Subjects and Methods

Subjects

Eight deafness patients (4 early-onset, 4 late-onset) were randomly selected from among 150 CI or EAS patients (69 male and 81 female, aged 0 to 91), without common *GJB2, SLC26A4,* or mitochondrial 1555A>G or 3243A>G mutations determined by direct sequencing. Four patients with early-onset deafness received CI, and 4 late-onset patients had residual hearing at lower frequencies and received EAS. All subjects or next of kin, caretakers, or guardians on the behalf of the minors/children gave prior written informed consent for participation in the project, and the Ethical Committee of Shinshu University approved the study and the consent procedure.

Auditory behavioral development was assessed by IT-MAIS and LittlEARS, both of which are parent questionnaires regarding a young infant or toddler's auditory behavior [5,6]. IT-MAIS consists of 10 questions, each scored on a 5-point scale: 0 = never, 1 = rarely, 2 = occasionally, 3 = frequently, and 4 = always. LittlEARS has 35 questions, each scored as 1 = yes, and 0 = no.

Amplicon Library Preparation

An Amplicon library of the target exons was prepared with an Ion AmpliSeq™ Custom Panel (Applied Biosystems, Life Technologies., Carlsbad, CA) designed with Ion AmpliSeq™ Designer (https://www.ampliseq.com/browse.action) for 58 genes reported to be causative of non-syndromic hearing loss listed in Table S1 (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/) by using Ion AmpliSeq™ Library Kit 2.0 (Applied Biosystems, Life Technologies) and Ion Xpress™ Barcode Adapter 1–16 Kit (Applied Biosystems, Life Technologies) according to the manufacturers' procedures.

In brief, DNA concentration was measured with Quant-iT™ dsDNA HS Assay (Invitrogen, Life Technologies) and Qubit® Fluorometer (Invitrogen, Life Technologies) and DNA quality was confirmed by agarose gel electrophoresis. 10 ng of each genomic DNA sample was amplified, using Ion AmpliSeq™ HiFi Master Mix (Applied Biosystems, Life Technologies) and AmpliSeq™ Custom primer pools, for 2 min at 99°C, followed by 15 two-step cycles of 99°C for 15 sec and 60°C for 4 min, ending with a holding period at 10°C in a PCR thermal cycler (Takara, Shiga, Japan). After the Multiplex PCR amplification, amplified DNA samples were digested with FuPa enzyme at 50°C for 10 min and 55°C for 10 min and the enzyme was successively inactivated for 60°C for 20 min incubation. After digestion, diluted barcode adapter mix including Ion Xpress™ Barcode Adapter and Ion P1 adaptor were ligated to the end of the digested amplicons with ligase in the kit for 30 min at 22°C and the ligase was successively inactivated at 60°C for 20 min incubation. Adaptor ligated amplicon libraries were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The amplicon libraries were quantified by using Ion Library Quantitation Kit (Applied Biosystems, Life Technologies) and the StepOne plus realtime PCR system (Applied Biosystems, Life Technologies) according to the manufacturers' procedures. After quantification, each amplicon library was diluted to 20pM and the same amount of the 6 libraries for 6 patients were pooled for one sequence reaction.

Emulsion PCR and Sequencing

The emulsion PCR was carried out with the Ion OneTouch™ System and Ion OneTouch 200 Template Kit v2 (Life Technologies) according to the manufacturer's procedure (Publication Part Number 4478371 Rev. B Revision Date 13 June 2012). After the emulsion PCR, template-positive Ion Sphere™ Particles were enriched with the Dynabeads® MyOne™ Streptavidin C1 Beads (Life Technologies) and washed with Ion OneTouch™ Wash Solution in the kit. This process were performed using an Ion OneTouch™ ES system (Life Technologies).

After the Ion Sphere Particle preparation, MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system using the Ion PGM™ 200 Sequencing Kit and Ion 318™ Chip (Life Technologies) according to the established procedures (Publication Part Number 4474596 Rev. B Revision Date 14 July 2012).

Base Call and Data Analysis

The sequence data were processed with standard Ion Torrent Suite™ Software and Torrent Server successively mapped to human genome sequence (build GRCh37/hg19) with Torrent Mapping Alignment Program optimized to Ion Torrent™ data. The average of 412.93 Mb sequences with about 3,200,000 reads was obtained by one Ion 318 chip. The 98.0% sequences were mapped to the human genome and 94.9% of them were on the target region. Average coverage of depth in the target region was 326.5 and 94.2% of them were over 20 coverage.

After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. Selected variant candidates were filtered with the average base QV (minimum average base quality 25), variant frequency (40–60% for heterozygous mutations and 80–100% for homozygous mutations) and coverage of depth (minimum coverage of depth 10). After the filtrations, variant effects were analyzed with the wANNOVAR web site [7,8] (http://wannovar.usc.edu) including the functional prediction software for missense variants listed below. PhyloP (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html), MutationTaster (http://www.mutationtaster.org/), and GERP++ (http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html).

Algorithm

Flow of informatics analysis is shown in Fig. 1. Missense, nonsense, and splicing variants were selected among the identified variants. Variants were further selected as less than 1% of, 1) the 1000 genome database (http://www.1000genomes.org/), 2) the 5400 exome variants (http://evs.gs.washington.edu/EVS/), and 3) the 72 in-house controls. Candidate mutations were confirmed by Sanger sequencing and the responsible mutations were identified by segregation analysis using samples from family members of the patients.

Direct Sequence Analysis

Primers were designed with the Primer 3 plus web server (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Each genomic DNA sample (40 ng) was amplified using AmpliTaq Gold (Life Technologies) for 5 min at 94°C, followed by 30 three-step cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min, ending with a holding period at 4°C in a PCR thermal cycler (Takara, Shiga, Japan). The PCR products were treated with ExoSAP I (GE Healthcare Bio, Buckinghamshire, UK) and by incubation at 37°C for 30 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle sequencing reaction with ABI Big Dye terminators in an ABI 3130×l sequencer (Life Technologies).

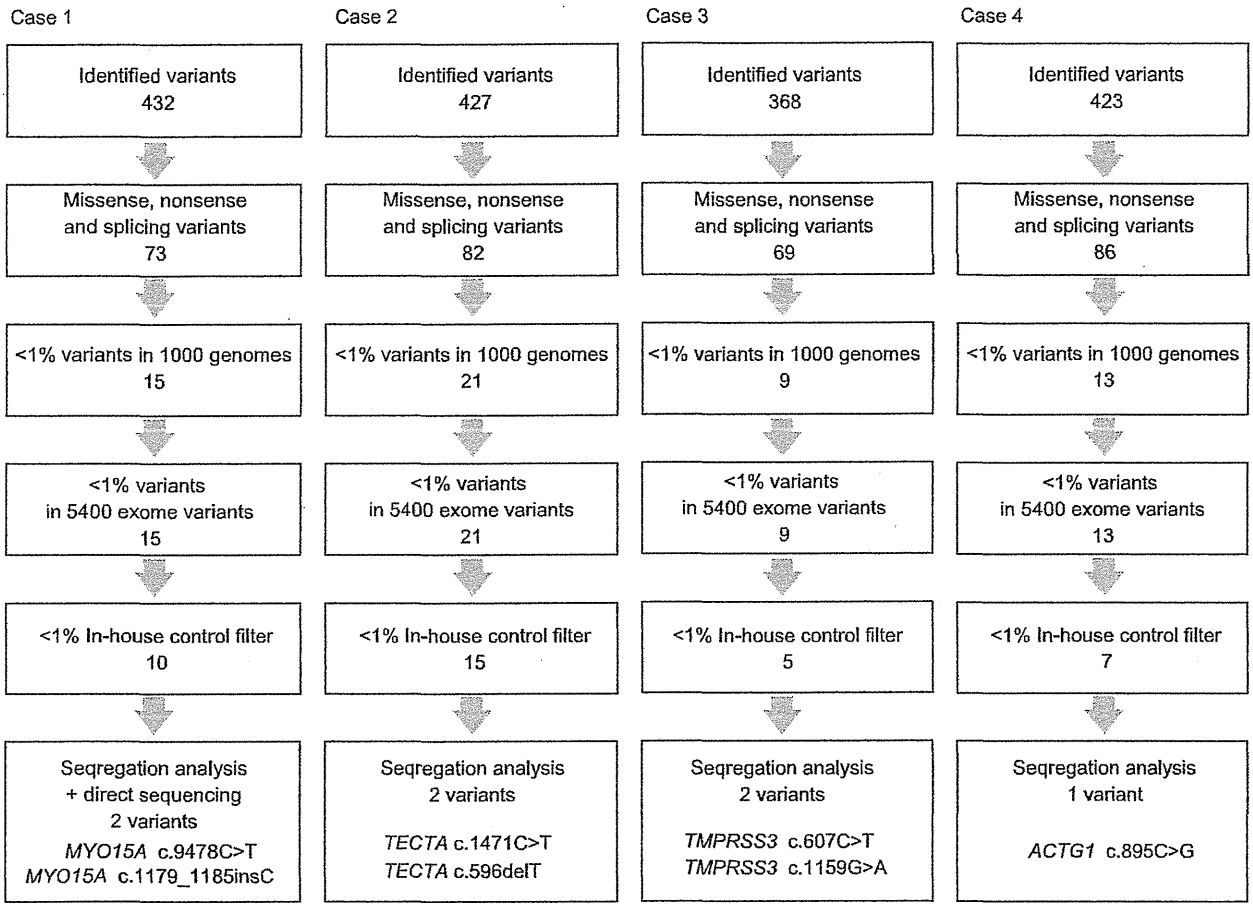

Figure 1. Flow of informatics analysis. Selected missense, nonsense, and splicing variants were filtered with 1) the 1000 genomes, 2) the 5400 exome variants, and 3) the in-house control. Responsible mutations were confirmed by segregation analysis.
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Results

After informatics analysis, several candidate variants were identified and segregation analysis confirmed responsible mutations in *MYO15A* (Case #1) and *TECTA* (Case #2) in pre-lingual patients with conventional CI, and mutations in *TMPRSS3* (Case #3) and *ACTG1* (Case #4) were identified in patients with post-lingual deafness with EAS (Fig. 1). All detected mutations were predicted to be pathologic by several software programs (Table 1). In the remaining four cases, there were no conclusive causative mutations found in this study.

Case #1: Severe Hearing Loss caused by *MYO15A* Mutations (Fig. 2)

As in Fig. 1, MPS identified 10 candidate variants in 9 genes. Among the 9 genes, *CDH23* and *MYO15A* are known to be inherited in a recessive manner. Sanger sequencing could not detect the *CDH23* variant. A *MYO15A* mutation (c.9478C>T (p.L3160F)) was confirmed by Sanger sequencing. Consecutive Sanger sequencing analysis identified another mutation, c.1179_1185insC, which was not found by MPS. The inconsistent results between the two methods were due to this mutation being located in the homo-polymer (poly C stretch) region, which is difficult to detect using this system [9] The patient (5y 5 m-old boy) had compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations (Fig. 2A). The frameshift mutation c.1179_1185insC, leading to a stop codon, was predicted to be causative, and the missense mutation, c.9478C>T, was predicted to be pathologic by several software programs (Table 1).

