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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

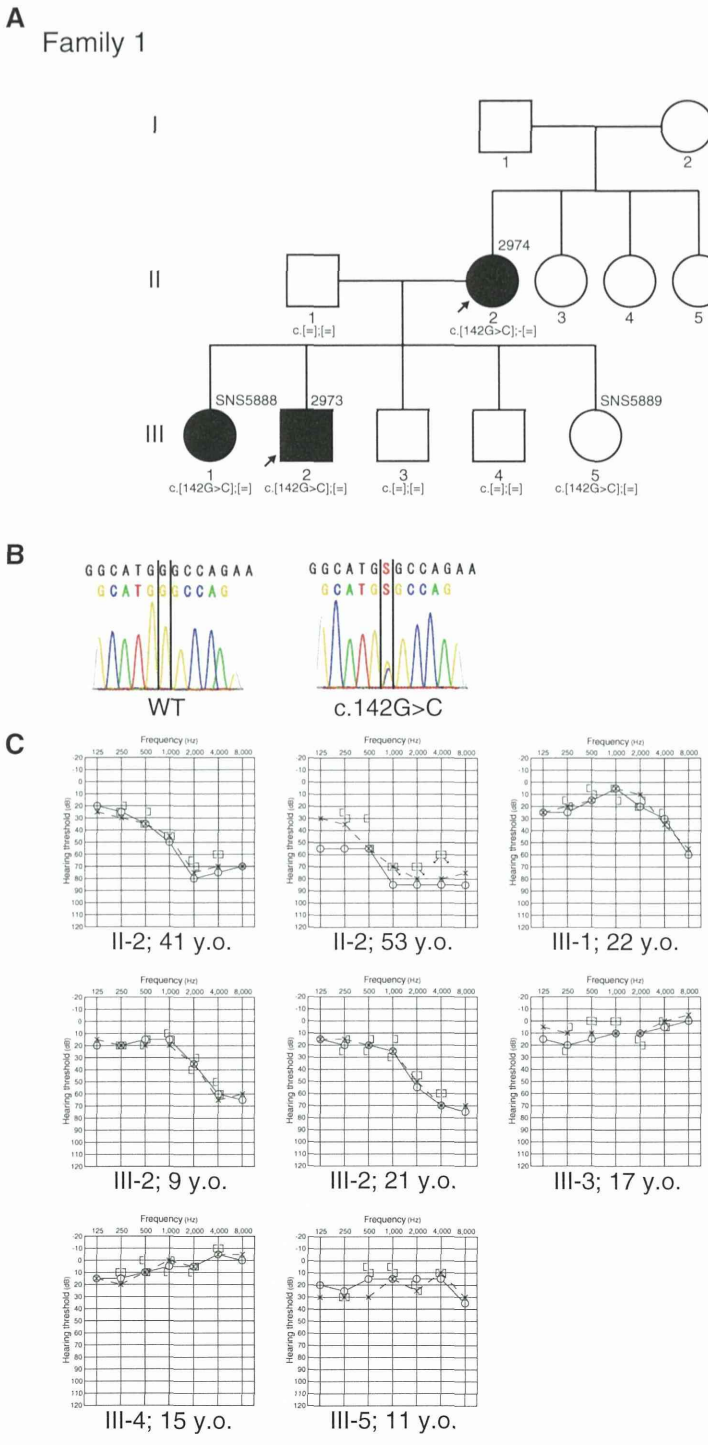

Figure 1. (A) Patient 2973 (a 21-year-old male), his mother (2974; 53 years), his older sister (22 years), and his young sister (11 years) had heterozygous c.142G>C (p.G48R) mutations. (B) The results of Sanger sequencing. (C) Audiograms of family members, showing high frequency–involved progressive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

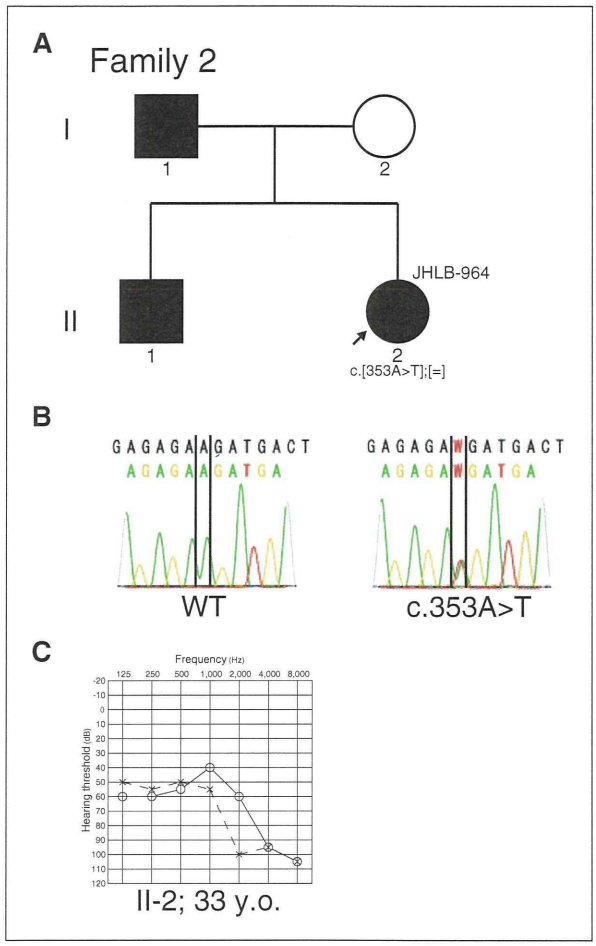

Figure 2. (A) The family tree of patient JHLB964 (a 33-year-old female). (B) The results of Sanger sequencing. (C) Audiogram of JHLB964. WT, wild type.

