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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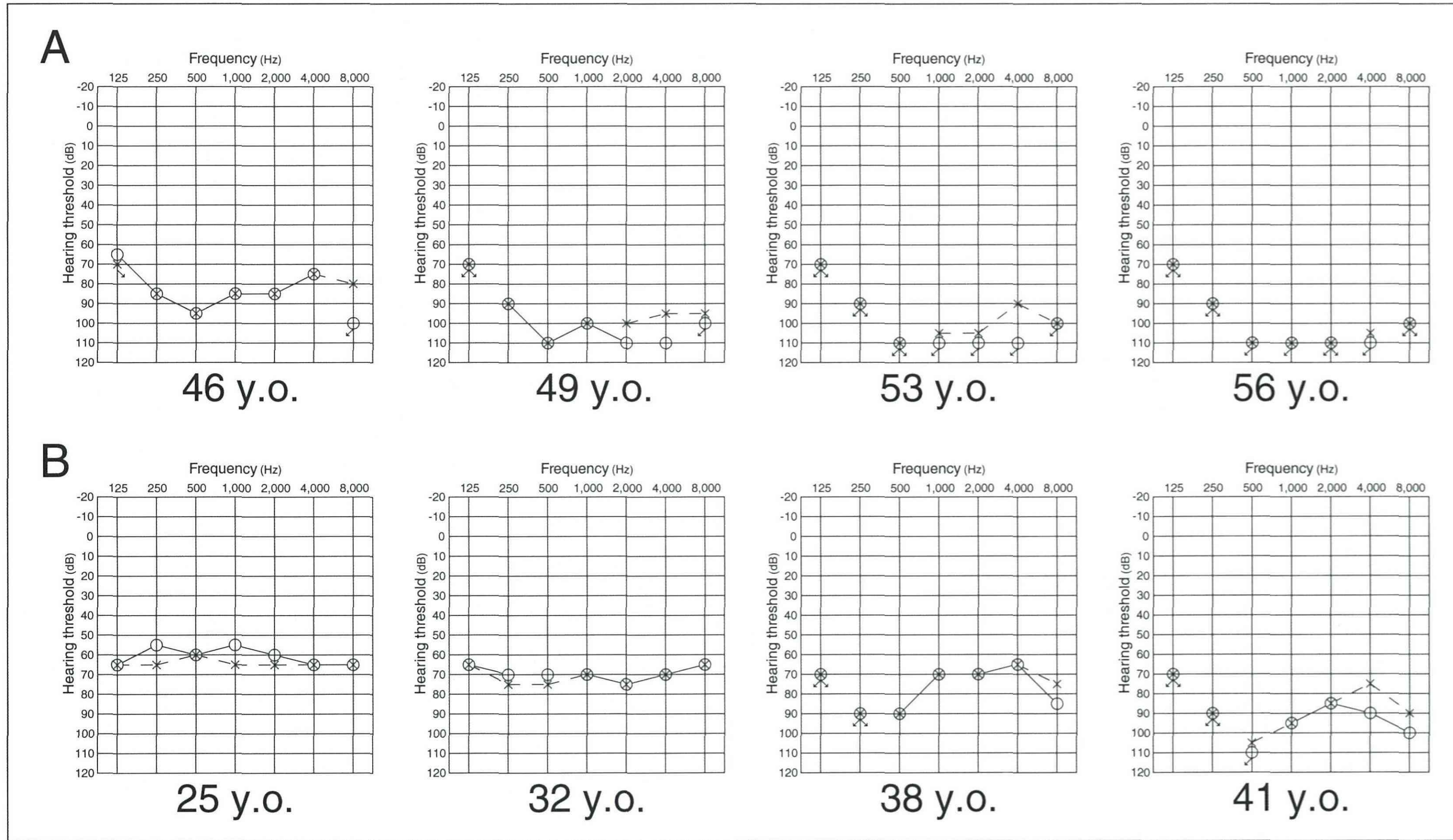

Figure 2. Serial audiograms of affected patients II-2 and III-2. (A) Audiograms of the patient (II-2) over a period of 10 years. Hearing loss rapidly deteriorated during the period from 46 to 49 years old. (B) Audiograms of the patient (III-2) over a period of 16 years. Hearing loss was relatively stable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

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.

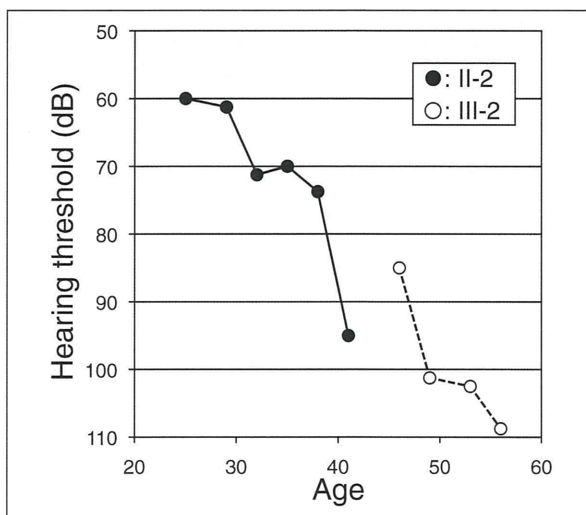

Figure 3. Progression of hearing loss in patients II-2 and III-2. Hearing loss rapidly deteriorated around age 40 to 50.

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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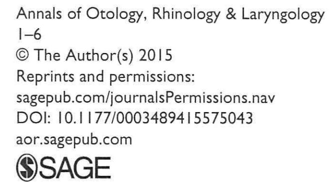

Novel Mutations in *LRTOMT* Associated With Moderate Progressive Hearing Loss in Autosomal Recessive Inheritance

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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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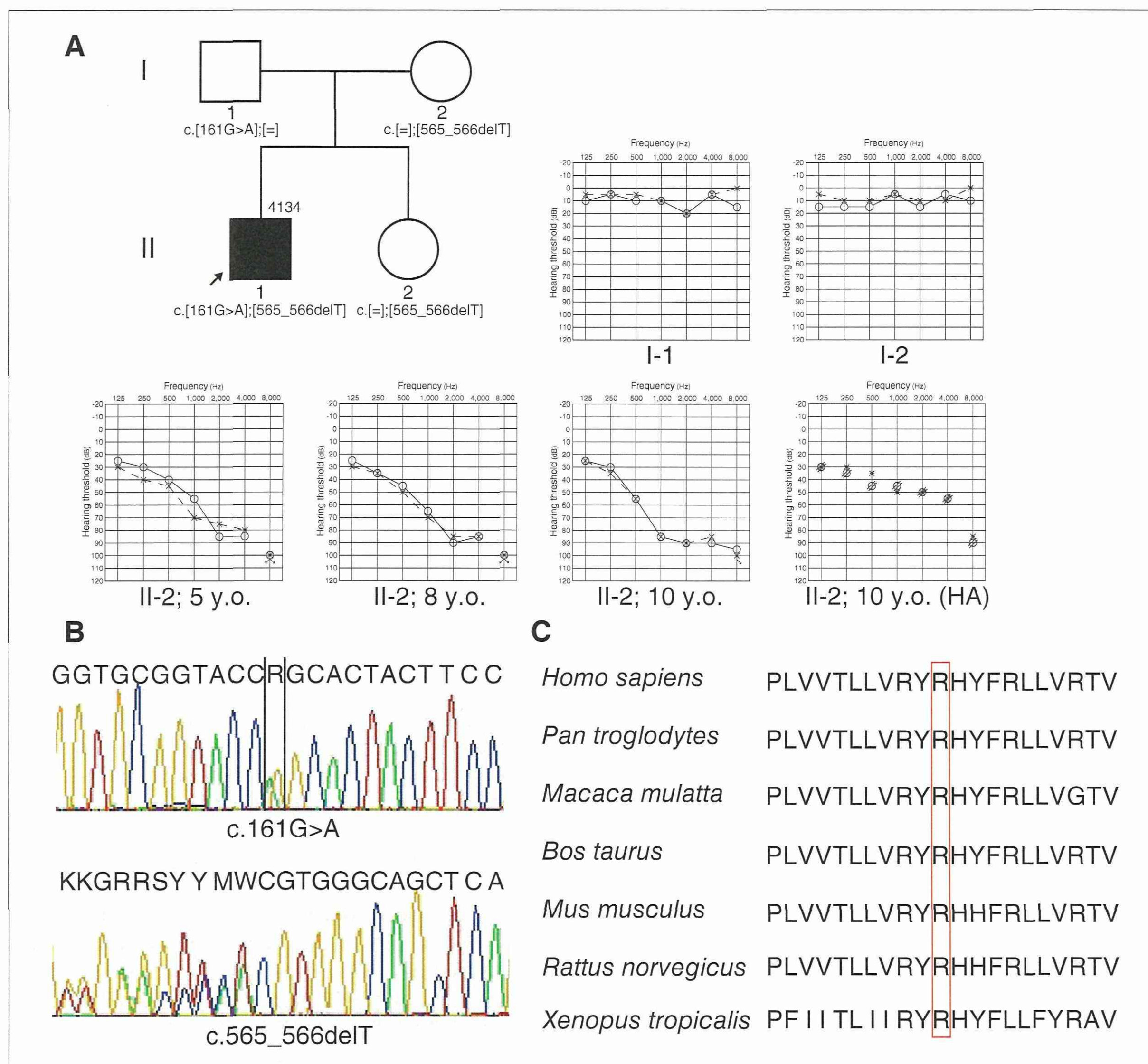

