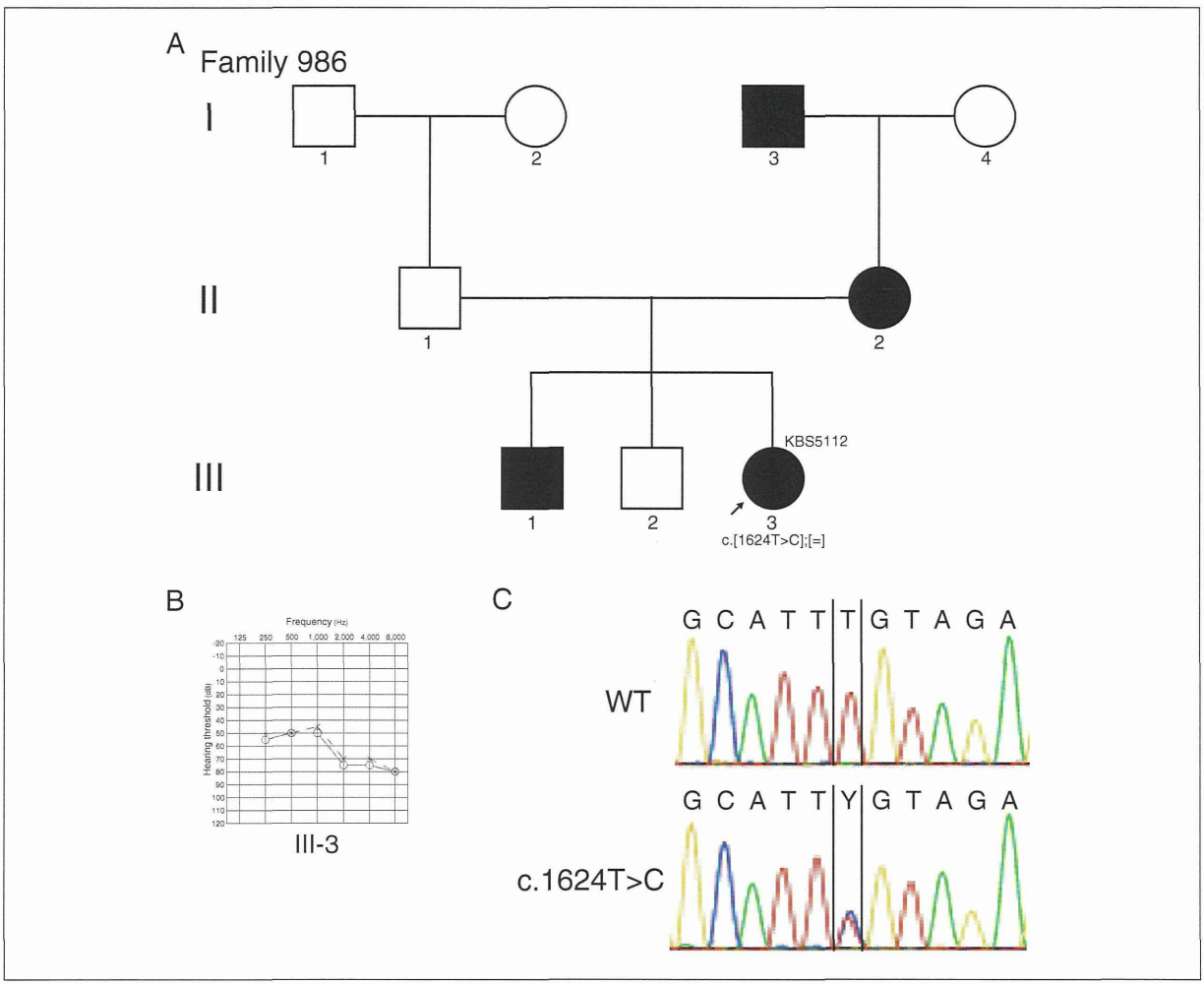

Figure 5. The (A) pedigree and (B) audiogram for the family with the p.C542R mutation. Chromatograms of the wild-type (WT) and identified p.C542R (c.T1624C) mutation in the *COCH* gene (C).

the misfolding of this domain.[20] In this study, we found 1 previous reported mutation in the LCCL domain and 2 novel mutations in the vWFA2 domain.

