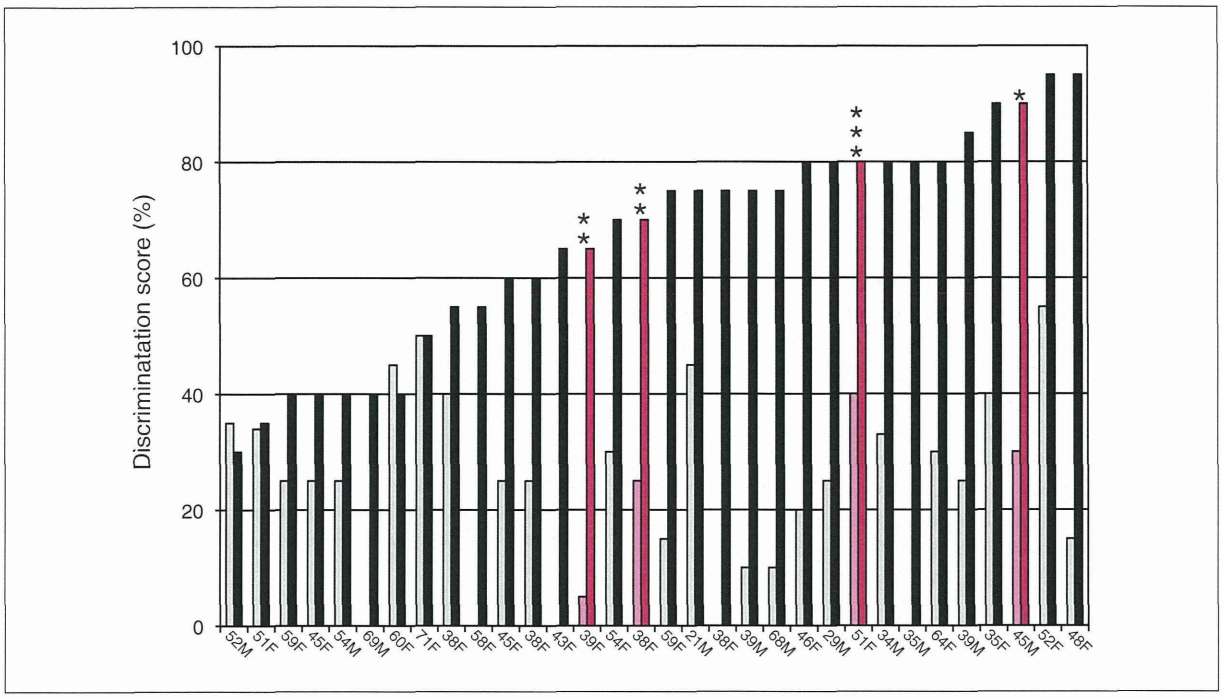

Figure 6. Speech discrimination scores (using the 67S Japanese monosyllable test, 70 dBSPL) preoperatively (grey) and at 12 months after the initial EAS (black). The 3 patients with *TMPRSS3* mutations (*SNS5355, **4541, ***SNS5134) showed significant improvement.

Discussion

In previous studies, the frequency of *TMPRSS3* mutations in hearing loss patients was found to be <0.5% in Europe, 3% in Pakistan, and 5.9% in Korea.[7,12,13] In this study, MPS technology successfully identified *TMPRSS3* mutations and the frequency of *TMPRSS3* mutations in a Japanese population. The rate of patients with *TMPRSS3* mutations was 0.36% (4/1120) among Japanese and 0.7% (4/600) in autosomal recessive sensorineural hearing loss (ARSNHL) patients, which are similar to the figures for Europe.

Five patients were detected with compound heterozygous mutations in this study, with no specific mutation found to occur at a significantly higher frequency. The existence of certain frequent mutations, such as c.916G>A (p.A306T), has been reported.[8,13,14] The mutation spectrum found in Japanese is quite different from those reported in other populations. Based on the present results, the carrier rate of *TMPRSS3* mutations is extremely low in Japan in comparison with *GJB2* or *SLC26A4*.

For such rare causative mutations/genes, targeted exon sequencing of selected genes using MPS technology was extremely useful. In fact, in this study we successfully identified 7 *TMPRSS3* mutations among 4 families. All of the patients with *TMPRSS3* mutations showed typical ski slope hearing loss with postlingual onset. However, the clinical characteristics varied among the patients.

