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).

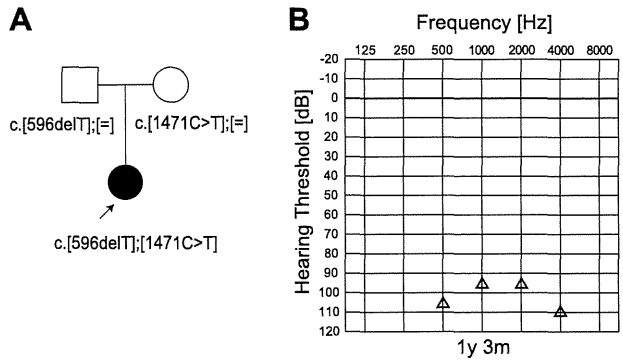

Figure 3. The CI patient with *TECTA* mutations. A: The patient has compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 9 m).
doi:10.1371/journal.pone.0075793.g003

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

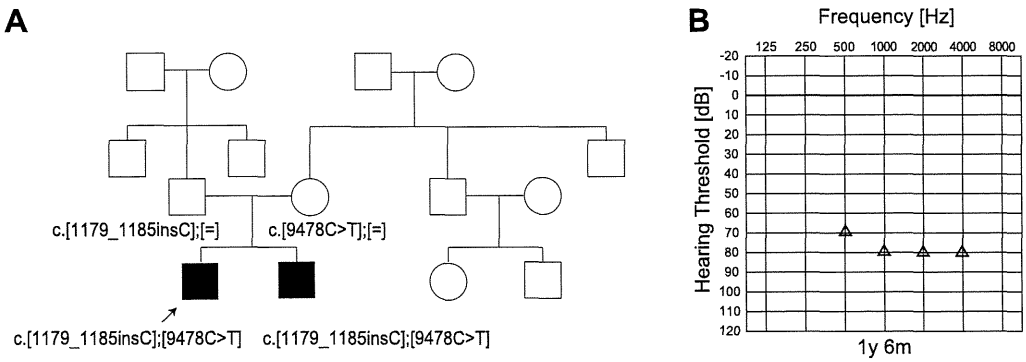

Figure 2. The CI patient with *MYO15A* mutations. A: The patient has compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 6 m).
doi:10.1371/journal.pone.0075793.g002

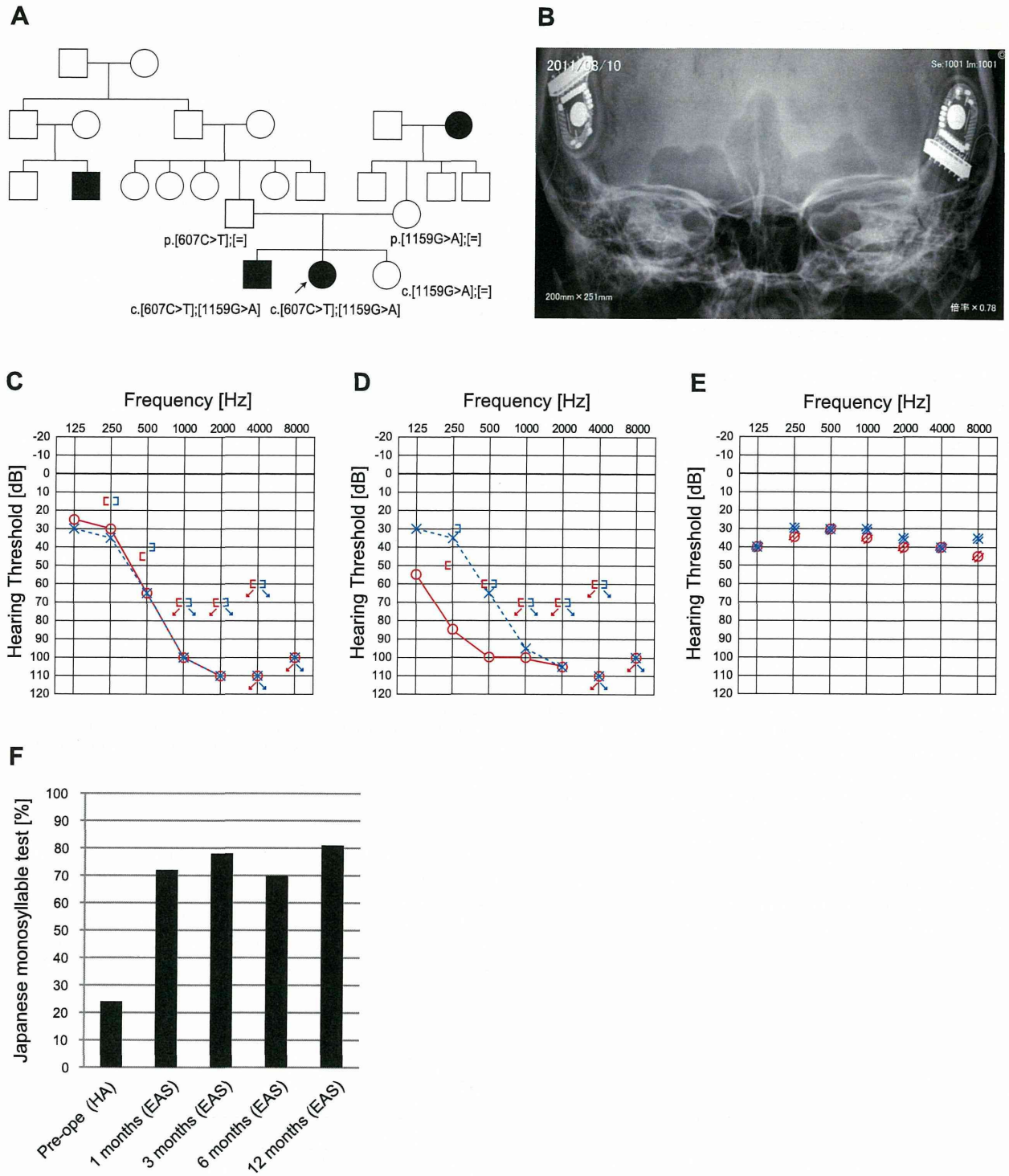

Figure 4. The EAS patient with *TMPRSS3* mutations. A: The patient has compound heterozygous *TMPRSS3* mutations, c.[607C>T];[1159G>A], and the parents were found to be carriers for these mutations. The patient's brother also has the same mutations. B: X-ray imaging after bilateral EAS. C: Pre-operative 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).