In the patients with *COCH* mutations, the onset age of the hearing loss ranged from the 20s to 70s,[21] whereas previous reports of the p.G88E mutation gave an estimated onset age of hearing loss in the late 40s to 50s, with the higher frequencies first affected.[2,19] In this study, the age at which the patients with p.G88E mutations became aware of their hearing loss was the early 40s to 50s, and the hearing loss started at the higher frequencies.

The onset age of the patients with the vWFA domain mutation, which was around their second to third decades, tended to be earlier than that in patients with the LCCL domain mutation at around 30 to 50 years of age. The patients with the novel vWFA domain p.I372T mutation became aware of their hearing loss in their 30s or early 40s. The onset ages of the hearing loss for these mutations were similar to those in previous reports. The patient with the p.C542R mutation became aware of her hearing loss during grade school, which is also similar to details presented in a previous report on p.C542F, a mutation at the same amino acid position, and is thought to be the lowest age of onset among those reported.

In most of the previous reports, the hearing loss was described as gradually progressing to a profound level across all frequencies at a rate of deterioration ranging from 1.8 dB to 5 dB annually.[4,19,22] However, the proband with the p.G88E mutation in the present report showed acute hearing deterioration at lower frequencies that occurred within 1 year, at 64 years old for the left and at 65 years old for the right ear, resulting in deafness. The audiological

Table 1. Clinical Features of Reported *COCH* Mutations.

Gene	Mutation	Effected protein	Domain	Phenotype	Onset age	Progressive	Vertigo	Other vestibular testing	Reference
COCH	c.151C>T	p.P51S	LCCL	Downward sloping	Third to sixth decade	Yes	Present in all	DC electrooculography	de Kok et al[4]
	c.197T>G	p.V66G	LCCL	Initially high progresses to all	Second to third decade	Yes	Present in one-third	Electronystagmography	Robertson et al[2]
	c.259G>T	p.G87W	LCCL	Initially high progresses to all	45	Yes	Present in all		Collin et al[31]
	c.260G>T	p.G87V	LCCL	Initially high progresses to all	Fourth decade	Yes	Present in all	Caloric vestibuloocular reflex	Chen et al[32]
	c.263G>A	p.G88E	LCCL	Initially high progresses to all	Fifth to sixth decade	Yes	Present in some	Electronystagmography	Robertson et al[2]
	c.266C>A	p.P89H	LCCL	Unilateral hearing loss	NA	NA	NA		Dodson et al[33]
	c.367-369del	p.V104del	LCCL	Initially high progresses to all	32	Yes	Present in all		Nagy et al[27]
	c.326T>A	p.I109N	LCCL	Initially high progresses to all	Second to third decade	Yes	Present in all		Kamarinos et al[34]
	c.326T>C	p.I109T	LCCL	Initially high progresses to all	30-43	Yes	Present in all		Pauw et al[35]
	c.349T>C	p.W117R	LCCL	Initially high progresses to all	Third decade	Yes	Present in some	Electronystagmography	Beak et al[36]
	c.355G>A	p.A119T	LCCL	Initially high progresses to all	35	Yes	Present in all	Caloric test	Usami et al[7]
	c.362T>C	p.F121S	LCCL	Initially high progresses to low and mid	Second to third decade	Yes	Present in all	Electronystagmography and caloric test	Hildebrand et al[21]
	c.889G>A	p.C162Y	Flanking vWFA1	Initially high progresses to low and mid	Around 17	Yes	Present in none		Gao et al[37]
	c.1115T>C	p.I372T	vWFA2	Downward sloping	Third to fourth decade	Yes	Present in none		This study
	c.1535T>C	p.M512T	vWFA2	Downward sloping	Second to fifth decade	Yes	Present in some		Yuan et al[38]
	c.1580T>G	p.F527Y	vWFA2	Downward sloping	Second decade	Yes	Present in none	Spontaneous nystagmus, posturography, head shaking test, Dix-Hallpike test, positional test, posturography, and rotation test	Cho et al[39]
	c.1600_1617del18		vWFA2	Initially high progresses to all	Second decade	Yes	Present in none		Gallant et al[40]
	c.1625G>T	p.C542F	Flanking vWFA2	Downward sloping	Grade school	Yes	Present in some	Oculomotor testing, computerized dynamic posturography, rotational testing, electronystagmography, VEMP, caloric testing	Street et al[29]
	c.1625G>A	p.C542Y	Flanking vWFA2	Downward sloping	Second to fifth decade	Yes	Present in some		Yuan et al[38]
	c.1624T>C	p.C542R	Flanking vWFA2	Downward sloping	Grade school	Yes	Present in 1		This study

features of the families in this study are similar to those previously reported in terms of onset age and the first affected frequencies, but the rate of progression in the patient with the p.G88E mutation differed from those of other reports.