Figure 1. Pedigree and pure-tone audiograms of family members as well as the results of *LRTOMT* mutation analysis. (A) Pedigree shows that the sporadic nature of the cases and allele segregation is compatible with autosomal recessive inheritance. Pure-tone audiograms show the deterioration of the threshold from 55 to 85 dB at 1000 Hz over 5 years, suggesting progressive hearing loss. HA, hearing thresholds with hearing aids. (B) The electropherogram of mutations in case ID 4134. (C) Conservation of the region of the LRTOMT2 protein including the missense mutation (c.161G>A) site.

in both ears, and behavioral observation audiometry (BOA) showed thresholds of 30 to 50 dB at around 500 to 2000 Hz. Computed tomography (CT) findings of the middle and inner ear were normal. He was diagnosed with bilateral symmetric moderate sloping hearing loss in the high frequencies. He was promptly fitted with bilateral hearing aids. At the age of 5 years, pure-tone audiometry (PTA) was performed, showing down sloping moderate SNHL. He received followed-up auditory assessment for 6 years, and his hearing loss progressed at 1000 Hz and higher frequencies. He did not suffer from tinnitus or dizziness during this period.

His parents and younger sister had normal hearing; there was no positive family history of hearing loss or other cognitive disorders. Audiologic assessment results and pedigree are shown in Figure 1A.

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Table 1. Known Mutations in the *LRTOMT* Gene and Associated Phenotypes.[a]

Nucleotide Change	Amino Acid Change	Type of Mutation	Zygosity	Hearing Loss Onset	Type of Hearing Loss	Population	Reference
c.47T>C	p.Leu16Pro	Missense	Homozygous	Prelingual	Severe-profound	Iranian	Du et al[5]
c.102G>A	p.Met34Ile	Missense	Homozygous	Prelingual	Profound	Iranian	Babanejad et al[6]
c.107delC	p.Ser35SerfsX13	Frameshift	Homozygous	Congenital	Profound	Iranian	Vanwesemael et al[7]
c.120G>T	p.Glu40Asp	Missense	Homozygous	Prelingual	Profound	Iranian	Babanejad et al[6]
c.121C>T	p.Arg41Trp	Missense	Homozygous	NA	NA	Iranian	Babanejad et al[6]
c.122G>A	p.Arg41Gln	Missense	Homozygous	Congenital	Severe	Tunisian	Ahmed et al[4]
c.122G>A	p.Arg41Gln	Missense	Homozygous	Congenital	Moderate-severe	Moroccan	Charif et al[8]
c.193T>C	p.Trp65Arg	Missense	Homozygous	NA	NA	Tunisian	Ahmed et al[4]
c.208G>A	p.Glu70Lys	Missense	Homozygous	Congenital	Profound	Pakistani	Ahmed et al[4]
c.213C>G	p.Tyr71X	Missense	Homozygous	Prelingual	Severe-profound	Iranian	Du et al[5]
c.238+4A>C	p.Ala29SerfsX54	Frameshift	Homozygous	Congenital	Severe-profound	Turkish	Ahmed et al[4]
c.161G>A	p.Arg54Gln	Missense	Compound heterozygous	Prelingual	Moderate	Japanese	This study
c.565_566delT	p.Ile188ThrfsX7	Frameshift		Prelingual	Moderate	Japanese	This study

Abbreviation: NA, not applicable or not reported.
[a]All nucleotide and amino acid changes are assigned to HGVS NM_001145309.

Mutation Analysis

We performed MPS and identified 1 novel frame-shift mutation and 1 missense mutation. The former mutation corresponded to c.565_566delT (NM_001145309) in exon 9 and led to a frameshift mutation and truncation (p.Ile188ThrfsX7). The second mutation was c.161G>A (p.Arg54Gln) located in exon 7, which was strongly suspected to be pathogenic. In silico prediction software (SIFT, MutationTaster, and Polyphen2) indicated the mutation as damaging (0.84, 0.98, and 1.00 [the maximum scores were 1.00], respectively). We also performed Sanger sequencing for the family segregation study and a confirmation of the variant MPS outputted result. As shown in Figure 1B, Sanger sequencing results revealed that the parents had 1 of either mutation in heterozygote and his younger sister had the heterozygous c.161G>A mutation. None of these mutations were identified in the 192 Japanese normal hearing controls. The residue is conserved as arginine in all sequenced vertebrates (Figure 1C).

Discussion

In this report, we identified a novel compound heterozygous mutation in the *LRTOMT* gene among sporadic hearing loss cases that were presumably autosomal recessive inherited. This is the first case reported to be affected by the compound heterozygous mutation (Table 1). Previously, there have been only 8 families reported with hearing loss caused by mutations in *LRTOMT*, and these were all homozygous mutations due to consanguineous families. In this study, we found a patient with hearing loss caused by a compound heterozygous mutation in a nonconsanguineous family. The c.161G>A mutation (NM_001145309) corresponded to a p.Arg54Gln substitution, which changed the basicity of arginine into a neutral glutamine residue. This arginine residue in the LRTOMT protein region of the mutated site was conserved among the other species. The other mutation corresponded to c.565_566delT (NM_001145309) and led to a frameshift mutation and a subsequent truncation of the protein (p.Ile188ThrfsX7) in exon 9. The mutated residues were segregated in both alleles of *LRTOMT*, present within the LRTOMT2 protein coding region. The LRTOMT2 protein has a transmembrane catechol-O-methyltransferase (COMT) domain and is also known as COMT2. This is strongly expressed in inner and outer hair cells and also in the vestibular organ.[5] Du et al[5] generated a mouse model of *Comt2* mutation (*add* mice) and found that the mice were profoundly deaf and had vestibular defects. Degeneration of the organ of Corti and disorganization of the stereocilia were observed by 8 weeks of age. Thus, these findings support the argument that mutations in LRTOMT2 are associated with hair cell defects and lead to SNHL. As shown in Figure 2, all previously reported mutations are assigned to the LRTOMT2 (NM_001145309) region, not to the LRTOMT1 (NM_145309) region. The majority of mutations, including the missense mutation that we identified, accumulate in exon 7 of the LRTOMT2 coding region. Therefore, the mutations in *LRTOMT2* are more likely to affect hearing loss through hair cell degeneration, and the region that exon 7 encodes might be a mutational hot spot in the *LRTOMT* gene.