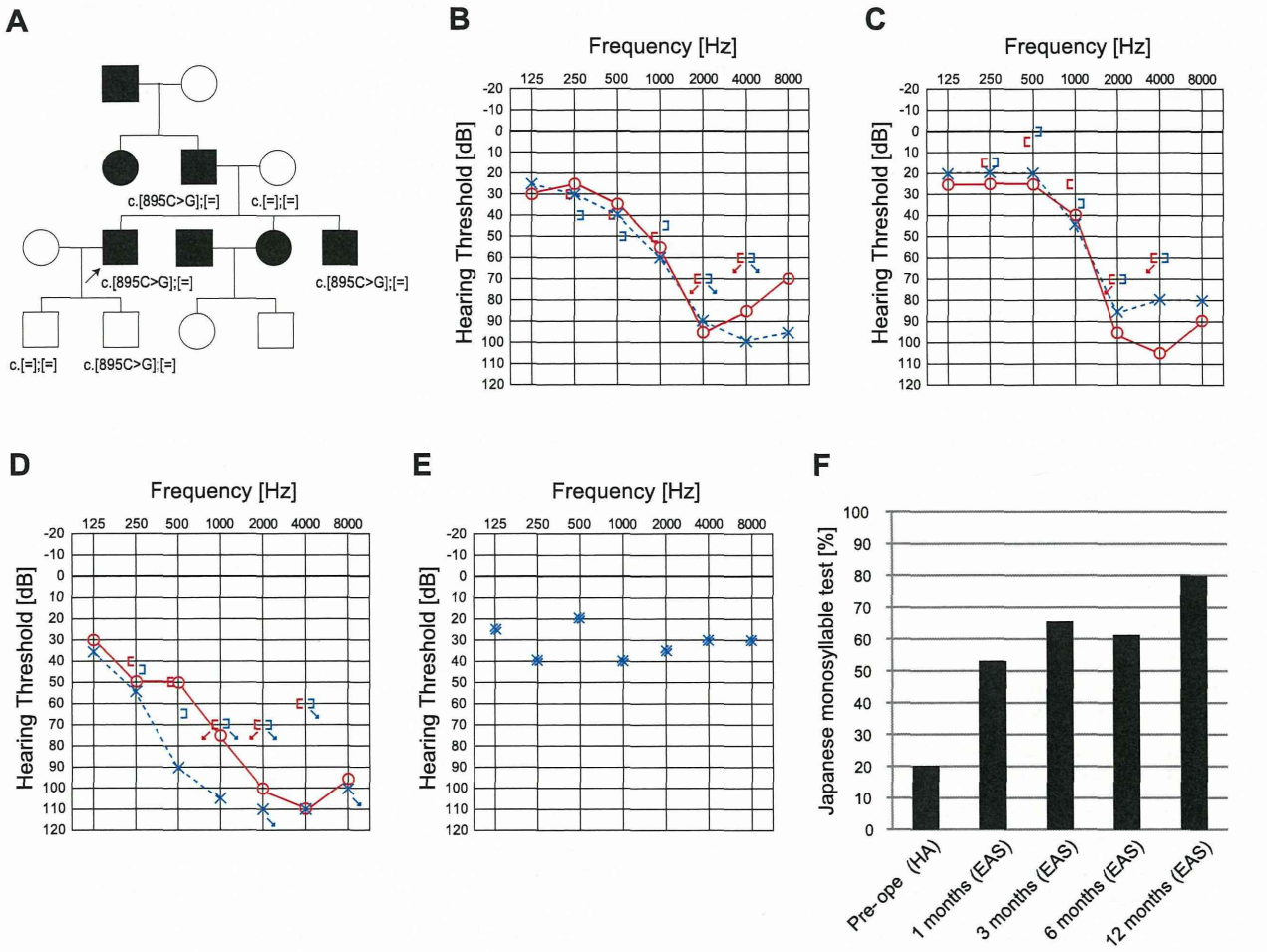

Figure 5. The EAS patient with *ACTG1* mutation. A: The patient has heterozygous *ACTG1* mutation, c.895C>G. Pedigree is compatible with autosomal dominant hearing loss. His father and brother carried the same mutation. B: Pre-operative audiogram. C: Audiogram of brother. D: Post-operative audiogram (6 months after EAS). E: Hearing threshold with EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with EAS.
doi:10.1371/journal.pone.0075793.g005

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 58-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.

References

1. Wilson BS, Dorman MF (2008) Cochlear implants: a remarkable past and a brilliant future. Hear Res. 242: 3–21.
2. Eppsteiner RW, Shearer AE, Hildebrand MS, Deluca AP, Ji H, et al. (2012) Prediction of cochlear implant performance by genetic mutation: The spiral ganglion hypothesis. Hear Res. 292: 51–58.
3. Usami S, Nishio SY, Nagano M, Abe S, Yamaguchi T (2012) Deafness Gene Study Consortium: Simultaneous screening of multiple mutations by invader assay improves molecular diagnosis of hereditary hearing loss: a multicenter study. PLoS One. 7: e31276.
4. Miyagawa M, Naito T, Nishio SY, Kamatani N, Usami SI (2013) Targeted exon sequencing successfully discovers rare causative genes and clarifies the molecular epidemiology of Japanese deafness patients. PLoS ONE. *in press*.
5. Zimmerman-Phillips S, Osberger MJ, Robbins AM (1997) Infant-Toddler: Meaningful Auditory Integration Scale (IT-MAIS) Sylmar, CA: Advanced Bionics Corporation.
6. Coninx F, Weichbold V, Tsiakpini L (2003) LittlEARS Auditory Questionnaire. Innsbruck, Austria: MED-EL.
7. Chang X, Wang K (2012) wANNOVAR: annotating genetic variants for personal genomes via the web. J Med Genet. 49: 433–436.
8. Wang K, Li M, Hakonarson H (2010) ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Research 38: e164.
9. Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, et al. (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol. 30: 434–439.
10. Liburd N, Ghosh M, Riazuddin S, Naz S, Khan S, et al. (2001) Novel mutations of *MYO15A* associated with profound deafness in consanguineous families and moderately severe hearing loss in a patient with Smith-Magenis syndrome. Hum Genet. 109: 535–541.
11. Wang A, Liang Y, Fridell RA, Probst FJ, Wilcox ER, et al. (1998) Association of unconventional myosin *MYO15* mutations with human nonsyndromic deafness DFNB3. Science 280: 1447–1451.
12. Belyantseva IA, Boger ET, Friedman TB (2003) Myosin XVa localizes to the tips of inner ear sensory cell stereocilia and is essential for staircase formation of the hair bundle. Proc Natl Acad Sci U S A. 100: 13958–13963.
13. Hildebrand MS, Morín M, Meyer NC, Mayo F, Modamio-Hoybjor S, et al. (2011) DFNA8/12 caused by *TECTA* mutations is the most identified subtype of nonsyndromic autosomal dominant hearing loss. Hum Mutat. 32: 825–834.
14. Mustapha M, Weil D, Chardenoux S, Elias S, El-Zir E, et al. (1999) An alpha-tectorin gene defect causes a newly identified autosomal recessive form of