Weegerink et al[8] hypothesized that *TMPRSS3* mutations are associated with 2 types of hearing impairment phenotypes, (1) DFNB10: a severe congenital or early childhood onset type with prelingual hearing impairment caused by the presence of 2 severe mutations and (2) DFNB8: a later onset progressive but initially milder type with postlingual hearing impairment caused by the presence of 1 mild and 1 severe mutation. In this study, patient ID 4541 had early onset and relatively rapid progressive hearing loss, resembling the DFNB10 phenotype. Her brother (patient ID 4540) also had a more severe phenotype that demonstrated earlier onset and deteriorated to profound hearing loss from young adulthood. Therefore, the c.226C>T(p.Q76X) and c.778G>A(p.A260T) mutations identified in this family are thought to be associated with early onset hearing loss. Conversely, patients SNS5355 and SNS5134 showed late-onset hearing loss. Those mutations might be associated with milder mutations, leading to a DFNB8 phenotype.

With regard to the function of *TMPRSS3* in the inner ear, it was hypothesized that *Tmpress3* participates in the regulation of sodium homeostasis through its ability to activate the inner ear-expressed sodium channel (ENaC) in vitro.[5] In the *Tmpress3*-related deafness mouse model, degeneration

of hair cells in the organ of Corti starts at the basal turn at the onset of hearing loss (postnatal day 12) and progresses toward the apex within 2 days.[32] This phenomenon observed in the mouse model is in line with the human phenotype that presents as high-frequency hearing loss.

Concerning vestibular function, the patients showed normal vestibular function, as evaluated by caloric test and cVEMP (Figure 5), and none of the patients showed vestibular symptoms. Similarly, none of the patients reported previously were found to have vestibular symptoms, but the vestibular function in some patients was reported to be affected. Weegerink et al[8] reported that 5 out of 9 patients showed mild hyperreflexia/hyporeflexia on rotatory and caloric tests. It is not surprising to find this type of associated vestibular hypofunction as *Tmprss3* is expressed both in the cochlear and vestibular hair cells.[32] A similar discrepancy between gene expression, vestibular testing, and vestibular symptoms has also been reported for *GJB2*-associated deafness.[33] Vestibular compensation may be related to these complications, and this possibility should be further examined in future studies.

Three of the 5 patients with mutations detected in this study received EAS. A good outcome for EAS in a *TMPRSS3* patient (patient ID: 4541) was previously reported.[10] In this study, 2 additional patients with *TMPRSS3* mutations also showed good outcomes, further confirming that patients with *TMPRSS3* mutations are good candidates for EAS.

The outcomes for CI for *TMPRSS3* patients remain controversial.[8,10,14,34] A majority of cases (13/15) were reported to show good outcomes for CI, while 2 cases reported by Eppsteiner et al[34] showed a poorer performance. *Tmprss3* is reported to be expressed not only in the organ of Corti but also in the spiral ganglion, and the loss of ganglion cells has been reported.[32,34] Therefore, it is possible that neuronal cell loss may negatively affect CI performance. However, the majority of cases, including our 3 EAS cases, showed good performance, indicating that CI and/or EAS is a potential therapeutic option. If the progression of hearing loss results in the patients losing the benefits of EAS acoustic stimulation, it is possible to cover all frequencies by electric stimulation as is common in CI. A recent study on the human temporal bones indicated that the behavior of human ganglion cells is different from those in animals, being more resistant to degeneration.[35] Although no study of the temporal bone in relation to patients with *TMPRSS3* mutations is available, such behavioral differences may provide an explanation of why CI/EAS is effective in patients with *TMPRSS3* mutations.

Clinicians should keep in mind that hearing loss caused by *TMPRSS3* mutations may be progressive and should consider proper intervention for these patients.

In conclusion, the present study provided additional evidence that the patients associated with *TMPRSS3* mutations are good candidates for EAS. Genetic testing based on next-generation sequencing will facilitate candidate selection and personalized intervention.

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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Detailed Hearing and Vestibular Profiles in the Patients with *COCH* Mutations

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Abstract

Objectives: To evaluate the clinical features of Japanese DFNA9 families with mutations of the *COCH* gene.

Methods: Mutation screening was performed using targeted next-generation sequencing (NGS) for 63 previously reported deafness genes. The progression of hearing loss and vestibular dysfunction were evaluated by pure-tone audiometry, caloric testing, cVEMP, and computed dynamic posturography.

Results: We detected 1 reported mutation of p.G88E and 2 novel mutations of p.I372T and p.C542R. The patients with the novel mutations of p.I372T and p.C542R within the vWFA2 domain showed early onset progressive hearing loss, and the patients with the p.G88E mutation showed late onset hearing loss and acute hearing deterioration over a short period. Vestibular symptoms were reported in the patients with p.G88E and p.C542R. Vestibular testing was performed for the family with the p.G88E mutation. Severe vestibular dysfunction was observed in the proband, and the proband's son showed unilateral semicircular canal dysfunction with mild hearing loss.

Conclusions: Targeted exon resequencing of selected genes using NGS successfully identified mutations in the relatively rare deafness gene, *COCH*, in the Japanese population. The phenotype is compatible with that described in previous reports. Additional supporting evidence concerning progressive hearing loss and deterioration of vestibular function was obtained from our study.