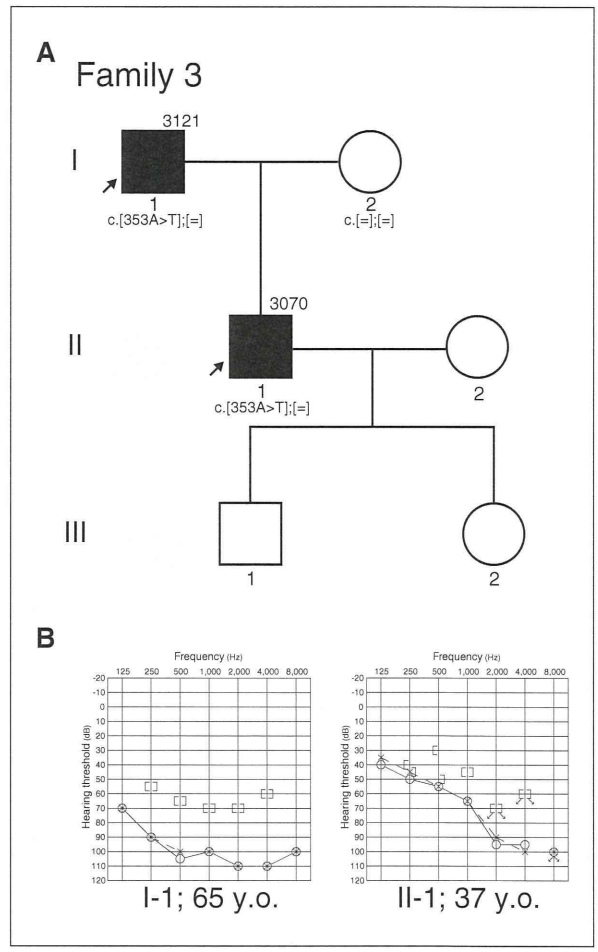

Figure 3. (A) The family tree of patient 3070 (a 37-year-old male) and his father (patient 3121). (B) Audiograms of affected members.