sensorineural pre-lingual non-syndromic deafness, DFNB21. Hum Mol Genet. 8: 409–412.
15. Guipponi M, Vuagniaux G, Wattenhofer M, Shibuya K, Vazquez M, et al (2002) The transmembrane serine protease (*TMPRSS3*) mutated in deafness DFNB8/10 activates the epithelial sodium channel (ENaC) in vitro. Hum Mol Genet. 11: 2829–2836.
16. Weegerink NJ, Schraders M, Oostrik J, Huygen PL, Strom TM, et al. (2011) Genotype-phenotype correlation in DFNB8/10 families with TMPRSS3 mutations. J Assoc Res Otolaryngol. 12: 753–766.
17. Elbracht M, Senderek J, Eggermann T, Thürmer C, Park J, et al. (2007) Autosomal recessive post-lingual hearing loss (DFNB8): compound heterozygosity for two novel *TMPRSS3* mutations in German siblings. J Med Genet. 44: e81.
18. Wattenhofer M, Sahin-Calapoglu N, Andreasen D, Kalay E, Caylan R, et al. (2005) A novel TMPRSS3 missense mutation in a DFNB8/10 family prevents proteolytic activation of the protein. Hum Genet. 117: 528–535.
19. Morín M, Bryan KE, Mayo-Merino F, Goodyear R, Mencía A, et al. (2009) In vivo and in vitro effects of two novel gamma-actin (ACTG1) mutations that cause DFNA20/26 hearing impairment. Hum Mol Genet. 18: 3075–3089.
20. von Ilberg CA, Baumann U, Kiefer J, Tillein J, Adunka OF. (2011) Electric-acoustic stimulation of the auditory system: a review of the first decade. Audiol Neurootol. Suppl. 2: 1–30.
21. Usami S, Miyagawa M, Suzuki N, Moteki H, Nishio S, et al. (2010) Genetic background of candidates for EAS (Electric-Acoustic Stimulation). Audiol Med. 8: 28–32.
22. Usami S, Miyagawa M, Nishio SY, Moteki H, Takumi Y, et al. (2012) Patients with *CDH23* mutations and the 1555A>G mitochondrial mutation are good candidates for electric acoustic stimulation (EAS). Acta Otolaryngol. 132: 377–384.

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Targeted Exon Sequencing Successfully Discovers Rare Causative Genes and Clarifies the Molecular Epidemiology of Japanese Deafness Patients

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Abstract

Target exon resequencing using Massively Parallel DNA Sequencing (MPS) is a new powerful strategy to discover causative genes in rare Mendelian disorders such as deafness. We attempted to identify genomic variations responsible for deafness by massive sequencing of the exons of 112 target candidate genes. By the analysis of 216randomly selected Japanese deafness patients (120 early-onset and 96 late-detected), who had already been evaluated for common genes/mutations by Invader assay and of which 48 had already been diagnosed, we efficiently identified causative mutations and/or mutation candidates in 57 genes. Approximately 86.6% (187/216) of the patients had at least one mutation. Of the 187 patients, in 69 the etiology of the hearing loss was completely explained. To determine which genes have the greatest impact on deafness etiology, the number of mutations was counted, showing that those in *GJB2* were exceptionally higher, followed by mutations in *SLC26A4, USH2A, GPR98, MYO15A, COL4A5* and *CDH23*. The present data suggested that targeted exon sequencing of selected genes using the MPS technology followed by the appropriate filtering algorithm will be able to identify rare responsible genes including new candidate genes for individual patients with deafness, and improve molecular diagnosis. In addition, using a large number of patients, the present study clarified the molecular epidemiology of deafness in Japanese. *GJB2* is the most prevalent causative gene, and the major (commonly found) gene mutations cause 30–40% of deafness while the remainder of hearing loss is the result of various rare genes/mutations that have been difficult to diagnose by the conventional one-by-one approach. In conclusion, target exon resequencing using MPS technology is a suitable method to discover common and rare causative genes for a highly heterogeneous monogenic disease like hearing loss.

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☯ These authors contributed equally to this work.

Introduction

Etiological studies have shown that approximately two-thirds of congenital/early-onset sensorineural hearing loss in developed countries is estimated to be due to genetic causes [1]. Deafness is an extremely heterogenous disorder and the involvement of nearly 60 distinct nonsyndromic deafness genes sometimes makes the precise diagnosis difficult. To clarify individual etiology in such heterogenous diseases, one-by-one gene screening based on conventional PCR-based direct sequencing of candidate genes has been developed, and currently *GJB2* has become the first to be screened, followed by several commonly encountered genes. As more comprehensive screening methods, micorarray-based screening [2,3] and Invader assay-based screening [4,5] have also been developed. Recent advances in exome sequencing using Massively Parallel DNA Sequencing (MPS) have revolutionized the elucidation of genetic defects causing monogenic disorders [6–8]. A number of papers regarding gene discovery and successful clinical application for identification of responsible genes for deafness using MPS have recently been published [9–17]. In this study, we have chosen 112 genes (including 54 known deafness causing genes, 22 known syndromic hearing loss causing genes and 36 possible candidate genes which expressed highly in the inner ear) and conducted genetic analysis to 1) confirm the potentiality of MPS -based genetic screening strategies for such a genetically heterogenous disease, and 2) clarify molecular epidemiology by identifying responsible/candidate genes in a large number of patients using MPS technology.

Materials and Methods

Subjects

Two hundred sixteen Japanese patients with bilateral sensorineural hearing loss from 33 ENT departments nationwide participated in the present study. With regard to onset age (the age of awareness), 120 patients had early-onset deafness (below 6 y.o.), and 96 had late-detected deafness. Thirty subjects were from autosomal dominant or mitochondrial inherited families (two or

more generations affected); 98 subjects were from autosomal recessive families (parents with normal hearing and two or more affected siblings) or had sporadic deafness (also compatible with recessive inheritance or non-genetic hearing loss). Hearing loss was evaluated using pure-tone audiometry (PTA) classified by a pure-tone average over 500, 1000, 2000 and 4000 Hz in the better hearing ears. For children who could not undergo PTA, we used an average over 500, 1000, 2000 Hz in either auditory steady-stem response (ASSR) or conditioned oriented reflex audiometry (COR), or the response threshold (dB) from auditory brainstem response (ABR). Computed tomography (CT) scans were performed to check for congenital inner ear anomalies.

The patients had already been evaluated by conventional PCR-based one-by-one gene screening and Invader-based multi-gene screening [5], and 61 out of the 216 (45/120 prelingual, 16/96 postlingual) patients were already found to have *GJB2* (n = 38), *SLC26A4* (n = 15), or mitochondrial 1555 (n = 3) and 3243 (n = 5) mutations. We chose these patients because 1) they were "randomly" selected, and 2) they had already been screened by Invader assay and further fully sequenced by Sanger sequencing for the previously found common and frequent deafness causing genes i.e., *GJB2, SLC26A4, KCNQ4,* and *CDH23*. Therefore, we could simultaneously use these 216 samples for both diagnostic purposes and for verification. As a control for pathogeneity of each genomic variation, 72 Japanese samples were used in this study, because they were 1) ethnically similar, 2) had normal hearing evaluated by pure-tone audiometry, and 3) were collected from throughout the nation, and were able to undergo identical procedures. 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 Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions of the Deafness Gene Study Consortium (Hokkaido University, Hirosaki University, Iwate Medical University, Tohoku University, Yamagata University, Fukushima Medical University, Jichi Medical University, Gunma University, Nihon University, Nippon Medical School, Nippon Medical School Tama Nagayama Hospital, Jikei University, Toranomon Hospital, Kitasato University, Hamamatsu Medical University, Mie University, Shiga Medical Center for Children, Osaka Medical College, Hyogo College of Medicine, Kobe City Medical Center General Hospital, Wakayama Medical University, Okayama University, Yamaguchi University, Ehime University, Kyushu University, Fukuoka University, Nagasaki University, Kanda ENT Clinic, Miyazaki Medical College, Kagoshima University, Ryukyus University) approved the study.