Histological analysis of the temporal bones of patients with *COCH* mutations has revealed cellular loss with acidophilic deposits, described as a mucopolysaccharide-like substance, in the cochlea and vestibular endorgans, including the loss of cochlear dendrites.[23-26] In addition, Nagy et al[27] reported that cochlear implantation had no effect in patients with *COCH* mutations. However, the proband with the p.G88E mutation underwent cochlear implantation and showed improved postoperative speech perception, while other reports have also described improved results after cochlear implantation for the treatment of deafness in patients with *COCH* mutations.[28,29] A previous temporal bone study[23] reported that the neuron cells of the spiral ganglion remain to some degree despite the severe loss of cochlear dendrites. As the cochlear implant stimulates the spiral ganglion, the remaining spiral ganglion cells can be stimulated, resulting in an improved performance after cochlear implantation in patients with *COCH* mutations.

Patients with *COCH* mutations in the LCCL domain show vestibular symptoms more frequently than do patients with mutations in the vWFA domain.[32] Our study likewise found that the patients with p.I372T mutations in the vWFA2 domain had no vestibular symptoms whereas the patients with p.G88E mutations in the LCCL domain had severe vestibular symptoms. We performed vestibular testing in 1 family with p.G88E mutations. The proband with the p.G88E mutation experienced vestibular dysfunction, with caloric testing revealing bilateral areflexia, cVEMP failing to induce any reaction, and SOT demonstrating severe dysfunction in overall balance and balance compensation. The proband's son, also with the p.G88E mutation, was shown by caloric testing to have unilateral areflexia without any defect in saccular function or any vestibular symptoms at the age of 42 years. Previous studies have reported that vestibular impairment and progression either starts earlier and progresses more rapidly than hearing does loss[30] or that it progresses simultaneously with hearing impairment.[19] Our study also showed earlier and more severe impairment in semicircular function than in hearing in patient IV-2 with a p.G88E mutation with the vestibular function progressing to a level shown in the proband. However, none of the patients with *COCH* mutations in the LCCL domain have been reported to show vestibular symptoms.[2,19] One of the reasons for this might be the compensation of vestibular function, as seen in the patients who showed good compensation in the SOT testing, despite the existence of a unilateral semicircular defect, and had no vestibular symptoms.

Previous cVEMP data were available for 2 patients from a family with mutations in the vWFA domain, and no cVEMP was observed in either patient despite 1 of them having well-preserved hearing at the lower frequencies.[29] In this study, the proband with profound hearing loss also demonstrated bilateral saccular defects. On the other hand, cVEMP reaction was well preserved bilaterally in the proband's son (IV-2) with a p.G88E mutation, whose hearing at the lower frequencies was also preserved. The reason for the intact saccular function in this patient, despite unilateral semicircular dysfunction, remains unclear. Further follow-up is necessary to clarify the change in saccular function.

In conclusion, NGS successfully identified mutations in the relatively rare deafness gene, *COCH*, in the Japanese hearing loss population. The phenotype is compatible with that described in previous reports. Additional supporting evidence concerning the progression of hearing loss and deterioration of vestibular function was obtained for the family in our study. Good outcomes after cochlear implantation indicated that it is an appropriate intervention option for patients with *COCH* mutations.

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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Mutations in *LOXHD1* Gene Cause Various Types and Severities of Hearing Loss

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Abstract

Objective: We present 2 families that were identified with novel mutations in *LOXHD1* as a cause of nonprogressive hearing loss.

Methods: One thousand three hundred fourteen (1314) Japanese subjects with sensorineural hearing loss from unrelated families were enrolled in the study. Targeted genomic enrichment and massively parallel sequencing of all known nonsyndromic hearing loss genes were performed to identify the genetic cause of hearing loss.

Results: Two patients in 1 family affected with homozygous mutation c.879+1G>A in *LOXHD1* showed profound congenital hearing loss, whereas 2 patients in another family with compound heterozygous mutations, c.5869G>T (p.E1957X) and c.4480C>T (p.R1494X), showed moderate to severe hearing loss.

Conclusion: Mutations in *LOXHD1* are extremely rare, and these cases are the first identified in a Japanese population. The genotype-phenotype correlation in *LOXHD1* is still unclear. The differences in phenotypes in each patient might be the result of the nature of the mutations or the location on the gene, or be influenced by a genetic modifier.

