Declaration of Conflicting Interests

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Article

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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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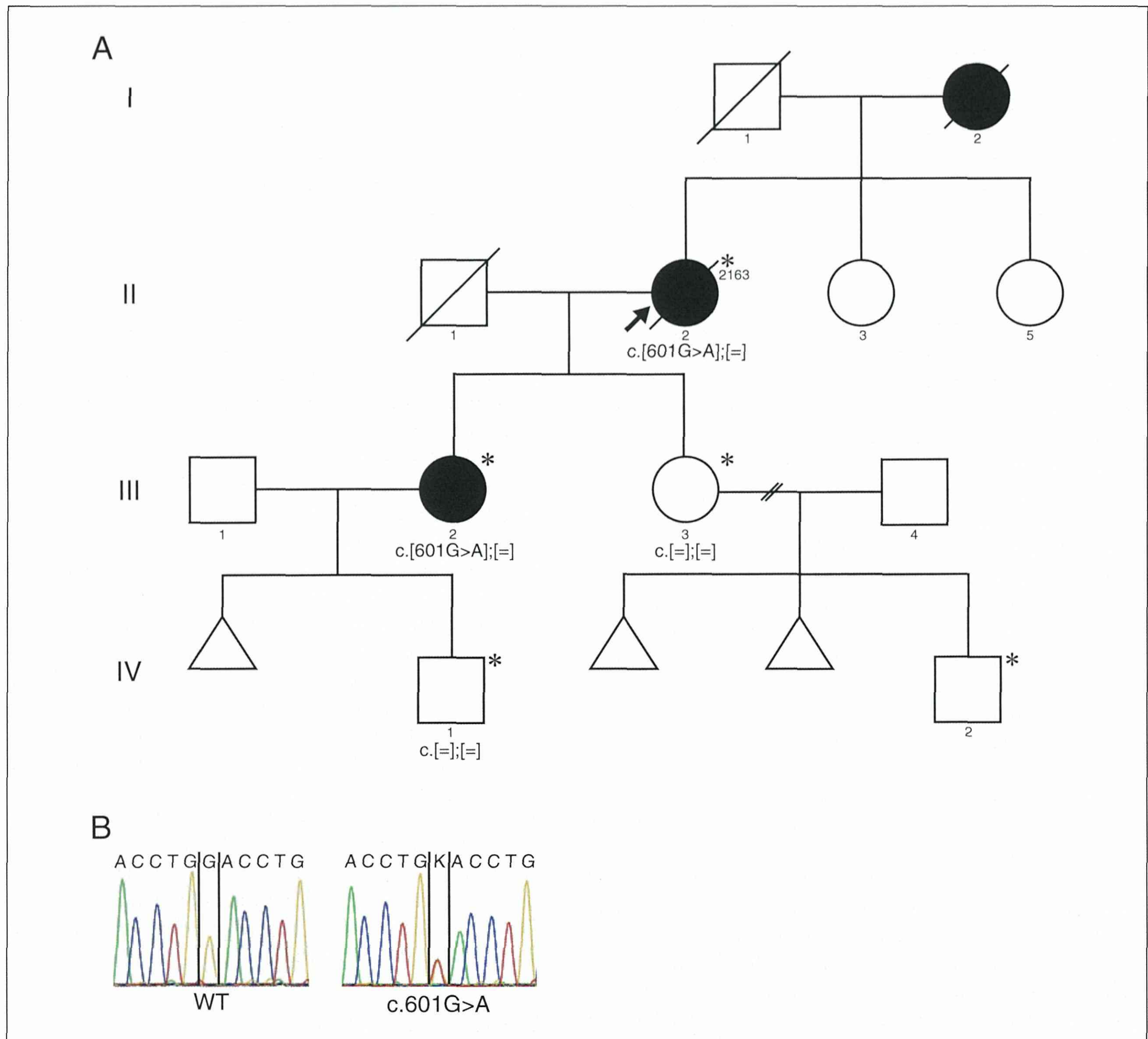

