In summary, we analyzed 1314 Japanese and identified 4 patients, and 3 mutations in *LOXHD1*. It seems extremely rare, 0.30% in Japanese hearing loss patients. Candidate gene testing is not applicable for such rare genes. MPS makes it possible to detect rare genes like *LOXHD1*.

Acknowledgments

We thank the participants of the Deafness Gene Study Consortium. We also thank Mr Jim George and Mr Sean Mehmet for their help in preparing the manuscript.

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.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by NIDCD RO1s DC003544, DC002842 and DC012049 to R.J.H.S. This study also was supported by a Health and Labour Sciences Research Grant for Research on Rare and Intractable Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.) and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

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Annals of Otology, Rhinology & Laryngology
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DOI: 10.1177/0003489415575061
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Novel Mutations in *GRXCR1* at DFNB25 Lead to Progressive Hearing Loss and Dizziness

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Abstract

Objective: We identified 2 patients in 1 family who had novel mutations in *GRXCR1*, which caused progressive hearing loss.

Methods: One thousand one hundred twenty Japanese hearing loss patients with sensorineural hearing loss from unrelated families were enrolled in this study. Targeted genomic enrichment with massively parallel sequencing of all known nonsyndromic hearing loss genes was used to identify the genetic causes of hearing loss.

Results: In this study, 2 affected individuals with compound heterozygous mutations—c.439C>T (p.R147C) and c.784C>T (p.R262X)—in *GRXCR1* were identified. The proband had moderate to severe hearing loss and suffered from dizziness with bilateral canal paralysis.

Conclusion: Our cases are the first identified in the Japanese population and are consistent with previously reported cases. The frequency of mutations in *GRXCR1* seems to be extremely rare. This study underscores the importance of using comprehensive genetic testing for hearing loss. Furthermore, longitudinal audiologic assessment and precise vestibular testing are necessary for a better understanding of the mechanisms of hearing loss and vestibular dysfunction caused by *GRXCR1* mutations.

Keywords

hearing loss, genetics, dizziness, *GRXCR1*, massively parallel sequencing

Introduction

Hearing loss is diagnosed in approximately 2 in every 1000 children in developed countries, and genetic causes account for at least 50% of all childhood nonsyndromic sensorineural hearing loss (SNHL).[1] Autosomal recessive (AR) SNHL occurs in 70% of cases and is characterized as typically congenital or prelingual, and severe to profound.[2] The most common causative gene is *GJB2*, which is affected with prelingual severe to profound SNHL, and most of these cases occur with nonprogressive hearing loss. However, the prevalence of SNHL increases to 2.7 per 1000 before the age of 5 years and reaches 3.5 per 1000 at adolescence.[3] This may suggest a potential for developing progressive SNHL with genetic causes. Several studies reported that only a few genetic causes of AR SNHL might be naturally occurring progressive hearing loss such as *SLC26A4* and *CDH23*.[4]

GRXCR1 is mapped to chromosome 4p13, which is known to be a cause of DFNB25.[5] Mutations in *GRXCR1* result in a progressive nonsyndromic SNHL. There have been only 2 reports in 6 families, from Pakistan, the Netherlands, and Iran.[5,6] According to these reports, SNHL caused by mutations in *GRXCR1* had a phenotypic feature showing early-onset progressive hearing loss and could be associated with vestibular dysfunction. The prevalence of *GRXCR1* hearing loss is deemed to be extremely rare, but the exact frequency is still unknown. Even though the phenotypic features are obtained, it could be hard to prioritize the candidate gene and move on to the sequencing of the whole *GRXCR1* gene. Recent advances in targeted genomic enrichment with massively parallel sequencing (TGE+MPS) have made possible the sequencing of all

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known causative genes simultaneously.[7-9] We performed genetic testing to analyze the genetic etiology of Japanese hearing loss patients using MPS. Here, we describe a family who was identified with novel mutations in *GRXCR1*, resulting in progressive hearing loss and vestibular dysfunction.

Patients and Methods

Patients

One thousand one hundred twenty Japanese hearing loss patients (autosomal dominant SNHL: 266, ARSNHL: 600, unknown: 254) from 53 ENT departments nationwide participated in this study. Written informed consent was obtained from all patients (or from their next of kin, caretaker, or guardian on the 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.