Previous studies have shown that affected individuals had severe to profound prelingual SNHL, whereas the case with *LRTOMT* mutations that we identified had only moderate SNHL. The frameshift mutation, c.565_566delT, is located in

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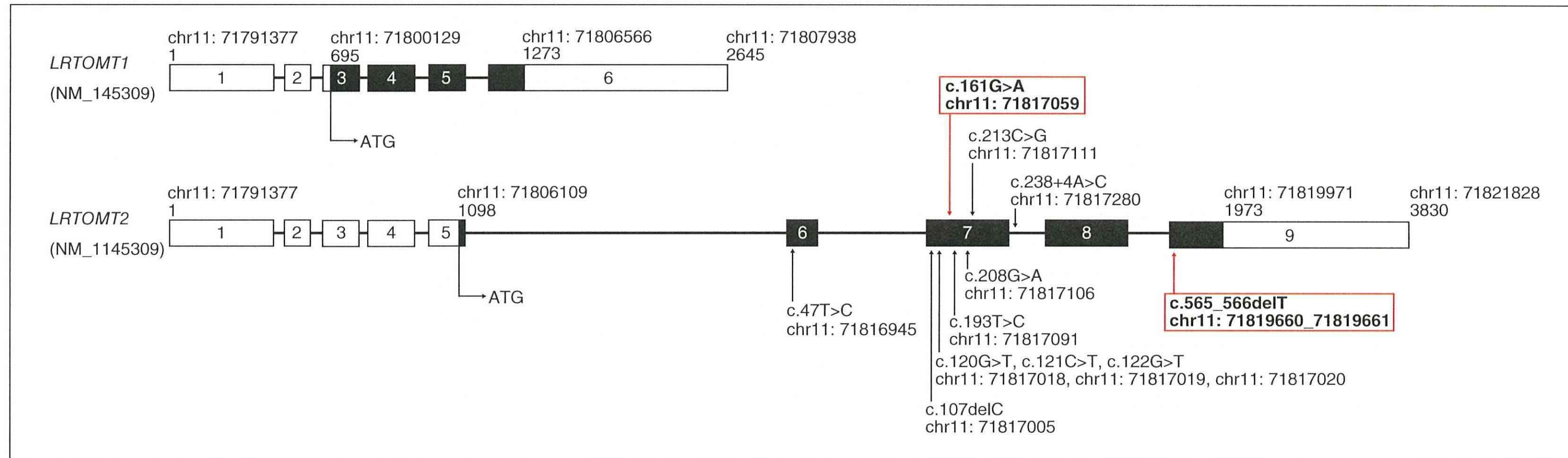

Figure 2. Two isoforms encoded by the *LRTOMT* gene. LRTOMT1 (NM_145309) and LRTOMT2 (NM_001145309) consist of 6 and 9 exons, respectively. Both are translated into 2 alternate reading frames using different exon sets; that of LRTOMT1 starts in exon 3 and that of LRTOMT2 starts in exon 5. CDS regions are colored black. Most of the previously reported mutations are located in exon 7 of the LRTOMT2 region.

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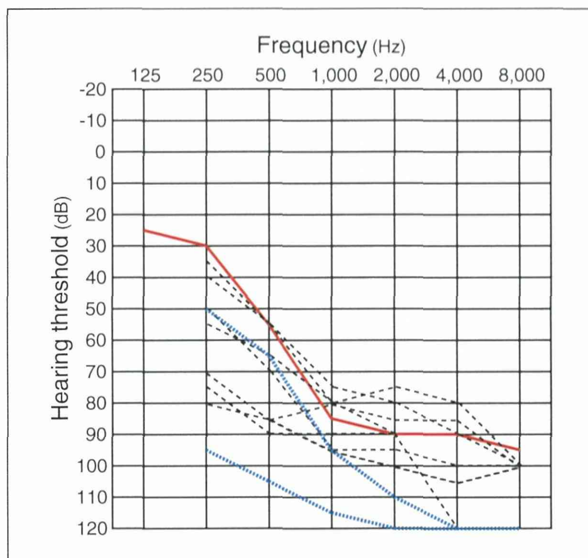

Figure 3. Overlapping audiogram of the affected individuals with *LRTOMT* mutations. Red line indicates the patient in this study. Dotted lines (in black) indicate the individuals reported by Kalay et al,[1] and dotted lines (in blue) indicate the individuals reported by Tlili et al.[3]

the region near the 3′-end and C-terminus of the LRTOMT2 region (Figure 2), in which the mutated allele might be translated partially. It can be supposed that an incomplete LRTOMT2 protein with residual activity was formed in the present case exhibiting moderate SNHL. Kalay et al[1] and Tlili et al[3] reported audiologic profiles for each individual showing high frequency sloping hearing loss. As shown in Figure 3, these overlapping PTA results were identical or similar to our case, but these studies had no longitudinal results. The serial audiologic findings for the 6 years of our study show the deterioration of hearing level in the middle frequencies. Our patient still had residual hearing in the lower frequencies, with hearing aids necessary, but hearing ability and speech perception with hearing aids are speculated to be further reduced due to the deterioration of mid- to high-frequency hearing. We suggest that careful management of hearing is necessary for *LRTOMT* hearing loss patients.

In conclusion, our results indicate that mutations in the *LRTOMT* gene lead to alterations in the LRTOMT2 (COMT2) protein and might be involved in progressive SNHL. Further studies, including a long-term follow-up and accurate characterization of phenotypic features, will afford a better understanding of the *LRTOMT* gene.

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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.

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Deafness Gene Variations in a 1120 Nonsyndromic Hearing Loss Cohort: Molecular Epidemiology and Deafness Mutation Spectrum of Patients in Japan

Shin-ya Nishio, PhD[1,2] and Shin-ichi Usami, MD, PhD[1,2]

Abstract

Objectives: To elucidate the molecular epidemiology of hearing loss in a large number of Japanese patients analyzed using massively parallel DNA sequencing (MPS) of target genes.

Methods: We performed MPS of target genes using the Ion PGM system with the Ion AmpliSeq and HiSeq 2000 systems using SureSelect in 1389 samples (1120 nonsyndromic hearing loss cases and 269 normal hearing controls). We filtered the variants identified using allele frequencies in a large number of controls and 12 predication program scores.

Results: We identified 8376 kinds of variants in the 1389 samples, and 409 835 total variants were detected. After filtering the variants, we selected 2631 kinds of candidate variants. The number of *GJB2* mutations was exceptionally high among these variants, followed by those in *CDH23, SLC26A4, MYO15A, COL11A2, MYO7A*, and *OTOF*.

Conclusions: We performed a large number of MPS analyses and clarified the genetic background of Japanese patients with hearing loss. This data set will be a powerful tool to discover rare causative gene mutations in highly heterogeneous monogenic diseases and reveal the genetic epidemiology of deafness.

Keywords

hearing loss, massively parallel DNA sequencing, next-generation DNA sequencer, molecular epidemiology

Introduction

Congenital hearing loss is one of the most common sensory disorders, occurring in 1 of 700 to 1000 newborns. Approximately 50% to 70% of cases are attributable to genetic causes,[1] and 10% to 25% of cases are attributable to congenital cytomegalovirus infection. More than 80 genes have been identified as a cause of hearing loss and an estimated 100 genes are involved in hearing loss.[2]

Despite such advances in gene identification, clinicians and/or geneticists sometimes encounter difficulties related to molecular diagnosis in a clinical setting; for example, the family size is not large enough to allow linkage analysis, meaning that only limited familial information for predicting the causative gene is available. In such cases, targeted exon sequencing of selected genes using massively parallel DNA sequencing (MPS) technology will potentially enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis.

An increasing number of articles regarding gene discovery and successful clinical application for the identification of genes responsible for deafness using MPS have recently been published.[3-16] We applied MPS technology to (1) discover causative mutations in relatively rare causative genes[12,13] and (2) clarify the molecular epidemiology.[12] Our results demonstrated that MPS-based screening is powerful in terms of identifying mutations in rare causative genes, and from an epidemiological view point, *GJB2* mutations are involved in 30% to 40% cases of deafness, while the remaining cases of hearing loss arise from various rare genes/mutations that were not easy to identify using the conventional one-by-one screening approach.

For clinical application to genetic heterogeneous diseases, systemic screening of known genes in a cost-effective manner is required. Hybridization-based capture is commonly used for genomic target enrichment, but for clinical application,

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polymerase chain reaction (PCR)–based technologies in combination with MPS have also been proposed.[11,13,16,17]

In the current study, on the basis of our PCR-based technologies in combination with MPS,[13,17] we increased the number of patients (1120 cases of nonsyndromic hearing loss) to establish a database for clinical molecular diagnosis and to confirm the molecular epidemiology of deafness. Data analysis concerning diagnostic sensitivity and specificity, which is important for clinical application, was also performed.