Keywords

COCH, DFNA9, progressive hearing loss, vestibular dysfunction, Massively Parallel Sequencing, next-generation sequencing

Introduction

The majority of genetic hearing loss is autosomally inherited, and autosomal dominant nonsyndromic hearing loss (ADNSHL) is estimated to be responsible for 20% of those with a genetic cause,[1] with 30 causative genes identified to date (Hereditary Hearing Loss Homepage, http://hereditaryhearingloss.org/). Mutations in the *COCH* gene are well known to cause ADNSHL with vestibular dysfunction (DFNA9).[2]

COCH encodes a 550 amino acid extracellular protein "cochlin" that includes 1 late gestation lung protein Lgl1 (LCCL) domain and 2 von Willebrand factor A (vWFA) domains.[3,4] Although the precise function of cochlin remains unclear, it is well known to be predominantly expressed in the inner ear[3,5] and constitutes 70% of all inner ear proteins,[6] suggesting that this protein plays an important role in the inner ear.

Our previous report showed a Japanese family with autosomal dominant progressive cochleo-vestibular dysfunction caused by a p.A119T mutation in the *COCH* gene.[7] However, mutations of the *COCH* gene are thought to be rare in the Japanese hearing loss patients. In this study, we identified 4 families with causative *COCH* mutations in a large cohort of hearing loss patients by the use of targeted next-generation sequencing (NGS) of deafness genes, and we herein summarize the clinical features observed for Japanese families with *COCH* mutations.

Subjects and Methods

Subjects

A total of 1120 Japanese hearing loss (HL) patients (ADSNHL, 266; ARSNHL, 600; unknown, 254) from 53 ENT departments nationwide participated in this study.

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Written informed consent was obtained from all subjects (or from their next of kin, caretaker, or guardian in the case of minors/children) prior to enrollment in the project. This study was approved by the Shinshu University Ethical Committee as well as the respective ethical committees of the other participating institutions.

Amplicon Library Preparation

Amplicon libraries were prepared using an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, California, USA), according to the manufacturer's instructions, for 63 genes reported to cause nonsyndromic hearing loss. The detailed protocol was described elsewhere.[8] After preparation, the amplicon libraries were diluted to 20 pM, and equal amounts of 6 libraries for 6 patients were pooled for 1 sequence reaction.

Emulsion Polymerase Chain Reaction and Sequencing

Emulsion polymerase chain reaction (PCR) and sequencing were performed according to the manufacturer's instructions. The detailed protocol was described elsewhere.[8] Massively Parallel Sequencing 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.[9,10] 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[11], (2) the 6500 exome variants, (3) the Human Genetic Variation Database (data set for 1208 Japanese exome variants)[12], and (4) the 269 in-house Japanese normal hearing controls.

The pathogenicity of the missense variants was predicted using the following functional prediction software: PhyloP[13], Sorting Intolerant from Tolerant (SIFT)[14], Polymorphism Phenotyping[15], LRT[16], MutationTaster[17], and GERP++.[18]

Candidate mutations were confirmed by Sanger sequencing, and the responsible mutations were identified by segregation analysis using samples from among the patients' family members.

Audiologic Evaluations

Audiometric evaluation from 125 to 8000 Hz was performed by pure-tone audiometry. To evaluate speech perception outcomes, speech discrimination scores (using the 67S Japanese monosyllable test) and speech perception scores (using the Japanese CI2004 word and sentence test) were used. Subjects sat 1 m away from and facing the loudspeaker (azimuth = 0°) and recorded monosyllable words presented at 70 dBSPL in quiet condition.

Vestibular Evaluations

Caloric Testing. Caloric testing involved the measurement of the maximum slow phase velocity (SPV) by cold water irrigation (20°C, 5 ml, 20 s). We defined a maximum SPV value below 10 deg/s as representing areflexia and a value between 10 to 20 deg/s as representing hyporeflexia.

cVEMP. For cVEMP testing, electromyography (EMG) was performed using a pair of surface electrodes mounted on the upper half and sternal head of the sternocleidomastoid (SCM) muscle, respectively. The electrographic signal was recorded using a Neuropack evoked potential recorder (Nihon Kohden Co Ltd, Tokyo, Japan). Clicks lasting for 0.1 ms at 105 dBnHL were presented through a headphone. The stimulation rate was 5 Hz, the bandpass filter intensity was 20 to 2000 Hz, and the analysis time was 50 ms. The responses to 100 stimuli were averaged twice.