Keywords

hearing loss, genetics, *LOXHD1*, DFNB77, massively parallel sequencing

Introduction

Sensorineural hearing loss (SNHL) associated with autosomal recessive (AR) inheritance is commonly supposed to have a phenotypic feature resulting in severe to profound hearing loss. The most common genetic cause of hearing loss is *GJB2*, which results in prelingual severe to profound SNHL, and most of these cases present with nonprogressive hearing loss.[1] Several studies have reported that some genetic causes associated with AR inheritance might be naturally occurring progressive hearing loss, such as *SLC26A4, CDH23*, and *MYO3A*. The *LOXHD1* gene mapped at chromosome 18q12-21 is known to be the cause of DFNB77, a progressive form of autosomal recessive nonsyndromic SNHL.[2] Mutations in *LOXHD1* are extremely rare: only 5 reports, 6 pedigrees. *LOXHD1* consists of 2211 amino acids that encodes 15 polycystin-1/lipoxygenase/alpha-toxin (PLAT) domains. Each single PLAT domain consists of about 120 amino acids and interacts with the plasma membrane. In a mouse study, *Loxhd1* was expressed along the membrane of mature hair cell stereocilia. It played an important role in maintaining normal hair cell function in the cochlea.[2] SNHL, caused by the mutations in *LOXHD1*, had a phenotypic feature showing progressive hearing loss at mid to high frequencies during childhood.[2,3] On the other hand, nonprogressive congenital profound hearing loss has been reported as well.[4]

Here, we describe 2 families that were identified with novel mutations in *LOXHD1* as a cause of progressive hearing loss using massively parallel sequencing (MPS).

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Subjects and Methods

Subjects

We recruited 2 groups from a Japanese hearing loss population for this study. All subjects had presumed nonsyndromic SNHL. For each proband, informed consent was obtained to participate in this study, which was approved by the human subjects ethical committee associated with each clinic. Clinical information and blood samples were obtained for each proband and for all consenting affected and unaffected relatives.

In the first group, 1120 Japanese hearing loss patients (autosomal dominant [AD] SNHL, 266; ARSNHL, 600; unknown, 254) from 53 ENT departments nationwide participated. For the second group, 194 Japanese subjects from unrelated families were ascertained through 33 otolaryngology clinics in 28 prefectures across Japan.

Methods

Group 1

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.[5] 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 (PCR) and sequencing. Emulsion PCR and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere.[5] MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using the Ion PGM 200 Sequencing Kit and Ion 318 Chip (Life Technologies).

Group 2

Targeted genomic enrichment and MPS. Genomic DNA was assessed for quality by gel electrophoresis and spectrophotometry (Nanodrop 1000; Thermo Fisher Scientific, Waltham, Massachusetts, USA; 260/280 ratio of 1.8-2.2) and for quantity by fluorometry (Qubit 2.0 Fluorometer; Life Technologies). Targeted genomic enrichment of all exons of all genes implicated in nonsyndromic SNHL, including nonsyndromic SNHL mimics, was completed as described, targeting 89 genes as part of the OtoSCOPE v5 platform. Libraries were prepared using a modification of the solution-based Agilent SureSelect target enrichment system (Agilent Technologies, Santa Clara, California).[6] Of the 194 samples, 58 samples were processed manually; the remainder were prepared robotically using the Sciclone NGS Workstation.

In brief, 3 μg of gDNA was randomly fragmented to an average size of 250 bp (Covaris Acoustic Solubilizer; Covaris Inc, Woburn, Massachusetts, USA), fragment ends were repaired, A-tails were added, and sequencing adaptors were ligated before the first amplification. Solid phase reverse immobilization purifications were performed between each enzymatic reaction. Hybridization and capture with RNA baits was followed by a second amplification before pooling for sequencing. Minimal amplification was used—typically 8 cycles for the prehybridization PCR (range, 8-10 cycles) using NEB Phusion HF Master Mix (New England BioLabs Inc, Ipswich, Massachusetts, USA) and 14 cycles for the posthybridization PCR (range, 12-16 cycles) using Agilent Herculase II Fusion DNA Polymerase. All samples were barcoded and multiplexed before sequencing on either an Illumina MiSeq or HiSeq (Illumina Inc, San Diego, California, USA) in pools of 4 to 6 or 48, respectively, using 100-bp paired-end reads.

