TABLE 2. COMPARISON OF THE TAQMAN ASSAY-BASED MUTATION SCREENING AND MASSIVELY PARALLEL DNA SEQUENCING-BASED COMPREHENSIVE SCREENING OF DEAFNESS GENES

| *Mutations* | *Number of patients with mutations detected by TaqMan genotyping* (n=*384*) | *Variant alleles detected by TaqMan genotyping* (n=*768*) | *Variant alleles detected by MPS* (n=*768*) |
|---|---|---|---|
| *CDH23*:NM_001171930:c.719C>T:p.P240L | 15 (3.9%) | 18 (2.3%) | 18 |
| *CDH23*:NM_022124:c.4762C>T:p.R1588W | 6 (1.6%) | 6 (0.8%) | 6 |
| *CDH23*:NM_022124:c.6085C>T:p.R2029W | 4 (1.0%) | 5 (0.7%) | 5 |
| *CDH23*:NM_022124:c.4249C>T:p.R1417W | 1 (0.3%) | 2 (0.3%) | 2 |
| *CDH23*:NM_022124:c.5147A>C:p.Q1716P | 2 (0.5%) | 2 (0.3%) | 2 |
| *CDH23*:NM_022124:c.5627G>A:p.S1876N | 2 (0.5%) | 2 (0.3%) | 2 |
| *CDH23*:NM_022124:c.5722G>A:p.V1908I | 2 (0.5%) | 2 (0.3%) | 2 |
| *CDH23*:NM_022124:c.4877A>C:p.D1626A | 1 (0.3%) | 1 (0.1%) | 0[a] |
| *CDH23*:NM_001171933:c.141T>G:p.N47K | 1 (0.3%) | 1 (0.1%) | 1 |
| *CDH23*:NM_022124:c.5131G>A:p.V1711I | 1 (0.3%) | 1 (0.1%) | 1 |
| *KCNQ4*:NM_004700:c.211delC:p.Q71fs | 6 (1.6%) | 6 (0.8%) | 0[b] |
| *MYO15A*:NM_016239:c.9478C>T:p.L3160F | 7 (0.9%) | 7 (0.9%) | 7 |
| *OTOF*:NM_194323:c.3515G>A:p.R1172Q | 2 (0.5%) | 2 (0.3%) | 2 |
| *OTOF*:NM_194248:c.1422T>A:p.Y474X | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM00441:c.2229_2301delGAA | 1 (0.3%) | 1 (0.1%) | 0[b] |
| *SLC26A4*:NM_000441:c.1315G>A:p.G439R | 1 (0.3%) | 1 (0.1%) | 1 |

[a]c.4877A>C mutation did not call by variant calling program (low depth).
[b]These mutations were located in the region not covered by AmpliSeq primers.

TABLE 3. COMPARISON OF THE DIRECT SEQUENCING ANALYSIS OF THE SELECTED GENES AND MASSIVELY PARALLEL DNA SEQUENCING-BASED COMPREHENSIVE SCREENING

| | *Number of patients with mutations detected by direct sequencing* (n=*384*) | *Variant alleles detected by direct sequencing* (n=*768*) | *Variant alleles detected by MPS* (n=*768*) |
|---|---|---|---|
| *GJB2*:NM_004004:c.95G>A:p.R32H | 2 (0.5%) | 2 (0.3%) | 2 |
| *GJB2*:NM_004004:c.11G>A:p.G4D | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.257C>T:p.T86M | 0[a] | 0[a] | 1 |
| *GJB2*:NM_004004:c.511_512insAACG:p.A171fs | 4 (1.0%) | 4 (0.5%) | 4 |
| *GJB2*NM_004004:c.595T>C:p.S199P | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.558_559ins46:p.E187_ K188delinsEKTVFTVFMIAVSGIX | 2 (0.5%) | 2 (0.3%) | 2 |
| *GJB2*:NM_004004:c.583A>G:p.M195V | 2 (0.5%) | 2 (0.3%) | 2 |
| *GJB2*:NM_004004:c.53C>G:p.T18S | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.379C>T:p.R127C | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.511G>A:p.A171T | 0[a] | 0[a] | 1 |
| *GJB2*:NM_004004:c.334_335del:p.112_112del | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.318C>A:p.F106L | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.637T>A:p.L213M | 1 (0.3%) | 1 (0.1%) | 1 |
| *GJB2*:NM_004004:c.223C>T:p.R75W | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.945T>A:p.Y315X | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.2123T>C:p.F708S | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.641A>G:p.Y214C | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.863T>A:p.L288X | 2 (0.5%) | 2 (0.3%) | 2 |
| *SLC26A4*:NM_000441:c.1264-2A>G:Splicing | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.918+1G>A:Splicing | 1 (0.3%) | 1 (0.1%) | 1 |
| *SLC26A4*:NM_000441:c.107_120del13ins16 | 1 (0.3%) | 1 (0.1%) | 0[b] |
| *SLC26A4*:NM_000441:c.147C>G:p.S49R | 1 (0.3%) | 1 (0.1%) | 0[b] |

[a]These mutations were not detected by direct sequencing in one case each (low signal intensity).
[b]These mutations were not detected by MPS (reason unknown).