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

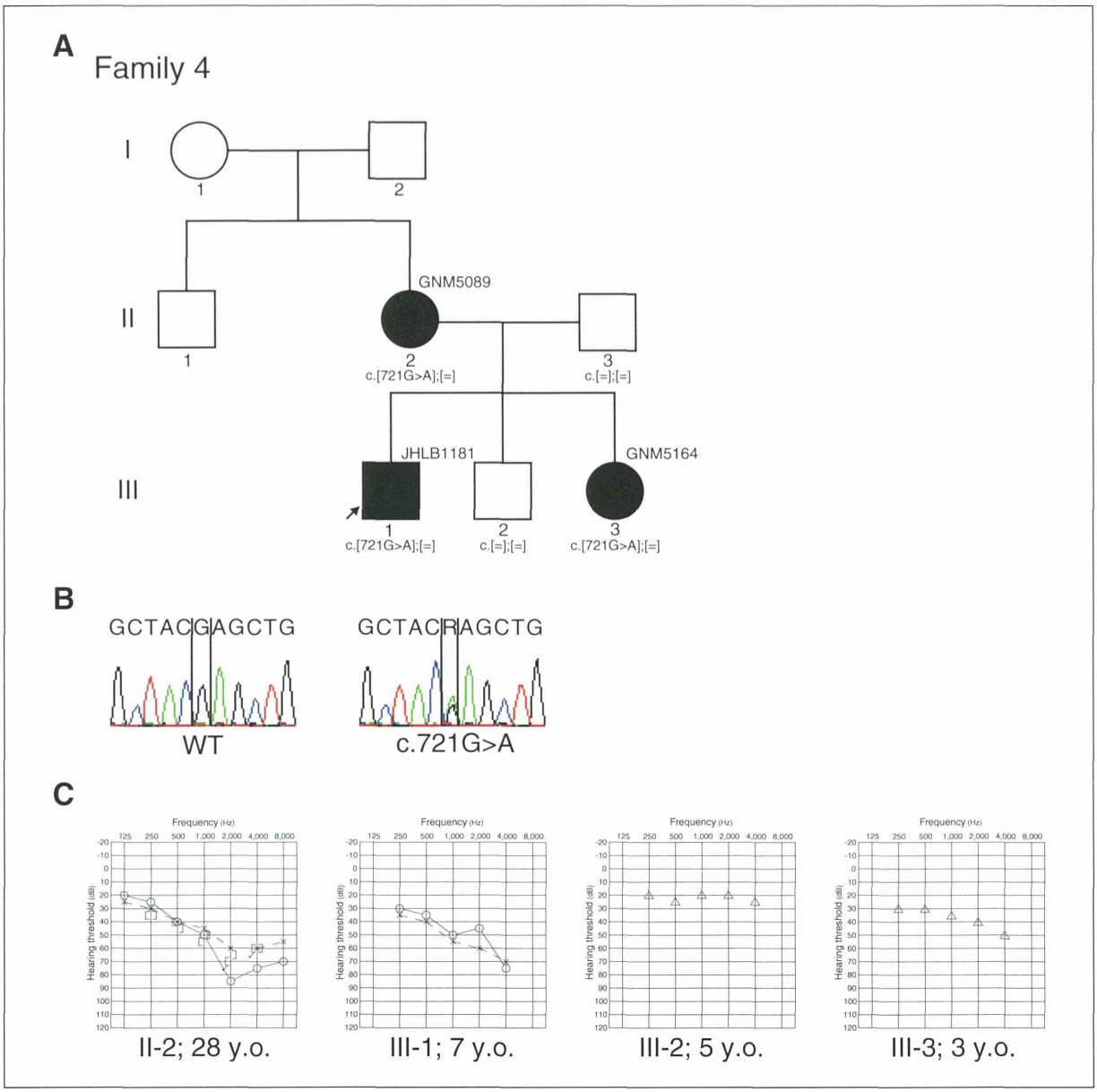

Figure 4. (A) The family tree of patient JHLB1181 (a 7-year-old boy). (B) The results of Sanger sequencing. (C) Audiograms of affected members. WT, wild type.

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

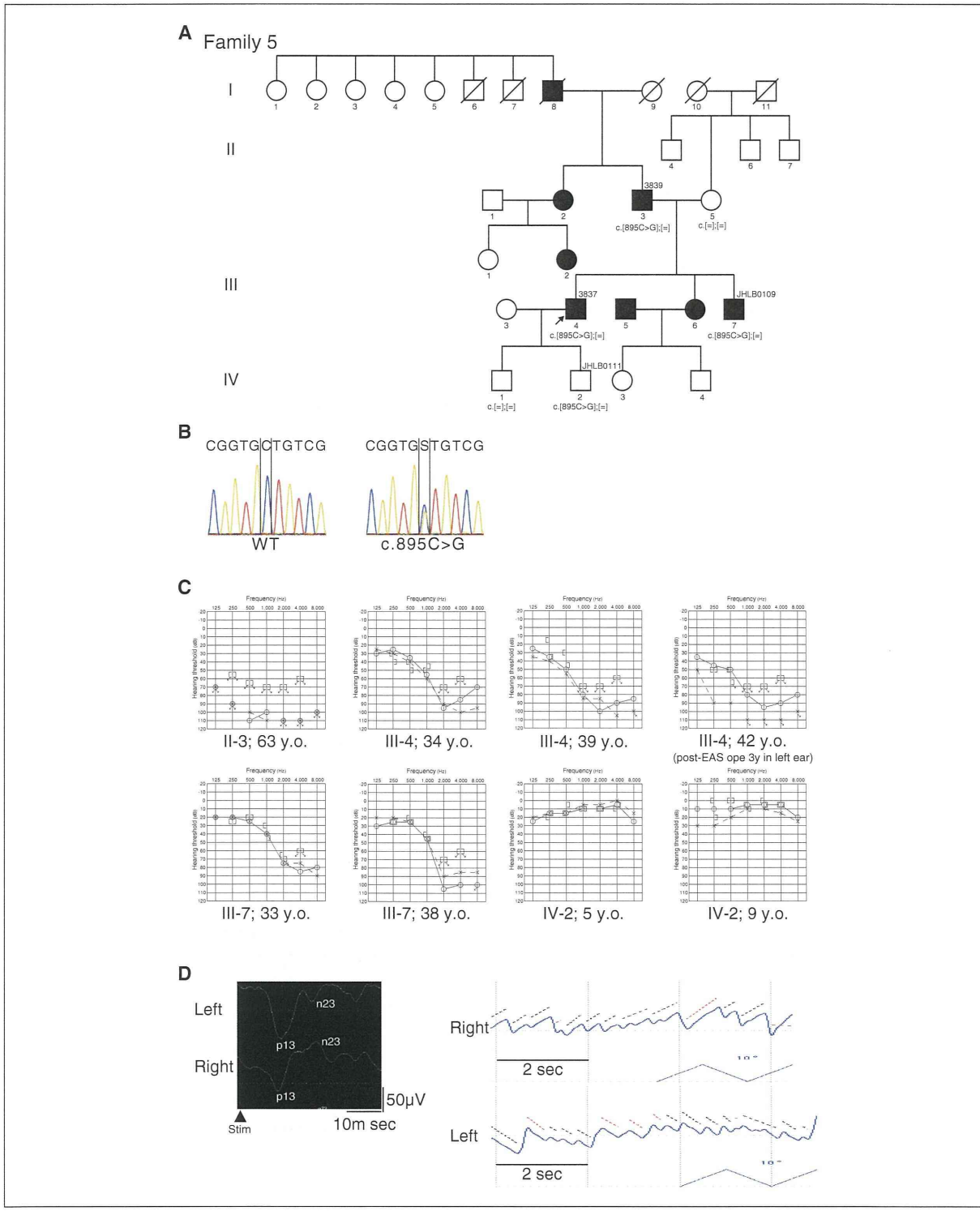

Figure 5. (A) The family tree of patient 3837 (a 42-year-old male). (B) The results of Sanger sequencing. (C) Audiograms of affected members showing progression. (D) Vestibular examination of patient 3837. His vestibular testing, including caloric testing and cervical vestibular evoked myogenic potential (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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Novel *PTPRQ* Mutations Identified in Three Congenital Hearing Loss Patients With Various Types of Hearing Loss

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Abstract

Objectives: We present 3 patients with congenital sensorineural hearing loss (SNHL) caused by novel *PTPRQ* mutations, including clinical manifestations and phenotypic features.

Methods: Two hundred twenty (220) Japanese subjects with SNHL from unrelated and nonconsanguineous families were enrolled in the study. Targeted genomic enrichment with massively parallel DNA sequencing of all known nonsyndromic hearing loss genes was performed to identify the genetic cause of hearing loss.