Subjects and Methods

Subjects

A total of 1120 Japanese patients (266 autosomal dominant or mitochondrial inheritance cases, 600 autosomal recessive inheritance or sporadic cases, and 253 unknown family history cases) with bilateral nonsyndromic sensorineural hearing loss from 53 ear, nose, and throat departments nationwide participated in this study. In addition, 269 normal hearing controls, confirmed by pure-tone audiometry, were also enrolled. Informed written consent was obtained from all subjects, their next of kin, caretakers, or guardians (in the case of minors) prior to participation. This study was approved by the Shinshu University Ethical Committee and the ethics committees of all other participating institutions listed in the Acknowledgments.

Genetic Analysis

We performed the MPS analysis using an Ion PGM with Ion AmpliSeq for 1174 samples (905 hearing loss cases and 269 normal hearing controls) and using HiSeq 2000 with SureSelect in 215 cases.

Amplicon Library Preparation and Ion PGM Platform Sequencing

Amplicon libraries of the target gene exons from 63 genes reported to cause nonsyndromic hearing loss[2] were prepared with an Ion AmpliSeq Custom Panel (Life Technologies, Foster City, California, USA). These libraries were designed with an Ion AmpliSeq Designer (Life Techologies), and amplicon libraries were prepared using an Ion AmpliSeq Library Kit 2.0 and an Ion Xpress Barcode Adapter 1-96 Kit (Life Technologies) according to the manufacturer's instructions. After the amplicon libraries were prepared, they were diluted to 20 pM, and the same amount of libraries from the 6 libraries of 6 patients were pooled for 1 sequence reaction. The emulsion polymerase chain reaction and sequencing were performed with an Ion Torrent Personal Genome Machine (PGM) system using an Ion PGM 200 Sequencing Kit and an Ion 318 Chip (Life Technologies) according to the manufacturer's instructions. The detailed protocol has been described elsewhere.[13,17] The sequence data were mapped to the human genome sequence (build GRCh37/hg19) with the Torrent Mapping Alignment Program. After sequence mapping, the DNA variant regions were piled up with the Torrent Variant Caller plug-in software version 4.0 (Life Technologies).

Targeted Enrichment and HiSeq Platform Sequencing

The SureSelect target enrichment kit, designed for the 112 potentially deaf-causing genes, including the 63 genes reported to cause nonsyndromic hearing loss, the 22 genes reported to cause syndromic hearing loss, and the 36 genes highly expressed in the adult human inner ear by microarray analysis, was used in this study.[18] The detailed gene list is described in our previous report.[12] A 3-μg DNA aliquot was fragmented using the Covaris S2 System (Covaris, Woburn, Massachusetts, USA) to a fragment length of about 200 bp. Furthermore, the target regions were enriched using the SureSelect Target DNA Enrichment kit with a barcode adapter (Agilent Technologies, Santa Clara, California, USA) according to the manufacturer's protocol. The same amount of libraries from each of 12 patients was pooled into 1 tube and analyzed in 1 lane of the Illumina HiSeq 2000 sequencer (Illumina, San Diego, California, USA) according to the manufacturer's protocol. The sequence data were processed by filtering the read quality to QV = 30 as a cutoff and duplicate reads removed. After the filtering process, sequence reads were mapped to the human genome sequence (build GRCh37/hg19) using BWA software.[19] After sequence mapping, the DNA variant regions were piled up with GATK software.[20]

Filtering Detected Variants

After detecting the variants, the effects of the variants were analyzed using ANNOVAR software.[21,22] The missense, nonsense, insertion/deletion, and splicing variants were selected among the identified variants. Variants were further selected as <1% of: (1) the 1000 genome database,[23] (2) the 6500 exome variants,[24] (3) the human genetic variation database (data set for 1208 Japanese exome variants),[25] (4) the 269 in-house Japanese normal hearing controls, and (5) 1000 control data in the deafness variation database.[26] The filtering process is shown in Figure 1.

Results and Discussion

DNA Sequencing Metrics and Accuracy of Each Sequencing System

MPS metrics used in this study are summarized in Supplemental Table 1 (available in the online journal). The

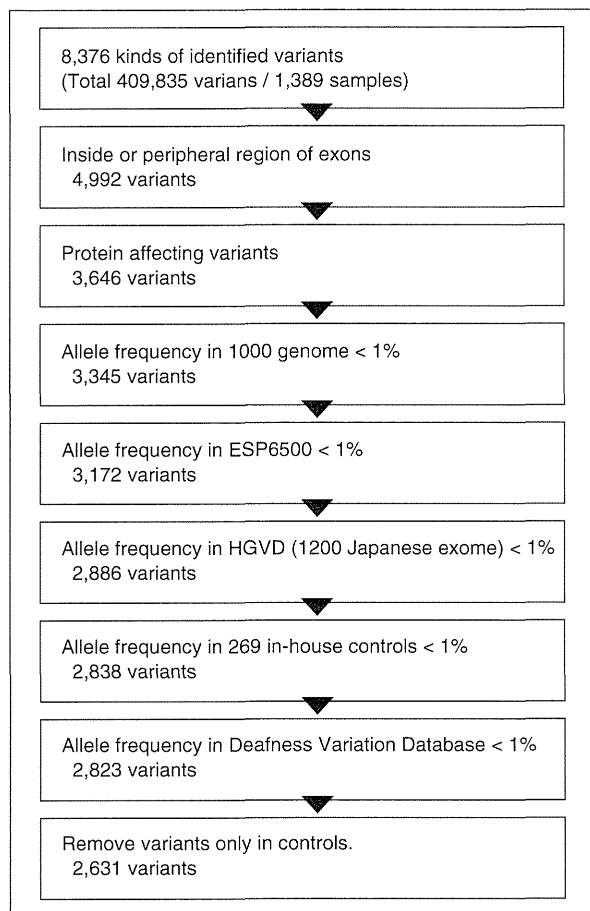

Figure 1. Algorithm applied in this study. The nonsense, splice-site, insertion-deletion, and missense variants were chosen according to this algorithm.

mean depth of coverage of the target region for 1174 samples analyzed by the Ion PGM sequencer was 284.3 ± 94.5× (range, 690.0-96.6×). The percentage of each region with more than 20× coverage (indicating the percentage of each region sequenced 20 times or more by MPS) was 97.6% ± 0.9% (range, 93.1%-99.2%). To reduce the risk of incorrect genotyping and missed *single nucleotide polymorphisms (*SNPs) in poor-coverage regions, we employed a minimum mean depth of coverage of 100 and a minimum percentage of 96% for regions with more than 20× coverage. The mean depth of coverage for 215 samples analyzed by the HiSeq 2000 sequencer was 1536.1 ± 538.4× (range, 206-5925×). The percentage of each region with more than 20× coverage (indicating the percentage of each region sequenced 20 times or more by MPS) was 98.8% ± 0.7% (range, 93.5%-99.4%).

To investigate the accuracy of the MPS used in this study, we compared the results of the Invader assay-based mutation screening[27] and MPS by blinded samples (384 samples were analyzed by both methods). As a result, 99.98% of results were identical in the Ion PGM system. Detailed information regarding this comparison was given in our recent report.[17]

DNA Variants Identified in the Large Japanese Nonsyndromic Hearing Loss Cohort

From the 1389 samples, including 1120 nonsyndromic sensorineural hearing loss cases and 269 controls, we identified 8376 kinds of variants, and 409 835 (average, 295.1 variants/sample; Figure 1) total variants were detected. Among the 8376 variants, 4992 were located in the exon region, 2 were located in exonic regions of micro-RNA MIR96, which is a causative micro RNA associated with DFNA50, and 92 were located in splicing junctions. The others were located in the 3'- untranslated region (UTR), 5'-UTR, intron, and intergenic regions. Among the exon region variants, 3646 affected proteins (2955 missense variants, 76 nonsense variants, 161 frame shift deletions, 71 frame shift insertions, 136 frame shift multibase substitutions, 89 non–frame shift deletions, 4 non–frame shift insertions, 149 non–frame shift multibase substitutions, 2 exonic splice junction substitutions, and 3 stop loss mutations). Together with the splicing junction and noncoding RNA mutations, 3742 variants remained for further analysis.