Figure 1. Pedigree of Patient ID 2163. (A) Pedigree showed maternal or autosomal dominant inheritance. Targeted genome enrichment and massively parallel sequence was carried out for the patient II-2. (B) The electropherogram of the mutation in the *P2RX2* gene. Asterisks indicate individuals affected 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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HCM, mild mental retardation, and bilateral progressive SNHL. The other daughter (III-3) had the same manifestations except SNHL, and she had completely normal hearing over time. They were overweight and of short stature. Unfortunately, the proband died of cardiac failure associated with HCM at the age of 57. Her grandson (IV-2) was also suspected of having MELAS; however, hearing assessment using auditory brainstem response (ABR) exhibited normal hearing bilaterally. More recently, her other grandson (IV-1), the son of III-2, was identified as having a mitochondrial 3243A>G mutation, and was suspected of MELAS due to fatigue and mild developmental delay. However, he had normal hearing at the age of 8.

With respect to hearing loss status, the proband's hearing loss rapidly deteriorated during 3 years (46-49 years old) and became undetectable. Her daughter's (III-2) hearing loss also progressed over a period of 16 years (25-41 years old). Pedigree and serial audiograms are shown in Figures 1 and 2. Manifestations of each family member are shown in Table 1.

Mutation Analysis

We performed comprehensive genetic testing using targeted genomic enrichment and massively parallel sequencing of all known nonsyndromic hearing loss genes as well as nonsyndromic mimic genes, as described previously.[15] We identified a novel missense mutation in the *P2RX2* gene that corresponded to c.601G>A (p.Asp201Tyr, NM_012226). We employed *in silico* pathogenicity prediction algorithms (PhyloP, SIFT, PolyPhen2, LRT, Mutation Taster, GERP), and all scores indicated "damaging" or "disease causing." We also performed Sanger sequencing for a family segregation study and confirmation of the variant in the proband. As shown in Figure 1, the Sanger sequencing results revealed that the proband and her elder daughter (III-2) had the mutation, although her younger daughter (III-3) did not. The *P2RX2* mutation (p.Asp201Tyr) segregated with only the patients who had hearing loss in the family.

Discussion

In this report, we identified a novel mutation in the *P2RX2* gene in Japanese hearing loss patients. The *P2RX2* gene is one of the latest identified as a cause of SNHL. There are only 2 previous reports on 3 families including their phenotypes of *P2RX2* mutations. It seems extremely rare as there have only been 2 reported mutations: c.178G>T (p.Val60Leu) and c.1057G>C (p.Gly353Arg), from China and Italy, respectively.[12,13]

The *P2RX2* gene encodes the P2X2 receptor, which plays an essential role in the cochlea as an ATP-gated ion channel receptor through ATP-mediated regulation.[9] P2X2 receptors are expressed in the epithelial cells surrounding the cochlear partition of the endolymphatic compartment, which includes the organ of Corti. Sustained noise exposure induces an up-regulation of P2X2 transcripts in the surface of cells.[20]

ATP is thought to have a neurotransmission effect at the synapse of the hair cells and contributes to regulation of the endocochlear potential. ATP is released into the endolymphatic compartment in which P2X2 receptors are expressed during noise stress and activates the P2X2 receptors, producing cation shunt conductance, which reduces the endocochlear potential. This mechanism has a protective effect on the cochlea by reducing sound transduction and hair cell sensitivity during noise exposure.[21,22] Based on these facts, Yan et al[12] clearly reported that exacerbation of hearing loss occurred among families with p.Val60Leu heterozygous mutations in the *P2RX2* gene and was more severe in the subjects having experienced noise exposure.