Targeted Enrichment and DNA Sequencing

One hundred twelve genes listed in **Table S1**, including 54 genes reported to be causative of non-syndromic hearing loss (Hereditary Hearing loss Homepage; http://hereditaryhearingloss.org/) and 22 reported to cause syndromic hearing loss were selected for sequencing. In hopes of finding novel causative genes, we added 36 genes that are highly expressed in the adult human inner ear by microarray analysis [18]. DNA from 12 patients was pooled and 3 μg of each pooled DNA was used as an input material for SureSelect target DNA enrichment (Agilent Technologies, Santa Clara, CA) and Illumina GAIIx sequencing (Illumina, San Diego, CA) according to the manufacturers' procedures. Each genomic DNA pool was fragmented using the Covaris™ S2 System (Covaris, Woburn, MA) to about 200 bp fragment length. After fragmentation, DNA fragments were blunt-ended and phosphorylated at the 5′ end using a Paired End Genomic DNA Sample Prep Kit (Illumina) and successively, adeninylated at the 3′ end and ligated to pre-capture adaptor olligonucleotides containing SureSelect target DNA enrichment kit. After adaptor oligonucleotide ligation, pre-capture amplification was performed with Heraculase II Fusion DNA polymerase (Agilent Technologies). Between each step of sample preparation, DNA pools were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The Capture library was designed with Agilent's eArray homepage (http://earray.vhem.agilent.com/earray/). The bait cRNA library contained all exons of 112 genes. Exons of selected genes of all variants were selected from RefSeq and Ensembl databases using the University of California Santa Cruz table browser (http://genome.ucsc.edu/). Adaptor ligated and pre-amplicated samples were hybridized to the Capture cRNA library at 65°C for 24 hours with SureSelect Hybridization buffer and successively captured with Dynabeads MyOne Streptavidin T1 beads (Invitrogen) and washed with SureSelect Wash buffer. After target capture, selected product from pooled DNA was post-amplified with Heraculase II Fusion DNA polymerase and Illumina Multiplexing Sample Preparation Oligonucleotide Kit and then submitted to the massive parallel sequencing in a lane on a Illumina GAIIx genome platform (Illumina).

Mapping and Filtering

The sequence data were processed with standard Illumina base calling procedure and successively mapped to human genome sequence (build hg 36) with the Bowtie program and BWA program [19,20]. The two programs were used consecutively, because the Bowtie program cannot detect insertion/deletion efficiently. A total of 55.4 and 8.5 Gb sequences with about 9,000,000 and 1,400,000 reads were obtained by the pair-end method for the patients and the controls, respectively. After alignment, the filtering algorithm shown in **Fig. 1** was applied to collect the responsible genes/mutations. First, because of usage of pooled DNA samples, potential single nucleotide variants (SNVs) were filtered by the frequency of variant reads at each position. For the number of variants in each position, we assumed a binomial distribution with the probability parameter of 1/24, and the size parameter of the number of coverage. The largest integer number that is not larger than the value giving the cumulative distribution function of 0.025 of the binomial distribution was used as the threshold value, and the position was selected when the number of the reads of the variant were not lower than the threshold value indicated in formula (1).

$$P(j \geq k) = 1 - \sum_{i=0}^{k-1} {}_nC_i p^i (1-p)^i \qquad (1)$$

In the formula, n denotes total depth (wild type+mutation allele) of each mapped position, j denotes the observed number of mutational alleles at each mapped position, and p denotes the relative frequency of the mutation allele in the pool. In this study DNA of 12 patients was pooled, and the minimal positive value of the relative frequency of the mutational allele in each pooled DNA sample should be 1/24. Therefore, we employed $p = 1/24$. To reduce false negative cases, we used $P = 95\%$ and after the calculation of this formula, k value indicated the number of minimal mutation allele copies that was used as the threshold for each mapped position. We fixed $p = 1/24$ and $P = 95\%$, and then, k value was dependent only on the total depth n.

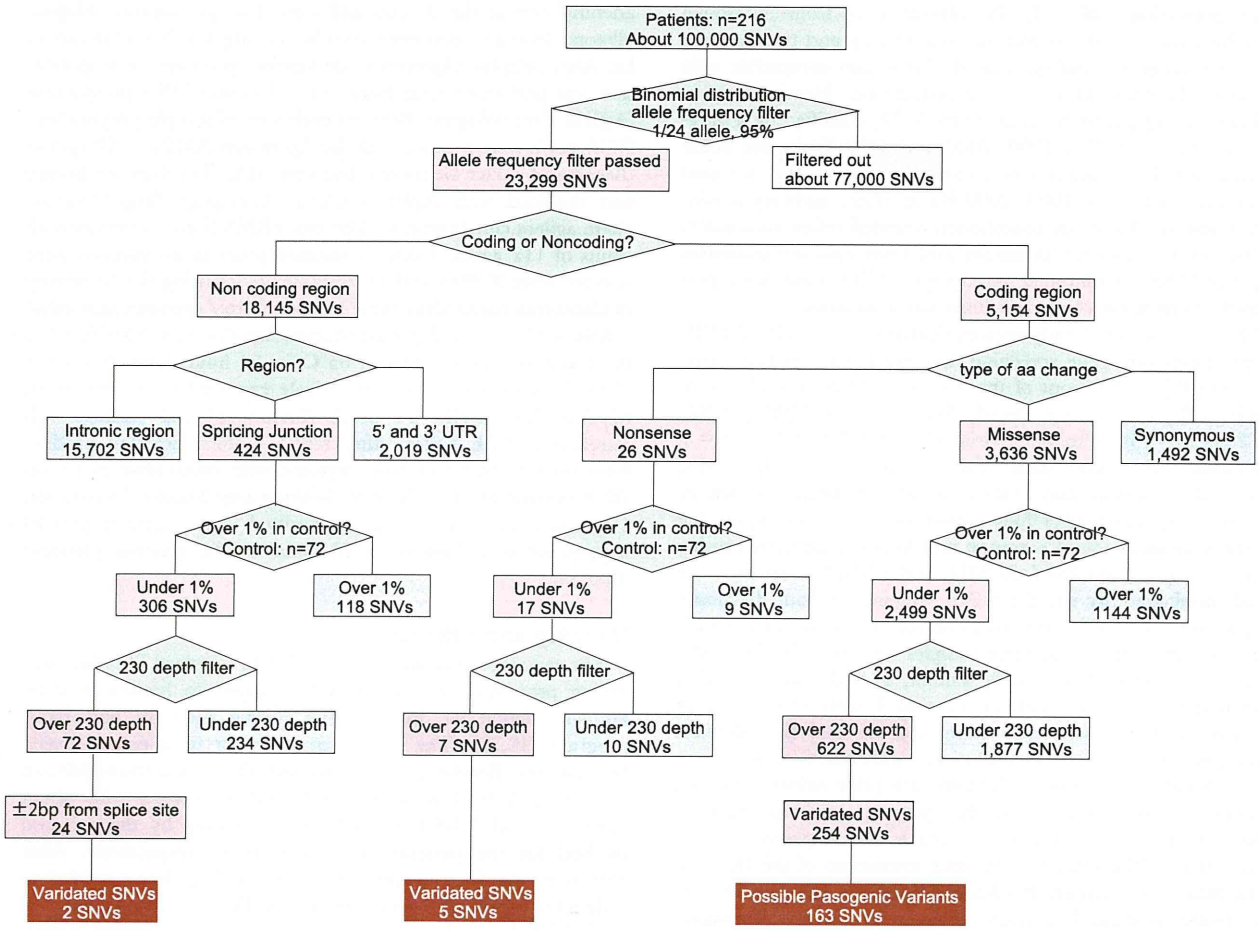


Figure 1. Algorithm applied in this study. Nonsense mutations, splice-site mutations, and missense mutations were chosen according to this algorithm.
doi:10.1371/journal.pone.0071381.g001

When compared with the mutations already identified by Sanger sequencing, this first filtering was effective to detect those mutations (**Fig. S1**).

