of anterior predominant lissencephaly. Other typical features include postnatal short stature and microcephaly, intellectual disability, seizures, and hearing loss. According to a previous report, 50% to 83% of Baraitser-Winter syndrome patients develop hearing loss.[33] Baraitser-Winter syndrome is rare, but Baraitser-Winter syndrome caused by *ACTB* is more severe than that caused by *ACTG1*. Missense mutations in *ACTG1* have been reported in association with Baraitser-Winter syndrome.

In this report, patients JHLB1181 and GNM5089 in family 4 showed symptoms other than hearing loss. Although the developmental disorder found in JHLB1181 and the neurological disorder found in GNM5089 were not confirmed to be due to the *ACTG1* mutation, such variations in symptoms need to be treated with due caution, and further clinical evaluation of larger populations of patients harboring *ACTG1* mutations will reveal the genotype-phenotype correlations of this gene.

In patients with the nonsyndromic form of hearing loss (cases without any other symptoms), the etiology of *ACTG1* is located within the cochlea, indicating that comparatively good outcomes for hearing aids or cochlear implants (CIs) can be expected. We recently demonstrated that EAS was an effective intervention for patient 3837 with *ACTG1* mutations as EAS is compatible with an intra-membranous labyrinth etiology.[12] EAS is a new trend in therapy for the patients with residual hearing in the lower frequencies.[34] Various genes were found to be involved in the patients with EAS,[12,35] and onset age as well as the rate of progress of hearing loss appeared to vary according to the etiology.

Identification of the responsible genes may be a good predictor when choosing therapeutic options. As the rate of progression may depend on the responsible gene, this information may be helpful in timing EAS/CI surgery and in the selection of the appropriate device and/or electrode.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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The Patients Associated With *TMPRSS3* Mutations Are Good Candidates for Electric Acoustic Stimulation

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Abstract

Objectives: To clarify the frequency of *TMPRSS3* mutations in the hearing loss population, genetic analysis was performed, and detailed clinical characteristics were collected. Optical intervention for patients with *TMPRSS3* mutations was also discussed.

Methods: Massively parallel DNA sequencing (MPS) was applied for the target exon-sequencing of 63 deafness genes in a population of 1120 Japanese hearing loss patients.

Results: Hearing loss in 5 patients was found to be caused by compound heterozygous *TMPRSS3* mutations, and their detailed clinical features were collected and analyzed. Typically, all of the patients showed ski slope type audiograms and progressive hearing loss. Three of the 5 patients received electric acoustic stimulation (EAS), which showed good results. Further, the onset age was found to vary, and there were some correlations between genotype and phenotype (onset age).

Conclusions: MPS is a powerful tool for the identification of rare causative deafness genes, such as *TMPRSS3*. The present clinical characteristics not only confirmed the findings from previous studies but also provided clinical evidence that EAS is beneficial for patients possessing *TMPRSS3* mutations.

Keywords

TMPRSS3, DFNB8/10, high-frequency hearing loss, massively parallel DNA sequencing, next generation sequencing, EAS

Introduction

Hearing impairment is a general sensory defect in humans. Based on the results of several etiological studies, it has been estimated that at least 50% of congenital hearing loss is of genetic etiology.[1] More than 80 genes have already been reported to be associated with sensorineural hearing loss (SNHL).

Cochlear implantation (CI), which electrically stimulates the spiral ganglion neurons, has been established as the standard therapy for severe to profound SNHL.[2] Electric acoustic stimulation (EAS) is a hearing implant system combining a cochlear implant and acoustic amplification technology in one device and has recently become a standard intervention for the patients with partial deafness, defined as a mild to moderate low-frequency sensorineural hearing loss sloping to a profound hearing loss in the higher frequencies.[3]

TMPRSS3 is responsible for autosomal recessive hearing loss, particularly high-frequency involved hearing loss. Interestingly, *TMPRSS3* is the cause of DFNB10 (severe and congenital) and DFNB8 (mild and postlingual) phenotypes.[4]

TMPRSS3 is a type-II transmembrane serine protease, structurally defined by a transmembrane domain located near the N terminus. In a previous study, *TMPRSS3* mRNA was detected in the cell bodies of spiral ganglion neurons, the entire epithelium supporting the organ of Corti, as well as the inner hair cells of the organ of Corti and in the lower levels of the stria vascularis.[5,6] *TMPRSS3* may be involved in processing proneurotrophins and, therefore, in the development and survival of cochlear neurons.