Results: Four novel causative *PTPRQ* mutations were identified in 3 cases. Case 1 had progressive profound SNHL with a homozygous nonsense mutation. Case 2 had nonprogressive profound SNHL with a compound heterozygous mutation (nonsense and missense mutation). Case 3 had nonprogressive moderate SNHL with a compound heterozygous mutation (missense and splice site mutation). Caloric test and vestibular evoked myogenic potential (VEMP) test showed vestibular dysfunction in Case 1.

Conclusion: Hearing loss levels and progression among the present cases were varied, and there seem to be no obvious correlations between genotypes and the phenotypic features of their hearing loss. The *PTPRQ* mutations appeared to be responsible for vestibular dysfunction.

Keywords

hearing loss, *PTPRQ*, DFNB84, massively parallel DNA sequencing

Introduction

Hearing loss is the most common sensory impairment in humans. Genetic causes account for the largest proportion of congenital sensorineural hearing loss (SNHL). Hearing loss is an extremely heterogeneous disorder, and approximately 75% of hereditary hearing loss is nonsyndromic. Therefore, it is difficult to predict the clinical course on the basis of clinical findings. Genetic testing is one way to resolve this problem. However, due to the extreme heterogeneity of SNHL, much labor and expense are required for analysis when using conventional Sanger sequencing.

Recent advances in targeted genomic enrichment with massively parallel DNA sequencing (TGE+MPS) have made it possible to sequence all known causative genes simultaneously.[1,2] This technology has been reported to afford an effective approach to the diagnosis of genetic hearing loss, particularly in terms of sensitivity, specificity, and reproducibility.[1]

In this study, we performed genetic testing using TGE+MPS to analyze the genetic etiology of Japanese hearing loss patients and identified mutations in the *PTPRQ* (protein tyrosine phosphatase receptor Q) gene. The *PTPRQ* gene is one of the latest identified as a cause of nonsyndromic SNHL. The locus had been mapped on chromosome 12q21.31 and was assigned DFNB84.[3] The *PTPRQ* gene is comprised of 58 exons and encodes the PTPRQ protein, which is one of

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the membrane proteins localized in the basal region of the stereocilia.[3-5] The PTPRQ protein has 3 domains: the extracellular domain (fibronectin type 3 domain), the membrane spanning domain (tansmembrane domain), and the cytoplasmic domain (phosphatase domain).[3,6,7] The PTPRQ protein is known to play key roles in the regulation of actin filaments reorganization, cell shape changes, and shaft connector formation of hair cell stereocilia.[4,8,9] Sakaguchi et al[5] reported that the PTPRQ protein appears to maintain the organization of the cell surface coat and the structure of the overall stereocilia bundle through interactions with Myosin VI.

Until now, only 3 families with *PTPRQ* mutations have been reported, and most of the phenotypic features remain unclear.[3,6] Here, we describe 3 Japanese patients with congenital SNHL caused by novel *PTPRQ* mutations.

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. This study was approved by the Ethical Committee of Shinshu University and Yokohama City University.

The first group:Yokohama samples. Twenty-six (26) Japanese subjects from unrelated and nonconsanguineous families were enrolled. These subjects visited Yokohama City University hospital for examination of hearing loss and participated in this study.

The second group: Shinshu samples. One hundred ninety-four (194) Japanese subjects from unrelated and nonconsanguineous families were ascertained through 33 otolaryngology clinics in 28 prefectures across Japan.

Methods

The First Group: Yokohama Samples

Amplicon library preparation. Amplicon libraries were prepared with 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. The 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 Ion 318 Chip (Life Technologies).

The Second Group: Shinshu Samples

Targeted genomic enrichment and massively parallel sequencing. TGE 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, USA).[11]

In brief, 3 μg 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

The first group: Yokohama samples. 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.[12,13] 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,[14] (2) the 6500 exome variants,[15] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[16] 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,[17] Sorting Intolerant from Tolerant (SIFT),[18] Polymorphism

Phenotyping (PolyPhen2),[19] LRT,[20] MutationTaster,[21] and GERP++.[22]

The second group: Shinshu samples. Data were analyzed as described using a local installation of the open-source Galaxy software and the following open-source tools: BWA[23] for read mapping, Picard for duplicate removal, GATK[24] for local realignment and variant calling, and NGSRich[25] for enrichment statistics.[2] We reported and annotated variants with custom software.

Variant Confirmation

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

We identified 3 cases that had the causative *PTPRQ* mutations in this study (220 hearing loss patients).

Mutation Analysis

We identified novel 1 nonsense mutation, 2 missense mutations, and 1 splicing junction mutation in the *PTPRQ* gene (NM_00145026). Case 1: AG 8960 had a homozygous mutation. This mutation corresponded to c.1261C>T, leading to p.Arg421stop (Figure 1). Case 2: SNS 2193 had a compound heterozygous mutation. This mutation corresponded to c.166C>G and 1261C>T, leading to p.Pro56Ala and Arg421Stop (Figure 2). Case 3: SNS 2912 had a compound heterozygous mutation. This mutation corresponded to c.6453+3delA and 4046T>C, which leads to p.Met1349Thr (Figure 3). As shown in Figures 1, 2 and 3, Sanger sequencing for family segregation was confirmed for each pedigree. None of these mutations were identified in the 1000 genome database, the 6500 exome variants, or the 1208 Japanese exome variants, in addition to the 269 in-house Japanese normal hearing controls database.