TABLE 4. PATHOGENIC MUTATION CANDIDATES COMBINED WITH ONE KNOWN PATHOGENIC VARIANT DETECTED BY THE INVADER ASSAY OR TAQMAN GENOTYPING ASSAY OF THE SAME GENES

| *Gene* | *Pathogenic mutations detected by Invader assay or TaqMan genotyping assays as heterozygous* | *MPS detected mutations found in the same gene* |
|---|---|---|
| *GJB2* | NM_004004:c.235delC:p.L79fs | NM_004004:c.511_512insAACG:p.A171fs |
| *GJB2* | NM_004004:c.235delC:p.L79fs | NM_004004:c.511_512insAACG:p.A171fs |
| *GJB2* | NM_004004:c.235delC:p.L79fs | NM_004004:c.C257T:p.T86M |
| *GJB2* | NM_004004:c.235delC:p.L79fs | NM_004004:c.T595C:p.S199P |
| *GJB2* | NM_004004:c.235delC:p.L79fs | NM_004004:c.558_559ins46:p.E187_K188delins |
| *GJB2* | NM_004004:c.C427T:p.R143W | NM_004004:c.A583G:p.M195V |
| *GJB2* | NM_004004:c.G109A:p.V37I | NM_004004:c.C379T:p.R127C |
| *GJB2* | NM_004004:c.C408A:p.Y136X | NM_004004:c.558_559ins46:p.E187_K188delins |
| *GJB2* | NM_004004:c.C257G:p.T86R | NM_004004:c.C53G:p.T18S |
| *GJB2* | NM_004004:c.176_191del:p.59_64del | NM_004004:c.511_512insAACG:p.A171fs |
| *SLC26A4* | NM_000441:c.A2168G:p.H723R | NM_000441:c.A641G:p.Y214C |
| *SLC26A4* | NM_000441:c.A2168G:p.H723R | NM_000441:c.T863A:p.L288X |
| *SLC26A4* | NM_000441:c.A2168G:p.H723R | NM_000441:c.T863A:p.L288X |
| *SLC26A4* | NM_000441:c.A2168G:p.H723R | NM_000441:c.T945A:p.Y315X |
| *SLC26A4* | NM_000441:c.A2168G:p.H723R | NM_000441:c.T2123C:p.F708S |
| *SLC26A4* | NM_000441:c.C2162T:p.T721M | NM_000441:exon7:c.918+1G>A |
| *SLC26A4* | NM_000441:c.C1229T:p.T410M | NM_000441:exon11:c.1264-2A>G) |
| *CDH23* | NM_001171930:c.C719T:p.P240L | NM_001171930:c.G1282A:p.D428N |
| *CDH23* | NM_001171930:c.C719T:p.P240L | NM_001171933:c.2079_2085del:p.693_695del |
| *CDH23* | NM_001171930:c.C719T:p.P240L | NM_001171933:c.2265dupT:p.H755fs |
| *CDH23* | NM_001171930:c.C719T:p.P240L | NM_022124:c.G4672A:p.G1558R |
| *CDH23* | NM_022124:c.C4762T:p.R1588W | NM_022124:c.G5419A:p.V1807M |
| *CDH23* | NM_022124:c.C4762T:p.R1588W | NM_001171933:c.G746A:p.R249H |
| *MYO15A* | NM_016239:c.C9478T:p.L3160F | NM_016239:c.A9938C:p.H3313P |
| *OTOF* | NM_194323:c.G3515A:p.R1172Q | NM_194322:c.G1186A:p.G396R |