Base Call and Data Analysis

Group 1. The sequence data were mapped to the human genome sequence (build GRCh37/hg19) with a Torrent Mapping Alignment Program. After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. After variant detection, variant effects were analyzed using ANNOVAR software.[7,8] 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,[9] (2) the 6500 exome variants,[10] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[11] 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,[12] Sorting Intolerant from Tolerant,[13] Polymorphism Phenotyping (PolyPhen20),[14] LRT,[15] MutationTaster,[16] and GERP++.[17]

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

Group 2. Data were analyzed as described using a local installation of the open-source Galaxy software and the following open-source tools: BWA[18] for read mapping, Picard for duplicate removal, GATK[19] for local realignment and variant calling, and NGSRich[20] for enrichment statistics.[21] We reported and annotated variants with custom software.

Variant Confirmation

All pathogenic variants were confirmed by Sanger sequencing and segregation analysis using exon-specific custom primers.

Results

We identified 4 patients, from 2 families, who had causative mutations in *LOXHD1* in 1314 hearing loss Japanese.

Case Details

Family No. 963; AH 4029, AH 4028. AH 4029 was a 9 year-old boy. He had not undergone newborn hearing screening. At the age of 6, hearing loss was detected at a school check-up. He had been referred to the Iwate Medical University Hospital, Department of Otolaryngology for further examinations. His tympanic membranes were normal. An auditory brain stem response (ABR) with click stimuli showed bilateral hearing loss that was approximately 40 dBnHL in both ears, and pure-tone audiometry (PTA) showed bilateral moderate sensorineural hearing loss (Figure 1). He was promptly fitted with a left hearing aid when he was 7 years, 3 months old. He showed 100% in word scores at the 50 dB sound field threshold. His hearing was unchanged from 6 to 9 years old. He didn't have any episodes of dizziness or vertigo attacks. Computed tomography (CT) findings of the middle and inner ear showed no abnormalities.

AH 4028 was a 3-year-old girl. She was a younger sister of AH 4029, and her parents noticed that she didn't have any intelligible words until she was 1 year, 6 months old. She visited the Iwate Medical University Hospital at the same time that AH 4029 received his check-up. Her bilateral tympanic membranes were normal. A previous ABR test at 2 years of age had shown bilateral severe hearing loss that was approximately 80 dBnHL in both ears, and PTA had also shown bilateral severe SNHL at the age of 3 (Figure 1). Her hearing had been unchanged from 3 to 5 years old. She started to wear a right hearing aid when she was 3 years old. CT findings of the middle and inner ear showed no abnormalities.

Family No. 143; 2061, 2059. Patient 2061 was a 70-year-old male. He was born in 1929. PTA showed bilateral profound SNHL (Figure 2). In his medical examination by interview, he demonstrated bilateral congenital nonprogressive hearing loss. He didn't have any episodes of vertigo. He had not undergone CT of the temporal bone.

Patient 2059 was a 60-year-old female. She was the younger sister of patient 2061 and was born in 1939. PTA showed bilateral profound SNHL (Figure 2). In her medical examination by interview, she also demonstrated congenital nonprogressive hearing loss. She didn't have any episodes of vertigo.

As shown in Figure 2, the pedigree tree showed consanguineous parental ancestry of patient 2061 and patient 2059 with profound hearing loss. They were both unmarried. We didn't obtain any information about the symptoms of hearing loss during childhood because of World War II.

Mutation Analysis

We identified compound heterozygous mutations, c.5869G>T, p.E1957X and c.4480C>T, p.R1494X in the *LOXHD1* gene in 2 patients from family No. 963.

We also identified homozygous splice site mutations: c.879+1G>A in the *LOXHD1* gene in 2 patients from family No. 143.

Discussion

We identified causative compound heterozygous p.R1494X and p.E1957X and homozygous c.879+1G>A mutations in the *LOXHD1* gene. These are the first cases found in the Japanese population.