ATP is derived from mitochondria, and mitochondrial disease is attributed to dysfunctions in the oxidative phosphorylation of the cell resulting in a marked reduction in ATP production. Mit.3243A>G mutation generally causes MELAS; affecting multiple biological aspects including ATP loss, increase of lactate, and reactive oxygen species; and leads to systemic defects in various organs. In this family member, manifestations of MELAS presented as DM, short stature, stroke episodes, weakness, lactic acidosis, and intellectual disability. Nevertheless, there were only 2 patients in this family who were affected with severe progressive SNHL, and they had relatively low heteroplasmy of Mit.3243A>G mutation (Table 1). It is noteworthy that only these 2 patients carried the mutation in the *P2RX2* gene and had significantly severe SNHL, while other members with MELAS who did not carry the *P2RX2* mutation had normal hearing.

With regard to hearing level and its progression, 2 affected patients (II-2, III-2) exhibited more severe hearing loss. Besides, hearing loss deteriorated more rapidly as compared with the progression of *P2RX2* hearing loss reported by Yan et al[12] (Figure 3). It is speculated that that hearing loss caused by the *P2RX2* mutation might be influenced by the decrease in ATP production due to MELAS. Scuderi et al[23] have reported a similar case that had a nuclear gene *DCX* mutation coexisting with MELAS, a Mit.3243A>G mutation, and the manifestations of the *DCX* mutation were exacerbated by the mitochondrial dysfunction causing the MELAS. We also suggest that nuclear genetic factors may play a modifying role in the mitochondrial dysfunction.

Previously, genetic testing to identify mitochondrial mutations was carried out based on clinical findings. If a corresponding mutation was identified, further testing was not deemed necessary. As such, even if patients with mitochondrial DNA mutations did not express different types of hearing loss, it could be interpreted as a variability in

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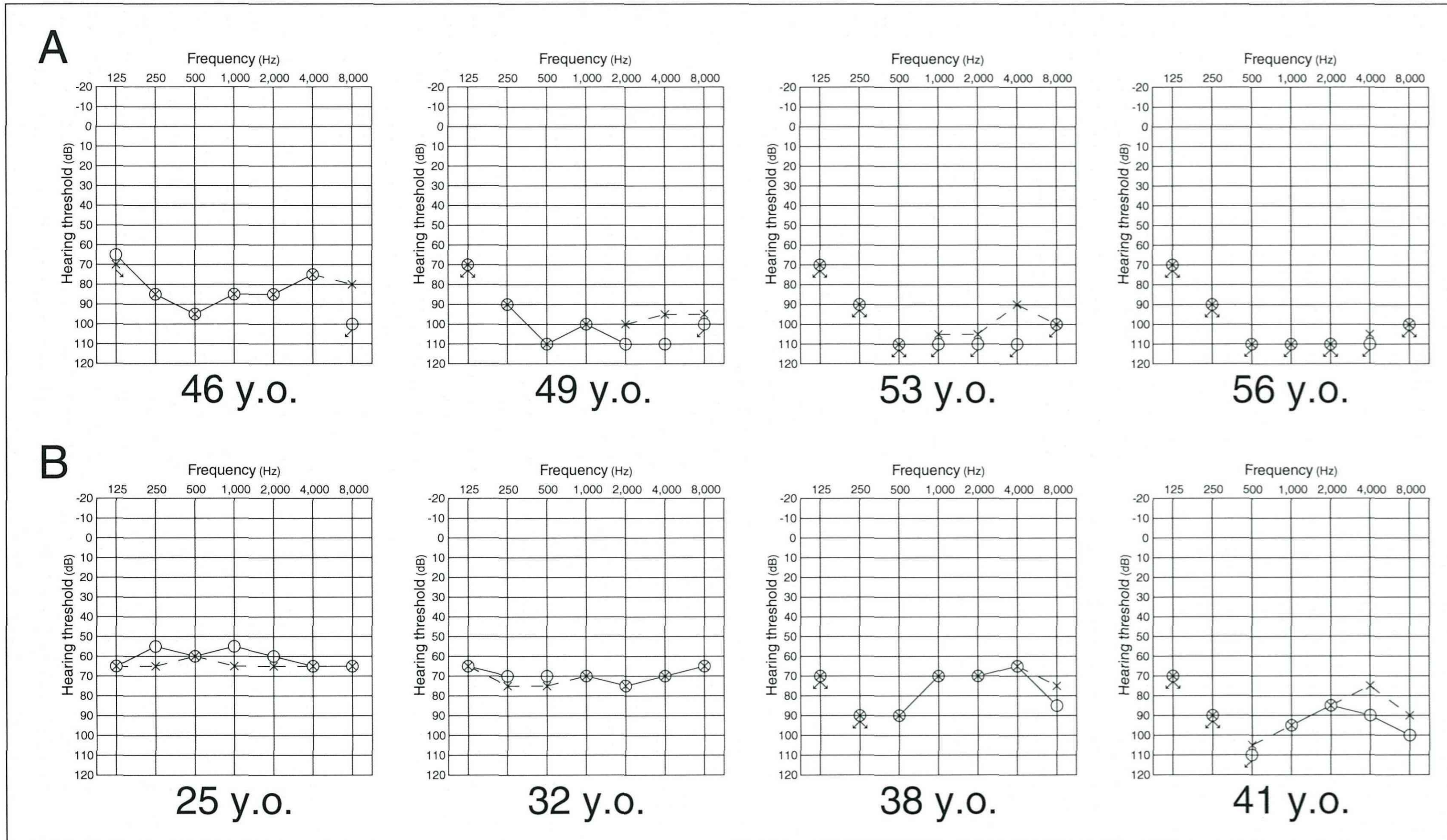