Invader assay, it is possible that other mutations might exist in the coding region of the same genes, but the Invader assay did not detect these mutations. Among the 384 patients, 36 heterozygous mutations of autosomal recessive deafness genes were detected by the Invader assay (27 *GJB2* heterozygous and nine *SLC26A4* heterozygous mutations). Among these 36 patients, MPS detected an additional 16 mutations in the same genes, leading to a final diagnosis of compound heterozygous mutations (10 *GJB2* and seven *SLC26A4* mutations, Table 4). A similar situation was observed for TaqMan genotyping assay target mutations. Among the 384 patients, 34 heterozygous mutations of autosomal recessive deafness genes were detected by TaqMan genotyping assay (24 *CDH23*, seven *MYO15A*, two *SLC26A4*, and one *OTOF* mutation). Among these 34 patients, MPS detected eight additional mutations in the same genes, leading to a final diagnosis of compound heterozygous mutations (six *CDH23*, one *MYO15A*, and one *OTOF* mutation, Table 4). MPS, therefore, improved the diagnostic rate in 24 cases (6.3%). In addition, MPS-based genetic testing was able to identify previously reported pathogenic mutations, also contributing to an improved diagnostic rate. Among the 384 patients, MPS found 20 previously reported pathogenic mutations not identified in the Invader or TaqMan genotyping assays listed in Table 5. Of course, it was difficult to distinguish whether the variants detected by MPS were really pathogenic or benign, so most of the mutations identified by MPS were considered to be variations of uncertain significance, and further examination is needed to elucidate the pathogenicity of the variants found in this study.

## Discussion

In our previous study, MPS analysis of 63 genes known to cause deafness using an Ion PGM system and Ion AmpliSeq was able to identify rare gene mutations responsible for hearing loss in patients with cochlea implantation (Miyagawa *et al.*, 2013).

Before the clinical application of such new diagnostic tools, the uniformity of the results and the reliability/accuracy of the method should be confirmed in a clinical setting, but most of the previous reports regarding MPS focused mainly on the detection of novel gene mutations or rare causative mutations (Rehman *et al.*, 2010; Shearer *et al.*, 2010; Walsh *et al.*, 2010; Brownstein *et al.*, 2011; Lin *et al.*, 2012). In this study, we focused on the uniformity and the accuracy of the MPS-based genetic test in comparison with the results of Invader assay-based genetic screening, TaqMan genotyping assays, and direct sequencing.

With regard to uniformity, most of the samples were sequenced deeply enough for accurate genotyping (average depth of coverage 241×) and the percentage samples with greater than 20×was also sufficient (97.72% of the target region was sequenced with an average depth of coverage of over 20×). Furthermore, only 14 (3.6%) of the 384 samples did not fulfill the minimum coverage (average coverage of over 100×) or minimum depth of coverage (over 96% of the target region must be sequenced at a depth of over 20×) criteria. However, all of these 14 samples could be analyzed by another sequence run to fulfill the minimum criteria. Therefore, all samples could be analyzed by the MPS-based genetic analysis used in this study. One of the advantages of

TABLE 5. PREVIOUSLY REPORTED PATHOGENIC VARIANTS DETECTED BY MASSIVELY PARALLEL DNA SEQUENCING, WHICH WERE NOT IDENTIFIED IN THE INVADER AND TAQMAN GENOTYPING ASSAYS

| *Gene name* | *Reported pathogenic mutation* | | *Reference* |
|---|---|---|---|
| Autosomal dominant inheritance mutations | | | |
| *ACTG1* | NM_001199954:c.A353T:p.K118M | | Zhu *et al.* (2003) |
| *ACTG1* | NM_001199954:c.G721A:p.E241K | | Morín *et al.* (2009) |
| *KCNQ4* | NM_004700:c.C546G:p.F182L | | Su *et al.* (2007) |
| *KCNQ4* | NM_004700:c.C546G:p.F182L | | Su *et al.* (2007) |
| *KCNQ4* | NM_004700:c.C546G:p.F182L | | Su *et al.* (2007) |
| *MYH9* | NM_002473:c.G2114A:p.R705H | | Dong *et al.* (2005) |
| *TECTA* | NM_005422:c.C5597T:p.T1866M | | Sagong *et al.* (2010) |
| *WFS1* | NM_001145853:c.G1846T:p.A616S | | Liu *et al.* (2005) |
| *WFS1* | NM_001145853:c.G2185A:p.D729N | | Domènech *et al.* (2002) |
| *WFS1* | NM_001145853:c.G2590A:p.E864K | | Eiberg *et al.* (2006) |
| *Gene name* | *Reported pathogenic mutation* | *Novel mutation found by MPS* | *Reference* |
| Autosomal recessive inheritance mutations | | | |
| *CDH23* | NM_001171930:c.C805T:p.R269W | NM_001171933:c.C2407T:p.R803W | Oshima *et al.* (2006) |
| *MYO7A* | NM_000260:c.G635A:p.R212H | NM_000260:c.G3475A:p.G1159S | Weil *et al.* (1997) |
| *MYO15A* | NM_016239:c.G6731A:p.G2244E | NM_016239:c.6457delG:p.A2153fs | Nal *et al.* (2007) |
| *SLC26A4* | NM_000441:c.T2228A:p.L743X | NM_000441:c.C1208A:p.A403D | Yuan *et al.* (2009) |

Among the autosomal recessive causative genes, only the reported pathogenic variants with other mutation candidates in the same genes detected by MPS were listed.