Computed Dynamic Posturography (Equitest) by Sensory Organization Testing. Sensory Organization Testing (SOT), which was used to assess overall balance and compensation of balance, was performed using the Balance Manager system (NeuroCom, Clackamas, Oregon, USA) under 6 sensory conditions, each of which was performed in triplicate, as follows: condition 1: eyes open, fixed platform surface and background; condition 2: eyes closed, fixed platform surface and background; condition 3: eyes open, moving background (directly proportional to patient antero-posterior sway); condition 4: eyes open, moving platform surface (directly proportional to patient antero-posterior sway); condition 5: eyes closed, moving platform surface; and condition 6: eyes open, moving platform and background.

Equilibrium scores (ES), which indicate the amplitude of the sway angle based on the maximum displacement of the center of gravity, were calculated from the ratio of the estimated antero-posterior sway to the theoretical range of normal antero-posterior sway (12.5°), and the mean ES from 3 trials was used. When a trial ended with a fall or the mean ES was beyond the 95% confidence interval of age-specific normative data, it was considered a failure. Sensory analysis (SA) was also used to assess total balance and

compensation. Based on average balance scores in specific pairs of trials, SA classifies sensory dysfunction and the abnormal sensory preference including (1) somatosensory (SOM; the ratio between conditions 2 and 1), (2) visual (VIS; conditions 4:1), (3) vestibular (VEST; conditions 5:1), and (4) visual preference (PREF; conditions 3+6:2+5).

Results

We identified 4 families among the study cohort that had causative mutations of the *COCH* gene.

Mutation Analysis

We found 1 previously reported causative mutation, p.G88E, in 1 family (Figure 1) as well as 2 novel *COCH* missense mutations. One of the novel mutations detected in 2 of the Japanese families, FAM 475 and FAM 535, is a heterozygous c.T1115C (NM_004086) (Figure 4A) mutation in exon 11 that substitutes a threonine residue for an isoleusine (p.I372T) in the vWFA2 domain. We performed Sanger sequencing as part of a family segregation study and confirmed the co-segregation of p.I372T and the disease phenotype in FAM 475. In addition, p.I372T was predicted to be deleterious by both PolyPhen2 (score of 1.0) and SIFT (score of 0.996) programs.

The other heterozygous c.T1624C (NM_004086) mutation (Figure 5A) in exon 12, leading to a p.C542R substitution in the vWFA2 domain, was found in a member of family FAM 986. Both PolyPhen2 and SIFT predicted that the substitution caused "probably damaging" with a highest possible score of 1.0 and 0.998, respectively.

To exclude common polymorphisms, the 1000 genome database, the 6500 exome variants, and the Human Genetic Variation Database (data set for 1208 Japanese exome variants and 269 in-house Japanese normal hearing controls) were analyzed. These mutations were not found in any of the databases. Therefore, we concluded that the p.I372T and p.C542R mutations were causative mutations associated with hearing loss.

Details of a Case of p.G88E

The pedigree and audiogram of the family of the subject with a p.G88E mutation are shown in Figure 1A and 1B, respectively.

Audiologic Profile

The proband was a 70-year-old man (III-2), and changes in his hearing are shown in Figure 2A. He first noticed hearing loss without tinnitus or vertigo in his early 50s, and his first visit to the Shinshu University Hospital was at the age of 57. Pure-tone audiograms showed hearing loss in the higher frequencies (an average threshold of 45 dB, bilaterally), and his speech discrimination scores were 80% in the right ear and 90% in the left ear. The patient experienced a sudden deterioration in hearing in his left ear with tinnitus, resulting in profound hearing loss in all frequencies at age 64. Moreover, acute progression of hearing loss in his right ear occurred soon after that event, and he developed bilateral deafness with a speech discrimination score of 16% even with hearing aid at age 65. Cochlear implantation (CI) was therefore performed for the right ear at age 68. He received a MED-EL PULSAR with FLEXSOFT electrode (MED-EL, Innsbruck, Austria) through a round window approach. The speech perception scores using the Japanese CI2004 word and sentence test in quiet thereafter improved from 43% and 63% preoperatively using a hearing aid to 76% and 75% with CI, respectively (Figure 2B).

The proband's son (IV-2), aged 42, showed mild to moderate hearing loss at the higher frequencies (2 kHz, 4 kHz, and 8 kHz) with tinnitus in the left ear without awareness of hearing loss (Figure 1B). Otoacoustic emissions (OAEs) showed an abnormal response at the higher frequencies.

Vestibular Profiles

The detailed results of vestibular testing are shown in Figure 3.

The proband (III-2) experienced an onset of dizziness at the age of 64, and caloric testing, cVEMP, and SOT were subsequently performed on the proband and his son. The proband showed no response bilaterally in the caloric testing and cVEMP. In the SOT, he fell into a safety harness under conditions 2, 3, and 4, and could not continue under conditions 5 and 6.

Although the proband's son (IV-2) had no symptoms of vestibular dysfunction and a normal bilateral response in cVEMP, caloric testing revealed areflexia in the left ear (MSPV: right, 22.2 deg/s; left, 7.3 deg/s) and a fall during 1 of 3 trials under condition 5 occurred in the SOT.