His hearing loss was found through newborn hearing screening using OAE. Auditory steady state response (ASSR) and conditioned orientation reflex (COR) evaluated at the ages of 1y 6 m, 2y 3 m, 2y 8 m, and 3y 6 m showed progressive hearing loss. He used hearing aids and some language development was seen, but due to progressive hearing loss, hearing aid amplification was insufficient, and he received a left CI (MEDEL PULSAR CI100/standard electrode) at the age of 4y 9 m. To obtain the final outcome, long-term follow up will be needed, but language was developed after 3 months of CI use (Scores of IT-MAIS: 16/40>25/40, LittlEar: 28>33).

Case #2: Profound Hearing Loss caused by *TECTA* Mutations (Fig. 3)

The patient (a 2-year-old boy) had compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations (Fig. 3A). The frameshift mutation, c.596delT, leading to a stop codon, was predicted to be pathologic. The missense mutation, c.1471C>T

Table 1. Missense mutations found in this study.

Gene	Base Change	AA Change	ESP5400	1000g2012feb	dbSNP135	PhyloP	SIFT	PolyPhen2	LRT	MutationTaster	GERP++
MYO15A	c.9478C>T	p.L3160F	0.007618	0.01	rs140029076	N (0.885983)	D (0.97)	NA (0.754167)	NA (0.981216)	D (0.99518)	0.651
TECTA	c.1471C>T	p.R491C	–	–	–	C (0.998333)	D (0.97)	D (1)	D (1)	D (0.684828)	4.88
TMPRSS3	c.1159G>A	p.A387T	–	–	–	C (0.997807)	D (0.96)	B (0.074)	D (1)	N (0.364687)	4.62
ACTG1	c.895C>G	p.L299V	–	–	–	C (0.978424)	NA (0.750464)	B (0.006)	D (0.99998)	D (0.999635)	1.2

SIFT, Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores in which increasing values indicate a probable mutation. ESP5400 and 100g2012feb are the allele frequency in each 5400 exome and 1000 genome project.
Abbreviations: C, conserved; N, not-conserved or neutral D, damaging or deleterious; B, benign; NA, not applicable.
doi:10.1371/journal.pone.0075793.t001

(p.R491C), was predicted to be pathologic by several software programs (Table 1).

His hearing loss was found through newborn hearing screening using OAE. ASSR and COR evaluated at the age of 8 m, 1 y 3 m, and 1 y 9 m showed progressive hearing loss. He used hearing aids, but due to insufficient amplification, he received a left CI at the age of 2. Language was developed after 4 months of CI use (Scores of IT-MAIS: 9/40>23/40).

Case#3: Late Onset Hearing Loss with Residual Hearing in Low Frequencies caused by *TMPRSS3* Mutations (Fig. 4)

The patient (a 40-year-old woman) had compound heterozygous *TMPRSS3* mutations c.[607C>T];[1159G>A] (p.[Q203X];[A387T]) (Fig. 4A). The nonsense mutation p.Q203X was predicted to be causative, and the missense mutation (p.A387T) was predicted to be pathologic by several software programs (Table 1). The parents were found to be carriers for these mutations. She had hearing loss detected by mass screening in primary school. It appeared to slowly progress, and by age 25 she suffered inconvenience in hearing and communication. EAS (MEDEL PULSAR FLEXeas) was applied at the ages of 38 and 39. Residual hearing for acoustic amplification could be preserved, and hearing level with bilateral EAS was around 30dB (Fig. 4C–E). Japanese monosyllable test (65dB SPL in quiet) showed dramatic improvement with bilateral EAS from 18% to 90% one year after receiving the second EAS (Fig. 4F).

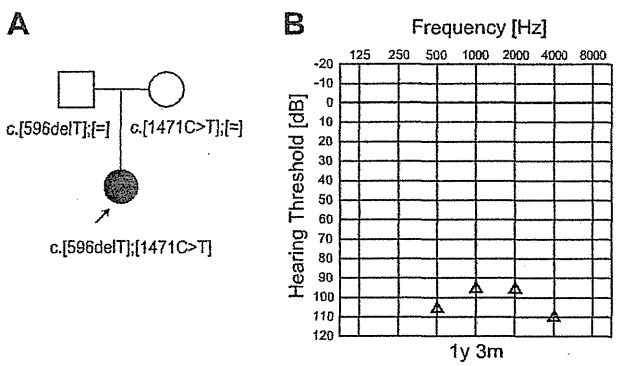

Figure 3. The CI patient with *TECTA* mutations. A: The patient has compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 9 m).
doi:10.1371/journal.pone.0075793.g003

Case #4: Late Onset Hearing Loss with Residual Hearing in Low Frequencies caused by *ACTG1* Mutation (Fig. 5)

The patient (a 41-year-old man) had a heterozygous *ACTG1* mutation, c.895C>G (p.L299V) (Fig. 5A). His pedigree was compatible with autosomal dominant hearing loss. A missense mutation, p.L299V, was predicted to be pathologic by several

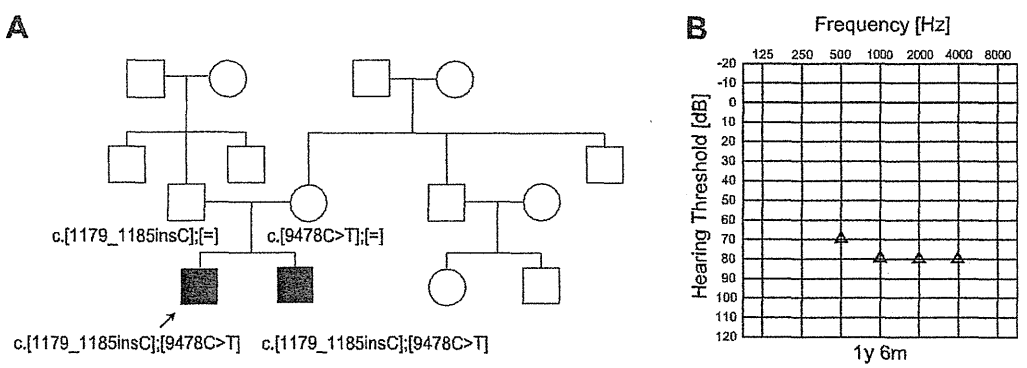

Figure 2. The CI patient with *MYO15A* mutations. A: The patient has compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 6 m).
doi:10.1371/journal.pone.0075793.g002

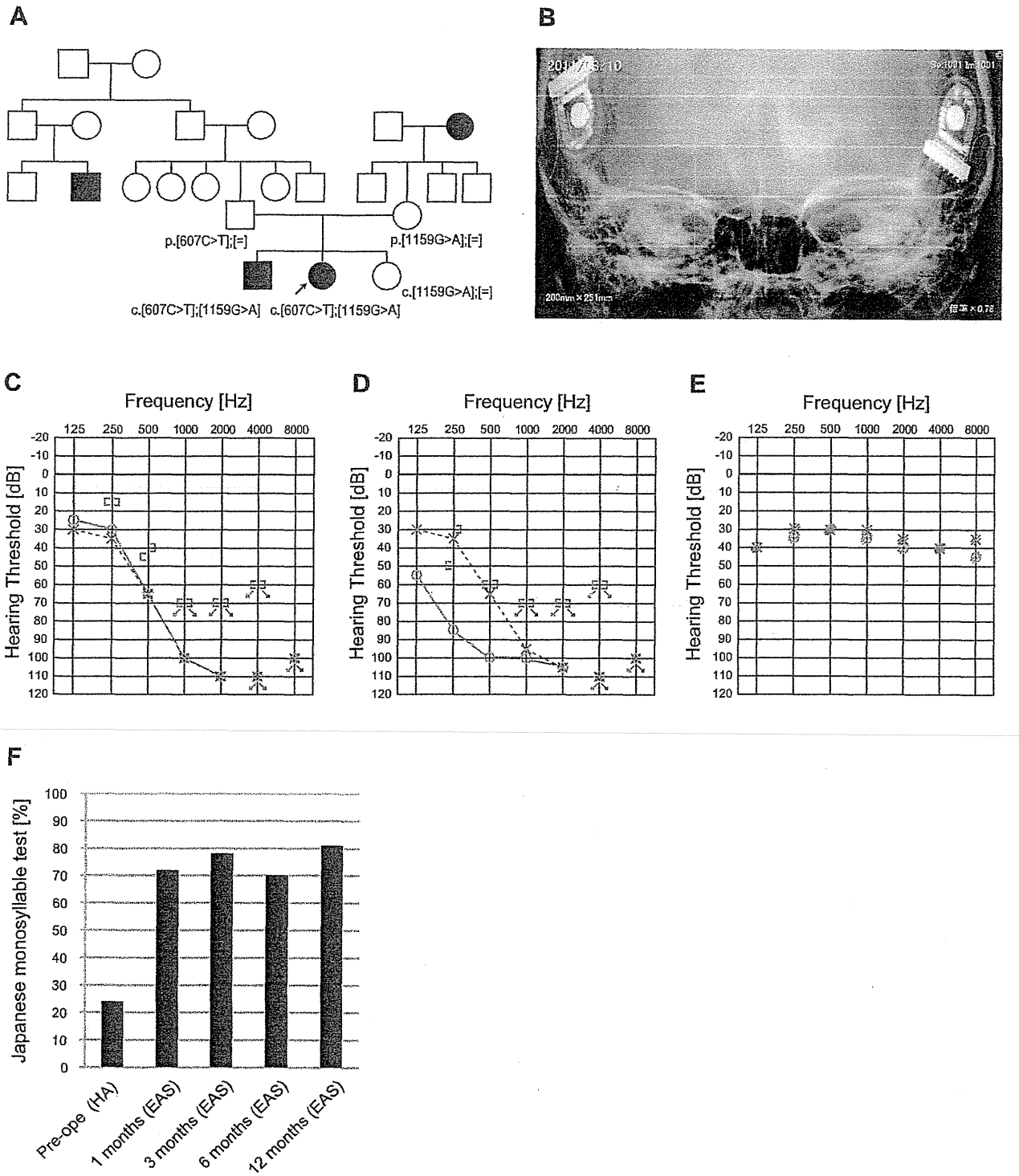

Figure 4. The EAS patient with *TMPRSS3* mutations. A: The patient has compound heterozygous *TMPRSS3* mutations, c.[607C>T];[1159G>A], and the parents were found to be carriers for these mutations. The patient's brother also has the same mutations. B: X-ray imaging after bilateral EAS. C: Pre-operative audiogram. D: Post-operative audiogram (left: 24 months after first EAS, right: 4 months after second EAS). E: Hearing threshold with bilateral EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with bilateral EAS.
doi:10.1371/journal.pone.0075793.g004

software programs (Table 1). He noticed his hearing loss at around age 20. He received EAS due to progressive hearing loss. Residual hearing for acoustic amplification could be preserved, and hearing level with bilateral EAS was around 30dB (Fig. 5B, D, E). Japanese

monosyllable test (65dB SPL in quiet) showed dramatic improvement from 20% to 80% one year after receiving EAS (Fig. 5F). His father and brother carried the same mutation. The audiogram of the brother is shown in Fig. 5C. His father also has hearing loss based on anamnestic evaluation. Neither of the patient's sons (aged 10 and 12) have any hearing loss evaluated by pure tone audiogram, although the younger son has the same mutation.