Methods

Amplicon library preparation. Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Grand Island, New York, USA), according to the manufacturer's instructions, for 63 genes reported to cause nonsyndromic hearing loss. The detailed protocol was described elsewhere.[8] 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.[8] 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.[10,11] 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,[12] (2) the 6500 exome variants,[13] (3) the Human Genetic Variation Database (dataset for 1208 Japanese exome variants),[14] 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,[15] Sorting Intolerant from Tolerant,[16] Polymorphism Phenotyping (PolyPhen2),[17] LRT,[18] MutationTaster,[19] and GERP++.[20] Candidate mutations were confirmed by Sanger sequencing and the responsible mutations were identified by segregation analysis using samples from among the patients' family members.

Protein modeling. A 3-dimensional model was built by comparative modeling using the SWISS-MODEL workspace[21] and with coordinates of human GLRX5 (PDB accession number 2wul) as a structural template. The results of the 3-dimensional analysis were visualized by MacPyMOL version 0.99rc.6 (http://www.pymol.org).

Variant Confirmation

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

Results

For the hearing loss patient cohort, we identified a family (family number 972) who had causative mutations in the *GRXCR1* gene.

Mutation Analysis

We identified compound heterozygous mutations—c.439C>T (p.R147C) and c.784C>T (p.R262X)—in *GRXCR1* in 2 hearing loss patients in the family (Figure 1A). c.439C>T (NM_001080476) is located in exon 2, which was strongly suspected as pathogenic. In silico prediction software (SIFT, PolyPhen2, LRT, and MutationTaster) indicated the mutation as pathogenic (0.98, 1.00, 1.00, and 1.00, respectively). c.784C>T (NM_001080476), which is located in exon 4, leads to a premature stop codon and truncation (p.R262X).

Case Details

Family number 972; II-1: AH 2888, II-5. The proband (II-1: AH 2888) was a 41-year-old male. He noticed bilateral hearing loss in infancy. He had normal motor development as an infant. He acquired spoken language through special classes for hearing loss students in his elementary school, but sometimes he needs lip-reading to communicate in his daily life. His pronunciation was clear, and his speech was almost completely intelligible. He began to use bilateral hearing aids around the age of 20. As shown in Figure 1C, his pure-tone audiometry (PTA) showed bilateral moderate sensorineural hearing loss at the age of 25 years. His