As shown in Table 1, we summarized the mutations in *LOXHD1* that have been previously reported. The mutations of p.E1957X and c.879+1G>A were novel, and p.R1494X had been reported in an American family.[22]

LOXHD1 is expressed along the plasma membrane of the stereocillia. It is considered that *LOXHD1* may couple the plasma membrane to the underlying F-actin cytoskeleton. Although stereocilliary development was unaffected in a mouse model (*samba* mouse), hair cell function was disturbed and hair cells eventually degenerated.[2]

In this study, affected individuals in family No. 143 showed profound congenital SNHL, but affected individuals in family No. 963 showed different degrees of SNHL; hearing loss in AH 4028 was about 30 dB more severe than in her older sibling. Vozzi et al[3] reported 3 patients, in a consanguineous family, who had early-onset progressive SNHL, which differed in degree in spite of having the same genotype, homozygous nonsense alleles (c.1588G>T, p.E530X). On the other hand, nonprogressive congenital SNHL was also reported in other homozygous nonsense alleles (c.4714C>T, p.R1572X).[4] In *samba* mice, a homozygous missense mutation in *Loxhd1* caused profound deafness shortly after birth. Nevertheless, homozygous nonsense mutations in *Loxhd1* caused progressive hearing loss.[2] The cases in family No. 143 had splice site mutations and were totally deafened by their sixties. It is possible that their hearing deteriorated in childhood, resulting in profound hearing loss at a younger age. The genotype-phenotype correlation in *LOXHD1* is still unclear. The differences in phenotypes in each affected individual might be the result of the nature of the mutations and the location on the gene, or result from agenetic modifier.[23]

Two affected individuals in family No. 963 were fitted with hearing aids, and both of them were able to benefit from them. If their hearing loss progresses in future, a cochlear implant could be considered for them to acquire hearing ability. Eppsteiner et al[22] reported that a patient with compound heterozygous mutations in *LOXHD1* was a good CI performer (HINT[90], CNC[73], combined[81.3]).