Discussion

The present MPS-based genetic analysis efficiently identified rare causative mutations in four genes, *MYO15A*, *TECTA*, *TMPRSS3*, and *ACTG1*. All except *TMPRSS3* were first reported in patients with CI/EAS.

MYO15A has been reported mainly in severe to profound hearing loss [10]. Therefore, it is not surprising the patient with the *MYO15A* mutation was found among the CI patients. However, probably due to being too large to be screened by conventional direct sequencing, the routine screening of this particular gene was hampered in spite of its importance in this particular population. *MYO15A* is known to be responsible for DFNB3 [11]. *Myosin 15a* localizes to the tips of inner ear sensory cell stereocilia and is essential for staircase formation of the hair bundle [12]. Since the etiology is located within the sensory hair cells, comparatively better outcomes can be predicted. This case in fact showed better performance after CI.

TECTA encodes α-tectorin, the major component of non-collagenous glycoprotein of the tectorial membrane. *TECTA* has been reported to be responsible for both autosomal dominant non-syndromic sensorineural hearing loss (ADNSHL) (DFNA8/12) and autosomal recessive non-syndromic sensorineural hearing loss (ARNSHL) (DFNB21). Dominant *TECTA* mutations can cause mid-frequency, high-frequency progressive HL, and *TECTA* is reported to be the commonest causative gene among ADNSHL [13]. Dominant inherited deafness caused by this gene has not been reported to reach the level of profound hearing loss. In contrast, recessive *TECTA* mutations cause more profound hearing loss [14]. The etiology is located within the cochlea, therefore comparatively better outcomes can be predicted. This is the first report of a patient with mutations in this gene showing good outcome as prospected from intra-membranous labyrinth etiology.

In this study, *TMPRSS3* was identified in a patient with post-lingual deafness with EAS (Case #3).

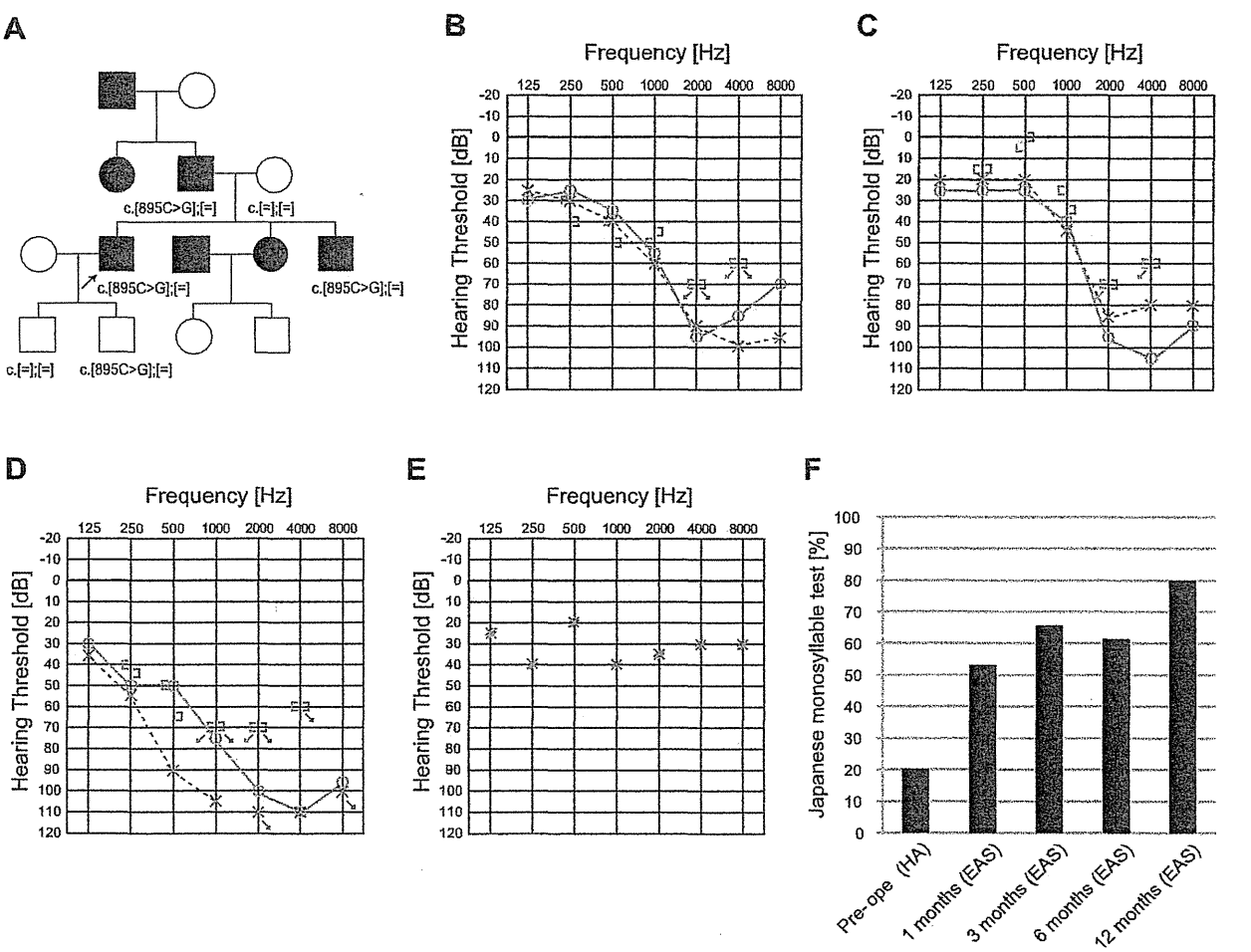

Figure 5. The EAS patient with *ACTG1* mutation. A: The patient has heterozygous *ACTG1* mutation, c.895C>G. Pedigree is compatible with autosomal dominant hearing loss. His father and brother carried the same mutation. B: Pre-operative audiogram. C: Audiogram of brother. D: Post-operative audiogram (6 months after EAS). E: Hearing threshold with EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with EAS.
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TMPRSS3 is a member of the Type II Transmembrane Serine Protease family.

TMPRSS3 may be involved in processing proneurotrophins and therefore in the development and survival of the cochlear neurons [15].

TMPRSS3 has been reported to be responsible for DFNB8/10. Typically, the patients show ski-slope type audiograms and progressive HL [16], being compatible with the phenotype of the present patient. Outcome of CI for patients with *TMPRSS3* is controversial [2,16,17]. Two older papers reported good outcome of CI, while a recent report described poorer performance. Eppsteiner et al. [2] reported two cases of 38-year-old patients with a history of progressive hearing loss starting at the age of 5–6 years. Both of their outcomes were poorer compared with other patients, and the authors hypothesized that it was because the encoded protein is also expressed in the spiral ganglion. However, the present 40-year-old patient showed completely different performance after EAS, indicating that CI is not a contraindication and CI and/or EAS can be a recommended therapeutic option. Especially, the previously reported typical phenotype is high frequency involved hearing loss, which is a good indication for EAS. In the literature, there is also a severe phenotype with all frequencies affected [18]. Our 40-year-old patient did not have rapid progressive hearing loss (only 24 dB (125+250+500 Hz/3) during the 7-year follow-up period), supporting that this patient was a good candidate for EAS. Within this family, intra-familial variation was observed, i.e., an elder brother with the same mutations showed early onset (10 y.o.) profound hearing loss. Therefore, other factors may also potentially be involved in determining the phenotype (including severity and progression).

ACTG1 was identified in a patient with post-lingual deafness with EAS (Case #4).

His brother (35 y.o.) also showed similar high frequency involved progressive hearing loss. Together with the previous literature, high frequency involved progressive nature is one of the characteristic features of the patients with *ACTG1* mutations. The present study proved that EAS is a good therapeutic option for the patients with this gene mutation. *ACTG1* is known to be responsible for DFNA20/26. *ACTG1*, encoding gamma-actin, is the predominant actin isoform in auditory hair cells, more specifically in the cuticular plate, adherens junctions and stereocilia [19]. The etiology is located within the cochlea, therefore comparatively better outcomes can be predicted. Our patient's successful performance after EAS is compatible with the intra-membranous labyrinth etiology. The younger son who carried the same mutation will potentially have progressive hearing loss and his hearing is currently checked semiannually.

EAS is a new trend in therapy for the patients with residual hearing in the lower frequencies [20]. Various genes may be involved in the candidates [21], and we have found the mitochondrial 1555 A>G mutation and *CDH23* mutations in the patients receiving EAS [22], suggesting that the patients with those etiologies may have a potential to show good outcomes. Using the new MPS platform based on new generation sequencing enabled us to add two responsible genes, *TMPRSS3*, and *ACTG1*, in the patients with EAS. Identification of those genes may be good predictor when choosing the therapeutic options. Since the speed of progression may depend on the responsible gene, this information may be helpful for timing of EAS surgery and the selection of the electrode.

Overall, the current findings confirmed the importance of genetic information for predicting outcome of the CI/EAS patients, i.e., relatively good performance would be expected if the pathology exists within the cochlea. Such molecular diagnosis is important for the decision making process for selection of appropriate intervention, such as conventional cochlear implantation, EAS, hearing aid, or combination with other communication modes.

In spite of difficulty in discovery of the responsible gene for each individual patient, genetic testing using MPS may be a breakthrough. In the current series, MPS successfully discovered rare causative genes in CI patients and in EAS patients. These genes have not usually been screened and therefore mutations in them have not been diagnosed by the conventional approach. From that point of view, MPS has the potential power to identify such rare genes/mutations.

Supporting Information

Table S1 58 genes reported to be causative of non-syndromic hearing loss.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: MM SN. Analyzed the data: MM SN. Contributed reagents/materials/analysis tools: MM TI KF. Wrote the paper: SU.

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Comprehensive Genetic Screening of *KCNQ4* in a Large Autosomal Dominant Nonsyndromic Hearing Loss Cohort: Genotype-Phenotype Correlations and a Founder Mutation

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Abstract

The present study of *KCNQ4* mutations was carried out to 1) determine the prevalence by unbiased population-based genetic screening, 2) clarify the mutation spectrum and genotype/phenotype correlations, and 3) summarize clinical characteristics. In addition, a review of the reported mutations was performed for better understanding of this deafness gene. The screening using 287 probands from unbiased Japanese autosomal dominant nonsyndromic hearing loss (ADNSHL) families identified 19 families with 7 different disease causing mutations, indicating that the frequency is 6.62% (19/287). While the majority were private mutations, one particular recurrent mutation, c.211delC, was observed in 13 unrelated families. Haplotype analysis in the vicinity of c.211delC suggests existence of a common ancestor. The majority of the patients showed all frequency, but high-frequency predominant, sensorineural hearing loss. The present study adds a new typical audiogram configuration characterized by mid-frequency predominant hearing loss caused by the p.V230E mutation. A variant at the N-terminal site (c. 211delC) showed typical ski-slope type audiogram configuration. Concerning clinical features, onset age was from 3 to 40 years old, and mostly in the teens, and hearing loss was gradually progressive. Progressive nature is a common feature of patients with *KCNQ4* mutations regardless of the mutation type. In conclusion, *KCNQ4* mutations are frequent among ADNSHL patients, and therefore screening of the gene and molecular confirmation of these mutations have become important in the diagnosis of these conditions.