Ion AmpliSeq library preparation is thought to be this high assay success rate. The Ion AmpliSeq library preparation used in this study required only 20 ng DNA samples, and the quality of the DNA samples did not affect the sequence results. This robustness with regard to DNA quality was also found to apply to the MPS analysis of fragmented DNA samples obtained from Formalin-Fixed Paraffin-Embedded (FFPE) samples (Tsongalis *et al.*, 2014).

With regard to the accuracy of MPS-based genetic screening, we confirmed that it was sufficient for clinical diagnosis by comparison of the test results of the MPS-based genetic test to the Invader assay or direct sequencing. Another advantage of this MPS genetic test is thought to be in its potential for the efficient detection of short insertion and deletion mutations such as *GJB2* c.176_191del16, c.511_512insAACG, and c.558_559ins46. As the IonPGM sequencer had a longer read length (200 bp for Amplicon resequencing), this might assist the mapping process of the read fragments of such insertion and deletion mutations.

With regard to the improvement in the diagnostic rate, MPS improved the diagnostic rate by 11.5% (MPS identified an additional mutation in the same gene in 24 cases of heterozygous mutations detected by the Invader or TaqMan genotyping assays, and 20 cases of previously reported pathogenic mutations were found by MPS) over those for the Invader assay and TaqMan genotyping assays in the most conservative setting (this improvement did not include any novel mutations without clues identified by the Invader or TaqMan genotyping assays or in previous reports). Of course, various novel candidate causative variants as well as the previously reported variants were found by MPS analysis, but it is difficult to determine the pathogenicity of these mutations. We are now analyzing family samples for such candidate causative mutations and intend to report our results at a later date.

In conclusion, the MPS-based comprehensive mutation screening for deafness genes had high uniformity, high assay success rate, and sufficient accuracy for clinical use. In addition, this screening method affords an improved diagnostic rate among hearing loss patients. This genetic analysis system is expected to facilitate more precise clinical genetic diagnosis, appropriate genetic counseling, and proper medical management for auditory disorders.

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Address correspondence to:
*Shin-Ichi Usami, MD, PhD*
*Department of Otorhinolaryngology*
*Shinshu University School of Medicine*
*3-1-1 Asahi*
*Matsumoto 390-8621*
*Japan*

*E-mail:* usami@shinshu-u.ac.jp

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# The Patients Associated With *TMPRSS3* Mutations Are Good Candidates for Electric Acoustic Stimulation

**Maiko Miyagawa, MD, PhD[1,2], Shin-ya Nishio, PhD[1,2], Yuika Sakurai, MD, PhD[3], Mitsuru Hattori, PhD[1], Keita Tsukada, MD, PhD[1], Hideaki Moteki, MD, PhD[1,2], Hiromi Kojima, MD, PhD[3], and Shin-ichi Usami, MD, PhD[1,2]**

## Abstract

**Objectives:** To clarify the frequency of *TMPRSS3* mutations in the hearing loss population, genetic analysis was performed, and detailed clinical characteristics were collected. Optical intervention for patients with *TMPRSS3* mutations was also discussed.

**Methods:** Massively parallel DNA sequencing (MPS) was applied for the target exon-sequencing of 63 deafness genes in a population of 1120 Japanese hearing loss patients.

**Results:** Hearing loss in 5 patients was found to be caused by compound heterozygous *TMPRSS3* mutations, and their detailed clinical features were collected and analyzed. Typically, all of the patients showed ski slope type audiograms and progressive hearing loss. Three of the 5 patients received electric acoustic stimulation (EAS), which showed good results. Further, the onset age was found to vary, and there were some correlations between genotype and phenotype (onset age).

**Conclusions:** MPS is a powerful tool for the identification of rare causative deafness genes, such as *TMPRSS3*. The present clinical characteristics not only confirmed the findings from previous studies but also provided clinical evidence that EAS is beneficial for patients possessing *TMPRSS3* mutations.

## Keywords

*TMPRSS3*, DFNB8/10, high-frequency hearing loss, massively parallel DNA sequencing, next generation sequencing, EAS

## Introduction

Hearing impairment is a general sensory defect in humans. Based on the results of several etiological studies, it has been estimated that at least 50% of congenital hearing loss is of genetic etiology.[1] More than 80 genes have already been reported to be associated with sensorineural hearing loss (SNHL).