We filtered these variants using allele frequency <1% of (1) the 1000 genome project; (2) the exome variant server; (3) the human genetic variation database, which contains 1200 Japanese exome data; (4) the 269 in-house Japanese normal hearing controls; and the (5) 1000 controls in the deafness variation database. For this filtering step, we employed <1% frequency as a cutoff line because the most frequent pathogenic variants observed in the Japanese population were *GJB2*: c.235delC and *GJB2*: c.109C>G, and the allele frequencies in the Japanese control population were estimated as 0.4% and 0.6%.[28] However, there were some possibilities to filter out the frequent autosomal recessive (AR)-pathogenic variants; therefore, we did not filter out the pathogenic or likely pathogenic variants previously reported in the deafness variation database[26] and ClinVar.[29]

After filtering the many ethnic controls, 2823 variants remained (Figure 1). Among them, we removed variants only found in controls as probable polymorphisms. Finally, 2631 variants were selected as candidates (Figure 1; 2017 missense mutations, 72 nonsense mutations, 2 stop loss mutations, 131 frame shift deletions, 30 frame shift insertions, 129 frame shift multibase substitutions, 21 non–frame shift deletions, 4 non–frame shift insertions, 138 non–frame shift multibase substitutions, 2 exon split junction substitutions, 77 splicing junction regions, and 2 micro-RNA MIR96 exonic regions).

Table 1. Previously Reported Pathogenic Variants Detected in This Study.

Identified Variants	ESP6500	1000g	HGVD	dbSNP138	SIFT	PP2 HDVI	PP2 HVAR_	ClinVar	DVD	Disease	PMID	Pat NUM	CNT Num
ACTG1:NM_001614:c.353A>T:p.K118M				rs104894544	D	B	P	Pathogenic (DFNA20)	Pathogenic	NSHL-Dominant	13680526	3	0
ACTG1:NM_001614:c.721G>A:p.E241K				rs267606631	D	D	D	Pathogenic (DFNA20)	Pathogenic	NSHL-Dominant	19477959	1	0
CDH23:NM_022124:c.2407G>A:p.V803I			0.00141		T	B	B		Pathogenic	NSHL-Recessive	22899989	0	1
CDH23:NM_022124:c.2866G>A:p.E956K					D	D	D		Pathogenic	NSHL-Recessive	22899989	4	0
CDH23:NM_022124:c.4249C>T:p.R1417W			0.001255		D	D	P		Pathogenic	NSHL-Recessive	22899989	6	2
CDH23:NM_022124:c.5131G>A:p.V1711I	0.000079	0.000399361	0.001247	rs181611778	T	D	D		Pathogenic	NSHL-Recessive	22899989	2	0
CDH23:NM_022124:c.5147A>C:p.Q1716P					T	D	D		Pathogenic	NSHL-Recessive	17850630	4	0
CDH23:NM_022124:c.5627G>A:p.S1876N			0.003179		T	P	P		Pathogenic	NSHL-Recessive	22899989	6	0
CDH23:NM_022124:c.6085C>T:p.R2029W			0.002271		D	D	D		Pathogenic	NSHL-Recessive	17850630	19	1
CDH23:NM_022124:c.6319C>T:p.R2107X					T	.	.		Pathogenic	Usher syndrome	11090341	1	0
CDH23:NM_022124:c.6389C>T:p.A2130V					T	B	B		Pathogenic	NSHL-Recessive	22899989	2	0
CDH23:NM_022124:c.6861T>G:p.N2287K					D	D	D		Pathogenic	NSHL-Recessive	22899989	1	0
CDH23:NM_022124:c.719C>T:p.P240L		0.000199681	0.002725	rs121908354	T	D	D	Pathogenic (DFNB12)	Pathogenic	NSHL-Recessive	17850630	45	2
CDH23:NM_022124:c.902G>A:p.R301Q	0.000081			rs121908355	T	D	D	Pathogenic (Alport syndrome)	Pathogenic	NSHL-Recessive	17850630	2	0
CDH23:NM_022124:c.9127C>T:p.R3043W	0.00008				D	D	P		Pathogenic	Usher syndrome	21569298	1	0
COCH:NM_004086:c.263G>A:p.G88E				rs121908928	T	D	D	Pathogenic (DFNA9)	Pathogenic	NSHL-Dominant	9806553	1	0
COL11A2:NM_080680:c.2492C>T:p.S831L	0.000118			rs121912949	T	D	P		Pathogenic	Otospondylomegaepiphyseal dysplasia, AD	NULL	1	0
COL4A5:NM_000495:c.2215C>G:p.P739A		0.00344371	0.059811	rs104886164	T	B	B	Pathogenic (Alport syndrome)				19	0
COL4A5:NM_000495:c.2858G>T:p.G953V	0.000189	0.00794702	0.01005	rs78972735	.	.	.	Pathogenic (Alport syndrome)				2	0
CRYM:NM_001888:c.941A>C:p.K314T				rs104894512	D	P	B	Pathogenic (AD-NSHL)	Pathogenic	NSHL-Dominant	12471561	2	0
EYA1:NM_000503:c.1276G>A:p.G426S			0.00134	rs121909199	D	D	D	Pathogenic (BOR syndrome)	Pathogenic	BOR syndrome, AD	10655545	2	0
EYA1:NM_000503:c.1319G>A:p.R440Q				rs121909196	D	D	D	Pathogenic (Melnick-Fraser syndrome)	Pathogenic	BOR syndrome, AD	10464653	1	0
EYA1:NM_000503:c.724A>G:p.S242G		0.000199681	0.01083	rs191838840	T	B	B		Pathogenic	BOR syndrome, AD	12701758	4	0
GJB2:NM_004004:c.109G>A:p.V37I	0.001307	0.0153754	0.006806	rs72474224	T	D	D	Pathogenic (DFNB1A)	Pathogenic	NSHL-Recessive	10633133	47	4
GJB2:NM_004004:c.134G>A:p.G45E			0.00349	rs72561723	D	D	D	Pathogenic (DFNB1A)	Pathogenic	KID syndrome, DFNB1A	10501520	46	1
GJB2:NM_004004:c.146C>T:p.A49V			0.002494		D	P	B		Pathogenic	NSHL-Recessive	12560944	2	0
GJB2:NM_004004:c.212T>C:p.I71T			0.001166		D	D	P		Pathogenic	NSHL-Recessive	12560944	1	1
GJB2:NM_004004:c.223C>T:p.R75W				rs104894402	D	D	D	Pathogenic (DFNA3A)	Pathogenic	NSHL-Dominant	9856479	1	0
GJB2:NM_004004:c.235delC:p.L79fs		0.00159744		rs80338943				Pathogenic (DFNB1A)	Pathogenic	NSHL-Recessive	10501520	166	3
GJB2:NM_004004:c.257C>G:p.T86R					D	D	D		Pathogenic	NSHL-Recessive	12560944	11	0
GJB2:NM_004004:c.257C>T:p.T86M					D	D	D		Pathogenic	NSHL-Recessive	17041943	2	0
GJB2:NM_004004:c.29T>C:p.L10P					D	D	D		Pathogenic	NSHL-Recessive	12865758	1	0
GJB2:NM_004004:c.334_335del:p.K112fs									Pathogenic	NSHL-Recessive	9529365	1	0
GJB2:NM_004004:c.368C>A:p.T123N	0.000154	0.00179712	0.006146	rs111033188	T	B	B	Probable nonpathogenic	Pathogenic	NSHL-Recessive	10983956	4	2
GJB2:NM_004004:c.379C>T:p.R127C					D	D	B		Pathogenic	NSHL-Recessive	11587277	1	0
GJB2:NM_004004:c.389G>C:p.G130A					D	D	D		Pathogenic	NSHL-Recessive	12792423	1	0
GJB2:NM_004004:c.408C>A:p.Y136X			0.00349		T	.	.		Pathogenic	NSHL-Recessive	10501520	46	1
GJB2:NM_004004:c.427C>T:p.R143W	0.000231	0.000199681	0.002331	rs80338948	D	D	D	Pathogenic (DFNB1A)	Pathogenic	NSHL-Recessive	9471561	29	0
GJB2:NM_004004:c.511G>A:p.A171T	0.000154	0.000399361	0.001166	rs201004645	T	P	B	Probable nonpathogenic	Pathogenic	NSHL-Recessive	11438992	1	0

(continued)

Table 1. (continued)