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Introduction

Autosomal dominant nonsyndromic hearing loss (ADNSHL) is extremely heterogeneous. To date, more than 60 DFNA loci have been identified and 27 genes for DFNA have been identified (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org). Genetic testing has become crucial for precise diagnosis, progression estimation, and selection of ideal intervention. However, due to such genetic heterogeneity and lack of recurrent mutations, routine genetic testing for ADNSHL has lagged. Linkage analysis is a powerful tool to identify a responsible gene for ADNSHL, but in the usual clinical setting, only a limited number of samples are available and this is insufficient for linkage analysis. Among ADNSHL genes, several are frequent, for example, *WFS1, KCNQ4, COCH, GJB2, MYO1A,* and *TECTA* [1]. Based on the number of reported mutations, the *KCNQ4* gene (responsible gene for DFNA2) is known to be one of the most frequent responsible genes for ADNSHL [1]. *KCNQ4*, a member of the voltage-gated potassium channel family, plays a role in potassium recycling in the inner ear [2]. In this 695-amino acid protein there are six transmembrane domains and a hydrophobic P-loop region, which is between the transmembrane domains S5 and S6 (residues 259 to 296). A channel pore, containing a potassium ion-selective filter, is formed by the P-loop domain. Channel function of this selectivity filter is eliminated by pore region mutations [2]. DFNA2-associated hearing loss has been reported to be typically late onset high frequency-involved and progressive over time, as opposed to early onset and severe loss in recessive forms [3]. To date, more than ten pathologic mutations have been identified in *KCNQ4* and they are mostly

missense mutations with a dominant-negative mechanism [3]. It was a matter of interest to know the prevalence of *KCNQ4* mutations to be found through unbiased population-based genetic screening. In this study, we performed the screening in a comprehensive manner to establish the mutation spectrum and genotype/phenotype correlations associated with this type of ADNSHL. Also, we were interested to know whether there are any recurrent mutations. In addition, we reviewed the reported mutations for better understanding of this deafness gene. We found that *KCNQ4* is frequent among ADNSHL patients, and therefore an important causative gene to be screened.

Materials and Methods

Subjects and clinical evaluation

The subjects participating in this study were 287 probands, each from an independent Japanese ADNSHL family. Whether or not progression was present was based on anamnestic evaluation. None of the subjects had any other associated neurological signs, visual dysfunction or diabetes mellitus. The control group was 252 unrelated Japanese individuals with normal hearing evaluated by auditory testing. The average threshold in the conversation frequencies (0.5 kHz, 1 kHz, 2 kHz) was calculated for the better ear, and severity of hearing loss was noted to be normal (−19 dB) in 24 subjects, mild (20–39 dB) in 69 subjects, moderate (40–69 dB) in 132 subjects, severe (70–94 dB) in 23 subjects, and profound (≥95 dB) in 24 subjects. Subjects with high frequency hearing loss only at 4 kHz and 8 kHz were classified as normal because they had normal hearing at 0.5, 1 and 2 kHz. Hearing loss severity was not obtained for 15 subjects. All probands' pure-tone thresholds were recorded on the frequencies of 125, 250, 500, 1000, 2000, 4000, and 8000 Hz.

Ethics Statement

All subjects or next of kin, caretakers, or guardians on the behalf of the minors/children gave prior written informed consent for participation in the project, and the Ethical Committee of Shinshu University approved the study and the consent procedure.

Mutation analysis

All fourteen exons and flanking intronic sequences of the *KCNQ4* gene were amplified by polymerase chain reaction PCR. Primers were designed to flank all of the exon-intron boundaries through use of the Primer3 web based server. Each genomic DNA sample (40 ng) was amplified using Multiplex PCR Assay Kit (Takara, Shiga, Japan) for 5 min at 95°C, followed by 40 three-step cycles of 94°C for 30 s, 60–67.6°C for 90 s, and 72°C for 90 s, with a final extension at 72°C for 10 min, ending with a holding period at 4°C in a Perkin-Elmer thermal cycler. The PCR products varied in size at about 100–400 bp, and they were treated with 0.1 ul exonuclease I (Amersham) and 1 ul shrimp alkaline phosphatase (Amersham) and by incubation at 37°C for 30 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle sequencing reaction with ABI Big Dye terminators in an ABI 3100 autosequencer (Applied Biosystems).

Computer analysis to predict the effect of missense variants on the protein function was performed with wANNOVAR (http://wannovar.usc.edu) including the functional prediction software listed below. PhyloP (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html), and MutationTaster (http://www.mutationtaster.org/).

Haplotype analysis

Haplotype pattern within the 1Mbp region surrounding position c.211, where the frequent Japanese mutation c.211delC was found, was analyzed using a set of 48 single nucleotide polymorphisms (SNPs) (21 sites upstream and 27 sites downstream). Haplotype analysis was performed by the direct sequencing method described above.

Statistical analysis of progression of hearing loss

Each subject's ages at the time of examination and their pure tone thresholds were plotted for detailed progression analysis with 125, 250, 500, 1000, 2000, 4000, 8000 Hz, respectively. The average progressive rates of hearing loss (db/year) were calculated by linear regression lines, and analysis of difference of the rates was performed using analysis of covariance (ANCOVA) with SPSS ver19 software.

Results

Mutation analysis

Direct DNA sequencing identified 8 possible disease-causing mutations among 20 autosomal dominant families (Table 1). There were one deletion mutation (c.211delC), one insertion mutation (c.229_230*ins*GC), and 6 missense mutations (p.F182L, p.V230E, p.W276S, p.P291S, p.P291L, p.R297S) (Table 1). These included 5 novel and three previously reported pathologic mutations: c.211delC, p.F182L, and p.W276S (Table 1, Fig. 1). However, we excluded p.F182L as it is unlikely to be pathologic, according to the prediction program (Table 1). p.F182L was also found in a control sample with normal audiogram (Table 1). Therefore, 7 pathologic mutations from 19 families were found in a total of 287 ADNSHL families in this study (Fig. S1). Concerning the domains in which the 7 mutations were localized, 2 mutations were found in the N-terminal cytoplasmic domain, one mutation in the S4–S5 linker domain, 3 mutations in the pore region and the P-loop region, and one mutation in the S-6 transmembrane domain (Table 1, Fig. 1).

Frequency of KCNQ4 mutations

The frequency of *KCNQ4* mutations found in ADNSHL families in this study was 6.62% (19/287). The most prevalent mutation was c.211delC, at 4.53% (13/287) and it accounted for 68.4% (13/19) of all KCNQ4 mutations.

Haplotype analysis

Haplotype pattern within the 1Mbp region surrounding the position of the most frequent mutation c.211delC, was characterized using a set of 48 single nucleotide polymorphisms (SNPs) (21 sites upstream and 27 sites downstream). All patients from 6 families with c.211delC showed an exactly identical pattern in the allele with c.211delC, though the other allele showed a variety of haplotype patterns (Fig. 2).

Clinical characteristics

Table 2 summarizes clinical characteristics of 36 patients from 19 families with hearing loss caused by the *KCNQ4* mutations, including age at their first visit to the ENT clinic, onset age (age of awareness), audiogram configuration, progression of hearing loss, tinnitus, and vestibular symptoms. The ages at first clinic visits were from 0 to 78 years. Ages of onset (awareness age) ranged

Table 1. *KCNQ4* mutations found in this study together with previously reported mutations.

					Functional Prediction								
Nucleotide Change	**Amino Acid Change**	**Exon**	**Position**	**Alleles in Control Chr**	**SIFT**	**P2 D.S.**	**PhyloP**	**LRT**	**Mut Taster**	**GERP++**	**Study location**	**No of Fm**	**Reference**
c.211_223del13	p. Q71fs	1	N-term cyto	?	–	–	–	–	–	–	Belgium	1	Coucke, et al. (1999)
c.211delC	p. Q71fs	1	N-term cyto	0/252	–	–	–	–	–	–	Japan	14	Kamada, et al. (2006), This report
*** c.229_230insGC**	**p.H77fs**	**1**	**N-term cyto**	**0/252**	–	–	–	–	–	–	**Japan**	**1**	**This report**
c.546C>G	p.F182L	4	S3 trans	0/100, 1/252	T (0.00)	B (0.01)	C (0.97)	N (0.999853)	D (0.88)	3.43	Taiwan, Japan	3	Su, et al. (2007), This report
c.664_681del18	p.G215_220del6	4	S4-S5 linker	0/100	–	–	–	–	–	–	Korea	1	Baek, et al. (2010)
*** c.689T>A**	**p.V230E**	**4**	**S4–S5 linker**	**0/252**	**D (1.00)**	**D (0.97)**	**C (0.99)**	**D (0.999999)**	**D (0.99)**	**4.61**	**Japan**	**1**	**This report**
c.725G>A	p.W241X	5	S5 trans	0/100	–	–	–	–	–	–	USA	1	Hildebrand, et al. (2008)
c.778G>A	p.E260K	5	S5 trans	0/100	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (0.99)	4.73	USA	1	Hildebrand, et al. (2008)
c.785A>T	p.D262V	5	S5 trans	0/100	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (0.99)	4.73	USA	1	Hildebrand, et al. (2008)
c.821T>A	p.L274H	5	PR (P)	?	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (1.00)	4.73	Neth	2	Van Hauwe, et al. (2000), De Heer, et al. (2011)
c.827G>C	p.W276S	5	PR (P)	0/252	D (1.00)	D (1.00)	C (0.99)	D (1.00)	D (1.00)	4.73	Neth, Japan	4	Coucke, et al. (1999), Akita et al. (2001), Van Camp, et al. (2002), Topsakal, et al. (2005)
c.842T>C	p.L281S	6	PR (P)	0/96	D (1.00)	Pr (0.84)	C (0.99)	D (1.00)	D (1.00)	5.14	USA	1	Talebizadeh, et al. (1999)
c.853G>T	p.G285C	6	PR (P)	?	D (1.00)	D (1.00)	C (0.99)	D (0.999999)	D (1.00)	5.14	USA	1	Coucke, et al. (1999)
c.853G>A	p.G285S	6	PR (P)	0/150	D (1.00)	D (0.99)	C (0.99)	D (0.999999)	D (1.00)	5.14	France	1	Kubisch, et al. (1999)
c.859G>C	p.G287R	6	PR (P)	0/274	D (1.00)	D (0.99)	C (0.99)	D (1.00)	D (1.00)	5.14	USA	1	Arnett, et al. (2011)
*** c.871C>T**	**p.P291S**	**6**	**PR (P)**	**0/252**	**D (1.00)**	**D (1.00)**	**C (0.99)**	**D (1.00)**	**D (1.00)**	**5.14**	**Japan**	**1**	**This report**
*** c.872C>T**	**p.P291L**	**6**	**PR (P)**	**0/252**	**D (1.00)**	**D (1.00)**	**C (0.99)**	**D (1.00)**	**D (1.00)**	**5.14**	**Japan**	**1**	**This report**
c.886G>A	p.G296S	6	PR	0/100	D (0.99)	D (0.97)	C (0.99)	D (1.00)	D (0.99)	5.14	Spain	1	Mencia, et al. (2008)
*** c.891G>T**	**p.R297S**	**6**	**S6 trans**	**0/252**	**D (1.00)**	**D (0.99)**	**C (0.99)**	**D (1.00)**	**D (0.95)**	**3.89**	**Japan**	**1**	**This report**
c.961G>A	p.G321S	7	S6 trans	?	D (0.99)	Po (0.31)	C (0.99)	D (1.00)	D (0.99)	4.92	Neth	1	Coucke, et al. (1999)