After the first filtering, the selected SNVs were then classified into the targeted regions (coding region, non-coding region, splicing junction) and types of changes (nonsense mutation, missense mutation, insertion or deletion) (**Fig. 1**). SNVs were then filtered against the sequences observed at over 1% in control subjects because most common *GJB2* deafness causing mutations so far found in Japanese had shown <1% allele frequencies in the control population (**Fig. S2**). Then, the minimum cut off value for the depth was decided to be 230 for each 12-patient pool, based on the data obtained for all exons of the *GJB2, CDH23,* and *KCNQ4* genes by Sanger sequencing and parallel sequencing (**Fig. S2**). For splice-site mutations, 24 possible candidates for causative mutations were selected because SNVs within +/−2base from the exon-intron junction site were considered to be important for splicing [21,22]. After the application of all these filters, the candidate deafness causing mutations were selected, and verified by the subsequent Sanger sequencing. For missense mutations, the Polyphen2 [23] software program was applied to predict the influence on the protein structure by amino acid substitution. Family member genotypes were also used to validate the co-segregations of the deafness trait and the candidate mutations in individual families.

Comparison with Another Algorithm for Pooled DNA Samples

We also analyzed all the data with VIPR, a program established and validated for use with pooled samples [24].

Results

Of 7 selected nonsense mutations, after Sanger sequencing, 2 were not confirmed but 5 actual nonsense mutations in 12 families were identified in *GJB2, EYA1, MIA, TMPRSS3,* and *MYO6* (**Table S2, Fig. 2**).

Of 24 selected splice-site mutations, after Sanger sequencing, 22 were not identified but 2 actual splice-site mutations in 3 families were successfully identified in *KCNQ1* and *SLC26A4* (**Table S2, Fig. 3**). The pathogenic nature was confirmed by 1) segregation within the family and 2) phenotypic configuration (long-QT for *KCNQ1* and enlarged vestibular aqueduct for *SLC26A4*).

Of 27 selected insertion-deletion mutations, after Sanger sequencing, 6 actual mutations in 48 families were successfully identified in *GJB2, MYO15A* and *MYH9* (**Table S2, Fig. 4**).

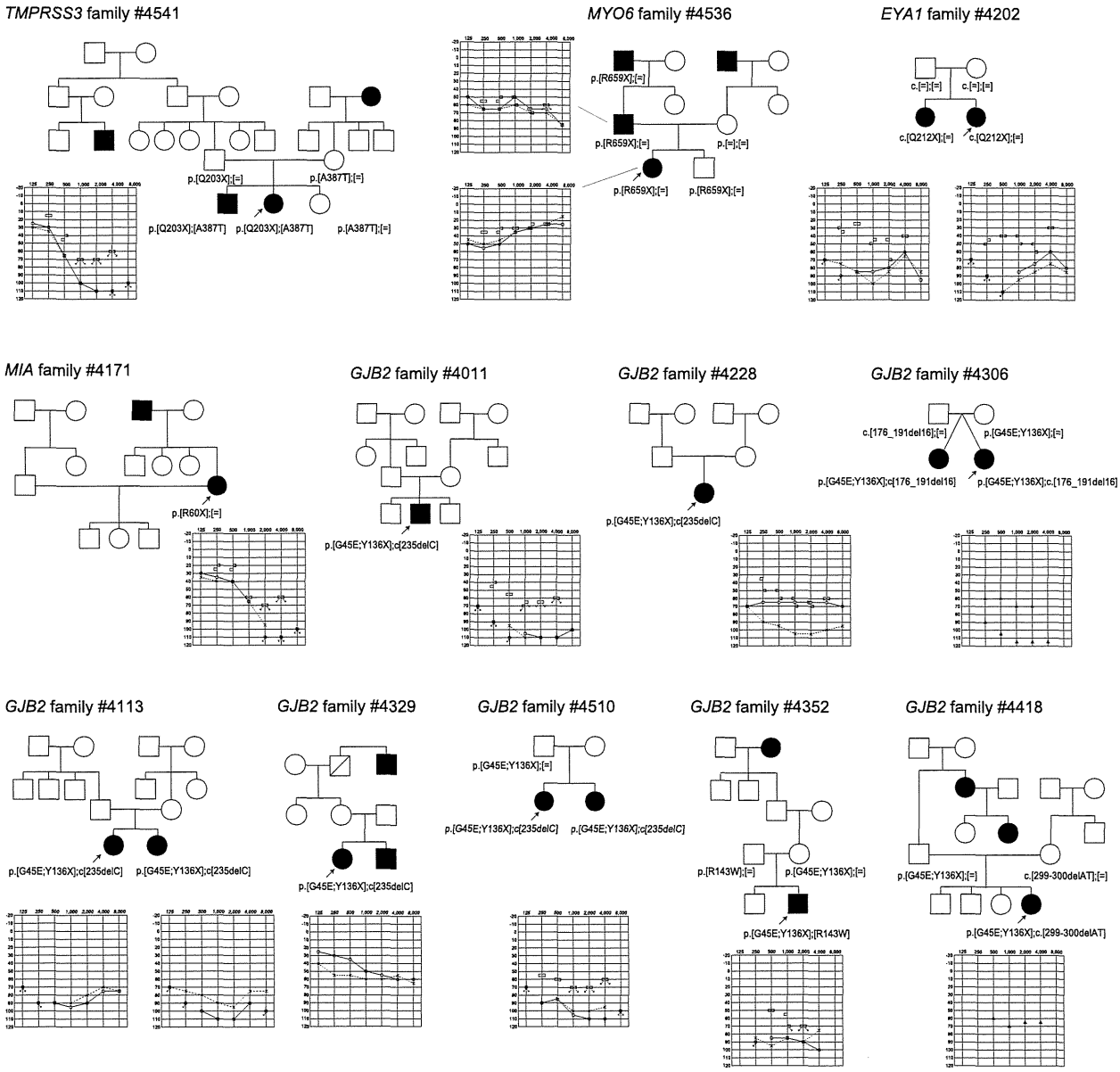


Figure 2. The pedigrees and audiograms of the patients with nonsense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g002

Of 622 missense mutations, 254 mutations were confirmed by Sanger sequencing. By using PolyPhen2 software, 167 were classified as "damaging" or "probably damaging" or "possibly damaging" and 87 were categorized as "benign" (**Table S2**). Of 167 selected missense mutations 163 were <1% allele frequencies in both the 1000 genome project (http://www.1000genomes.org/node/home) and the NHLB grand opportunity exome sequencing project: 6500 exomes (http://esp.gs.washington.edu/drupal/). *TMPRSS3, MYO15A, GJB2, SLC26A4* were found to be responsible for deafness in autosomal recessive or sporadic families. Examples of the families are shown in **Fig. 5, 6**. *TECTA, WFS-1, MYH9, EYA1, COL4A5, COL11A1* were identified as the responsible genes for deafness in autosomal dominant families (**Fig. 5, 6**).