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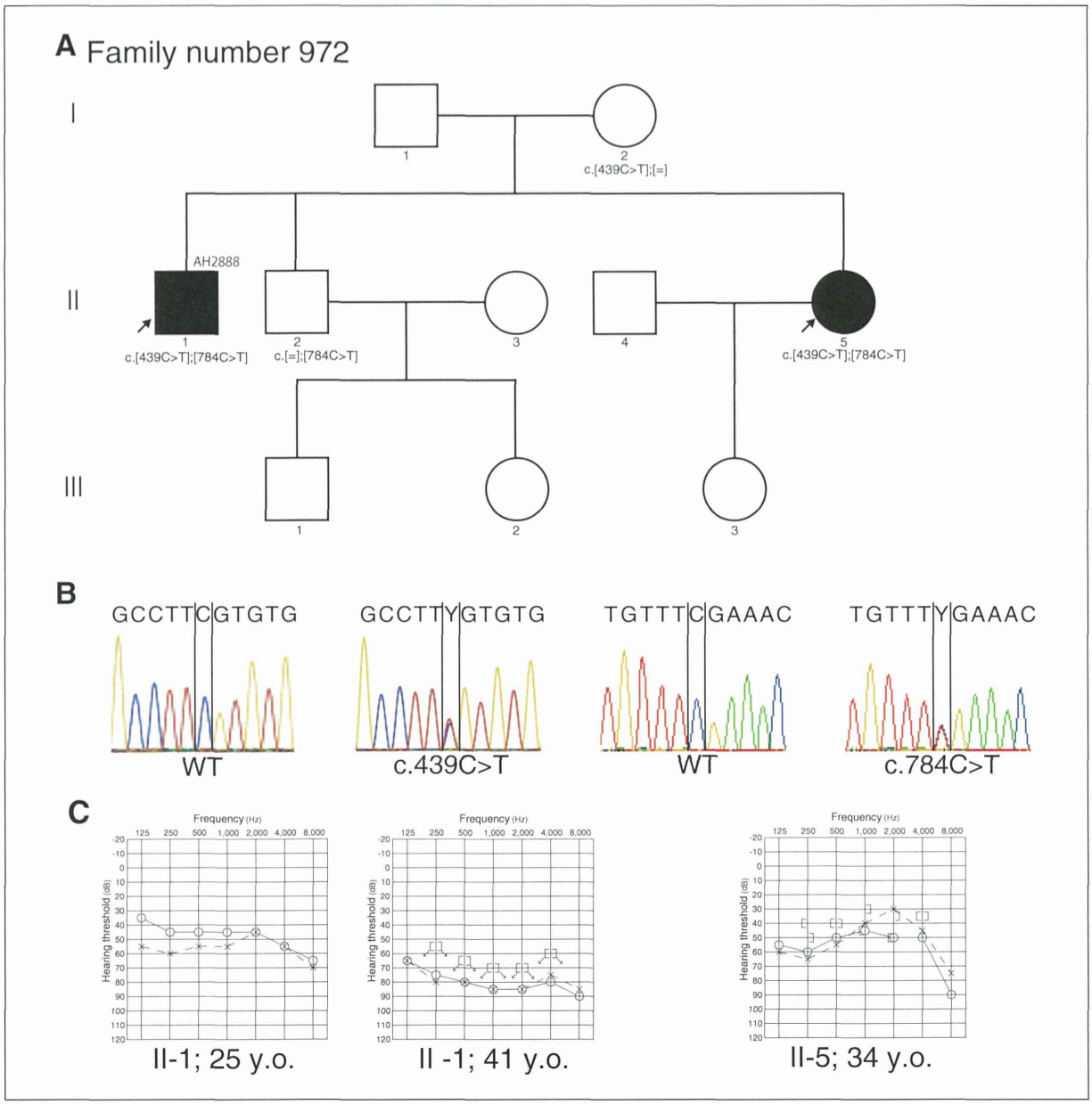

Figure 1. *Pedigree and clinical findings of family number 972.* (A) *Pedigree shows autosomal recessive inherited cases.* (B) The electropherograms show mutations in 2 cases. Target genome enrichment and massively parallel sequencing were carried out for II-1: AH 2888. Sanger sequencing for family segregation identified mutations in II-5. c.439C>T, (p.R147C) and c.784C>T, (p.R262X) were segregated in the family. (C) Pure-tone audiometry (PTA) shows bilateral sensorineural hearing loss in II-1: AH 2888 and II-5. PTA revealed that II-1: AH 2888 had progressive hearing loss.

hearing loss gradually deteriorated to become severe around the age of 40 years. For further examination of his progressive SNHL, he was referred to Kagoshima University Hospital, Department of Otolaryngology. He had some occasional episodes of dizziness, but no attacks of vertigo. Caloric testing with cold water irrigation (20°C, 5 mL) revealed his vestibular dysfunction; maximum slow phase velocity of the right side was 5°/sec, and that of the left side was 7°/sec. The computed tomography (CT) findings of the middle and inner ear were normal.

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Table 1. Known Mutations in the *GRXCR1* (DFNB25) Gene in Hearing Loss.