SIFT, Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores in which increasing values indicate a probable mutation.
Abbreviations: Chr, chromosomes; P2, PolyPhen2; MutTaser, Mutation Taser; Fm, family; cyto, cytoplasmic; trans, transmembrane; PR, Pore region; (P), P-loop; T, tolerated; D, damaging or deleterious; B, benign; Pr, probably damaging; Po, possibly damaging; C, conserved; N, neutral. Neth, Netherlands; *, Novel mutations found in this study.
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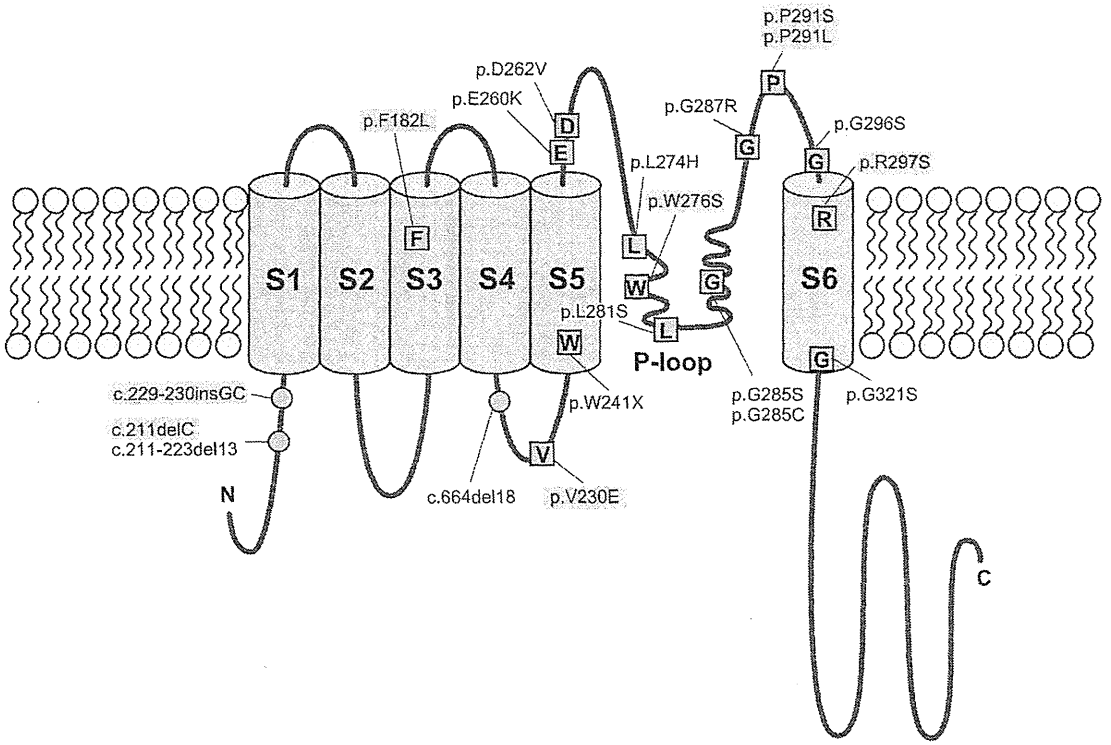

Figure 1. Localization of 20 *KCNQ4* mutations reported in previous studies in the protein. The 6 transmembrane domains (S1–S6) and the P-loop, located between S5 and S6, are shown. 5 mutations are concentrated in a narrow P-loop range. Mutations with pink and blue shadows; possible mutations detected in this study. Blue indicates novel mutations. Original schema is modified from Mencía A (2008) [14].
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from 3 to 40 years old, though the majority became aware when in their teens or younger. Most patients had associated tinnitus, but no vestibular symptoms except in a few cases.

Genotype/phenotype correlations

Concerning type of hearing loss, there were some correlations between genotype and phenotype (audiogram configuration). A variant at the N-terminal site (c. 211delC) showed ski-slope type configuration of audiogram with usually nearly normal hearing at 125–1000 Hz. We found this mutation in 20 patients from 13 families and their overlapped audiogram confirmed a similar configuration (Fig. 3). Onset age was from 10 to 40 years old, with most being in theirs teens and hearing loss was gradually progressive with age (Fig. 3, Table 2). The patients who had a variant in the P-loop region (W276S, P291L, P291S) also had high frequency involved hearing loss, but with some deterioration in the lower frequencies as well (Fig. 3). Most of the patients had earlier onset compared to the former phenotype and a progressive nature (Fig. 3, Table 2). The third audiogram configuration was mid-frequency involved hearing loss found in a family with a variant in the S4–S5 linker region (V230E) (Fig. 3). In most family members, onset was before age ten and gradually progressive (Fig. 3, Table 2). Overlapped audiograms were made for three mutations (W276S, c.211delC, V230E) for which there was a large enough number of patients to be analyzed (Fig. 3).

Therapeutic intervention

Sufficient amplification of hearing aids was obtained in all patients, and no patients received cochlear implantation. An affected subject with W276S (Family-Patient No. 16–2 in Table 2) had used a hearing aid from age 29. Similarly, affected subjects with P291L (Family-Patient No. 18–1) and V230E (Family-Patient No. 15–2) had used hearing aids. None of the affected subjects with c.211delC had a history of hearing aid usage.

Progression analysis

Detailed progression analysis in each frequency showed each affected member's age and their pure tone thresholds for 125, 250, 500, 1000, 2000, 4000, 8000 Hz, respectively (Fig. 4). Linear regression lines calculated by the plots are shown in the graph. Regarding the average progressive rates of hearing loss (db/year) for the patients with c.211delC, 125 (0.15) and 250 Hz (0.078) were shown to be significantly stable compared to the other two mutations (ANCOVA: $p<0.05$). They exhibited milder hearing loss at 500 and 1 KHz (ANCOVA: $p<0.05$). In contrast, at 4 KHz and 8 KHz, the patients with V230E mutations showed milder hearing loss compared to the other two mutations (ANCOVA: $p<0.05$).

Discussion

In this study, we have conducted a comprehensive genetic screening of *KCNQ4* using a large cohort of Japanese ADNSHL patients to establish the mutation spectrum. The *KCNQ4* mutations found in this study together with previously reported mutations (summarized in Table 1) represent an up-dated mutation spectrum for this gene. For missense mutations, we have gone through all reported missense mutations by computer analysis programs, SIFT and PolyPhen2, to predict the effect of

Distance from the c.211delC mutation (bp)	Fm 1		Fm2	Fm 5		Fm 10		Fm 11		Fm 13				Allele frequency				Marker
	Fa (+)	Dau (+)	Dau (+)	Mo (+)	Son (+)	Mo (+)	Son (+)	g.M (+)	Mo (+)	g.M (+)	Mo (+)	Son (+)	Dau (+)					
490912	C	C	T	T	T	C/T	C/T	C	C	T	T	T	T	C	0.80	T	0.20	rs10489431
468938	T	T	T	C/T	C/T	C/T	C/T	T	T	T	T	T	T	C	0.47	T	0.53	rs1846158
441312	A	A	A	A	A	A	A	A	A	A	A	A	A	T	0.31	A	0.69	rs12088482
422378	G	G	G	G	G	G	G	G	G	G	G	G	G	A	0.27	G	0.73	rs3013462
372705	A	A	A/G	A	A	A	A	A	A	A	A	A	A	G	0.68	A	0.32	rs16827291
339980	C	C	C	C/G	C/G	C/G	C/G	C	C	C	C	C	C	G	0.76	C	0.24	rs10489433
333758	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C	C	C	C	C	C	T	0.44	C	0.56	rs209607
333573	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.89	A	0.11	rs2076493
285371	C/G	C/G	C	C	C	C	C	C	C	C	C	C	C	C	0.48	G	0.52	rs12034162
215165	C/T	C/T	T	T	T	T	T	T	T	T	T	T	T	T	0.44	C	0.56	rs4660167
207908	G	G	G	G	G	A/G	A/G	A/G	A/G	G	G	G	G	G	0.41	A	0.59	rs4660436
201218	C/T	C/T	T	T	T	C/T	C/T	C/T	C/T	T	T	T	T	T	0.36	C	0.64	rs12128397
174767	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.59	A	0.41	rs500586
173410	A	A	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.56	A	0.44	rs12217146
168622	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.60	C	0.40	rs504242
151498	T	T	T	C/T	C/T	T	T	T	T	T	T	T	T	T	0.61	C	0.39	rs542214
140107	C	C	C	C/T	C/T	C	C	C	C	C	C	C	C	C	0.62	T	0.38	rs7520394
9505	T	T	T	T	T	T	T	T	T	T	T	T	T	A	0.42	T	0.58	rs823674
6548	C	C	C	C	C	C	C	C	C	C	C	C	C	T	0.39	C	0.61	rs1327887
3196	A	A	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.63	A	0.37	rs12405252
2353	T	T	T	T	T	T	T	T	T	T	T	T	T	T	0.70	C	0.30	rs17361386
0	--	--	--	--	--	--	--	--	--	--	--	--	--	-	--	-	--	c.211delC
17282	C	C	C	C	C	C	C	C	C	C/T	C/T	C/T	C/T	C	0.23	T	0.77	rs4660464
20187	A	A	A/T	A/T	A/T	A/T	A/T	A	A	A	A	A	A	T	0.87	A	0.13	rs12408769
25343	G	G	G	C/G	C/G	G	G	G	G	G	G	G	G	G	0.70	C	0.30	rs878043
34533	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.58	C	0.42	rs2361658
41555	A	A	A/G	A/G	A/G	A	A	A	A	A	A	A	A	A	0.50	G	0.50	rs3767942
43025	A	A	A	A/G	A/G	A/G	A/G	A	A	A	A	A	A	G	0.76	A	0.24	rs6697721
43513	T	T	C/T	C/T	C/T	T	T	T	T	T	T	T	T	T	0.73	C	0.27	rs3767944
43673	T	T	C/T	C/T	C/T	C/T	C/T	T	T	T	T	T	T	C	0.79	T	0.21	rs4660176
58166	C/T	C/T	T	C/T	C/T	T	T	T	T	T	T	T	T	C	0.23	T	0.77	rs1576122
58742	A	A	A/G	A	A	A	A	A	A	A	A	A	A	A	0.64	G	0.36	rs4660472
61431	A/C	A/C	A	A/C	A/C	A/C	A/C	A	A	A	A	A	A	C	0.33	A	0.67	rs4534368
65688	T	T	T	C/T	C/T	T	T	T	T	T	T	T	T	C	0.37	T	0.63	rs11209014
68464	A	A	G	A/G	A/G	A/G	A/G	G	G	G	G	G	G	A	0.46	G	0.55	rs4660473
73906	T	T	T	T	T	T	T	T	T	T	T	T	T	C	0.21	T	0.80	rs913382
75825	G	G	A	A/G	A/G	A	A	A	A	A	A	A	A	G	0.47	A	0.53	rs11209041
101565	A	A	A/T	T	T	A/T	A/T	T	T	A/T	A/T	A/T	A/T	T	0.60	A	0.40	rs6700929
121363	T	T	T	T	T	T	T	T	T	T	T	T	T	T	0.52	C	0.48	rs6684543
122261	T	T	T	T	T	T	T	C	C	T	T	T	T	C	0.68	T	0.32	rs11209145
233975	G	G	C	C/G	C/G	C/G	C/G	G	G	C	C	C	C	G	0.77	C	0.23	rs11209361
237645	A	A	C	A/C	A/C	A/C	A/C	A	A	C	C	C	C	A	0.86	C	0.14	rs6674450
250602	A	A	T	A/T	A/T	A/T	A/T	A	A	T	T	T	T	A	0.84	T	0.16	rs11580656
274693	A	A	A	A	A	A	A	A	A	A	A	A	A	G	0.09	A	0.91	rs4660500
322363	T	T	G/T	G/T	G/T	G/T	G/T	T	T	G	G	G	G	G	0.43	T	0.57	rs548007
334776	G	G	A	A/G	A/G	A	A	G	G	A	A	A	A	G	0.84	A	0.16	rs2284802
369918	G	G	A	A/G	A/G	A/G	A/G	G	G	A	A	A	A	A	0.23	G	0.77	rs213744
487513	C	C	C/T	C/T	C/T	T	T	C	C	T	T	T	T	C	0.48	T	0.52	rs11209779
503189	G	G	G	G	G	G	G	G	G	G	G	G	G	G	0.73	A	0.27	rs12029950