As in **Table S2**, a total of 57 responsible genes were found, and the number of mutations/mutation candidates is shown in **Fig. 1**. *GJB2* was exceptionally higher, followed by *SLC26A4, USH2A, GPR98, MYO15A, COL4A5,* and *CDH23*. In the early-onset group, *GJB2, SLC26A4, GPR98, MYO15A, USH2A, CDH23, and TECTA* were frequently found, in contrast to the late-detected group, where *GJB2, COL4A5, USH2A, MYO15A, CDH23, GPR98, EYA1, and TMPRSS3* were frequently found (**Fig. 7**). The number of possible mutations in the early-onset group vs. late-detected group was 54:22 for *GJB2*, 7:1 for *PCDH15*, 8:3 for *SLC26A4*, 18:2 for *TECTA*, and 3:5 for *TMPRSS3*.

Comparison data between the current algorithm and VIPR, which is widely used for pooled sample analysis due to its higher specificity in mutation detection compared to other programs for pooled samples, is shown in **Table S3.** VIPR is unable to detect

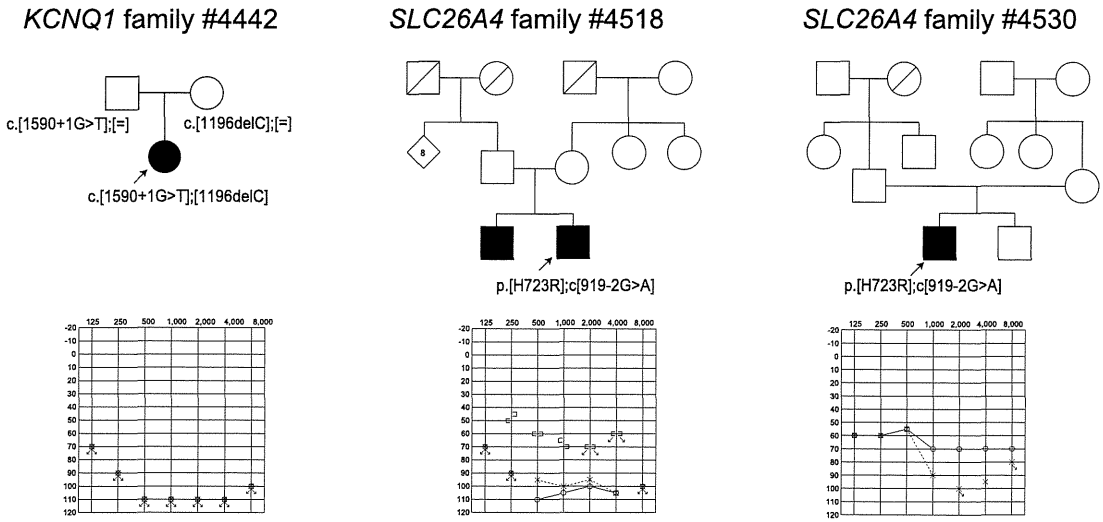


Figure 3. The pedigrees and audiograms of the patients with splice-site mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g003

deletion/insertion mutations, as well as some missense mutations. 93.5% (87/93) and 84.1% (37/44) of the mutations were detected in the *GJB2* and *SLC26A4* genes that had already been fully sequenced by Sanger sequencing, respectively.

Discussion

With regard to sensitivity and specificity, we placed priority on sensitivity because one of the main purposes was clarifying genetic epidemiology. In addition, we used pooled DNA samples because a large number of sample is needed for genetic epidemiology. With the cut off value setting in this study, we could obtain high sensitivity (93.5% sensitivity on the basis of *GJB2, SLC26A4*)(**Fig. S1**). We also analyzed all the data with VIPR, a program established and validated for use with pooled samples [24]. However, sensitivity (84.1%) was not as satisfactory as the current algorithm (**Table S3**). Also, because VIPR is unable to detect deletion/insertion mutations, we used our own algorithm in this study.

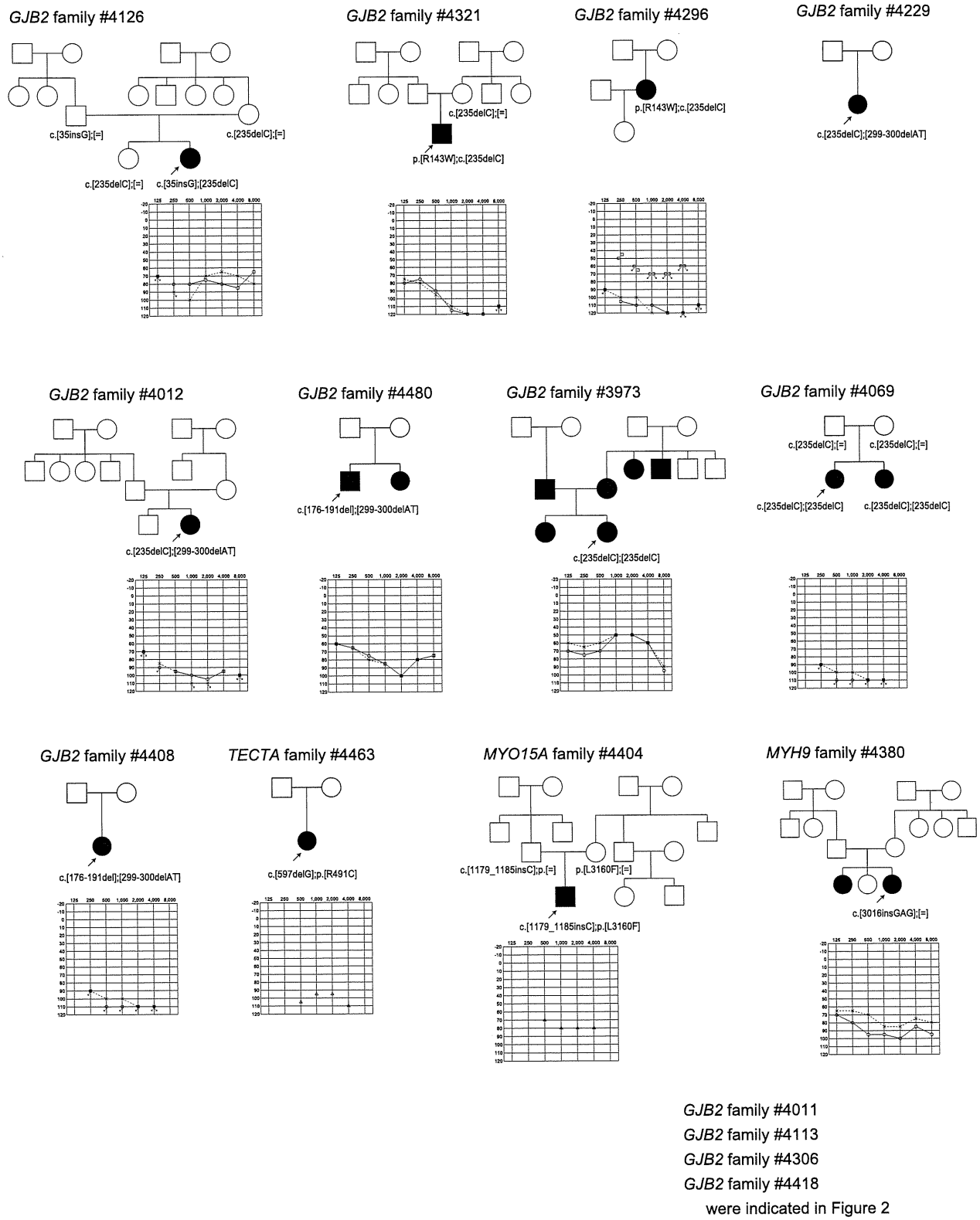
On the other hand, it is also true that one problem of the present algorithm is low specificity (high false positive rates: 16% for nonsense, 90% for splice-site, 75% for insertion-deletion mutations and 67% for missense mutations) necessitating time-consuming direct sequencing confirmation afterwards and making it unsuitable for diagnostic purposes. The low specificity was improved by using a more stringent cut off line in the minimum depth of coverage as well as a more stringent p-value in the binomial distribution filtering process. But for diagnosis, more sophisticated methods and algorithms with higher specificity such as bar-code procedures are available for genetic testing for individual patients.