Cochlear implantation (CI), which electrically stimulates the spiral ganglion neurons, has been established as the standard therapy for severe to profound SNHL.[2] Electric acoustic stimulation (EAS) is a hearing implant system combining a cochlear implant and acoustic amplification technology in one device and has recently become a standard intervention for the patients with partial deafness, defined as a mild to moderate low-frequency sensorineural hearing loss sloping to a profound hearing loss in the higher frequencies.[3]

*TMPRSS3* is responsible for autosomal recessive hearing loss, particularly high-frequency involved hearing loss. Interestingly, *TMPRSS3* is the cause of DFNB10 (severe and congenital) and DFNB8 (mild and postlingual) phenotypes.[4]

*TMPRSS3* is a type-II transmembrane serine protease, structurally defined by a transmembrane domain located near the N terminus. In a previous study, *TMPRSS3* mRNA was detected in the cell bodies of spiral ganglion neurons, the entire epithelium supporting the organ of Corti, as well as the inner hair cells of the organ of Corti and in the lower levels of the stria vascularis.[5,6] *TMPRSS3* may be involved in processing proneurotrophins and, therefore, in the development and survival of cochlear neurons.

Twenty-five mutations in *TMPRSS3* were previously reported in the Middle East, Europe, and East Asia (Table 1).[7-17] The function of the *TMPRSS3* gene in the auditory system remains unclear, but it has been reported to play a crucial role in the morphological and functional maturation of the

[1]Department of Otorhinolaryngology, Shinshu University School of Medicine Matsumoto, Japan
[2]Department of Hearing Implant Sciences, Shinshu University School of Medicine, Matsumoto, Japan
[3]Department of Otorhinolaryngology, Jikei University School of Medicine, Tokyo, Japan

**Corresponding Author:**
Shin-ichi Usami, MD, PhD, Department of Otorhinolaryngology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
Email: usami@shinshu-u.ac.jp

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inner ear as well as in the maintenance of the contents of the perilymph and endolymph.[5,18]

Recent advances in targeted exon sequencing of selected genes using massively parallel DNA sequencing (MPS) technology have enabled the successful identification of causative mutations in relatively rare genes such as *TMPRSS3*. In this study, we describe 5 patients from 4 families with *TMPRSS3* detected by MPS. We present the clinical features of the patients and discuss the appropriate forms of intervention for hearing loss caused by *TMPRSS3*.

## Subjects and Methods

### Subjects

A total of 1120 Japanese hearing loss (HL) patients (autosomal dominant sensorineural hearing loss, 266; autosomal recessive sensorineural hearing loss, 600; unknown, 254) from 53 otolaryngology departments nationwide participated in this study. Written informed consent was obtained from all subjects (or from their next of kin, caretaker, or guardian on 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.

### Amplicon Library Preparation

Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, California, USA) for 63 genes reported to cause nonsyndromic hearing loss according to the manufacturer's instructions. The detailed protocol was described elsewhere.[10] After preparation, the amplicon libraries were diluted to 20 pM, and equal amounts of 6 libraries for 6 patients were pooled for 1 sequence reaction.

### Emulsion Polymerase Chain Reaction and Sequencing

Emulsion polymerase chain reaction (PCR) and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere.[10] MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using an Ion PGM 200 Sequencing Kit and 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.[19,20] 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,[21] (2) the 6500 exome variants,[22] (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants),[23] 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,[24] Sorting Intolerant from Tolerant (SIFT),[25] Polymorphism Phenotyping (PolyPhen2),[26] LRT,[27] MutationTaster,[28] and GERP++.[29]

Candidate mutations were confirmed by Sanger sequencing, and the responsible mutations were identified by segregation analysis using samples from among the patients' family members. In cases identified as heterozygous, Sanger sequencing of the coding region of the *TMPRSS3* was performed.

### Outcome of EAS

Thirty-two consecutive hearing preservation surgeries in 30 of the 1120 patients with ski slope hearing loss were performed (for details, see Usami et al[30]). Twenty-nine ears in 27 patients received MED-EL PULSAR with a FLEX$^{24}$ electrode (24 mm), 2 ears in 2 patients received a FLEX$^{soft}$ electrode (31.5 mm), and 1 ear received a standard electrode (31.5 mm).

To evaluate speech perception outcomes, speech discrimination scores (using the 67S Japanese monosyllable test, 65dBSPL) preoperatively and at 12 months after the initial EAS stimulation were used. In this study, we compared the outcomes for 3 EAS patients with hearing loss resulting from *TMPRSS3* mutations with those for the remaining 27 patients with hearing loss from other etiologies.