Twenty-five mutations in *TMPRSS3* were previously reported in the Middle East, Europe, and East Asia (Table 1).[7-17] The function of the *TMPRSS3* gene in the auditory system remains unclear, but it has been reported to play a crucial role in the morphological and functional maturation of the

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inner ear as well as in the maintenance of the contents of the perilymph and endolymph.[5,18]

Recent advances in targeted exon sequencing of selected genes using massively parallel DNA sequencing (MPS) technology have enabled the successful identification of causative mutations in relatively rare genes such as *TMPRSS3*. In this study, we describe 5 patients from 4 families with *TMPRSS3* detected by MPS. We present the clinical features of the patients and discuss the appropriate forms of intervention for hearing loss caused by *TMPRSS3*.

Subjects and Methods

Subjects

A total of 1120 Japanese hearing loss (HL) patients (autosomal dominant sensorineural hearing loss, 266; autosomal recessive sensorineural hearing loss, 600; unknown, 254) from 53 otolaryngology departments nationwide participated in this study. Written informed consent was obtained from all subjects (or from their next of kin, caretaker, or guardian on behalf of minors/children) prior to enrollment in the project. This study was approved by the Shinshu University Ethical Committee as well as the respective ethical committees of the other participating institutions.

Amplicon Library Preparation

Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, California, USA) for 63 genes reported to cause nonsyndromic hearing loss according to the manufacturer's instructions. The detailed protocol was described elsewhere.[10] After preparation, the amplicon libraries were diluted to 20 pM, and equal amounts of 6 libraries for 6 patients were pooled for 1 sequence reaction.

Emulsion Polymerase Chain Reaction and Sequencing

Emulsion polymerase chain reaction (PCR) and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere.[10] MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using an Ion PGM 200 Sequencing Kit and an Ion 318 Chip (Life Technologies).

Base Call and Data Analysis

The sequence data were mapped against the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program. After sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. After variant detection, their effects were analyzed using ANNOVAR software.[19,20] The missense, nonsense, insertion/deletion, and splicing variants were selected from among the identified variants. Variants were further selected as less than 1% of (1) the 1000 genome database,[21] (2) the 6500 exome variants,[22] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[23] and (4) the 269 in-house Japanese normal hearing controls.

To predict the pathogenicity of missense variants, the following functional prediction software was used: PhyloP,[24] Sorting Intolerant from Tolerant (SIFT),[25] Polymorphism Phenotyping (PolyPhen2),[26] LRT,[27] MutationTaster,[28] and GERP++.[29]

Candidate mutations were confirmed by Sanger sequencing, and the responsible mutations were identified by segregation analysis using samples from among the patients' family members. In cases identified as heterozygous, Sanger sequencing of the coding region of the *TMPRSS3* was performed.

Outcome of EAS

Thirty-two consecutive hearing preservation surgeries in 30 of the 1120 patients with ski slope hearing loss were performed (for details, see Usami et al[30]). Twenty-nine ears in 27 patients received MED-EL PULSAR with a FLEX^{24} electrode (24 mm), 2 ears in 2 patients received a FLEX^{soft} electrode (31.5 mm), and 1 ear received a standard electrode (31.5 mm).

To evaluate speech perception outcomes, speech discrimination scores (using the 67S Japanese monosyllable test, 65dBSPL) preoperatively and at 12 months after the initial EAS stimulation were used. In this study, we compared the outcomes for 3 EAS patients with hearing loss resulting from *TMPRSS3* mutations with those for the remaining 27 patients with hearing loss from other etiologies.

Results

Detected Mutations

One nonsense and 5 missense mutations as well as 1 splice site mutation were identified (Table 1). The splice site mutation, c.617-4_-3dupAT (p.T205fs), was detected by additional Sanger sequencing. All of the detected mutations were confirmed by Sanger sequencing and were predicted to be pathologic by several software programs. Segregation analysis was consistent with them being plausible disease-causing mutations. All of the subjects with biallelic mutations were compatible with recessive inheritance patterns.

Table 1. *TMPRSS3* Mutations in Autosomal Recessive Sensorineural Hearing Loss (ARSNHL).