Novel p.I372T Mutation

The pedigrees and audiograms of patients with p.I372T are shown in Figure 4.

The proband (III-2) of FAM 475, a 38-year-old female, first noticed hearing loss at the age of 33, after which it progressed gradually. Hearing loss was more severe in the higher frequencies, with only mild impairment in the lower frequencies. His father (II-4), who started to lose his hearing at age 42, had a downward sloping audiogram that was worse than that of the proband. Neither subject complained of any vestibular symptoms at the time of testing.

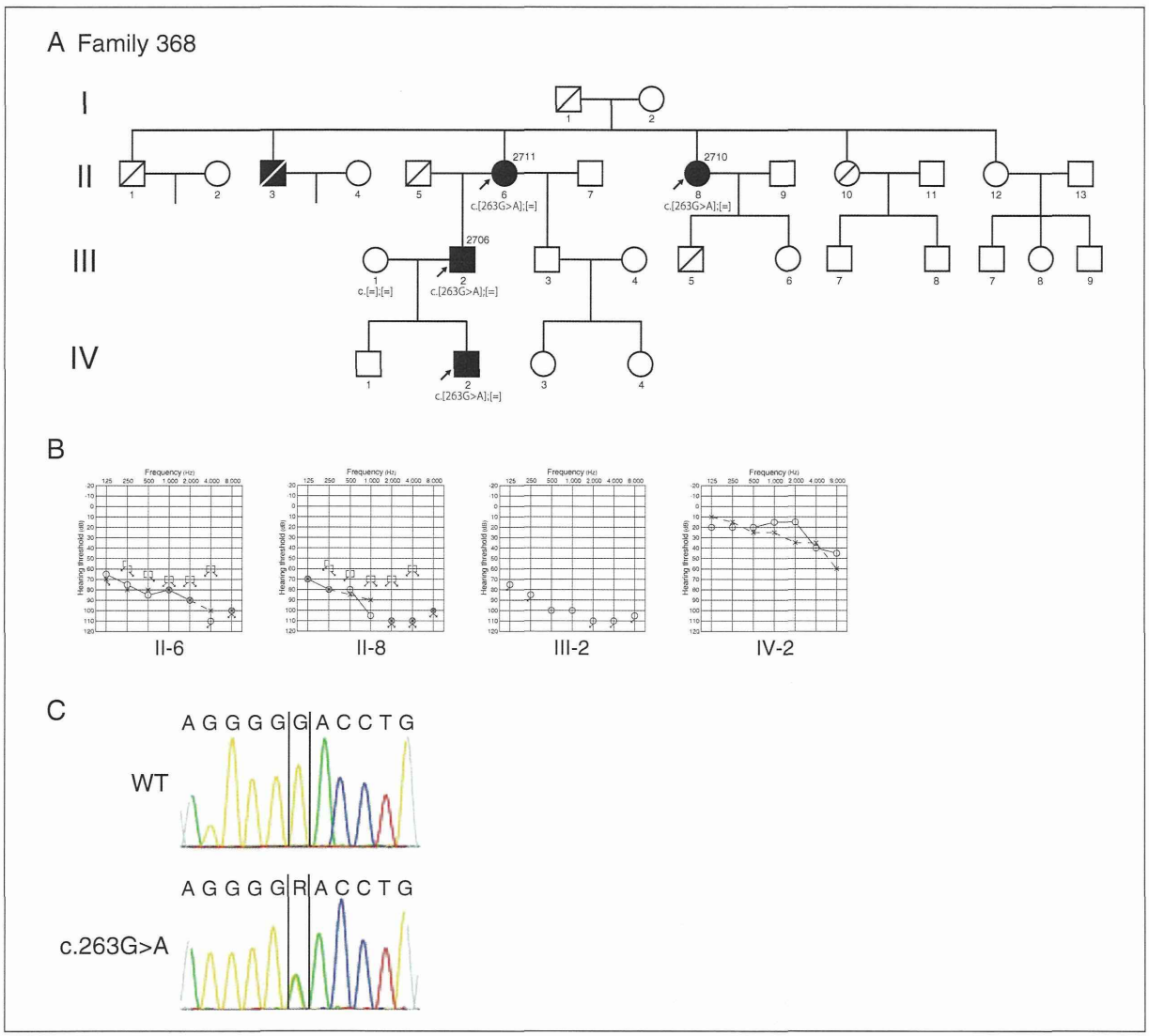

Figure 1. (A) Pedigree and (B) audiograms of the family with p.G88E mutations in the *COCH* gene. Chromatograms of the wild-type (WT) and identified p.G88E mutation in the *COCH* gene (C).