Figure 2. Serial audiograms of affected patients II-2 and III-2. (A) Audiograms of the patient (II-2) over a period of 10 years. Hearing loss rapidly deteriorated during the period from 46 to 49 years old. (B) Audiograms of the patient (III-2) over a period of 16 years. Hearing loss was relatively stable around age of 30.

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Table 1. Summary of Clinical Findings of Individuals With Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke-Like Episodes (MELAS).

Subject	Gender	Hearing	Diabetes Mellitus	Cardiac Disease	Mental Retardation	Heteroplasmy of 3243AG (%)
I-2	F	Severe HL	Yes	na	na	na
II-2	F	Severe HL	Yes	HCM	na	1
III-2	F	Severe HL	Yes	HCM	Yes	25
III-3	F	Normal	Yes	A-V block	Yes	2
IV-1	M	Normal	No	No	Yes	25
IV-2	M	Normal (ABR)	No	No	Yes	na

Abbreviations: ABR, auditory brainstem response; HCM, hypertrophic cardiomyopathy; HL, hearing loss; na, not applicable.

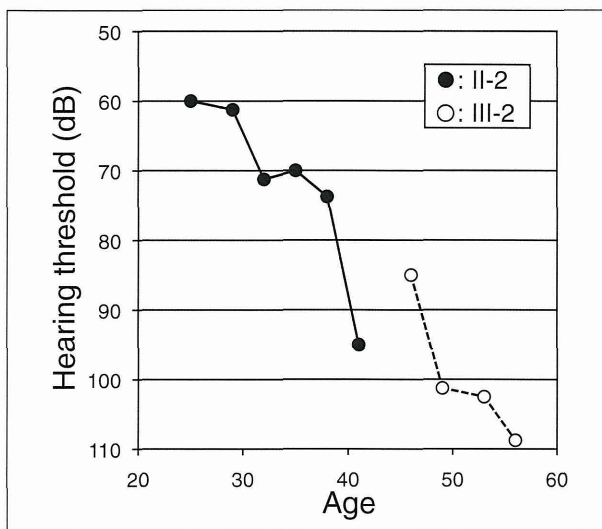

Figure 3. Progression of hearing loss in patients II-2 and III-2. Hearing loss rapidly deteriorated around age 40 to 50.

mitochondrial disease. In this family, the coexistence of the *P2RX2* mutation and the Mit.3243A>G mutation might occur accidentally. TGE and MPS allowed us to identify the disease-causing mutations, based on all known hearing loss genes screened. This study supports the use of comprehensive genetic diagnosis for SNHL cases to provide the highest chance of diagnostic success. In addition, further studies are necessary for cases that identify multiple pathogenic mutations, and investigations of these gene-gene interactions may help clarify the phenotype.