Identified Variants	ESP6500	1000g	HGVD	dbSNP138	SIFT	PP2 HDVI	PP2 HVAR_	ClinVar	DVD	Disease	PMID	Pat NUM	CNT Num
GJB2:NM_004004:c.571T>C:p.F191L		0.000199681	0.004115		D	D	D	Probable nonpathogenic	Pathogenic	NSHL-Recessive	12772454	0	1
GJB2:NM_004004:c.583A>G:p.M195V			0.001166		D	D	D		Pathogenic	NSHL-Recessive	20497192	4	0
GJB2:NM_004004:c.95G>A:p.R32H				rs111033190	D	D	D	Pathogenic (DFNB1A)	Pathogenic	NSHL-Recessive	11493200	3	0
GJB2:NM_004004c.299_300del:p.H100fs				rs111033204				Pathogenic (DFNB1A)	Pathogenic	NSHL-Recessive	10633133	14	0
GJB3:NM_024009:c.538C>T:p.R180X		0.000199681		rs74315319	T	.	.	Pathogenic (DFNA2B)	Benign*	NULL	NULL	1	0
GJB3:NM_024009:c.547G>A:p.E183K	0.000077	0.000998403	0.001361	rs74315318	D	D	D	Pathogenic (DFNA2B)	Benign*	NULL	NULL	2	0
GJB3:NM_024009:c.580G>A:p.A194T		0.00139776	0.01179	rs117385606	T	B	B	Pathogenic	Benign*	NULL	NULL	16	2
GJB6:NM_006783:c.689dupA:p.N230fs	0.000639							Pathogenic				9	0
KCNQ4:NM_004700:c.546C>G:p.F182L		0.000599042	0.006579	rs80358273	T	B	B	Pathogenic (DFNA2)	Pathogenic	NSHL-Dominant	17033161	8	1
LOXHD1:NM_144612:c.4480C>T:p.R1494X	0.001314	0.000199681		rs201587138	T	.	.		Pathogenic	NSHL-Recessive	23226338	2	0
LOXHD1:NM_144612:c.469C>T:p.R157C		0.000399361			.	.	.		Pathogenic	Fuchs corneal dystrophy		1	0
LOXHD1:NM_144612:c.4714C>A:p.R1572R	0.000657	0.0181709	0.051502	rs75949023				Pathogenic (DFNB77)	Benign*	NULL	NULL	83	18
MARVELD2:NM_001244734:c.1295+1G>A					.	.	.		Pathogenic	NSHL-Recessive	18084694	1	0
MYH9:NM_002473:c.2104C>T:p.R702C				rs80338826	D	D	D	Pathogenic (Fechtner syndrome)	Pathogenic	Epstein syndrome	10973259	1	0
MYH9:NM_002473:c.2114G>A:p.R705H				rs80338828	D	D	D	Pathogenic (DFNB17)	Pathogenic	NSHL/MYH9 related diseases, AD	11023810	1	0
MYO15A:NM_016239:c.6731G>A:p.G2244E					D	D	D		Pathogenic	NSHL-Recessive	17546645	2	0
MYO15A:NM_016239:c.8467G>A:p.D2823N					D	D	D		Pathogenic	NSHL-Recessive	22736430	1	0
MYO6:NM_004999:c.3496C>T:p.R1166X				rs121912558	T	.	.	Pathogenic (DFNB37)	Pathogenic	NSHL-Recessive	12687499	1	0
MYO7A:NM_000260:c.2005C>T:p.R669X	0.000081			rs111033201	T	.	.	Pathogenic (USH1B)	Pathogenic	Usher syndrome	9718356	1	0
MYO7A:NM_000260:c.2311G>T:p.A771S			0.003129		D	P	P		Pathogenic	Usher syndrome	20844544	4	1
MYO7A:NM_000260:c.3508G>A:p.E1170K				rs111033214	D	D	D	Pathogenic (USH1B)	Pathogenic	Usher syndrome	10425080	1	0
MYO7A:NM_000260:c.3602G>C:p.C1201S		0.000798722	0.002287	rs117966637	D	D	D	Unknown	Pathogenic	Usher syndrome	23237960	3	1
MYO7A:NM_000260:c.3718C>T:p.R1240W	0.000079	0.000199681		rs371374104	D	D	D		Pathogenic	Usher syndrome	16963483	1	0
MYO7A:NM_000260:c.3979G>A:p.E1327K	0.000079			rs373169422	D	D	D		Pathogenic	Usher syndrome	12112664	1	0
MYO7A:NM_000260:c.635G>A:p.R212H				rs28934610	D	D	D	Pathogenic (USH1B)	Pathogenic	Usher syndrome	7870171	2	0
MYO7A:NM_000260:c.652G>A:p.D218N	0.00008			rs201539845	D	D	D	Pathogenic (DFNA11)	Pathogenic	NSHL-Recessive	21150918	1	0
OTOF:NM_194248:c.1236delC:p.P412fs								Pathogenic (DFNB9)				2	0
OTOF:NM_194248:c.1273C>T:p.R425X					T	.	.	Pathogenic (DFNB9)				1	0
OTOF:NM_194248c.4023+1G>A		0.00179712	0.002269	rs186810296	.	.	.	Pathogenic				5	2
PCDH15:NM_033056:c.733C>T:p.R245X	0.000384			rs111033260	T	.	.	Pathogenic (USH1F)	Benign*	NULL	NULL	2	0
SIX1:NM_005982:c.386A>G:p.Y129C				rs104894478	D	D	D	Pathogenic (BOR syndrome 3)	Pathogenic	BOR syndrome, AD	15141091	1	0
SLC26A4:NM_000441:c.1001+1G>A	0.000461			rs80338849	.	.	.	Pathogenic (DFNB4)	Pathogenic	Pendred syndrome-Recessive	9618167	1	0
SLC26A4:NM_000441:c.1115C>T:p.A372V				rs121908364	D	D	D	Pathogenic (DFNB4)	Pathogenic	NSHL; NSHL with EVA, AR	10190331	1	0
SLC26A4:NM_000441:c.1174A>T:p.N392Y		0.000199681		rs201562855	D	D	D		Pathogenic	NSHL-Recessive	12676893	1	0
SLC26A4:NM_000441:c.1229C>T:p.T410M	0.000231	0.000199681	0.00134	rs111033220	D	D	D	Pathogenic (DFNB4)	Pathogenic	NSHL with EVA/Pendred syndrome	9618167	16	1
SLC26A4:NM_000441:c.1315G>A:p.G439R					D	D	D		Pathogenic	NSHL-Recessive	17851929	2	0
SLC26A4:NM_000441:c.1489G>A:p.G497S				rs111033308	D	D	D	Pathogenic (DFNB4)	Pathogenic	NSHL with EVA/Pendred syndrome	9500541	1	0
SLC26A4:NM_000441:c.1579A>C:p.T527P			0.00134		D	D	D		Pathogenic	NSHL-Recessive	17851929	3	0
SLC26A4:NM_000441:c.165-13T>G									Pathogenic	NSHL with EVA, AR	19645628	0	1
SLC26A4:NM_000441:c.1804-6G>A		0.000599042		rs377713770					Pathogenic	NSHL-Recessive	15574297	2	0

(continued)

Table 1. (continued)