Exon	Domain	NM No.	Nucleotide Change	Amino Acid Change	Family Origin	Reference
4	Truncation agter TM	NM_032405	c.208delC	p.His70ThrfsX19	Spanish, Greek, Pakistani, Canada, Dutch	7, 8, 9
4	LDLRA domain	NM_032405	c.212T>C	p.F71S	Japanese	This study
4	LDLRA domain	NM_032405	c.268G>A	p.A90T	UK, Moroccan	11
4	LDLRA domain	NM_032405	c.280G>A	p.G94R	Japanese	This study
4	LDLRA domain	NM_032405	c.308A>G	p.D103G	Greek	7
4	LDLRA domain	NM_032405	c.310G>A	p.E104K	Pakistani	9
4	LDLRA domain	NM_032405	c.310G>T	p.E104X	Pakistani	9
Intron 4	SRCR	NM_032405	c.323-6G>A	p.Cys107fs	Pakistani	4
5	LDLRA domain	NM_032405	c.325C>T	p.R109W	Pakistani, Korea	12, 13
5	SRCR domain	NM_032405	c.413C>A	p.A138G	UK, Dutch	8
7	SRCR domain	NM_032405	c.581G>T	p.C194F	Pakistani	12
7	SRCR domain	NM_032405	c.595G>A	p.V199M	Dutch	8
Intron 8	Serine protease domain	NM_032405	c.617-4_-3dupAT	p.T205fs	Japanese	This study
8	Just before senine protease	NM_032405	c.646C>T	p.R216C	German	14
8	Serine protease domain	NM_032405	c.743C>T	p.T248M	Korea	13
8	Serine protease domain	NM_032405	c.753G>C	p.W251C	Tunisian	16
8	Serine protease domain	NM_032405	c.767C>T	p.A256V	Pakistani	9
9	Serine protease domain	NM_032405	c.916G>A	p.A306T	German, Korea, Dutch	8, 13, 14
12	Serine protease domain	AB038157	c.1221C>T	p.P404L	Turkish, Tunisian	16
12	Serine protease domain	NM_032405	c.1219T>C	p.C407R	Pakistani	9, 12
4	LDLRA domain	NM_032404	c.226C>T	p.Q76X	Japanese	10, this study
5	SRCR domain	NM_032404	c.390C>G	p.H130R	Japanese	This study
7	Just before serine protease	NM_032404	c.647G>T	p.R216L	Turkish, Japanese	15, this study
9	Serine protease domain	NM_032404	c.778G>A	p.A260T	Japanese	10, this study
9	Serine protease domain	NM_032404	c.830C>T	p.P277L	Turkish, Tunisian	16
Intron 8	Serine protease domain	NM_024022	c.782+8insT		Pakistani	17
11	Serine protease domain	NM_024022	c.1180_1187del8ins68		Palestinian	4
11	Truncation of serine protease	NM_024022	c.1192C>T	p.Q398X	Turkish	15
12	Serine protease domain	NM_024022	c.1273T>C	p.C425R	Pakistani	9

Abbreviations: LDLRA, low-density lipoprotain recrptor; SRCR, scavenger receptor cysteine-rich.

The compound heterozygote mutations, c.[226C>T]; [778G>A](p.[Q76X];[A260T]), found in 1 family (patient 4541, 4540), were previously reported.[10,31] However, the other 4 mutations (c.212T>C [p.F71S], c.280G>A [p.G94R]), c.390C>G [p.H130R], and c.617-4_-3dupAT [p.T205fs]) were novel causative mutations.

Clinical Findings

The clinical features and genotypes for the 5 patients are shown in Figures 1, 2, 3, and 4 and Table 2. All pedigrees showed typical autosomal recessive inheritance patterns, and all affected patients displayed progressive, symmetrical