With regard to five nonsense mutations in 12 families (identified in *GJB2, EYA1, MIA, TMPRSS3, MYO6*), two selected splice-site mutations in three families (identified in *KCNQ1* and *SLC26A4*), and six insertion-deletion mutations (identified in *GJB2, MYO15A* and *MYH9*), segregation analysis confirmed they are plausible disease causing mutations (**Fig. 2–4**). For 163 selected missense mutations identified in 33 out of 54 known non-syndromic genes, it is difficult to reach a final conclusion about whether they are really disease causing mutations or not. Although some of the families were too small for segregation study or we failed to collect enough samples from familial members, most cases are consistent with the assumption that these are pathogenic mutations based on the software programs to predict the influence on the protein structure [20]. Actual causative mutations were successfully identified from the selected recessive as well as dominant families in which all the samples of family members were collected (Examples are shown in **Fig. 5, 6**). *TMPRSS3, MYO15A, GJB2, SLC26A4* were found to be responsible for deafness in autosomal recessive or sporadic families, while *TECTA, WFS1, MYH9, EYA1, COL4A5* and *COL11A1* were identified as the responsible genes for deafness in autosomal dominant families.

One interesting result is that a mutation in a novel putative responsible deafness gene, *MIA*, which is highly expressed in the inner ear, was identified in a dominant family (#4171), in the present study. Although the detailed function in the inner ear is currently unknown, genes that are highly expressed in the inner ear, as revealed by cDNA microarray analysis, may have a crucial functional role there [18].

The other interesting result was the mutations in the genes previously reported to be syndromic genes such as *EYA1*. Although re-contact was not possible in all cases, detailed genotype/phenotype correlation study will be an open question. One family was later found to be associated with ear pits (diagnosed as BOR syndrome) (family #4361 in Fig. 5), but the rest of the contacted families did not have any associated branchial disclosure. Interestingly, all families were associated with inner ear anomaly, and therefore these families have slightly different clinical phenotype from typical BOR syndrome. As in this case, the mutation analysis using MPS will potentially expand the phenotypic variations.

Based on the sensitivity, nonsense mutations, splice-site mutations, insertion-deletion mutations or selected missense mutations were found in 57 out of 112 genes (33/56 non-syndromic genes, 12/22 syndromic genes, and 12/36 genes highly expressed in the cochlea). The mutations previously found in Invader assays or direct sequencing were also confirmed effectively in our MPS algorithm. Of 93 previously found *GJB2* and *SLC26A4* mutations, we confirmed 87 (93.5%) of them (Table S3). Approximately 86.6% (187/216) of the patients had at least one mutation.



GJB2 family #4011
GJB2 family #4113
GJB2 family #4306
GJB2 family #4418
were indicated in Figure 2

Figure 4. The pedigrees and audiograms of the patients with insertion-deletion mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g004