Identified Variants	ESP6500	1000g	HGVD	dbSNP138	SIFT	PP2 HDVI	PP2 HVAR_	ClinVar	DVD	Disease	PMID	Pat NUM	CNT Num
SLC26A4:NM_000441:c.2162C>T:p.T721M				rs121908363	D	D	D	Pathogenic (DFNB4)	Pathogenic	NSHL with EVA/Pendred syndrome	10190331	4	0
SLC26A4:NM_000441:c.2168A>G:p.H723R		0.000399361	0.002264	rs121908362	D	D	D	Pathogenic (DFNB4)	Pathogenic	NSHL with EVA/Pendred syndrome	9618166	53	2
SLC26A4:NM_000441:c.2219G>T:p.G740V	0.000154			rs111033310	T	B	B	Unknown	Pathogenic	NSHL with EVA, AR	16570074	1	0
SLC26A4:NM_000441:c.2228T>A:p.L743X					T	.	.		Pathogenic	NSHL with EVA, AR	19954013	2	0
SLC26A4:NM_000441:c.225C>G:p.L75L	0.000231	0.000399361	0.002141	rs187447337					Pathogenic	NSHL-Recessive	23185506	1	1
SLC26A4:NM_000441:c.2283A>G:p.T761T		0.000399361	0.038462	rs202033028					Pathogenic	NSHL-Recessive	23185506	1	0
SLC26A4:NM_000441:c.367C>T:p.P123S			0.001166		T	D	D		Pathogenic	NSHL-Recessive	14508505	2	0
SLC26A4:NM_000441:c.439A>G:p.M147V			0.001667		D	D	D		Pathogenic	NSHL-Recessive	14508505	3	0
SLC26A4:NM_000441:c.601-1G>A			0.001166		.	.	.		Pathogenic	Pendred syndrome-Recessive	14508505	5	0
SLC26A4:NM_000441:c.678T>C:p.A226A									Pathogenic	NSHL-Recessive	23185506	1	0
SLC26A4:NM_000441:c.697G>C:p.V233L					T	D	D	Unknown	Pathogenic	NSHL-Recessive	17443271	1	0
SLC26A4:NM_000441:c.757A>G:p.I253V			0.001166		T	P	P		Pathogenic	NSHL-Recessive	23185506	2	0
SLC26A4:NM_000441:c.918+1G>A					.	.	.		Pathogenic	Pendred syndrome-Recessive	9618166	1	0
SLC26A4:NM_000441:c.919-18T>G									Pathogenic	NSHL-Recessive	20137612	3	0
SLC26A4:NM_000441:c.919-2A>G			0.00134	rs111033313	.	.	.	Pathogenic (DFNB4)	Pathogenic	NSHL with EVA/Pendred syndrome	10874637	8	0
SLC26A4:NM_000441:c.920C>T:p.T307M	0.000077	0.000199681	0.001166	rs144691257	D	D	D		Pathogenic	NSHL with EVA/Mondini, AR	16570074	2	0
SLC26A4:NM_000441:c.G1975G>C:p.V659L		0.000199681		rs200455203	D	P	B		Pathogenic	NSHL-Recessive	17443271	2	0
TECTA:NM_005422:c.1685C>T:p.T562M			0.00187		T	D	P		Pathogenic	NSHL-Dominant	21520338	0	1
TECTA:NM_005422:c.4198C>T:p.H1400Y		0.000199681	0.00271		T	D	P		Pathogenic	NSHL-Dominant	22718023	2	2
TECTA:NM_005422:c.5372C>G:p.P1791R					T	B	B		Pathogenic	NSHL-Dominant	21520338	1	0
TECTA:NM_005422:c.5597C>T:p.T1866M	0.000077			rs140236996	D	D	D		Pathogenic	NSHL-Dominant	20947814	1	0
TMC1:NM_138691:c.1165C>T:p.R389X	0.000077	0.000199681		rs151001642	T	.	.	Pathogenic	Pathogenic	NSHL-Recessive	15605408	1	0
TMIE:NM_147196:c.257G>A:p.R86Q					D	D	P		Pathogenic	NSHL-Recessive	20206386	1	0
TMPRSS3:NM_024022:c.916G>A:p.A306T		0.000199681	0.002058	rs181949335	.	D	D	Probable-pathogenic	Pathogenic	NSHL-Recessive	17551081	1	1
USH1C:NM_005709:c.1016G>A:p.R339Q					D	P	B		Pathogenic	Usher syndrome	22135276	0	1
USH2A:NM_206933:c.1876C>T:p.R626X					T	.	.		Pathogenic	Usher syndrome	10729113	1	0
USH2A:NM_206933:c.2802T>G:p.C934W		0.000798722	0.003333	rs201527662	D	D	D	Pathogenic (USH2A)	Benign*	NULL	NULL	3	1
USH2A:NM_206933:c.802G>A:p.G268R				rs111033280	D	D	D	Unknown	Pathogenic	Usher syndrome	18273898	1	0
USH2A:NM_206933:c.8254G>A:p.G2752R		0.000399361		rs201863550	D	D	D		Pathogenic	Usher syndrome	19737284	1	0
USH2A:NM_206933:c.8559-2A>G		0.000199681			.	.	.	Pathogenic (USH2A)	Pathogenic	Usher syndrome	19023448	6	0
WFS1:NM_006005:c.1846G>T:p.A616S		0.000199681	0.003411		T	B	B		Pathogenic	NSHL-Dominant	16408729	1	0
WFS1:NM_006005:c.1957C>T:p.R653C	0.000231	0.000199681	0.1	rs201064551	D	D	D		Pathogenic	Diabetes, AD		1	0
WFS1:NM_006005:c.2051C>T:p.A684V					D	D	D	Pathogenic (Wolfram-like syndrome, AD)	Pathogenic	Wolfram-like syndrome (deafness with optic atrophy), AD		1	0
WFS1:NM_006005:c.2146G>A:p.A716T				rs28937893	T	D	P	Pathogenic (Wolfram-like syndrome, AD)	Pathogenic	Wolfram-like syndrome (deafness with optic atrophy), AD	11709537	1	0
WFS1:NM_006005:c.2171C>T:p.P724L				rs28937890	D	D	D	Pathogenic (Wolfram-like syndrome, AD)	Pathogenic	Wolfram-like syndrome (deafness with optic atrophy), AD	9771706	0	1
WFS1:NM_006005:c.2507A>C:p.K836T					T	D	D		Pathogenic	NSHL-Dominant	19877185	1	0
WFS1:NM_006005:c.2590G>A:p.E864K				rs74315205	T	D	D	Pathogenic (Wolfram-like syndrome, AD)	Pathogenic	Wolfram-like syndrome (deafness with optic atrophy), AD		2	0
WFS1:NM_006005c.2185G>A:p.D729N		0.000399361			T	B	B		Pathogenic	Wolfram syndrome, AR	12107816	1	0

Abbreviations: 1000g, 1,000 genome database[23]; ClinVar, Clinical variation database29; DVD: Deafness variation database[26]; ESP6500, 6500 exome variants[24]; HGVD, Human Genetic Variation Database[25]; NUM, identified allele number in 269 normal hearing controls; Pat NUM, identified allele number in 1120 hearing loss cases; CNT NUM, identified allele number in 269 controls; Benign*, recently re-categorized variants (from pathogenic to benign) using a large number of many ethnic controls allele frequencies[26]; AD, autosomal dominant; AR autosomal recessive; NSHL, non-syndromic hearing loss; BOR, Branchio-oto-renal; EVA, enlarged vestibular aqueduct; PP2, PolyPhen2; PMID, PubMed ID.

Of the 2631 variants, 1694 (64.4%) were found in 1 patient (Figure 2). A total of 392 variants (14.9%) were found in 2 patients, 139 (5.3%) were found in 3, 92 (3.5%) were found in 4, 47 (1.8%) were found in 5, and 267 (10.1%) were found in 6 or more.

Previously Reported and Identified Pathogenic Variants in the Large Japanese Nonsyndromic Hearing Loss Cohort

Of the 2631 candidate variants, 105 were categorized as pathogenic variants in the deafness variation database, and 49 were categorized as pathogenic variants in ClinVar (Table 1). Thirty-seven variants were categorized as pathogenic in both databases, and 6 variants (*GJB3*: NM_001005752: c.538C>T:p.R180X, *GJB3*:NM_024009: c.547G>A:p.E183K, *GJB3*:NM_024009:c.580G>A:p.A194T, *LOXHD1*:NM_144612:c.4714C>A:p.R1572R, *USH2A*: NM_007123: c.2802T>G:p.C934W, and *PCDH15*:NM_00 1142767:c.622C>T:p.R208X) were categorized as pathogenic variants in ClinVar, but categorized as nonpathogenic in the deafness variation database. In contrast, 3 variants (*GJB2*:NM_004004: c.368C>A:p.T123N, *GJB2*:NM_004004: c.511G>A:p.A171T, and *GJB2*:NM_ 004004: c.571T>C:p.F191L) were categorized as pathogenic variants in the deafness variation database; however, they were categorized as nonpathogenic variants in ClinVar.

Among these previously reported pathogenic variants, 26 were autosomal dominant mutations in *ACTG1, COCH, COL11A2, CRYM, EYA1, GJB2, GJB3, KCNQ4, MYH9, SIX1, TECTA*, and *WFS1*; 88 were autosomal recessive mutations in *CDH23, GJB2, GJB3, GJB6, LOXHD1, MARVELD2, MYO15A, MYO6, MYO7A, OTOF, SLC26A4, OTOF, TMC1, TMIE, TMPRSS3, USH1C, USH2A*, and *WFS1*; and 2 were X-linked mutations in *COL4A5*.