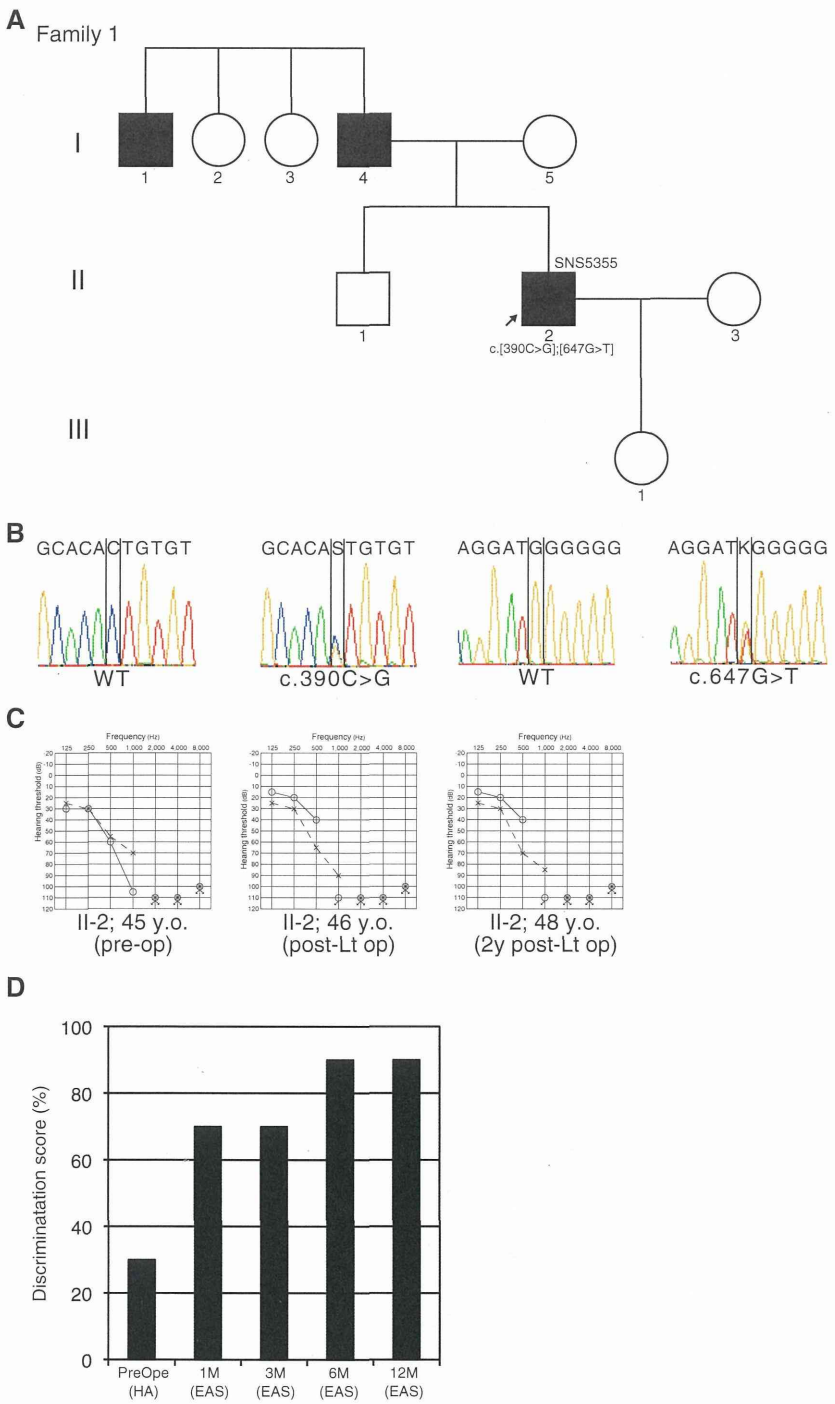

Figure 1. (A) The patient (SNS5355) shows compound heterozygous *TMPRSS3* mutations, c.[390C>G];[647G>T](p.[H130R];[R216L]). His father also developed age-related hearing loss with a different type of audiogram (not shown). (B) The results of Sanger sequencing. (C) Pre- and postoperative audiograms indicating the progressive nature of hearing loss and achievement of hearing preservation after EAS. (D) Japanese monosyllable test (65dB SPL in quiet) with bilateral EAS showing a good speech discrimination outcome after EAS. EAS, electric acoustic stimulation.