Details of Cases

Case 1 sample: ID no. AG 8960. The affected patient was a 19-year-old male. Newborn hearing screening was not performed at his birth. He had no particular complications in the perinatal period. His parents noticed his speech delay given that he only had a few spoken words at the age of 3. He had been referred to Yokohama City University Hospital, Department of Otolaryngology for hearing examinations. Play audiometry showed bilateral moderate hearing loss that was approximately 50 dBHL in the right ear and 75 dBHL in the left ear, which occurred together with otitis media with effusion. He was promptly fitted for hearing aids bilaterally. As a result of the hearing aids, he acquired age-appropriate spoken language. When he was 14 years old, he was aware of his own deterioration in hearing. Pure-tone audiometry (PTA) showed sloping high frequency SNHL that was on average 75 dBHL in both ears. Over a period of 5 years, his high frequency hearing gradually deteriorated. His hearing loss accelerated, and his hearing aids were ineffective by the time he was 19. Bilateral congenital progressive hearing loss was diagnosed.

He suffered from tinnitus and had no history of vertigo, but his elementary school teachers pointed out that he fell down frequently. Otoscopic examination revealed a normal tympanic membrane. Computed tomography (CT) and magnetic resonance imaging (MRI) of the temporal bone showed no abnormal malformations. Caloric test and the vestibular evoked myogenic potential (VEMP) test were performed at the age of 19 years old. These tests showed a hypo-caloric response on the right side and no caloric response or VEMP response on the left side. His parents, brother, and other relatives had no history of hearing impairment.

He underwent cochlear implantation, MED-EL FLEX28, in his right ear at the age of 19 and obtained adequate hearing level. Preoperative sound field threshold levels with hearing aids were approximately 60 dBSPL at 500 to 2000 Hz. Postoperative sound field threshold levels with cochlear implant were 40 dBSPL at 125 through 4000 Hz.

His pedigree, hearing level, and vestibular test results are shown in Figure 1.

Case 2 sample: ID no. SNS 2193. The patient was a 16-year-old female. She had no particular complications in the perinatal period. However, at age 1 year, 5 months, her mother suspected hearing loss because of her poor response to sound. She underwent a hearing examination, and an auditory brainstem response (ABR) with click stimuli showed no response to 100 dBnHL in both ears. Conditioned orientation response (COR) audiometry showed a threshold above 90 dBHL in all frequencies. Congenital severe-profound SNHL was suspected, and she was fitted for bilateral hearing aids at the age of 2. Over a period of 14 years, her hearing loss was unchanged. At the age of 16, PTA showed high frequency sloping profound SNHL. She had no history of vertigo in the following years. CT showed no abnormality of the inner or middle ears. Her parents, sister, brother, and other relatives had no history of hearing impairment. Her pedigree and hearing levels are shown in Figure 2.

Case 3 sample: ID no. SNS 2912. The affected patient was an 18-year-old female. She had no particular complications in the perinatal period. Bilateral hearing loss was identified