The most frequent mutation was *GJB2*:c.235delC, which was found in 166 alleles from 1120 patients with hearing loss and 3 alleles in the 269 normal hearing controls. *SLC26A4*:c.2168A>G (p.H723R) were the second most frequent; 53 alleles were found in 1120 hearing loss cases, and 2 alleles were found in the 269 controls.

Comparison of Previously Reported Pathogenic Mutations and Newly Identified Variants

To determine whether the missense mutations affect prediction cutoffs of the computer programs for protein function, we compared the prediction scores of the pathogenic variants previously reported to those of the newly identified variants using 12 computer programs including ANNOVAR.[21,22] As a result, the previously reported pathogenic variants were predicted to cause more severe effects (or damage) to protein function than those of the newly identified variants. The average SIFT* prediction score for the previously reported pathogenic variants for autosomal dominant nonsyndromic hearing loss (AD-NSHL: 25 variants) was 0.86 ± 0.24, that for autosomal recessive inheritance nonsyndromic hearing loss (AR-NSHL: 59 variants) was 0.88 ± 0.23, and that for the newly identified missense variants (1926 variants) was 0.74 ± 0.32 (Figure 2, *SIFT scores from the ANNOVAR software were converted to 1-SIFT scores; therefore, a higher score indicated a more damaging variant). The PolyPhen2 results were similar to the SIFT results (Polyphen2 HVID: the AD-NSHL variant score was 0.83 ± 0.32, the AR-NSHL variant score was 0.87 ± 0.29, and the newly identified variant score was 0.60 ± 0.43; Polyphen2 HVAR: the AD-NSHL variant score was 0.77 ± 0.35, the AR-NSHL variant score was 0.79 ± 0.34, and the newly identified variant score was 0.50 ± 0.43). The LRT, Mutation Taster, Mutation Assessor, FATHMM, Radial SVM, LR, GERP++, PhyloP, and SiPhy 29-way log odds scores were similar (Figure 2). All prediction programs are based on some similar strategies and are not completely independent of each other. However, each prediction program estimates the effect of amino acid changes from different viewpoints to some extent (some programs estimate the homology among many species, while others estimate the properties of amino acids). Therefore, it is conceivable that combining the results of multiple prediction programs might be better than using the results of each individual prediction program.

To maximize prediction appropriateness, we converted the results of each prediction program to a *z*-score (using all missense variant results: AD-NSHL variants + AR-NSHL variants + novel variants = 2010 variants) and calculated the average *z*-score of the 12 prediction programs (Figure 2, Table 2). As a result, the *z*-score of the AD-NSHL variants was 0.65 ± 0.45, that of the AR-NSHL variants was 0.60 ± 0.55, and that of the newly identified variants was −0.27 ± 0.65. These results clearly reveal differences between the previously reported pathogenic variants and the newly identified variants, including both the pathogenic variants and rare polymorphisms. As a result of the statistical analysis, the average *z*-score of 12 prediction programs indicated a *P* value lower than that of each of 12 prediction programs (AD-pathogenic vs novel: $P = 4.2 \times 10^{-7}$, AR-pathogenic vs novel: $P = 5.1 \times 10^{-9}$, Tukey's HSD test). As a notable result, the *GJB2*:c.368C>A (p.T123N) variant revealed the lowest score of −1.14 in the previously reported pathogenic variant group. This variant was recategorized as a rare polymorphism in our previous report.[28] Of course, in silico analysis has a limitation in the prediction of the pathogenicity and segregation analysis for family samples, and in vitro or in vivo studies are required to make conclusions about the pathogenicity of each variant.

From these results, we further selected the missense variants with average *z*-scores >0.05 (average −1 standard deviation of previously reported AR-NSHL variants) as candidates and analyzed the molecular epidemiology and mutation spectrum in Japanese patients with hearing loss.

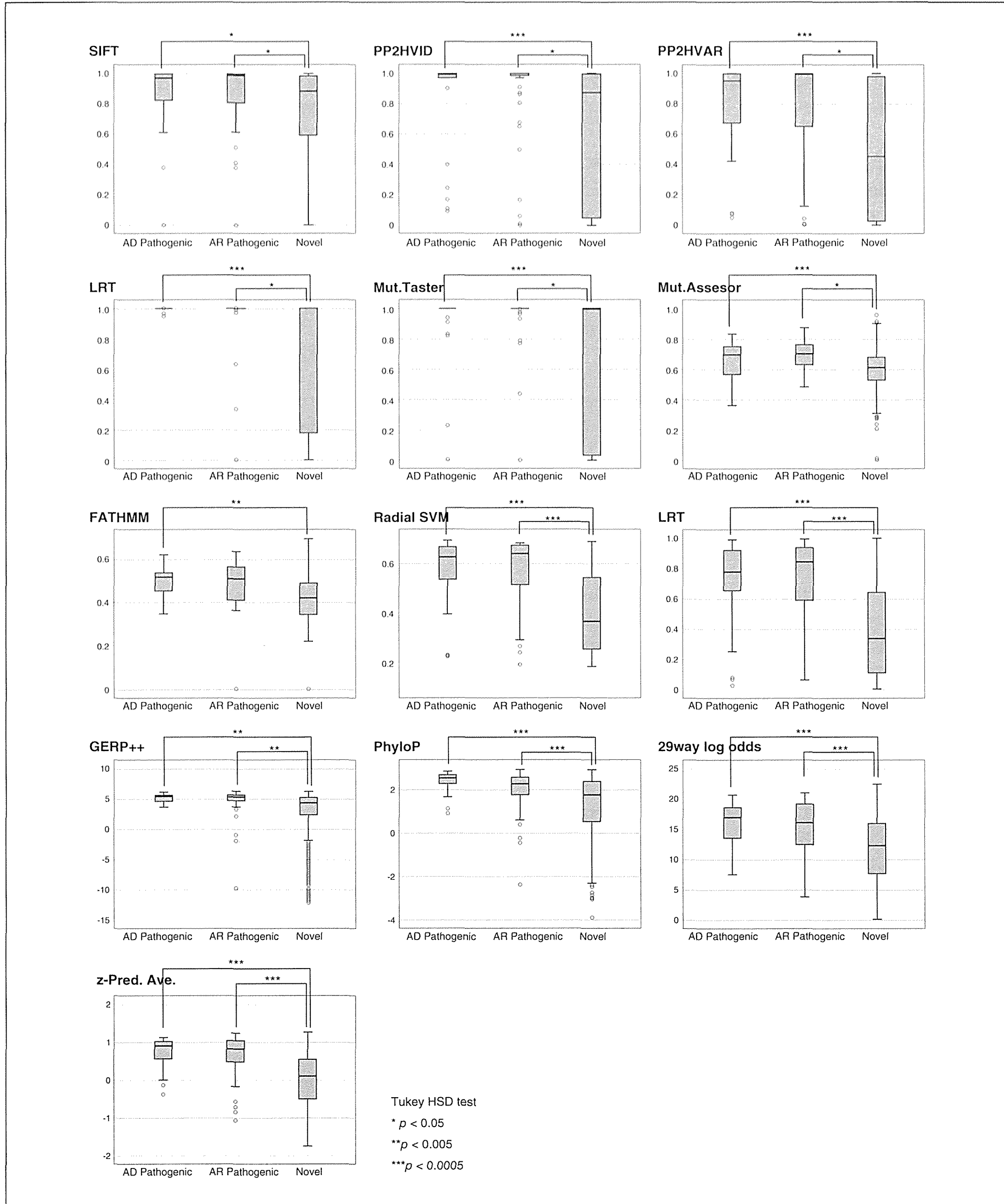

Figure 2. Distributions of the 12 computer prediction software programs (SIFT, Polyphen2 HVID, Polyphen2 HVAR, LRT, Mutation Taster, Mutation Assessor, FATHMM, Radial SVM, LR, GERP++, PhyloP, and SiPhy 29-way log odds) for identifying the missense mutations and the average *z*-score of the 12 prediction programs described in the text. The statistical analysis was performed using SPSS version 18 (SPSS Inc, Chicago, Illinois, USA).

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

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

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

Molecular Epidemiology of Japanese Patients With Hearing Loss

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

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

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

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

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

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