## Results

### Detected Mutations

One nonsense and 5 missense mutations as well as 1 splice site mutation were identified (Table 1). The splice site mutation, c.617-4_-3dupAT (p.T205fs), was detected by additional Sanger sequencing. All of the detected mutations were confirmed by Sanger sequencing and were predicted to be pathologic by several software programs. Segregation analysis was consistent with them being plausible disease-causing mutations. All of the subjects with biallelic mutations were compatible with recessive inheritance patterns.

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**Table 1.** *TMPRSS3* Mutations in Autosomal Recessive Sensorineural Hearing Loss (ARSNHL).

| Exon | Domain | NM No. | Nucleotide Change | Amino Acid Change | Family Origin | Reference |
|---|---|---|---|---|---|---|
| 4 | Truncation agter TM | NM_032405 | c.208delC | p.His70ThrfsX19 | Spanish, Greek, Pakistani, Canada, Dutch | 7, 8, 9 |
| 4 | LDLRA domain | NM_032405 | c.212T>C | p.F71S | Japanese | This study |
| 4 | LDLRA domain | NM_032405 | c.268G>A | p.A90T | UK, Moroccan | 11 |
| 4 | LDLRA domain | NM_032405 | c.280G>A | p.G94R | Japanese | This study |
| 4 | LDLRA domain | NM_032405 | c.308A>G | p.D103G | Greek | 7 |
| 4 | LDLRA domain | NM_032405 | c.310G>A | p.E104K | Pakistani | 9 |
| 4 | LDLRA domain | NM_032405 | c.310G>T | p.E104X | Pakistani | 9 |
| Intron 4 | SRCR | NM_032405 | c.323-6G>A | p.Cys107fs | Pakistani | 4 |
| 5 | LDLRA domain | NM_032405 | c.325C>T | p.R109W | Pakistani, Korea | 12, 13 |
| 5 | SRCR domain | NM_032405 | c.413C>A | p.A138G | UK, Dutch | 8 |
| 7 | SRCR domain | NM_032405 | c.581G>T | p.C194F | Pakistani | 12 |
| 7 | SRCR domain | NM_032405 | c.595G>A | p.V199M | Dutch | 8 |
| Intron 8 | Serine protease domain | NM_032405 | c.617-4_-3dupAT | p.T205fs | Japanese | This study |
| 8 | Just before senine protease | NM_032405 | c.646C>T | p.R216C | German | 14 |
| 8 | Serine protease domain | NM_032405 | c.743C>T | p.T248M | Korea | 13 |
| 8 | Serine protease domain | NM_032405 | c.753G>C | p.W251C | Tunisian | 16 |
| 8 | Serine protease domain | NM_032405 | c.767C>T | p.A256V | Pakistani | 9 |
| 9 | Serine protease domain | NM_032405 | c.916G>A | p.A306T | German, Korea, Dutch | 8, 13, 14 |
| 12 | Serine protease domain | AB038157 | c.1221C>T | p.P404L | Turkish, Tunisian | 16 |
| 12 | Serine protease domain | NM_032405 | c.1219T>C | p.C407R | Pakistani | 9, 12 |
| 4 | LDLRA domain | NM_032404 | c.226C>T | p.Q76X | Japanese | 10, this study |
| 5 | SRCR domain | NM_032404 | c.390C>G | p.H130R | Japanese | This study |
| 7 | Just before serine protease | NM_032404 | c.647G>T | p.R216L | Turkish, Japanese | 15, this study |
| 9 | Serine protease domain | NM_032404 | c.778G>A | p.A260T | Japanese | 10, this study |
| 9 | Serine protease domain | NM_032404 | c.830C>T | p.P277L | Turkish, Tunisian | 16 |
| Intron 8 | Serine protease domain | NM_024022 | c.782+8insT | | Pakistani | 17 |
| 11 | Serine protease domain | NM_024022 | c.1180_1187del8ins68 | | Palestinian | 4 |
| 11 | Truncation of serine protease | NM_024022 | c.1192C>T | p.Q398X | Turkish | 15 |
| 12 | Serine protease domain | NM_024022 | c.1273T>C | p.C425R | Pakistani | 9 |

Abbreviations: LDLRA, low-density lipoprotain recrptor; SRCR, scavenger receptor cysteine-rich.

The compound heterozygote mutations, c.[226C>T];[778G>A]( p.[Q76X];[A260T]), found in 1 family (patient 4541, 4540), were previously reported.[10,31] However, the other 4 mutations (c.212T>C [p.F71S], c.280G>A [p.G94R]), c.390C>G [p.H130R], and c.617-4_-3dupAT [p.T205fs]) were novel causative mutations.