The proband (II-3) of FAM 535 was a 41-year-old male. He became aware of his hearing loss at 34 years of age, after which it progressed gradually. He showed a ski slope–like audiogram and had residual hearing in the lower frequencies. As with the subjects described previously, he did not complain of vestibular symptoms.

Novel p.C542R Mutation

The pedigree and audiogram of FAM 986 are shown in Figure 5.

The 27-year-old proband had moderate-severe hearing loss with a downward sloping audiometric configuration. Her hearing loss was first identified at grade school and progressed gradually with episodes of vertigo.

Discussion

Targeted exon resequencing of selected genes using NGS successfully identified 3 *COCH* mutations in a large cohort of hearing loss patients in Japan, indicating that this technology is a powerful tool for the identification of causative mutations in relatively rare genes. In this report, we

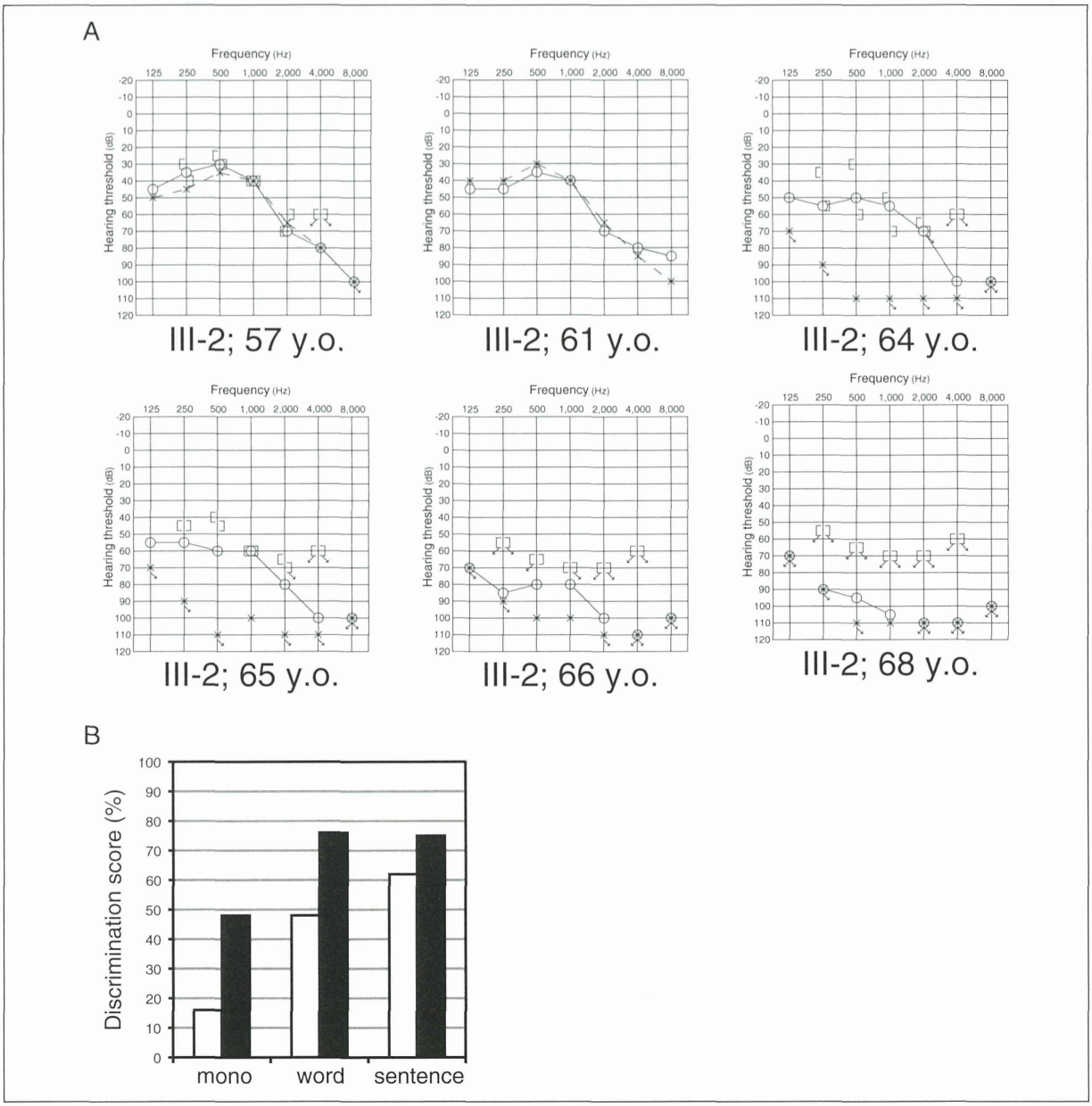

Figure 2. (A) The hearing progression of proband III-2 from Family 368 showed acute deterioration in the left ear at 64 years of age and a gradual to acute deterioration in the right ear around and after age 65. (B) The speech perception scores for monosyllables (mono), words, and sentences obtained using the Japanese CI2004 showed better speech perception after cochlear implantation. White and black bars indicate before and after cochlear implantation, respectively.

identified 3 missense mutations of p.G88E, p.I372T, and p.C542R in the *COCH* gene among the members of 4 Japanese autosomal dominant hearing loss families. The p.G88E mutation was previously reported in a US family[2] and a Dutch family[19] with progressive hearing loss and vestibular dysfunction, whereas p.I372T and p.C542R were both novel mutations.