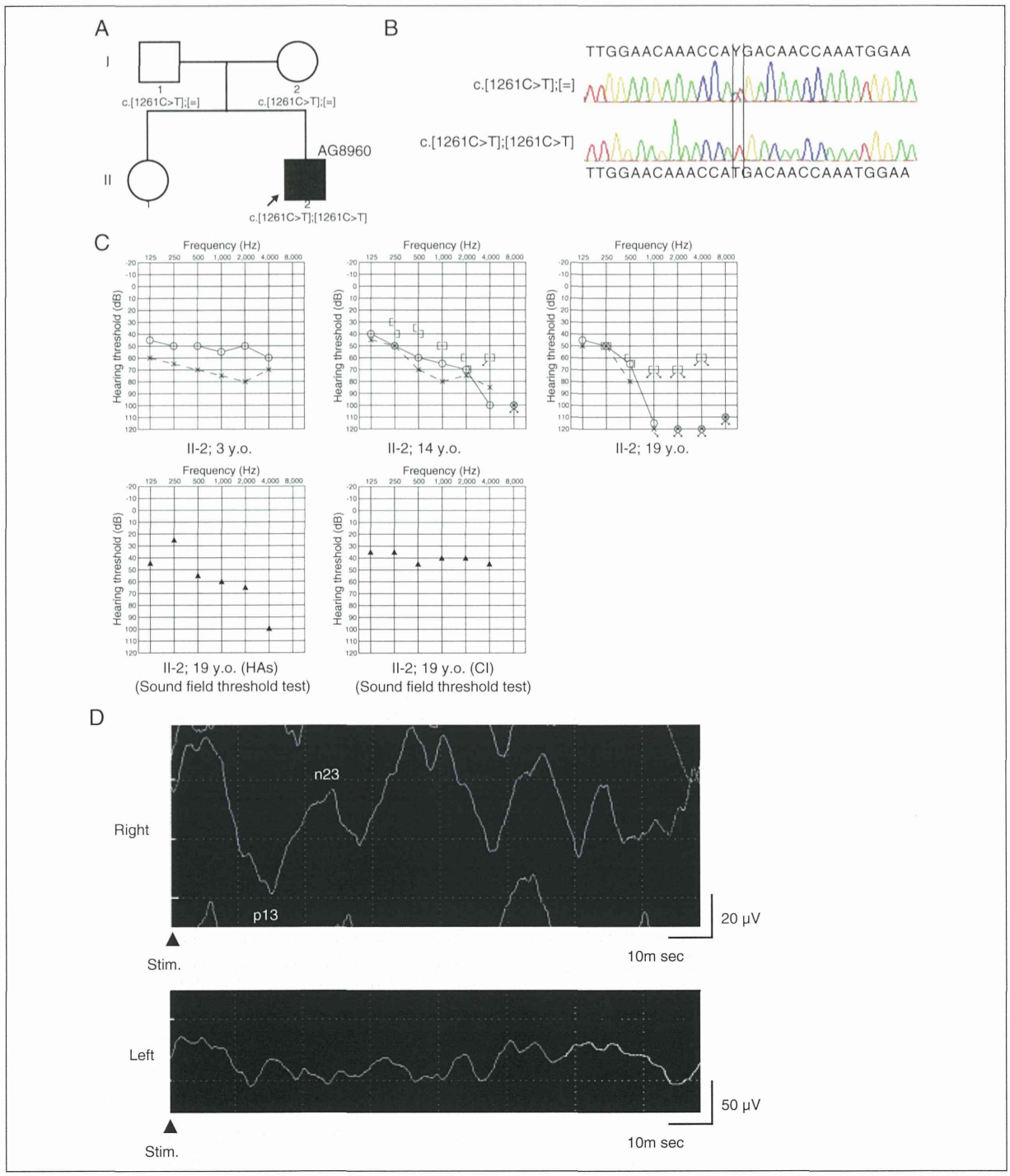

Figure 1. Pedigree and clinical findings for Case 1: AG 8960. (A) Pedigree showed a single case in this family. (B) Sanger sequencing and segregation analysis showed Case 1 had the homozygous mutation, and the parents and brother had the heterozygous mutation. (C) Pure-tone audiometry showed moderate sensorineural hearing loss (SNHL) at age 3 and deterioration to profound SNHL by age 19. Preoperative sound field threshold test with hearing aids (HAs) showed 60 dBSPL at 500 to 2000 Hz. After cochlear implantation (CI) in the right ear, sound field threshold test with CI showed 40 dBSPL. (D) Vestibular evoked myogenic potential (VEMP) test showed no response on left side.

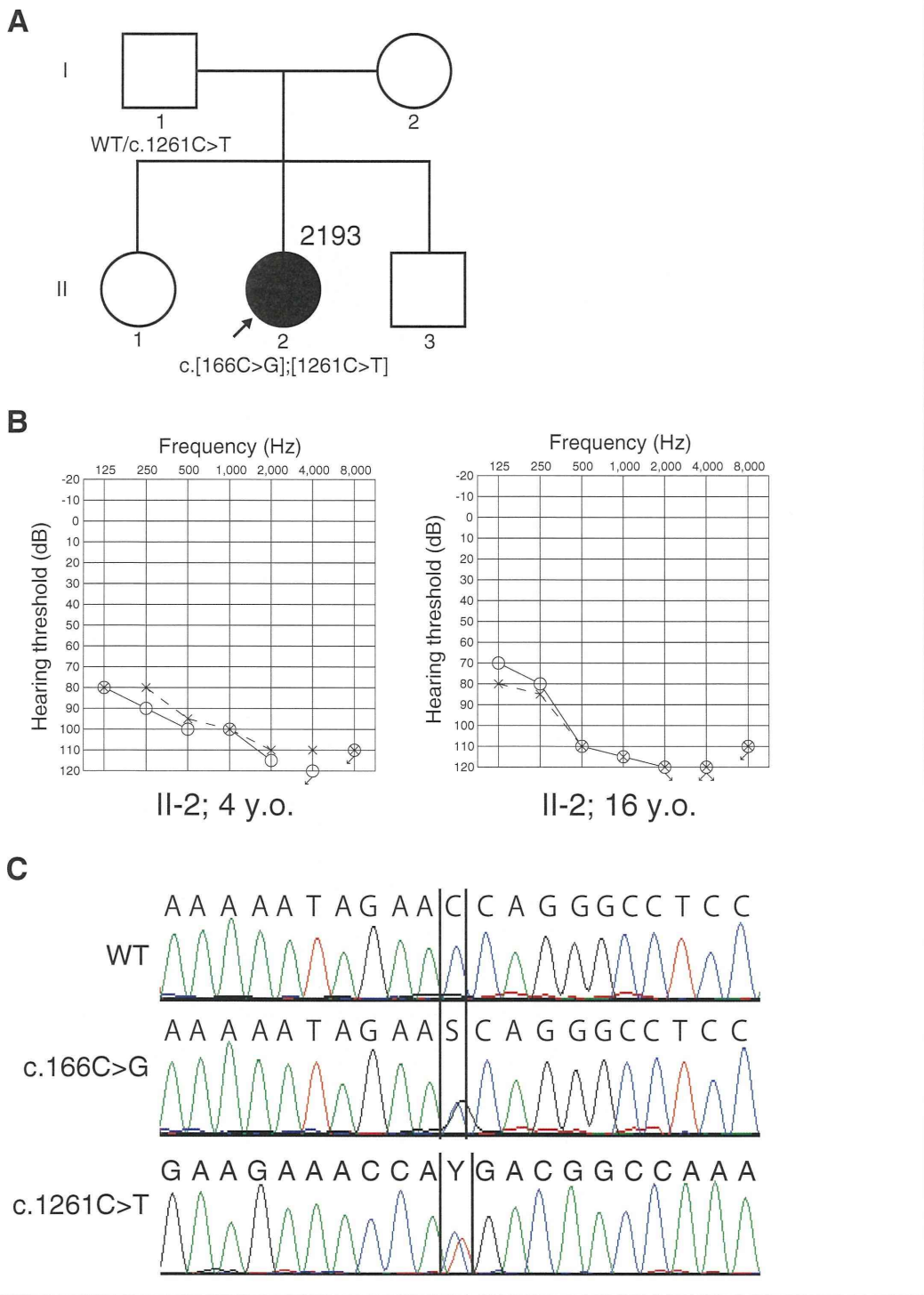

Figure 2. Pedigree and clinical findings for Case 2: SNS 2193. (A) Pedigree showed a single case in this family. (B) Pure-tone audiometry showed nonprogressive profound sensorineural hearing loss (SNHL). (C) Sanger sequencing and segregation analysis showed Case 2 had the compound heterozygous mutation, and her father had the heterozygous mutation.