Nucleotide Change	Amino Acid Change	Domain	Zygosity	NM Number	Hearing Loss Onset	Type of Hearing Loss	Progression	Vestibular Dysfunction	Population	Reference
c.113C>T	p.P38L	—	Homozygosity	NM_001080476	Congenital	Severe to profound	No evidence	No evidence	Iranian	Odeh et al, 2010
c.190G>A	p.G64S	—	Homozygosity	NM_001080476	Congenital	Severe to profound	No evidence	No evidence	Iranian	Odeh et al, 2010
c.229C>T	p.Q77X	—	Homozygosity	NM_001080476	Very early-onset or congenital	Severe	No evidence	No evidence	Pakistani	Schraders et al, 2010
c.412C>T	p.R138C	Glutaredoxin domain	Homozygosity	NM_001080476	Very early-onset or congenital	Severe	No evidence	No evidence	Pakistani	Schraders et al, 2010
c.439C>T	p.R147C	Glutaredoxin domain	Heterozygosity	NM_001080476	Childhood	Moderate to severe	Yes	Yes	Japanese	This study
c.457T>G	p.F153V	Glutaredoxin domain	Homozygosity	NM_001080476	Congenital	Severe to profound	No evidence	No evidence	Iranian	Odeh et al, 2010
c.627+19A>T	p.G210VfsX14	Glutaredoxin domain	Homozygosity	NM_001080476	Very early-onset or congenital	Mild to moderate	Yes	No	Dutch	Schraders et al, 2010
c.628-9C>A	p.G210LfsX5	Glutaredoxin domain	Homozygosity	NM_001080476	Very early-onset or congenital	Severe to profound	Yes	Yes	Dutch	Schraders et al, 2010
c.784C>A	p.R262X	—	Heterozygosity	NM_001080476	Childhood	Moderate to severe	Yes	Yes	Japanese	This study

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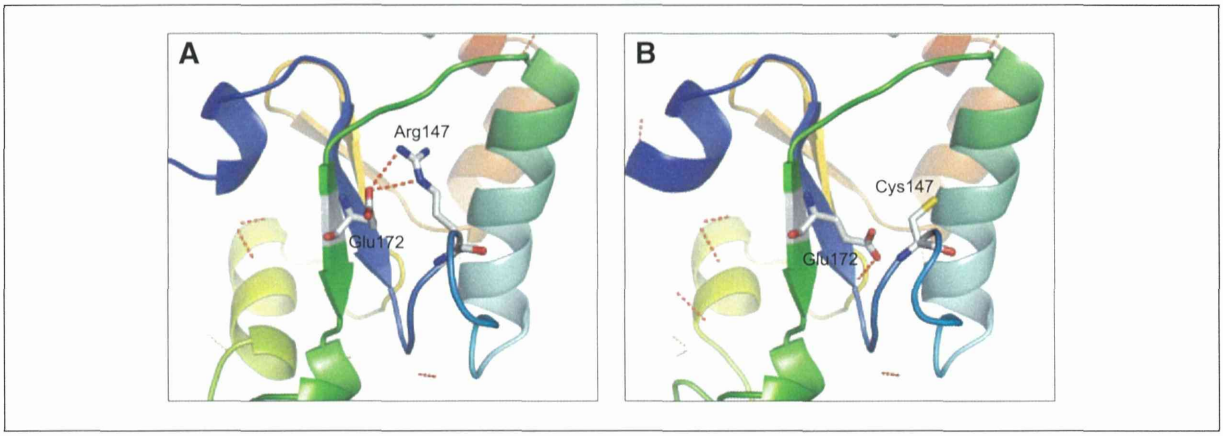

Figure 2. Three-dimensional model of the structure of glutaredoxin domain built by SWISS-MODEL workspace. (A) Wild type of the structure between arginine 147 (Arg147) and glutamic acid 172 (Glu172) showing a hydrogen bond. (B) The c.439C>T leads to a missense mutation from an arginine to a cysteine at the position 147 (Cys147), resulting in disruption of the hydrogen bond.

He had a younger sister (II-5): a 34-year-old female who began to use a hearing aid 2 years earlier. Her PTA showed bilateral moderate sensorineural hearing loss, but it was less severe than that of her brother (II-1) (Figure 1C).

Discussion

In this report, we identified novel causative mutations with compound heterozygous c.439C>T (p.R147C) and c.784C>T (p.R262X) mutations in the *GRXCR1* gene. *GRXCR1* encodes a 290-residue protein cysteine-rich C-terminal region and a predicted glutaredoxin (GRX) domain. GRX domains are the catalytic domains in glutaredoxins and are responsible for the modulation of the reversible S-glutathionylation of proteins.[22] We summarized the mutations in *GRXCR1* and the corresponding phenotypes that have been previously reported[5] in Table 1. Each affected family member had differing degrees of hearing loss severity and onset times. Mutations located near the N terminus may tend to be associated with a very early onset and severe SNHL. However, our study identified novel mutations that result in milder SNHL. We suggest that c.784C>T, located near the C terminus, might express an incomplete GRXCR1 protein with residual activity that appears to form in cases exhibiting moderate SNHL. We also suggest that c.439C>T, located in the GRX domain, might lead to the denaturation of glutaredoxins that reduce oxidized cysteines in cellular proteins and that this domain is crucial for the maintenance of enzymatic functions. In addition, 4 of the 7 previously reported mutations were located in the GRX domain. As shown in Figure 2A, our 3-dimensional model shows that arginine 147 and glutamic acid 172 are coupled by hydrogen bonds.[21] The c.439C>T mutation leads to a missense mutation from an arginine to a cysteine at position 147 of the protein, resulting in the disruption of the hydrogen bond and playing a role in the alteration of the protein structure (Figure 2B).