*Fm (n), Family number (n); Mo, Mother; Fa, Father; Dau, Daughter, g. M, grand mother,

Figure 2. The haplotypes around c.211delC mutation of six families constructed using SNPs are shown. Each column shows an affected allele. Each base is defined by pure segregation analysis in the family. Allele frequencies of SNPs are derived from HapMap JPT+CHB samples. Families 2, 5, 10, and 13 shared a large common region of about more than 1 Mb in their haplotypes (blue). Abbreviation: Fm, Family.
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missense variants on *KCNQ4* protein function. A missense mutation (p.F182L) was found in one control patient with normal audiogram and the results showed that it is not likely to be a pathologic mutation.

The present study identified 7 possible disease-causing mutations, including 5 novel mutations, in 19 autosomal dominant families. Based on our unbiased population-based genetic screening, the frequency is 6.62% (19/287) of the overall ADNSHL population. These data indicated that *KCNQ4* is one of the important causative genes among ADNSHL patients, particularly in patients with high frequency-involved hearing loss. This frequency is higher than our recently reported frequency (4/139:

Table 2. *Clinical features of affected family members associated with KCNQ4 mutations found in this study.*

Amino Acid Change	Family – Patient No.	HL onset age (years)	Age at the first visit (years)	Audiogram frequencies	Progression	Tinnitus	Vertigo
Q71fs	1–1	40	48	Ski slope	N/A	N/A	N/A
	1–2	15	15	Ski slope	+	–	–
	2–1	30	47	Ski slope	+	+	–
	3–1	N/A	31	Ski slope	N/A	–	–
	4–1	12	37	Ski slope	+	+	–
	5–1	32	42	Ski slope	–	+	–
	5–2	10	15	Ski slope	+	+	–
	6–1	14	40	Ski slope	+	+	–
	7–1	11	35	Ski slope	+	+	–
	8–1	18	25	Ski slope	+	+	–
	9–1	18	29	Ski slope	+	+	–
	10–1	17	22	Ski slope	+	+	–
	10–2	20	52	Ski slope	+	+	–
	11–1	40	43	Ski slope	+	–	–
	11–2	N/A	73	Ski slope	N/A	–	–
	12–1	22	38	Ski slope	+	+	–
	13–1	35	55	Ski slope	+	+	–
	13–2	25	33	Ski slope	+	+	+
	13–3	11	14	Ski slope	N/A	+	+
	13–4	–	6	Normal (*)	N/A	N/A	N/A
H77fs	**14**	**22**	**27**	**Ski slope**	**+**	**+**	–
V230E	**15–1**	**40**	**78**	**mid freq**	**+**	**+**	–
	15–2	**12**	**39**	**mid freq**	**+**	–	–
	15–3	**5**	**5**	**mid freq**	**+**	–	–
	15–4	**3**	**3**	**mid freq**	**N/A**	**N/A**	**N/A**
	15–5	**N/A**	**0**	**mid freq**	**N/A**	**N/A**	**N/A**
W276S	16–1	8	65	high freq	+	–	+
	16–2	12	46	high freq	+	–	–
	16–3	7	42	high freq	+	–	–
	16–4	8	8	high freq	+	–	+
	16–5	8	6	high freq	+	–	–
P291S	**17–1**	**20**	**33**	**high freq**	**+**	**N/A**	**N/A**
P291L	**18–1**	**17**	**40**	**high freq**	**N/A**	**N/A**	**N/A**
	18–2	**17**	**15**	**high freq**	**N/A**	**N/A**	**N/A**
R297S	**19–1**	**39**	**39**	**high freq**	–	**+**	–
	19–2	**5**	**5**	**high freq**	**+**	–	–

Abbreviations: HL, hearing loss; mid, middle; freq, frequency; N/A, not applicable.
(*) Six-year-old boy's hearing is normal in spite of having the mutation.
doi:10.1371/journal.pone.0063231.t002

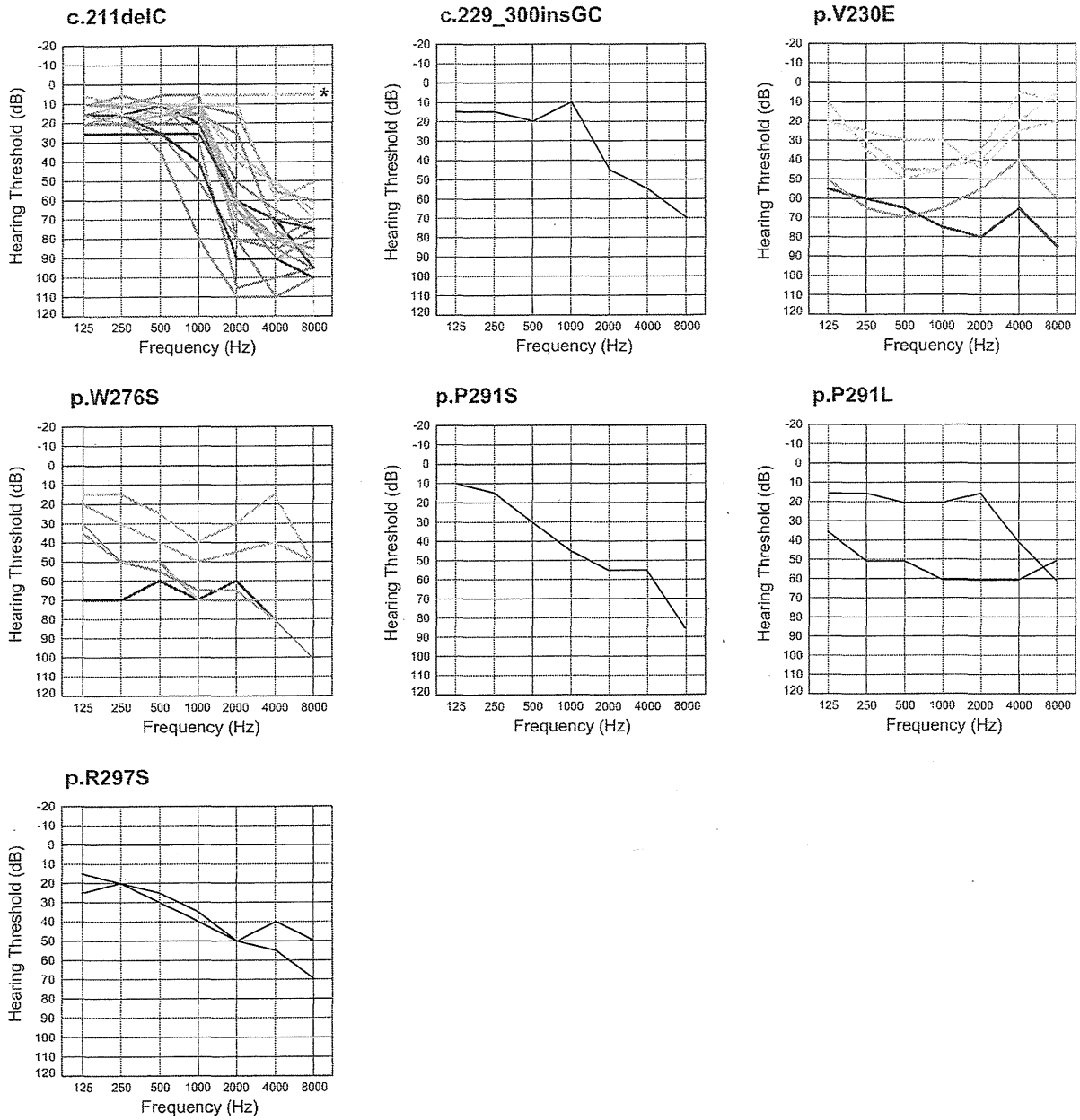

Figure 3. Overlapping audiograms from the better ear for each genotype. In cases of W276S, c.211delC, or V230E, light colored audiograms (green, blue, red) were from individuals aged 19 and under. Dark colored audiograms (green, blue, red) were from the patients aged 20–49 years old, and deep colored audiograms (green, blue, red) are from the patients in their 50 s and over. In family #13 with c.211delC, (*) a six-year-old boy's hearing is normal in spite of having the mutation.
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2.9%) of *TECTA* in Japanese ADNSHL families [4], therefore *KCNQ4* is found to be currently the most prevalent gene responsible for Japanese ADNSHL patients, and should be the first in line to be analyzed for ADNSHL patients.

Mutations lie in various domains of the *KCNQ4* protein. While the majority are private mutations, one particular recurrent mutation, c.211delC, was observed in 13 unrelated families. In this gene, we have reported that there is a hot spot mutation, p.W276S, in Belgian, Dutch, and Japanese families [5]. Based on haplotype analysis, in the case for c.211delC, it is not likely a hot spot but rather is suggested to be due to a common ancestor. Such recurrent mutations are common in recessive genes such as 235delC, 35delG, 167delT in *GJB2* [6][7], H723R in *SLC26A4* [8], and P204L in *CDH23* [9]. They are rare in dominant genes, though a mutation in *DFNA5* that causes autosomal dominant sensorineural hearing loss was reported to arise from a common ancestor [10]. Together with specific audiogram configuration, this may facilitate genetic testing for ADNSHL with a particular phenotype.