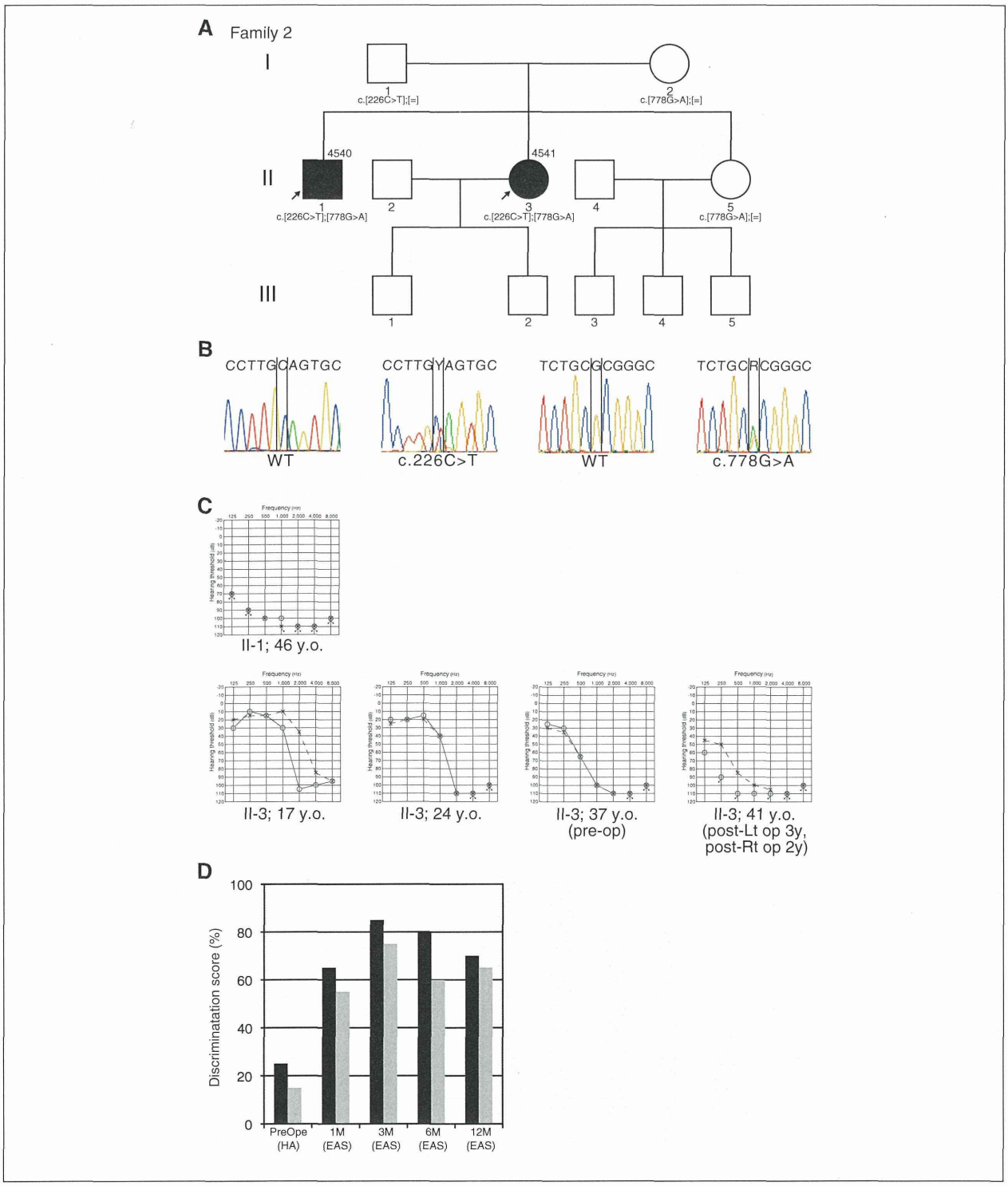

Figure 2. (A) The patient (4541) shows compound heterozygous *TMPRSS3* mutations, c.[226C>T];[778G>A](p.[Q76X];[A260T]), and the parents were found to be carriers for these mutations. The patient's brother (4540) has the same mutations. (B) The results of Sanger sequencing. (C) Audiograms of the 2 affected family members at different ages. Serial audiogram of the proband indicates the progressive nature of the hearing loss. (D) Japanese monosyllable test (65 dB SPL in quiet) for patient 4541 showing a dramatic improvement after bilateral EAS. Black, left side; gray, right side. EAS, electric acoustic stimulation.