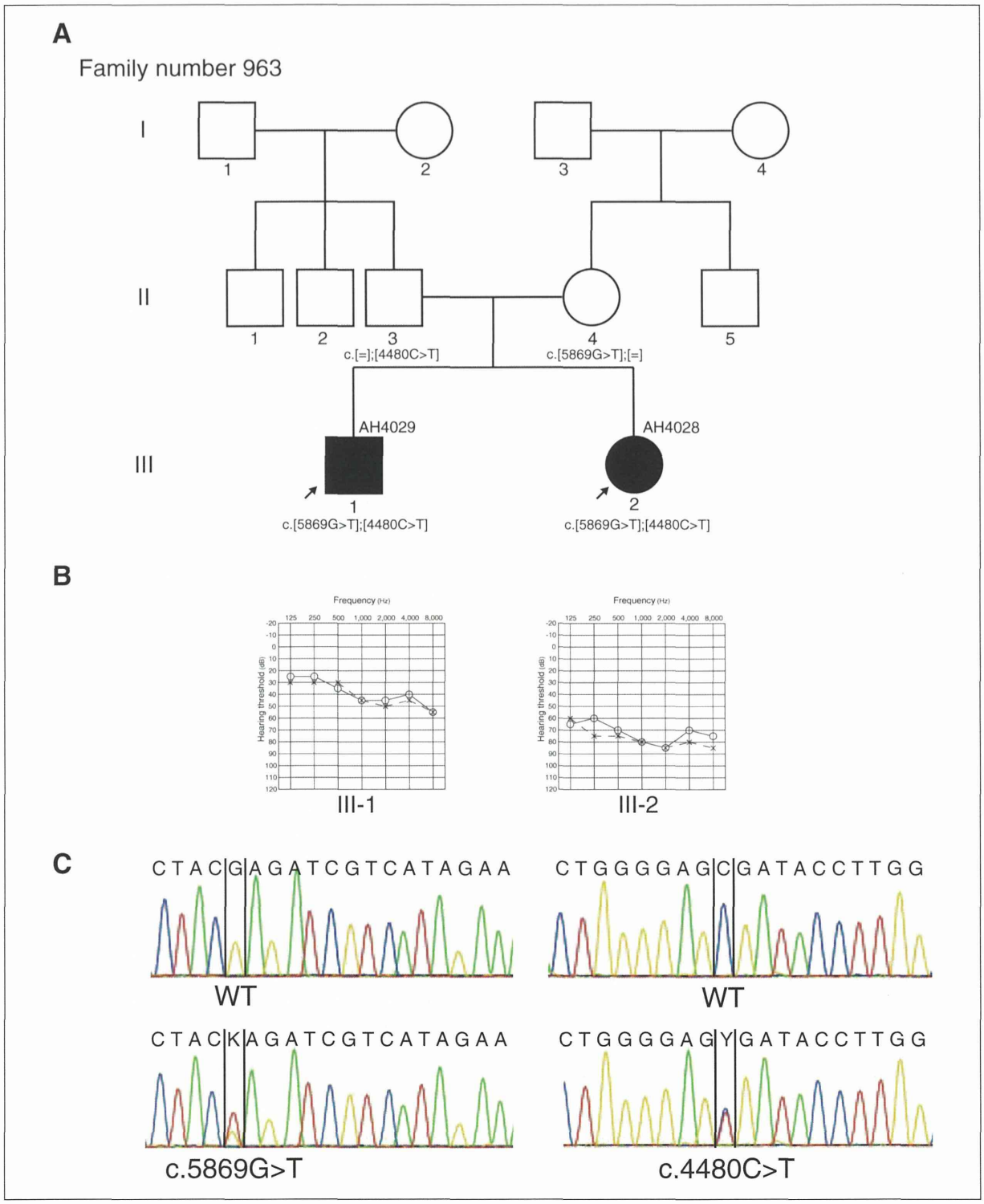

Figure 1. Pedigree and clinical findings of family No. 963. (A) Pedigree shows autosomal recessive inherited cases in this family. (B) Pure-tone audiometry (PTA) shows bilateral moderate sensorineural hearing loss in AH 4029 and bilateral severe sensorineural hearing loss in AH 4028. (C) The electropherogram shows mutations in 2 cases. AH 4029 and AH 4028 had identical compound heterozygous mutations, c.5869G>T, p.E1957X and c.4480C>T, p.R1494X. Each parent had 1 of the heterozygous mutations.