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Declaration of Conflicting Interests

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Novel Mutations in *LRTOMT* Associated With Moderate Progressive Hearing Loss in Autosomal Recessive Inheritance

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Abstract

Objective: We present a patient who was identified with novel mutations in the *LRTOMT* gene and describe the clinical features of the phenotype including serial audiological findings.

Methods: One hundred six Japanese patients with mild to moderate sensorineural hearing loss from unrelated and nonconsanguineous 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: Compound heterozygotes with a novel frame-shift mutation and a missense mutation were identified in the *LRTOMT* gene. The mutated residues were segregated in both alleles of *LRTOMT*, present within the LRTOMT2 protein coding region. The patient had moderate sloping hearing loss at high frequencies, which progressed at 1000 Hz and higher frequencies over a period of 6 years.

Conclusion: Hearing loss caused by mutations in the *LRTOMT* gene is extremely rare. This is the first case report of a compound heterozygous mutation in a nonconsanguineous family.

Keywords

hearing loss, genetics, *LRTOMT*, DFNB63, massively parallel sequencing

Introduction

DFNB63 was mapped to human chromosome 11q13.3-q13.4 reported from different study groups in 2007.[1-3] For affected patients with nonsyndromic sensorineural hearing loss (SNHL), this region was found to be segregated in 1 Turkish, 4 Pakistani, and 1 Tunisian consanguineous family. In 2008, Ahmed et al,[4] through genomic DNA sequencing of these affected individuals, revealed that the causative gene on the DFNB63 locus was *LRTOMT* and identified 4 pathogenic homozygous mutations in the *LRTOMT* gene. In addition, positional cloning of the *LRTOMT* gene had been performed from human liver cDNA, and it was revealed that the *LRTOMT* gene (NM_001145309) consisted of 9 exons and was transcribed into 5 different alternative splicing transcripts. The distinctive feature of the *LRTOMT* gene is that it primarily encodes 2 different proteins: LRTOMT1 and LRTOMT2 (also called COMT2).[4,5] Both proteins are produced by being translated into 2 alternate reading frames using different exon sets, of which LRTOMT1 starts in exon 3, and LRTOMT2 starts in exon 5.[4] LRTOMT2, also called COMT2, is expressed in sensory hair cells in the inner ear and is thought to be important for auditory function.[5] Several studies have also reported that SNHL caused by mutations in the *LRTOMT* gene might be more attributable to the LRTOMT2 (COMT2) region than to LRTOMT1.[6,7]

Hearing loss patients with mutations in the *LRTOMT* gene were found to be segregating in only Middle Eastern consanguineous families who carried the homozygous mutation.[1-3,6-9] These studies showed that the mutation results in severe-profound SNHL; however, clinical information, including serial audiograms, was lacking. In this study, we analyzed the genetic etiology for mild-moderate hearing loss families using massively parallel sequencing (MPS) for all known hearing loss genes simultaneously and identified novel mutations in the *LRTOMT* gene in the Japanese autosomal recessive SNHL population. Here, we

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report DFNB63 for the first time in the Japanese hearing loss population and provide a detailed description of the clinical features of its phenotype.