The GRXCR1 protein is localized in the hair bundles and stereocilia and plays a role in actin organization in hair cells toward the development of normal diameter and growth of stereocilia.[23] *Grxcr1* is expressed along the entire length of the stereocilia in the outer hair cells, inner hair cells, and all vestibular hair cells. In their study of the pirouette (*pi*) mouse model of *Grxcr1* mutants, Odeh et al[6] reported that loss of function of *Grxcr1* resulted in abnormally thin and slightly shortened stereocilia. It was also reported that a defective mouse with the *Grxcr1* gene had deafness and showed circling behavior. In this study, patient II-1 (AH 2888) had early-onset progressive SNHL and vestibular dysfunction. Schraders et al[5] reported that individuals with mutations in *GRXCR1* were also affected with vestibular dysfunction, and they concluded that mutations in *GRXCR1* caused congenital or early-onset moderate to severe hearing loss and could be associated with vestibular dysfunction. Our cases are consistent with previously reported cases.

We analyzed 1120 Japanese with hearing loss and identified only 1 patient (0.09%) with 2 novel mutations in *GRXCR1*. Candidate gene testing is not applicable for such a rare gene. Further evolution of genetic testing will make accurate diagnosis of hearing loss possible with less labor and expense. Furthermore, longitudinal audiologic assessment and precise vestibular testing are necessary for a better understanding of the mechanisms of hearing loss and vestibular dysfunction caused by *GRXCR1* mutation.

Acknowledgments

The authors thank the participants of the Deafness Gene Study Consortium. They also thank Mr David Callaghan for his help in preparing the manuscript.

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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.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by a Health and Labour Sciences Research Grant for Research on Rare and Intractable Diseases and Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.), and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (S.U.).

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Annals of Otology, Rhinology & Laryngology
2015, Vol. 124(5S) 177S–183S
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DOI: 10.1177/0003489415575045
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Hearing Loss Caused by a *P2RX2* Mutation Identified in a MELAS Family With a Coexisting Mitochondrial 3243AG Mutation

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Abstract

Objectives: We present a family with a mitochondrial DNA 3243A>G mutation resulting in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), of which some members have hearing loss in which a novel mutation in the *P2RX2* gene was identified.

Methods: One hundred ninety-four (194) Japanese subjects 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 causes of hearing loss.

Results: A novel mutation in the *P2RX2* gene that corresponded to c.601G>A (p.Asp201Tyr) was identified. Two patients carried the mutation and had severe sensorineural hearing loss, while other members with MELAS (who did not carry the *P2RX2* mutation) had normal hearing.

Conclusion: This is the first case report of a diagnosis of hearing loss caused by *P2RX2* mutation in patients with MELAS. A potential explanation is that a decrease in adenosine triphosphate (ATP) production due to MELAS with a mitochondrial 3243A>G mutation might suppress activation of P2X2 receptors. We also suggest that hearing loss caused by the *P2RX2* mutation might be influenced by the decrease in ATP production due to MELAS.