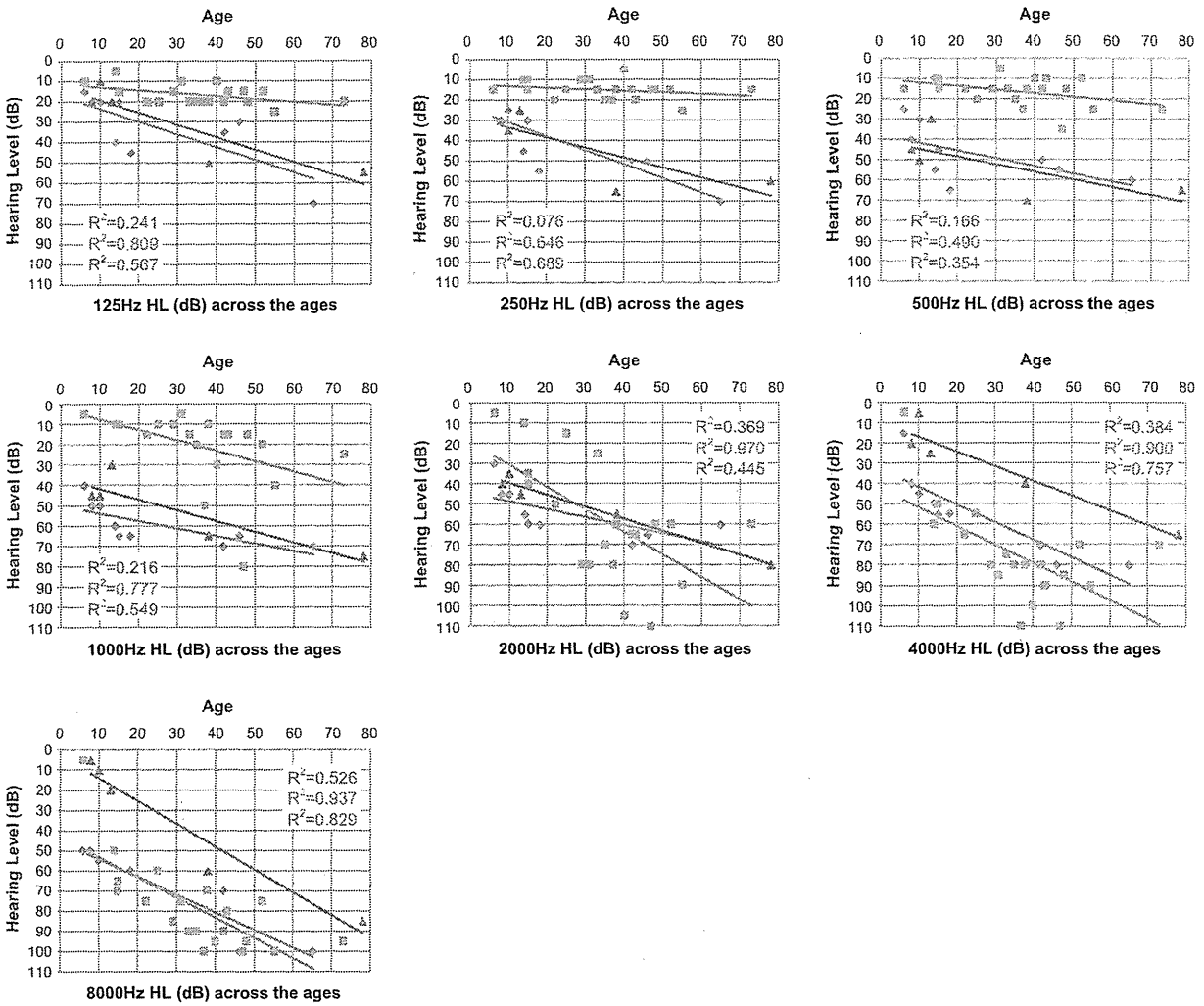

Figure 4. Detailed progression analysis in each frequency. A single audiogram (the better ear) from a single patient was plotted. Gradual progression is characterized regardless of frequency. Average progressive rates of hearing loss (db/year) for the patients with c.211delC, for 125 (0.15) and 250 Hz (0.078) were significantly stable compared to the other two mutations (ANCOVA: $p<0.05$) and they had milder hearing loss at 500 and 1 KHz (ANCOVA: $p<0.05$). In contrast, at 4 KHz and 8 KHz, patients with V230E mutations had milder hearing loss compared to the other two mutations (ANCOVA: $p<0.05$). Each color (green, blue, red) indicates W276S, c.211delC, or V230E, respectively.
doi:10.1371/journal.pone.0063231.g004

Table 2 summarizes clinical characteristics including hearing threshold, severity, onset age (age of awareness), progressiveness of hearing loss, and vestibular symptoms. Age of onset (awareness of hearing loss) ranged from 3 to 40 years old, though the majority of the patients were in their first decade of life. Many of the mutations were accumulated in the P-loop region as described before [3][11][12], but mutations were also found in the other domains (Table 1, Fig. 1). There were some correlations between genotype and phenotype (Fig. 3). Overlapped audiograms showed characteristic high frequency involved hearing loss in the majority of the patients with *KCNQ4* mutations. Unique audiograms were shown in the patients with c.211delC and p.V230E. The patients associated with c.211delC showed so-called ski slope hearing loss (high frequency involved hearing loss with nearly normal hearing at lower frequencies). Patients with p.V230E showed mid-frequency involved hearing loss.

It has been known that DFNA2 shows high-frequency involved hearing loss [3][13][14]. Based on collected audiograms from the patients with *KCNQ4*, an effective selection algorithm named "Audioprofile" has been proposed and many mutations have actually been successfully identified [13]. The present large cohort study allowed us to confirm and extend the genotype-phenotype correlations. It added a new type of audiogram configuration characterized by mid-frequency predominant hearing loss caused by a *KCNQ4* mutation (Fig. 3). Family #15 had a heterozygous T>A transition at nucleotide 689 in exon 4, which results in a Val to Glu substitution (V230E). This mutation was present in all five affected individuals, and not present in two unaffected family members. None of the 252 normal controls had this mutation. Prediction programs indicated that this mutation is likely to be pathologic. So far mid-frequency predominant hearing loss has been reported with *TECTA* mutations [4]. In this family, we sequenced for *TECTA* to find a mutation, but none were found

(data not shown). A different *KCNQ4* mutation (c.664_681del) within the same domain as this mutation was reported to cause high-frequency involved hearing loss, suggesting that the phenotype is not domain-specific [15]. The V230E mutation is a missense mutation that substitutes a nonpolar and aliphatic valine for a negatively charged glutamate. This single base substitution is located adjacent to the S4 transmembrane domain that has a key role as a voltage sensor. The V230E mutation may therefore change sensitivity of voltage sensor and have an affect on passage of potassium through the cell membrane.

The ski-slope type audiogram configuration found in the patients with c.211delC is also a striking characteristic phenotype (Fig. 3). Single families associated with c.211delC [16] and c.211_223del13 [17] have previously been reported to show ski-slope audiograms. The audiogram collection in this study further generalized this phenotype in the N-terminal site.

Analysis of the different frequencies found evident quickly progressive hearing loss in the middle frequencies, therefore those patients may be at risk for rapid deterioration of speech understanding during the time course. Patients with ski-slope type audiograms sometimes have difficultly in being fitted with hearing aids, but Electric Acoustic Stimulation (EAS) has recently been shown to be effective for those patients with high frequency involved hearing loss [18]. The present data on progression speed showed more stable hearing at low frequencies (125 and 250Hz) (Fig. 4), indicating EAS will be the potential therapeutic intervention for the patients with this particular mutation.

Progressive nature is a common feature of the patients with *KCNQ4* mutations regardless of the particular mutation (Fig. 3). Overlapped audiograms of all subjects with W276S, c.211delC, or V230E mutations showed the progressive nature of hearing loss regardless of the mutation type. However, no patients received cochlear implants in this cohort, suggesting that profound hearing loss may seldom be seen though their hearing loss has a progressive nature.

In conclusion, *KCNQ4* is frequent among ADNSHL patients, and therefore screening for this gene and molecular confirmation of *KCNQ4* mutations have become important in the diagnosis of these conditions.

Supporting Information

Figure S1 Pedigrees of the *KCNQ4* mutation families and detected mutations.
(PDF)

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: TN SN YI TY. Analyzed the data: TN SN YI TY SU. Contributed reagents/materials/analysis tools: TN KK SA KI HK AN CO. Wrote the paper: SU TN.

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OTOF mutation screening in Japanese severe to profound recessive hearing loss patients

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Abstract

Background: Auditory neuropathy spectrum disorder (ANSD) is a unique form of hearing loss that involves absence or severe abnormality of auditory brainstem response (ABR), but also the presence of otoacoustic emissions (OAEs). However, with age, the OAEs disappear, making it difficult to distinguish this condition from other nonsyndromic hearing loss. Therefore, the frequency of ANSD may be underestimated. The aim of this study was to determine what portion of nonsyndromic hearing loss is caused by mutations of *OTOF*, the major responsible gene for nonsyndromic ANSD.

Methods: We screened 160 unrelated Japanese with severe to profound recessive nonsyndromic hearing loss (ARNSHL) without *GJB2* or *SLC26A4* mutations, and 192 controls with normal hearing.

Results: We identified five pathogenic *OTOF* mutations (p.D398E, p.Y474X, p.N727S, p.R1856Q and p.R1939Q) and six novel, possibly pathogenic variants (p.D450E, p.W717X, p.S1368X, p.R1583H, p.V1778I, and p.E1803A).

Conclusions: The present study showed that *OTOF* mutations accounted for 3.2–7.3% of severe to profound ARNSHL patients in Japan. *OTOF* mutations are thus a frequent cause in the Japanese deafness population and mutation screening should be considered regardless of the presence/absence of OAEs.

Keywords: Auditory neuropathy spectrum disorder, DFNB9, Nonsyndromic hearing loss

Background

Auditory neuropathy (AN), a unique form of hearing loss, involves absence or severe abnormality of auditory brainstem response (ABR), but presence of otoacoustic emissions (OAE) and/or cochlear microphonic (CM). This disorder was defined by Starr [1], and also reported as "Auditory nerve disease" [2] and "Auditory dys-synchrony" [3]. AN was renamed "auditory neuropathy spectrum disorder (ANSD)" in 2008, due to the heterogeneous and multifaceted nature [4].

The prevalence of ANSD in sensorineural hearing loss is reported to be 0.5-15% [5]. The etiologies of ANSD are various; patients range from infants to adults, 42% of which are associated with hereditary neurological disorders, 10% with toxic, metabolic, immunological and infectious causes, and 48% with unknown causes [6]. Although the exact percentage of nonsyndromic ANSD is unclear, responsible genes have been gradually revealed. To date, mutations of *AUNA1, OTOF, PJVK, GJB2* and mitochondrial 12S rRNA are reported to be causal for nonsyndromic ANSD [7].

The *OTOF* gene (DFNB9) is mainly expressed in cochlear inner hair cells, and is necessary for synaptic exocytosis at the auditory ribbon synapse [8]. It encodes both long and short isoforms with the long isoform containing six C2 domains and the C-terminal transmembrane domain, and the short isoform containing only the last three C2 domains [9]. Mutations in the *OTOF* gene, encoding otoferlin, are reported to be the major causes of nonsyndromic recessive ANSD [10-12]. In Japanese, mutations in *OTOF* account for 56. 5% (13/23) of ANSD [13]. Although ANSD can be characterized by the presence of OAEs in the first two years of life, OAEs later disappear and the hearing loss then resembles other types of nonsyndromic hearing loss [14]. Because of expected good outcomes of cochlear implantation for

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patients with *OTOF* mutations [15,16], it is important to perform mutation screening for *OTOF* to select the appropriate intervention. Although some reports have described *OTOF* mutations in severe to profound autosomal recessive hearing loss patients in other populations [11,12], there has been no literature available regarding the screening of *OTOF* mutations using a large cohort in a comprehensive manner. The goal of this study was therefore to reveal the frequency of ANSD and to identify *OTOF* mutations in Japanese ARNSHL patients.