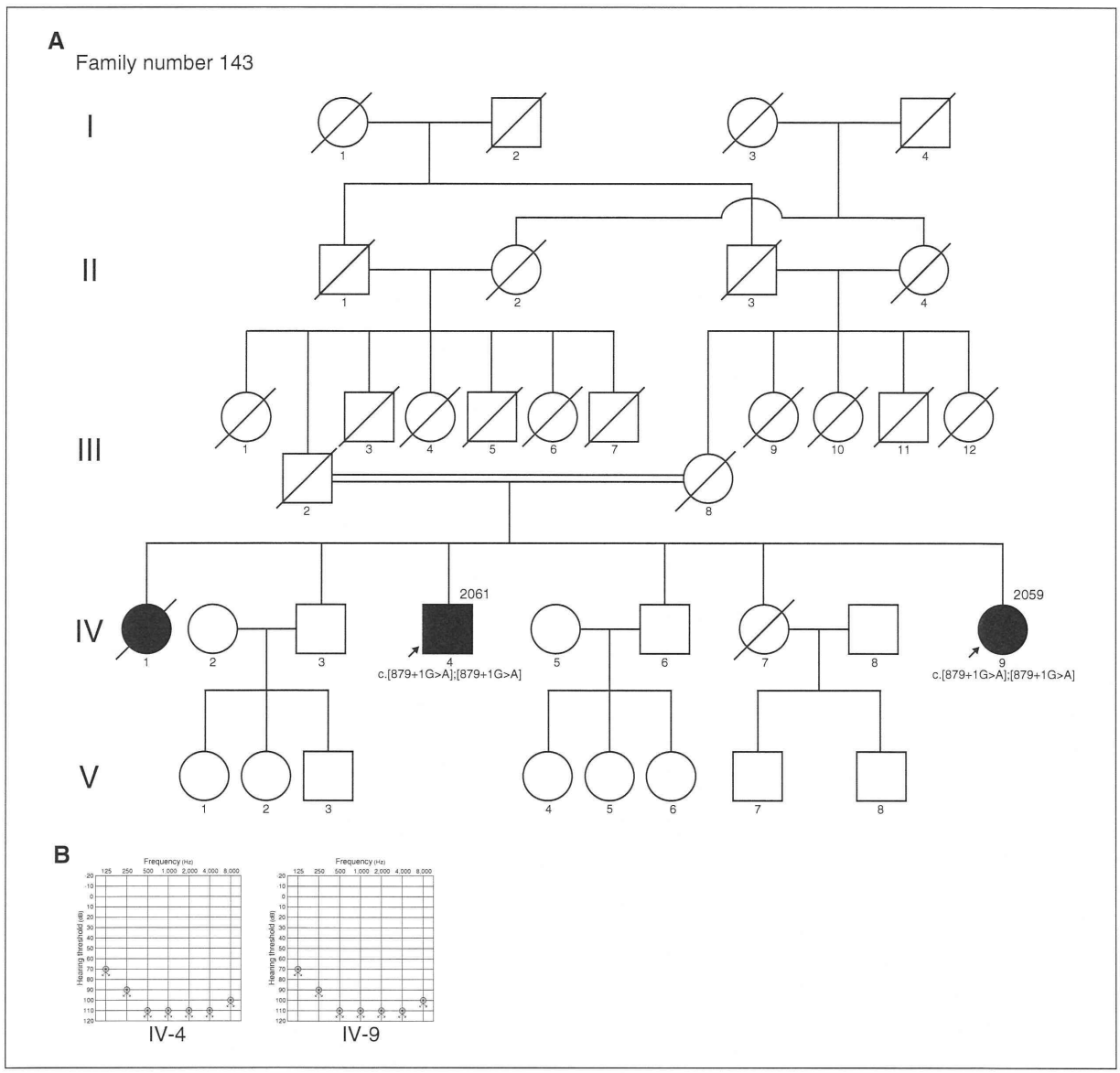

Figure 2. Pedigree and clinical findings of family No. 143. (A) Pedigree shows autosomal recessive inherited cases in this family. (B) Pure-tone audiometry (PTA) shows bilateral profound sensorineural hearing loss in both cases.

Affected individuals in family No. 963 and family No. 143 had no episodes of dizziness or vertigo attacks. No vestibular dysfunction is yet to be reported. Grillet et al[2] described *Loxhd1* expression was detected in the stereocillia of vestibular hair cells. However, under immunofluorescence microscopy, the expression level in vestibular hair cells was much weaker than that in cochlear hair cells.[2] Thus, *LOXHD1* might not associate with the vestibular system.

Mutations in *LOXHD1* have been reported to be responsible for late-onset Fuchs corneal dystrophy (FCD).[24] FCD is a genetic disorder of the corneal endothelium and is the most common cause of corneal transplantation. Recently, Stehouwer et al[25] reported that the rate of hearing disability in the FCD group was higher than that in the control group. This suggested an association between FCD and hearing loss. It is important that patients with hearing loss caused by *LOXHD1* have an ophthalmology check-up. Furthermore, FCD cases with hearing loss should be screened for *LOXHD1* mutation. These phenotypic features are important for genetic counseling. Further following examination should be necessary for the relevance to hearing loss and FCD caused by *LOXHD1* mutations.

Table 1. Known Mutations in the *LOXHD1* Gene in Hearing Loss.

Nucleotide Change	Amino Acid Change	Domain	Type of Mutation	Zygosity	NM No.	HL Onset	Type of HL	Progressiveness	Population	Reference
c.879+1G>A			Canonical-splice	Homozygous	NM_001145472	Congenital	Profound	Nonprogressive	Japanese	This study
c.1588C>T	p.E530X	PLAT4	Nonsense	Homozygous	NM_144612	Childhood	Severe-profound	Progressive	Qatar	Vozzi et al,[3] 2014
c.2008C>T	p.R670X	PLAT_repeat	Nonsense	Homozygous	NM_144612	Childhood	Moderate-profound	Progressive	Iranian	Grillet et al,[2] 2009
c.2863G>T	p.E955X	PLAT 7	Nonsense	Homozygous	NM_144612	na	na	na	Turkey	Diaz-Horta et al,[26] 2012
c.4480C>T	p.R1494X	PLAT 11	Nonsense	Homozygous	NM_144612	na	na	na	Turkey	Diaz-Horta et al,[26] 2012
c.4480C>T	p.R1494X	PLAT 11	Nonsense	Heterozygous	NM_144612	40 y.	Severe-profound	Progressive	American	Eppsteiner et al,[22] 2012
c.4480C>T	p.R1494X	PLAT 11	Nonsense	Heterozygous	NM_144612	na	Moderate-severe	Nonprogressive	Japanese	This study
c.4526G>A	p.G1509E	PLAT 11	Missense	Heterozygous	NM_144612	40 y.	Severe-profound	Progressive	American	Eppsteiner et al,[22] 2012
c.4714C>T	p.R1572X	PLAT 11	Nonsense	Homozygous	NM_144612	Prelingual	Severe-profound	Nonprogressive	Ashkenazi Jews	Edvardson et al,[4] 2011
c.5869G>T	p.E1957X	PLAT 14	Nonsense	Heterozygous	NM_144612	na	Moderate-severe	Nonprogressive	Japanese	This study

Abbreviations: HL, hearing loss; na, not available; PLAT, polycystin/lipoxygenase/alpha-toxin.