To date, 18 *COCH* mutations have been reported in DNFA9 hearing loss families (Table 1). Most of the *COCH* mutations occur in the LCCL domain, leading to

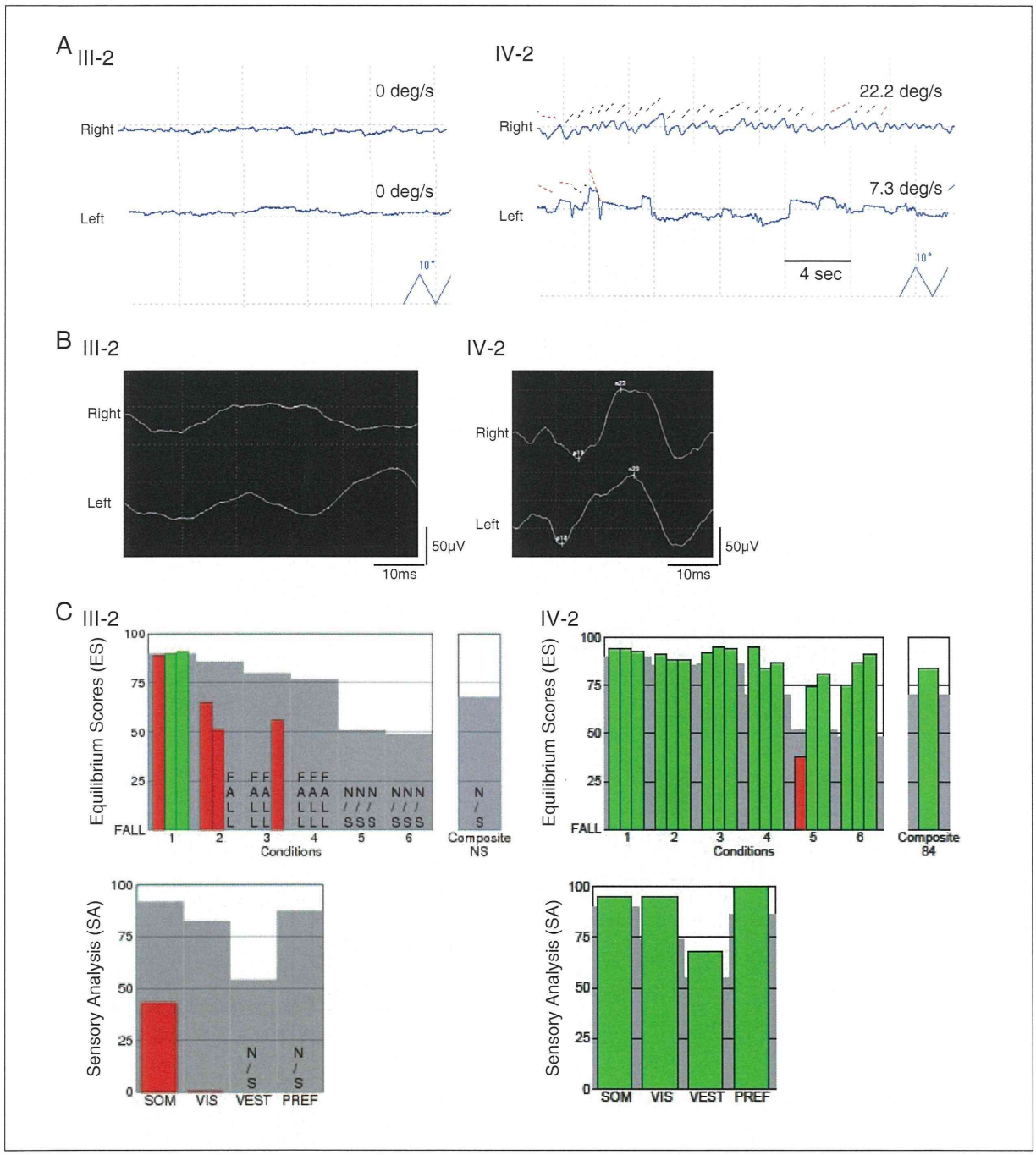

Figure 3. The results of caloric testing of (A, left panel) proband III-2 and (A, right panel) his son IV-2 from Family 368 showed no bilateral response in the proband and unilateral areflexia in the son. The VEMP results for the (B, left panel) proband and (B, right panel) son also showed no bilateral response in the proband and normal bilateral function in the son. Equilibrium scores (ES) and sensory analysis (SA) based on sensory organization training (SOT) for the (C, left panel) proband III-2 and (C, right panel) son IV-2 were evaluated. The (C, left panel) proband fell into the safety harness under conditions 2, 3, and 4. Although the (C, right panel) son fell during 1 of 3 trials under condition 5, the results of SA showed good compensation. The shaded area indicates the 95% confidence interval of age-specific normative data for each condition and sensory dysfunction. N/A, not available.