Methods

Subjects

Among the 1511 Japanese independent hearing loss patients registered in our DNA sample bank, 469 were congenital severe to profound sensorineural hearing loss (above 71 dB average over 500, 1000, 2000 and 4000 Hz in the better hearing ear) patients compatible with autosomal recessive inheritance (including sporadic cases). From those, we randomly selected 160 patients. All ANSD cases were sporadic (compatible with autosomal recessive inheritance). They were diagnosed as ANSD by evaluation of OAE response. We excluded autosomal dominant families because in previous studies *OTOF* mutations were not found in such groups [17]. Pure tone audiometry was used for adults (N= 32) and ABR, auditory steady-state responses (ASSR), and conditioned orientation response audiometry (COR) were used for pediatric patients (n=128). The control group was composed of 192 unrelated Japanese individuals who had normal hearing shown by auditory testing. All subjects gave prior informed written consent for participation in the project and the Ethical Committee of Shinshu University approved the study.

Mutation analysis

We designed 43 pairs of primers to amplify DNA fragments containing all exons in the coding regions of the *OTOF* gene (ENST00000403946). Primer3Plus (http://www.bioinformatic.nl/cgi-bin/primer3plus/primer3plus.cgi) was used to design primers to flank all the exon-intron boundaries. Each genomic DNA sample (40 ng) was amplified, using Ampli Taq Gold (Applied Biosystems, Foster City, CA), for 5 min at 95°C, followed by 30 three-step cycles of 95°C for 30s, 60°C for 30s, and 72°C for 60s, with a final extension at 72°C for 7 min, ending with a holding period at 4°C in a PCR thermal cycler (Takara, Shiga, Japan). PCR products were treated with ExoSAP-IT® (GE Healthcare Bio, Santa Clara, CA) by incubation at 37°C for 60 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle-sequencing reactions with ABI Big Dye® terminators in an ABI PRISM 3100 Genetic Analyzer autosequencer (Applied Biosystems, Foster City, CA).

Computer analysis to predict the effect of missense variants on the protein function was performed with wANNOVAR [18-20] (http://wannovar.usc.edu) including functional prediction software listed below. PhyloP (http://hgdownload. cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html), and MutationTaster (http://www.mutationtaster.org/).

Results

We found a total of 11 probable pathogenic variants in the patients (Table 1). Among them, five mutations were previously reported: p.D398E, p.Y474X, p.N727S, p.R1856Q and p.R1939Q. The other six probable pathogenic variants were novel: 2 nonsense mutations (p.W717X, p.S1368X) and 4 missense mutations (p.D450E, p.R1583H, p.V1778I, p. E1803A). Based on the prediction programs, it is most likely that p.D450E (c.1350C>G), p.R1583H (c.4748G>A), p.V1778I (c.5332G>A), and p.E1803A (c.5408A>C) were pathogenic. In addition, they were absent (or in very few numbers) in the controls, and located in C2 domains, which are highly conserved among species (Figure 1). In addition, polymorphic changes were also identified (Table 2). p.R1676C (c.5026C>T) was previously reported to be pathogenic [21], but we excluded p.R1676C as it is unlikely to be pathologic because of high frequencies in the control population (Table 2). Among the 16 patients with *OTOF* mutations, 4 were homozygous, 3 were compound heterozygotes, and 9 were heterozygous without second mutation (Table 3). After clinical re-evaluation, we recategorized cases with OAE as ANSD.

Discussion

So far, more than 90 pathologic mutations have been reported in *OTOF* [25]. The present study identified 11 possibly pathogenic *OTOF* variants in Japanese patients with nonsyndromic hearing loss, and 6 of them were novel mutations (p.D450E, p.W717X, p.S1368X, p.R1583H, p.V1778I, and p.E1803A). Concerning pathogenicity of the four novel missense mutations, p.R1583H is more likely to be a disease causing mutation, because 1) it was found in compound heterozygosity with p.R1939Q, 2) it was absent in controls, 3) it affects a C2 domain, and 4) the scores provided by prediction programs also agree with the pathogenicity. The pathogenic potential of the three other variants (p.D450E, p.V1778I, and p.E1803A) is less clear, because 1) all of them have been found in the heterozygous state without accompanying mutation in the other allele, and 2) p.D450E was found in controls. But it is also true that 1) they affect C2 domains, and 2) the scores of the prediction programs would support their classification as pathogenic variants.

Table 1 Probable pathogenic and uncertain pathogenic variants of OTOF identified in this study

Exon	DNA level	Protein level	Occurrence in this work (chromosome)	Control (chromosome)	Functional prediction						References
					PhyloP	SIFT (p-value)	P2 D.S.	LRT	Mutation taster	GERP ++	
Probable pathogenic variants											
Exon 14	c.1422T>A	p.Y474X	2/320	0/374	N (0.072941)	NA (0.829813)	NA (0.58309)	D (1)	A (1)	−3.78	[13]
Exon 18	c.2151G>A	p.W717X	1/320	0/344	C (0.994764)	NA (0.90345)	NA (0.734698)	D (0.999998)	A (1)	3.83	This study
Exon 34	c.4103C>G	p.S1368X	1/320	0/364	N (0.944413)	NA (0.915)	NA (0.554899)	NA (0.026679)	A (1)	0.571	This study
Exon 38	c.4748G>A	p.R1583H	1/320	0/366	C (0.997935)	D (1)	D (0.999)	D (1)	D (0.999661)	4.69	This study
Exon 44	c.5567G>A	p.R1856Q	1/320	0/380	C (0.99611)	T (0.91)	P (0.813)	D (1)	D (0.999517)	4.1	[11]
Exon 46	c.5816G>A	p.R1939Q	11/320	0/382	N (0.996658)	T (0.92)	NA (0.746672)	NA (1)	D (0.999886)	1.38	[22]
Uncertain pathogenic variants											
Exon 12	c.1194T>A	p.D398E*	1/320	1/380	N (0.232793)	T (0.77)	D (0.853)	D (1)	D (0.995165)	0.981	[23]
Exon 13	c.1350C>G	p.D450E*	1/320	1/380	C (0.986229)	T (0.74)	D (0.853)	D (1)	D (0.991594)	3.54	This study
Exon 18	c.2180A>G	p.N727S*	2/320	1/344	C (0.992986)	T (0.27)	P (0.386)	D (1)	D (0.95528)	3.98	[21]
Exon 43	c.5332G>A	p.V1778I	1/320	0/378	C (0.997116)	T (0.54)	P (0.289)	D (1)	D (0.994783)	4.38	This study
Exon 43	c.5408A>C	p.E1803A	1/320	0/378	C (0.994555)	D (1)	D (0.995)	D (1)	D (0.999914)	4.26	This study

*the variants found in controls.
Exon number was named based on ENST00000403946.
A, disease causing automatic; C, conserved; D, damaging or disease causing; N, not conserved; NA, not applicable; P, possibly damaging; T, tolerated; P2 D.S., Polyphen-2 damaging score. Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores that indicate a probable mutation with increasing value.

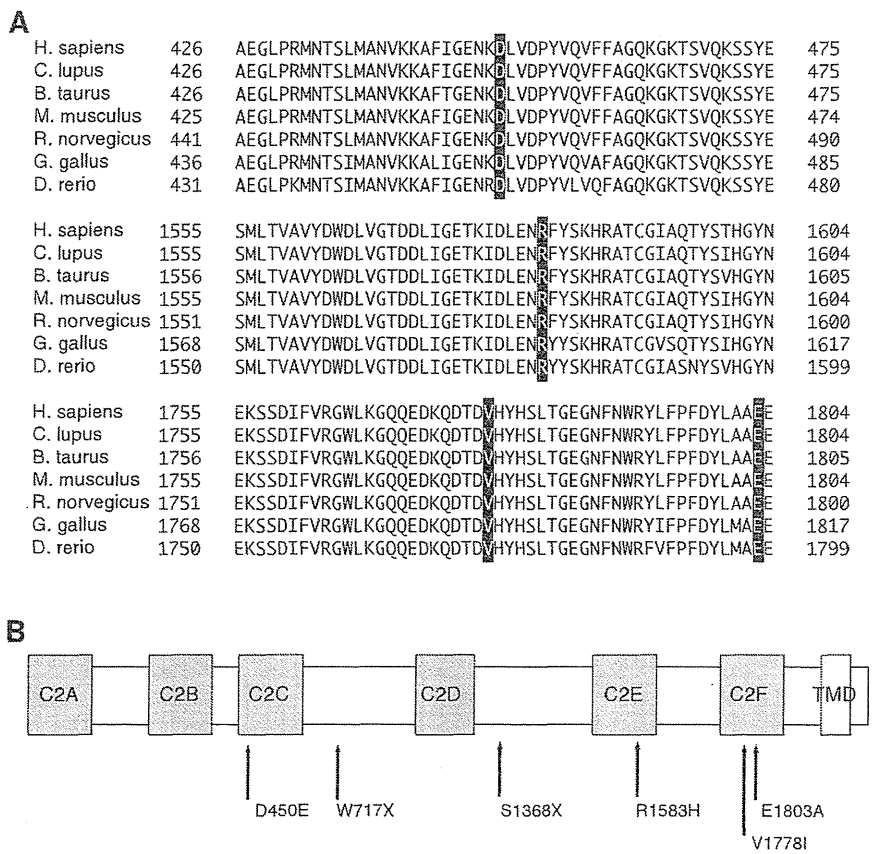

Figure 1 The location of mutations in otoferlin protein and the evolutionary conservation of the amino acids. (A) Evolutionary conservation. The locations of mutations are boxed. **(B)** Novel pathogenic *OTOF* mutations found in this work and relation to the functional domains of otoferlin. C2A-F: C2 domains. TMD: transmembrane domain.

As with other genes, the spectrum of *OTOF* mutations found in the Japanese population was quite different from those reported in Caucasians [13,26-28].

With regard to recurrent mutations, p.Q829X especially has a high frequency in Spanish people, being present in about 3% of all cases of recessive prelingual deafness [24]. C.2905-2923delinsCTCCGAGCGGCA is also common in Argentineans [12] and p.E1700Q is reported to be frequent in Taiwanese [29]. p.R1939Q, previously identified in the United States [22] and most recently reported as a frequent mutation in Japanese [13], was also frequently found in this study. Among 160 patients, 8 (5.0%) had this mutation, confirming it is indeed a recurrent mutation in Japanese.

Those recurrent mutations have been proved to be due to founder effects [13,24,29].

Out of 16 patients with *OTOF* mutations, 7 showed ANSD phenotype, confirming that *OTOF* mutations are major causes of ANSD. In this study, 9 were heterozygous without second mutation. A hallmark of recessive mutations is the detection of two mutations in the paternal and maternal alleles and the parents having normal hearing.

Table 2 Non-pathogenic variants of OTOF identified in this study

Exon	DNA level	Protein level	Occurrence in this work (chromosome)	Control (chromosome)	References
Exon 3	c.145C>T	p.R49W	5/320	10/238	[13]
Exon 3	c.157G>A	p.A53T	2/320	3/238	[23,24]
Exon 3	c.158C>T	p.A53V	42/320	110/238	[23]
Exon 4	c.244C>T	p.R82C	14/320	27/376	[23]
Exon 21	c.2452C>T	p.R818W	1/320	3/356	[12]
Exon 40	c.5026C>T	p.R1676C	1/320	3/356	[21]