Keywords

hearing loss, genetics, P2X2, MELAS, massively parallel sequencing

Introduction

Hearing loss affects over 300 million people worldwide[1] and is the most common sensory deficit. Genetic factors account for at least 50% of childhood sensorineural hearing loss (SNHL). The majority of genetic hearing loss (about 75%) is autosomal recessive (AR) inherited, with 20% autosomal dominant (AD), and X-linked estimated to be 1% to 5% of genetic cases.[2] Genetic SNHL is mainly categorized into 2 forms, nonsyndromic SNHL (70%) and syndromic SNHL accompanied by other specific manifestations (30%).[2]

Among mitochondrial mutations, there have been many manifestations recognized as mitochondrial diseases involving various organs. SNHL is one of the most common manifestations in patients with mitochondrial diseases, and several mutations have been found to be maternally inherited SNHL.[3] A 3243A>G mutation in the mitochondrial DNA is associated with maternally inherited diabetes combined with mitochondrial myopathy, encephalopathy,

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lactic acidosis, and stroke-like episodes (MELAS), which also frequently involves SNHL.[4]

With regard to nonsyndromic hearing loss, most of these cases are affected with severe and congenital prelingual deafness with AR inheritance. Meanwhile, AD SNHL is represented by a mostly late onset, mild to moderate, and progressive hearing loss with distinctive phenotypical audiological features correlating to the causative genes.[5] Thirty-two loci and 31 genes have been implicated in autosomal dominant SNHL (DFNA).[6] DFNA 41 harbors the *P2RX2* gene that encodes the P2X2 receptor expressed in the cochlear sensory epithelium and the spiral ganglion neurons.[7-9] It comprises a channel gated by extracellular adenosine triphosphate (ATP).[10,11] The *P2RX2* gene has recently been identified as a cause of late-onset and progressive SNHL in 2 Chinese families and 1 Italian family.[12,13]

Concerning modes of inheritance, it is difficult to distinguish between mitochondrial maternal inheritance and AD inheritance. Furthermore, some patients with mitochondrial diseases present variable symptoms, including different levels of hearing loss, as clinical expression may be altered by heteroplasmy. AD hearing loss may also have different levels at different ages due to its progressive nature. It can be difficult to recognize what gene would be a candidate, including mitochondrial gene mutations, and move on to the analysis using conventional DNA sequencing based on polymerase chain reaction (PCR). Recent advances in targeted genomic enrichment with massively parallel sequencing (TGE+MPS) have made the sequencing of all known causative genes simultaneously possible.[14,15]

Here, we describe a family with a mitochondrial DNA 3243A>G mutation resulting in MELAS, of which some members have hearing loss where we identified a novel mutation in the *P2RX2* gene. This is the first report of a diagnosis of hearing loss caused by *P2RX2* in patients with MELAS and highlights the importance of comprehensive genetic testing for concomitant genomic and mitochondrial DNA mutations.

Subjects and Methods

Subjects

One hundred ninety-four (194) Japanese subjects (114 females) from unrelated and nonconsanguineous families were ascertained through 33 otolaryngology clinics in 28 prefectures across Japan. 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.

Targeted Genomic Enrichment and Massively Parallel Sequencing

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, Carlsbad, California, USA). 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).[16] Of the 198 samples, 58 samples were processed manually; the remainder was prepared robotically using the Sciclone NGS Workstation.

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.

Bioinformatics Analysis

Data were analyzed as described using a local installation of the open-source Galaxy software and the following open-source tools: BWA[17] for read mapping, Picard for duplicate removal, GATK[18] for local realignment and variant calling, and NGSRich[19] for enrichment statistics.[15] 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 1 family that had a causative mutation in *P2RX2* in the cohort of this study (194 hearing loss patients).