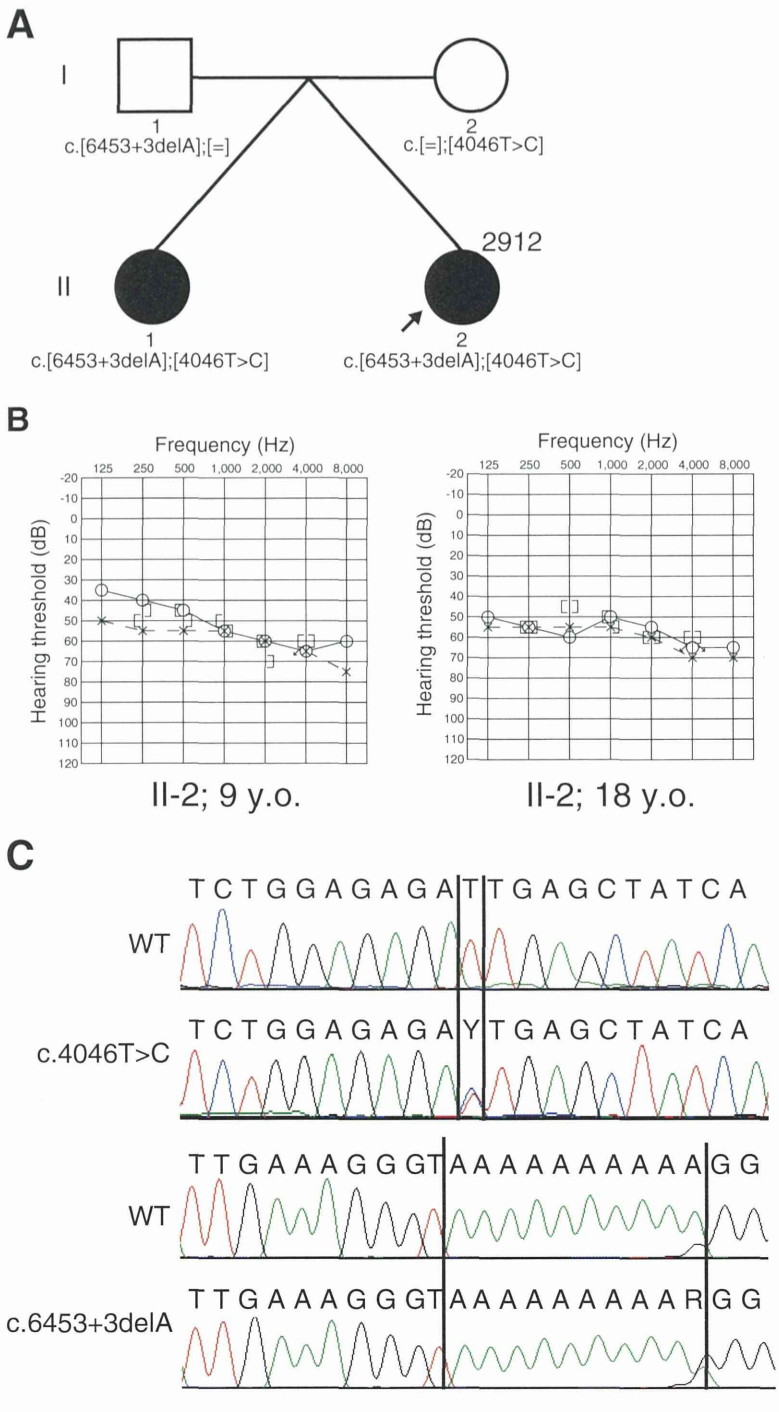

Figure 3. Pedigree and clinical findings for Case 3: SNS 2912. (A) Pedigree showed 2 cases in this family. (B) Pure-tone audiometry showed nonprogressive moderate sensorineural hearing loss (SNHL). (C) Sanger sequencing and segregation analysis showed Case 3 and her twin sister had the compound heterozygous mutation, and her parents had the heterozygous mutation.