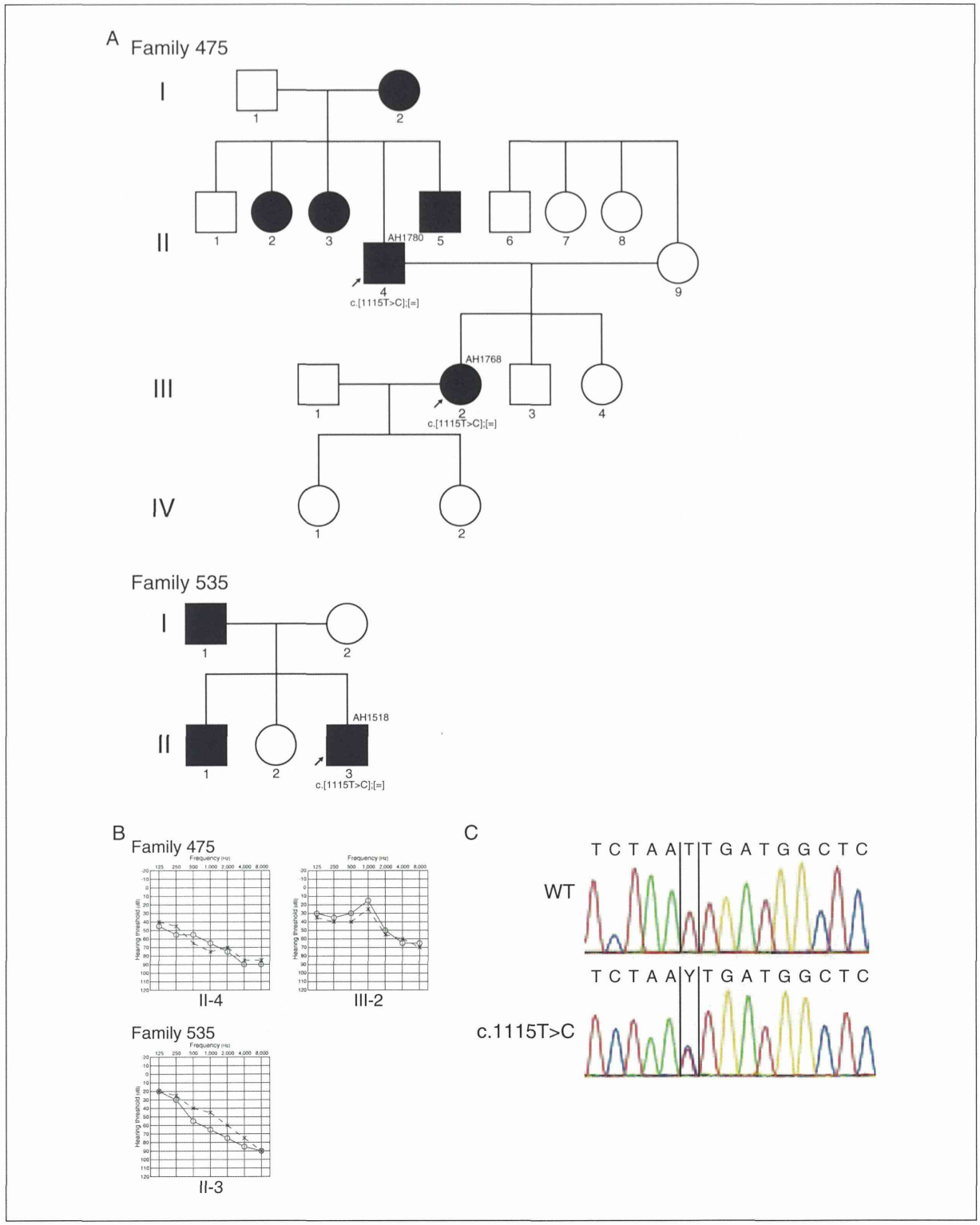

Figure 4. The (A) pedigree and (B) audiograms for the family with the p.I372T mutation. Chromatograms of the wild-type (WT) and identified p.I372T (c.T1115C) mutation in the *COCH* gene (C).