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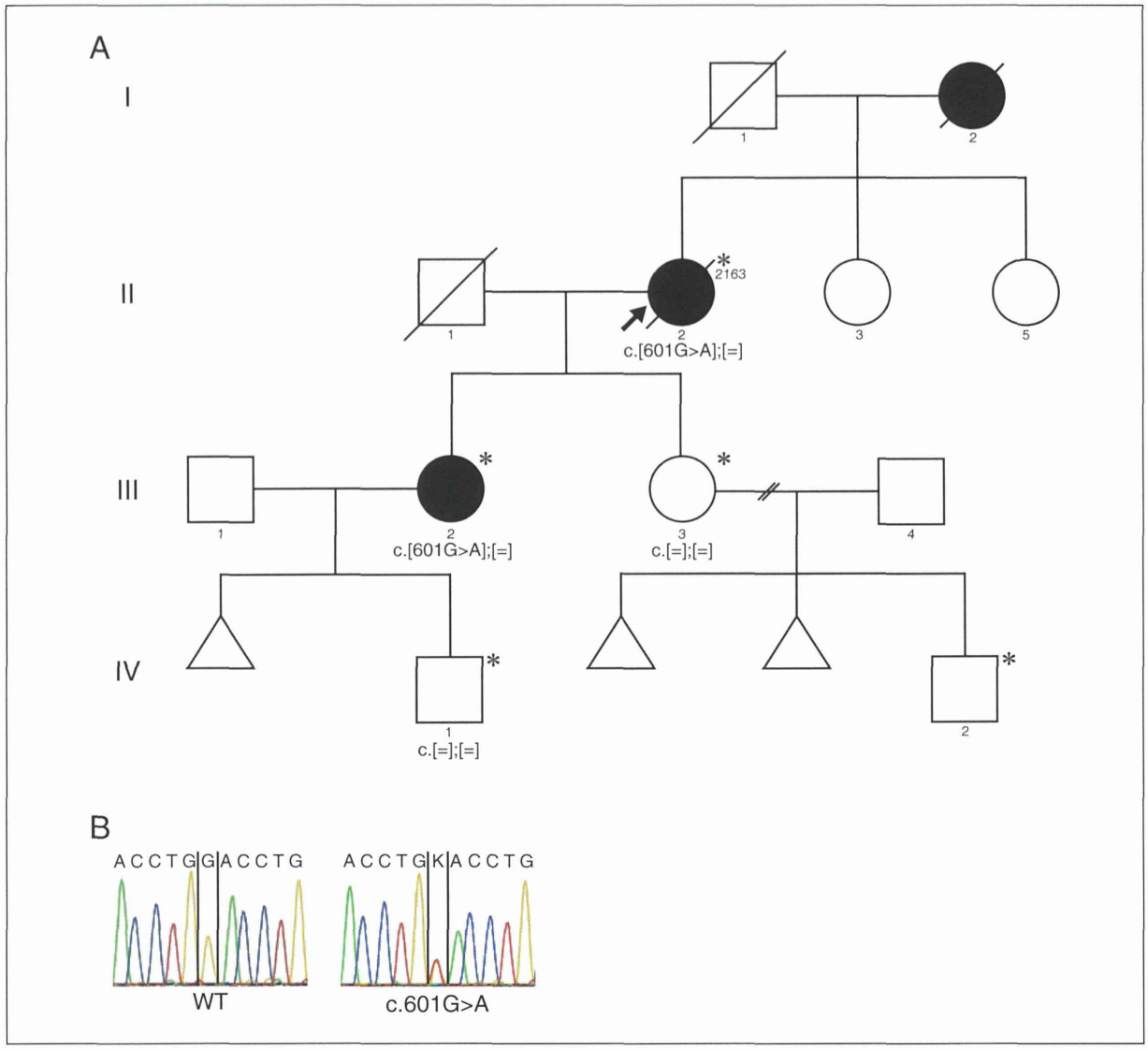

Figure 1. Pedigree of Patient ID 2163. (A) Pedigree showed maternal or autosomal dominant inheritance. Targeted genome enrichment and massively parallel sequence was carried out for the patient II-2. (B) The electropherogram of the mutation in the *P2RX2* gene. Asterisks indicate individuals affected with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS).

Case Details

The proband (ID: 2163) is a 46-year-old female in a Japanese family spanning 4 generations (Figure 1). She became aware of hearing loss around the age of 12, and her hearing loss progressed gradually. She was diagnosed with diabetes mellitus (DM) and started insulin therapy. Furthermore, hypertrophic cardiomyopathy (HCM) was also pointed out at the age of 32. At the age of 46, she visited the Department of Otolaryngology at Shinshu University Hospital for a hearing examination. An audiogram showed severe SNHL by this time. She had a positive family history of DM, which was harbored in her mother and 2 daughters. Also her elder daughter (III-2) had bilateral hearing loss. At the age of 50, she and her 2 daughters were referred to the Department of Internal Medicine at Shinshu University Hospital for control of their DM. A mitochondrial DNA 3243A>G mutation was identified at this time, and they were diagnosed with MELAS. The mother of the proband also suffered from DM and severe hearing loss, and died of cerebral embolism at age 68. The proband's daughter (III-2) had an unstable status of DM,

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