## *Clinical Findings*

The clinical features and genotypes for the 5 patients are shown in Figures 1, 2, 3, and 4 and Table 2. All pedigrees showed typical autosomal recessive inheritance patterns, and all affected patients displayed progressive, symmetrical

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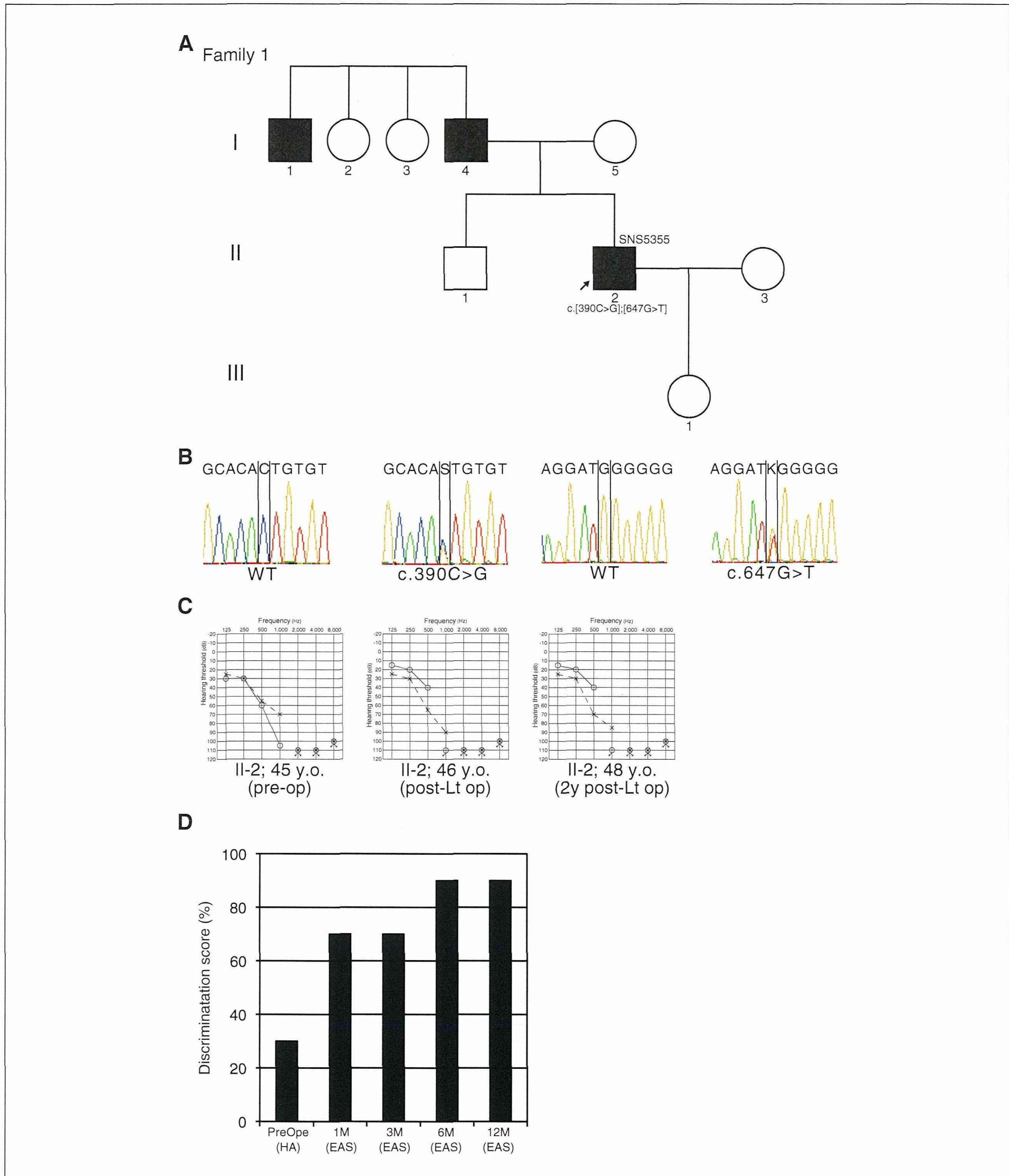


**Figure 1.** (A) The patient (SNS5355) shows compound heterozygous *TMPRSS3* mutations, c.[390C>G];[647G>T](p.[H130R]; [R216L]). His father also developed age-related hearing loss with a different type of audiogram (not shown). (B) The results of Sanger sequencing. (C) Pre- and postoperative audiograms indicating the progressive nature of hearing loss and achievement of hearing preservation after EAS. (D) Japanese monosyllable test (65dB SPL in quiet) with bilateral EAS showing a good speech discrimination outcome after EAS. EAS, electric acoustic stimulation.