Table 1. All Known Causative Mutations in the *PTPRQ* Gene

Nucleotide Change	Protein Change	Domain	Type of Mutation	Zygosity	Time of Onset	Type of HL (Age)	Progression	Family Origin	Reference
c.166C>G	p.Pro56Ala	EC	Missense	Compound heterozygous	Congenital	Profound (16 y)	Stable	Japanese	This study
c.1261C>T	p.Arg421Stop	EC	Nonsense						
c.1261C>T	p.Arg421Stop	EC	Nonsense	Homozygous	Congenital	Profound (19 y)	Progressive	Japanese	This study
c.1285C>T	p.Gln429Stop	EC	Nonsense	Homozygous	NR	Moderate (11 y)	NR	Palestinian	Shahin et al[6]
c.1285C>T	p.Gln429Stop	EC	Nonsense	Homozygous	NR	Moderate (15 y)	NR	Palestinian	Shahin et al[6]
c.1285C>T	p.Gln429Stop	EC	Nonsense	Homozygous	NR	NR	NR	Palestinian	Shahin et al[6]
c.1285C>T	p.Gln429Stop	EC	Nonsense	Homozygous	NR	Severe (5 y)	NR	Palestinian	Shahin et al[6]
c.1285C>T	p.Gln429Stop	EC	Nonsense	Homozygous	NR	Severe (14 y)	NR	Palestinian	Shahin et al[6]
c.1369A>G	p.Arg457Gly	EC	Missense	Homozygous	Congenital	Moderate (9 y)	Stable	Moroccan	Schraders et al[3]
c.1369A>G	p.Arg457Gly	EC	Missense	Homozygous	Congenital	Moderate (6 y)	Stable	Moroccan	Schraders et al[3]
c.1491T>A	p.Tyr497Stop	EC	Nonsense	Homozygous	Congenital	Profound (61 y)	Progressive	Dutch	Schraders et al[3]
c.1491T>A	p.Tyr497Stop	EC	Nonsense	Homozygous	Congenital	Profound (56 y)	Progressive	Dutch	Schraders et al[3]
c.4046T>C	p.Met1349Thr	EC	Missense	Compound heterozygous	Congenital	Moderate (18 y)	Stable	Japanese	This study
c.6453+3delA		CP	Splice site						

Abbreviations: Age, age at the time of hearing examination; CP, cytoplasmic domain; EC, extracellular domain; HL, hearing loss; NR, not reported.

at an elementary school health check-up, and she was referred to a general hospital clinic of otolaryngology at the age of 7. PTA showed bilateral moderate hearing loss, approximately 60 dBHL in both ears. She started to wear hearing aids bilaterally. Over a period of 11 years, her hearing loss did not deteriorate. Bilateral moderate SNHL was diagnosed; possibly congenital or early onset was suspected. She suffered from bilateral tinnitus when she turned 11. She had no history of vertigo in the following years. CT showed no abnormality of the inner or middle ears. She had a dizygotic twin, and this twin sister had the same level of SNHL. Her parents and other relatives had no history of hearing impairment. Her pedigree and hearing levels are shown in Figure 3.

Discussion

We identified novel causative mutations in the *PTPRQ* gene as a cause of congenital SNHL in a Japanese population using TGE+MPS.

As shown in Table 1, there are a total of 12 SNHL cases with *PTPRQ* mutations, including 3 cases identified in this study and 9 previously reported cases.[3,6] Each affected family had various degrees of hearing loss severity and progressiveness. With respect to Case 1: AG 8960, he had progressive and profound SNHL mainly affected at high frequencies, with a homozygous nonsense mutation. Four of the 7 cases with homozygous nonsense mutations were described as having severe to profound SNHL: 1 case with a flat audiogram and 3 cases with a down-sloping audiogram. Another 2 Palestinian cases with a homozygous nonsense mutation were described as having moderate SNHL, although clinical information on the deterioration of their hearing was lacking.[4] Schraders et al[3] also reported that hearing loss progressed over a period of 10 to 30 years, becoming profound SNHL in 2 Dutch cases with a homozygous nonsense mutation. Thus, there might be variations in hearing levels and progression even among the cases with homozygous nonsense mutations. Thus, we suspect that there is no obvious correlation between genotypes and the phenotypic features of their hearing loss.

In this study, we performed vestibular assessment (caloric test and VEMP test) for Case 1 only. The caloric test revealed a hypo-response on the right side and no response on the left side. The VEMP test revealed no response on the left side (Figure 1D). Schraders et al[3] also reported that caloric test showed either no or hypo-responses on both sides in 4 cases with *PTPRQ* homozygous mutations. A *Ptprq* knockout mouse study revealed deformation of the stereocilia and hair bundles in the utricle and defects in the hair bundles in the saccule and ampullae.[26] Vestibular evoked potentials (VsEPs) were absent in the majority of *Ptprq* knockout mice.[26] These findings suggested that the *PTPRQ* mutations might cause dysfunctions in the vestibular organs. However, Case 1 had not experienced any episodes of vertigo or dizziness. The reason for this incompatibility is unclear, but the *Ptprq* knockout mice showed no obvious abnormal behavior, except when swimming.[26]

Case 1 received a cochlear implant in the right ear at the age of 19, by which time his hearing loss had gradually become severe. His sound field threshold levels were improved after implantation. We suggest that cochlear implantation could be the intervention of choice for cases with *PTPRQ* mutations.

This study was the first to identify compound heterozygous mutations. Case 2 had profound SNHL with a compound heterozygote for missense and nonsense mutations. Case 3 had moderate SNHL with a compound heterozygote for splicing site mutation and missense mutation. Taken together with

the results of previous reports (Table 1), there does not appear to be any obvious genotype-phenotype correlation.

In summary, we performed TEG+MPS in this study, and we believe this method could be useful for identifying rare causative gene mutations, such as the *PTPRQ* gene. The *PTPRQ* mutations also appeared to be responsible for the vestibular dysfunction. However, the vestibular symptoms might be almost unrecognizable, even though vestibular tests showed a hypo-response. The hearing loss caused by the *PTPRQ* mutations appeared to be congenital. With regard to the hearing levels and progression, we observed variations among 3 cases. More precise studies are necessary for better understanding the molecular basis of the genotypes, and the hearing loss was progressive in some cases, so that the follow-up of the patients needed to be lengthy to clarify their phenotypic features.

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Declaration of Conflicting Interests

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