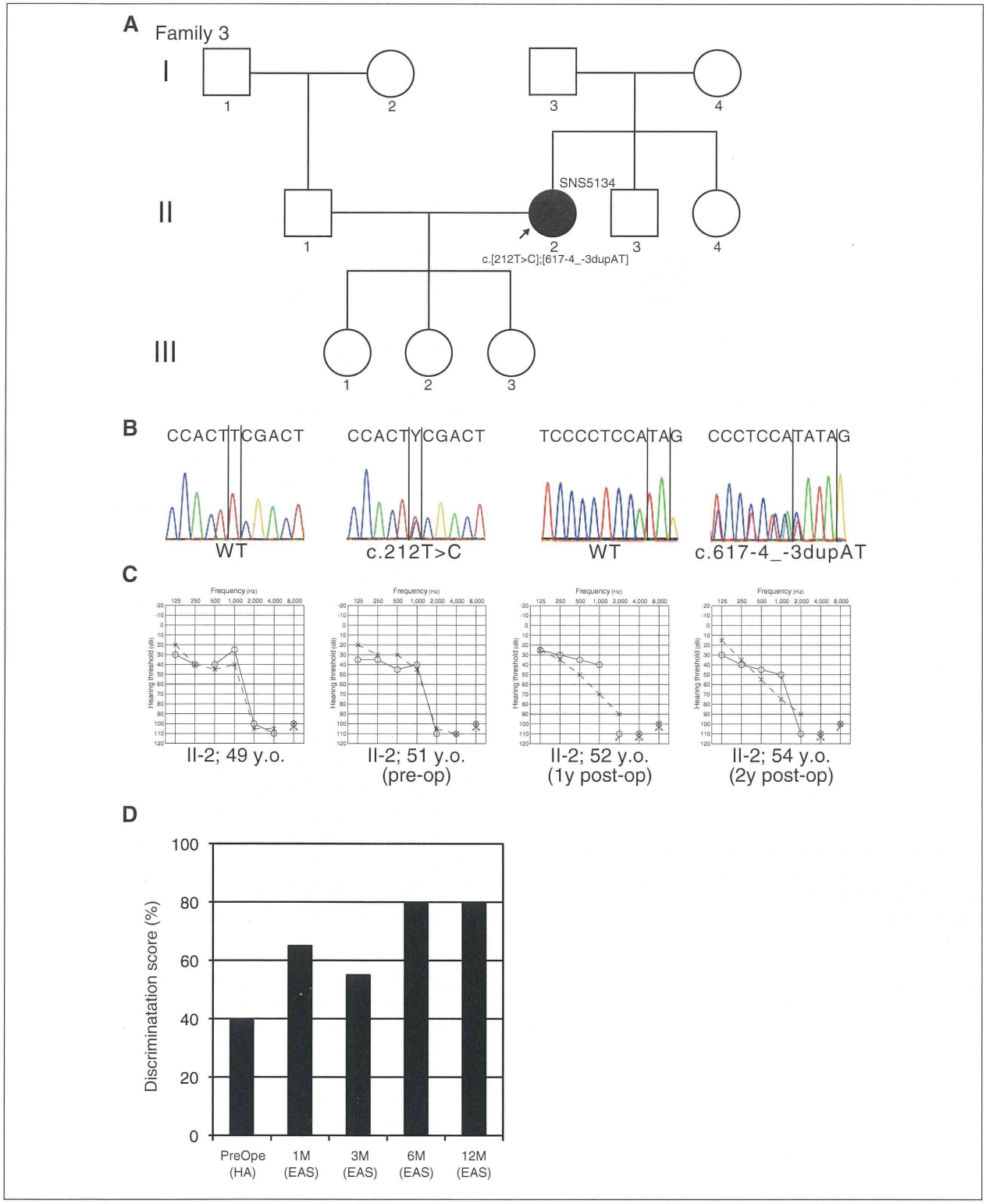

Figure 3. (A) The patient (SNS5134) shows compound heterozygous *TMPRSS3* mutations, c.[212T>C];[617-4_-3dupAT] (p.[F71S];[T205fs]). (B) The results of Sanger sequencing. (C) Pre- and postoperative audiograms indicating good hearing preservation after EAS. (D) Japanese monosyllable test (65 dB SPL in quiet) after bilateral EAS showing a dramatic improvement. EAS, electric acoustic stimulation.