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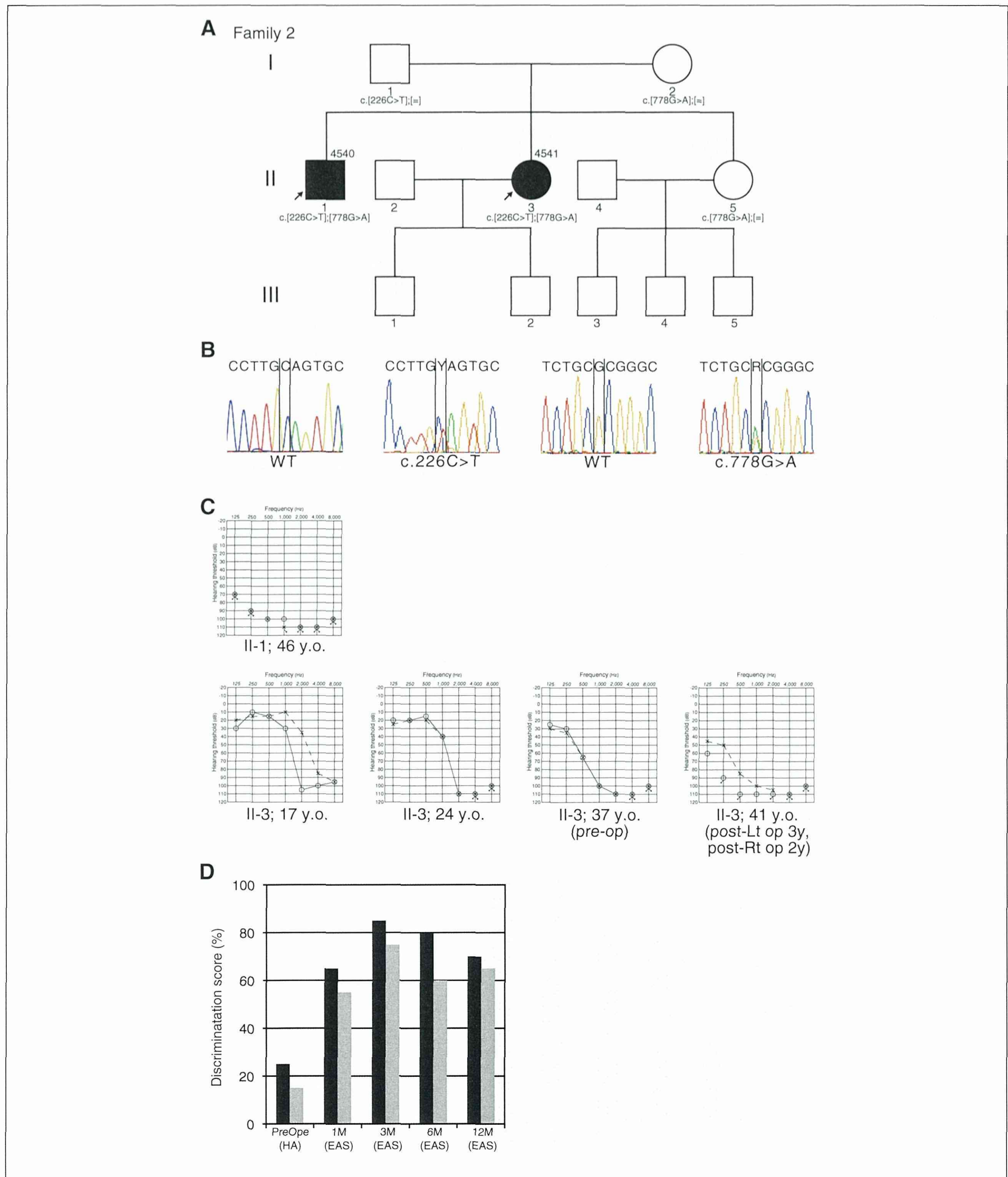


**Figure 2.** (A) The patient (4541) shows compound heterozygous *TMPRSS3* mutations, c.[226C>T];[778G>A](p.[Q76X];[A260T]), and the parents were found to be carriers for these mutations. The patient's brother (4540) has the same mutations. (B) The results of Sanger sequencing. (C) Audiograms of the 2 affected family members at different ages. Serial audiogram of the proband indicates the progressive nature of the hearing loss. (D) Japanese monosyllable test (65 dB SPL in quiet) for patient 4541 showing a dramatic improvement after bilateral EAS. Black, left side; gray, right side. EAS, electric acoustic stimulation.

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