Patients and Methods

Patients

One hundred six Japanese patients were selected as part of a large project, based in 33 otolaryngology clinics across Japan from 1995 to 2012, to identify causative genes for mild to moderate hearing loss. All patients had presumed nonsyndromic SNHL and were from unrelated and nonconsanguineous families. Severity of hearing loss was classified as mild to moderate (21-70 dB) by pure-tone or behavioral audiometry average (> 500, 1000, 2000, and 4000 Hz). Forty-four patients were from autosomal dominant or mitochondrial inherited families, 56 patients were from autosomal recessive families (parents with normal hearing and 2 or more affected siblings), and 7 patients were unknown. The control group consisted of 192 unrelated Japanese individuals with normal hearing as evaluated by auditory testing. 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

One hundred twelve genes, including the 54 genes reported to be causative of nonsyndromic hearing loss (Hereditary Hearing Loss Homepage; http://hereditaryhearingloss.org/) and the 22 reported to cause syndromic hearing loss, were selected for sequencing. Each genomic DNA pool was fragmented using the Covaris S2 System (Covaris Inc, Woburn, Massachusetts, USA) to about 200 bp fragment length. After fragmentation, DNA fragments were blunt-ended and phosphorylated at the 5'- end using a Paired-End DNA Sample Prep Kit (Illumina Inc, San Diego, California, USA) and, subsequently, adeninylated at the 3'- end and ligated to pre-capture adaptor oligonucleotides containing SureSelect target DNA enrichment kit (Agilent Technologies, Santa Clara, California, USA). After adaptor ligation, pre-capture amplification was performed with Heraculase II Fusion DNA polymerase (Agilent Technologies). The capture library was designed using Agilent's eArray homepage (http://earray.vhem.agilent.com/earray/). The bait cRNA library contained all exons of the 112 genes. Exons of selected genes of all variants were selected from the RefSeq and Ensembl databases using the University of California Santa Cruz genome browser (http://genome.ucsc.edu/). Adaptor ligated and pre-amplicated samples were hybridized to the Capture cRNA library at 65°C for 24 hours with SureSelect Hybridization buffer and successively captured with Dynabeads MyOne Streptavidin T1 beads (Invitrogen by Life Technologies, Carlsbad, California) and washed with SureSelect Wash buffer. After target capture, selected products were post-amplified with Agilent Heraculase II Fusion DNA polymerase and an Illumina Multiplexing Sample Preparation Oligonucleotide Kit, and then processed in the Illumina HiSeq2000 system (Illumina Inc).

Bioinformatics Analysis

The sequence data were processed with a standard Illumina base calling procedure and successively mapped to the human genome sequence (build hg 19) with both the Bowtie program and BWA program.[10] After the application of all of these filters, the candidate deafness-causing mutations were selected and verified by subsequent Sanger sequencing. For missense mutations, SIFT, MutationTaster, and PolyPhen2[11] software programs were applied to predict the influence on the protein function by amino acid substitution.

Variant Confirmation

All pathogenic variants were confirmed by Sanger sequencing and segregation analysis using exon-specific custom primers. PCR amplification and sequencing of the fragments were performed using the forward primer: 5′-CTTTCTGAGCCGTGGTTTGT-3′ and the reverse primer: 5′-CAAGAACCTGCCCAATTCAT-3′ for exon 7, and the forward primer: 5′-GGACCTGGCATGAAGTAAGC-3′ and the reverse primer: 5′-GAAAGGGAGGGGATTTTGAG-3′ for exon 9. Family member genotypes were also used to validate the co-segregations of the deafness trait and the candidate mutations in individual families.

Results

We identified a single case of compound heterozygous causative mutation in the *LRTOMT* gene in the cohort of this study (106 hearing loss patients with mild to moderate SNHL).

Case Details

The affected patient was a 12-year-old male (patient ID: 4134). No perinatal complications were noted, and newborn hearing screen was not performed. Parents suspected that he might have had speech delay at the age of 3 years, and he was referred to Shinshu University Hospital, Department of Otolaryngology for audiologic examinations. An auditory brainstem response (ABR) showed bilateral hearing loss that was approximately 60 dB nHL

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