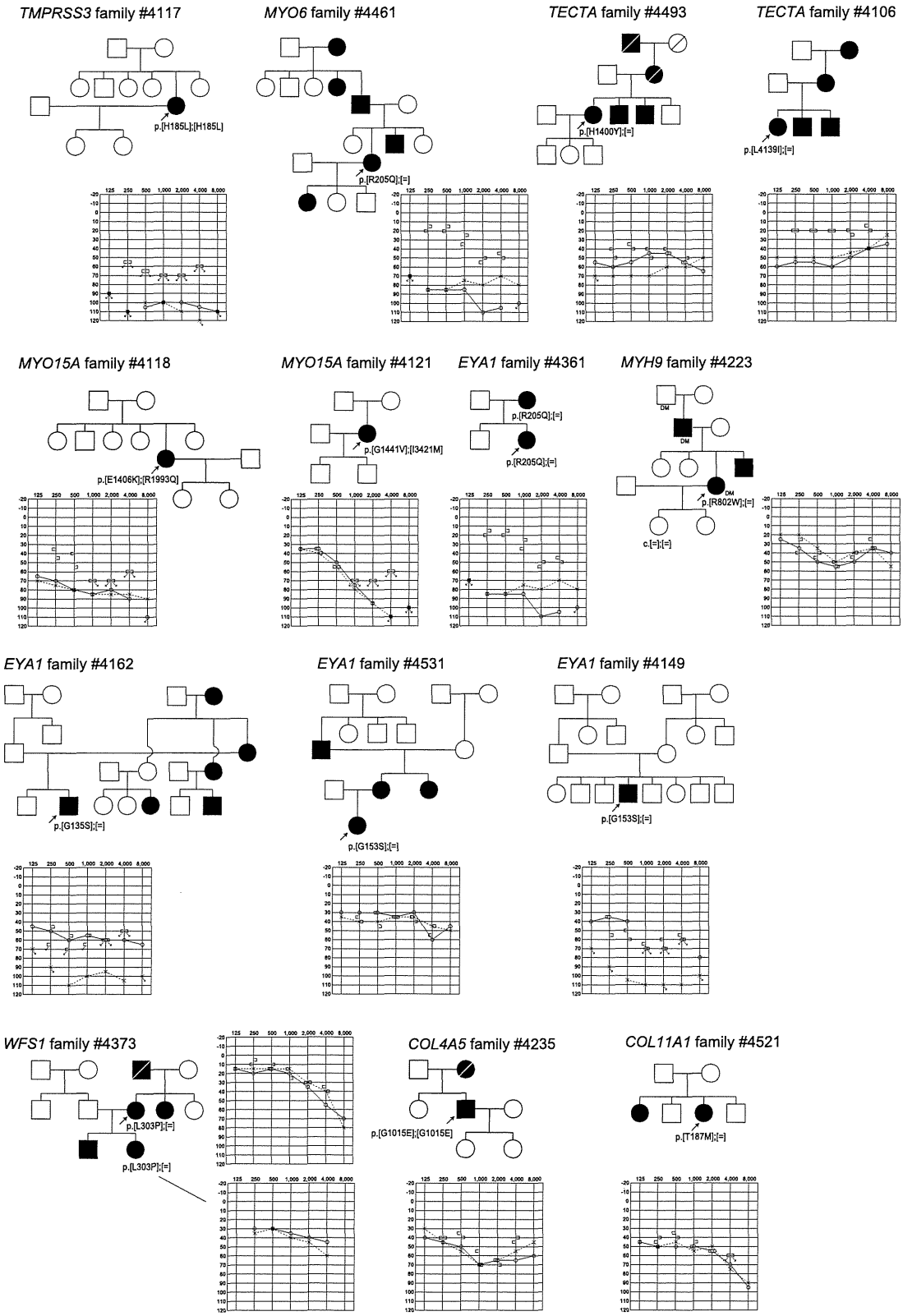


Figure 5. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g005

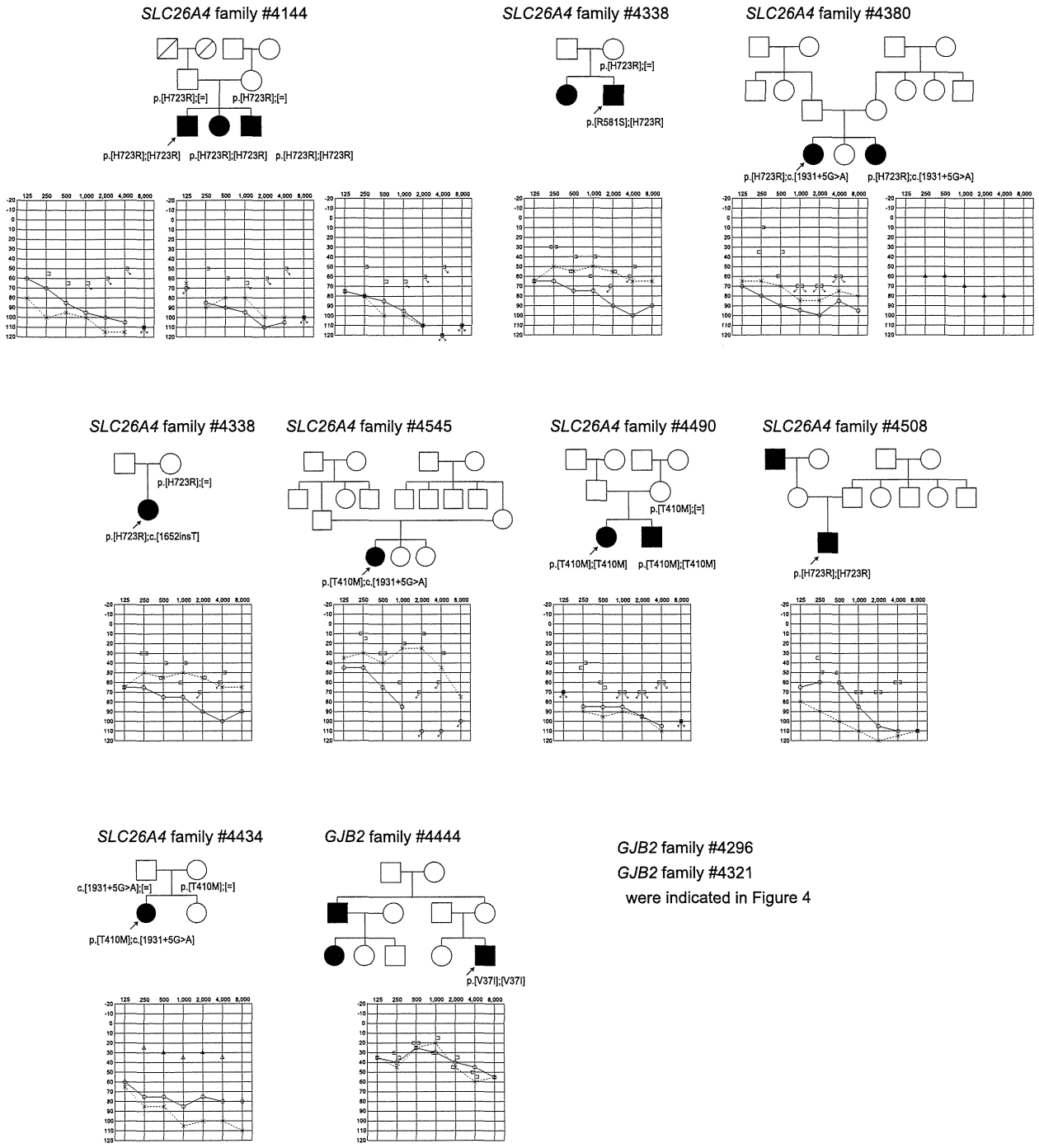
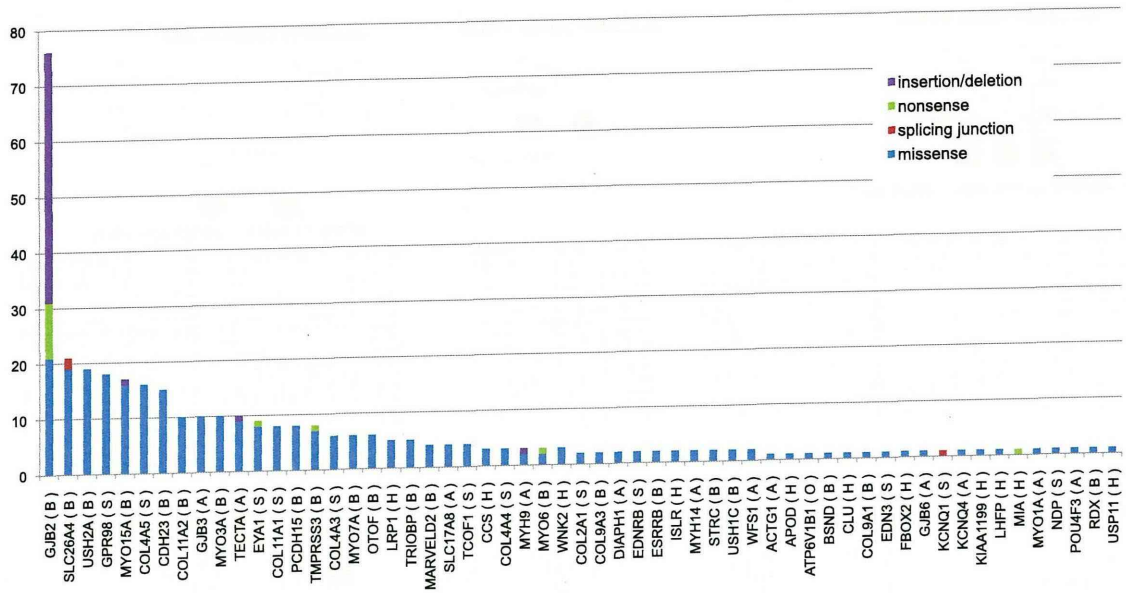
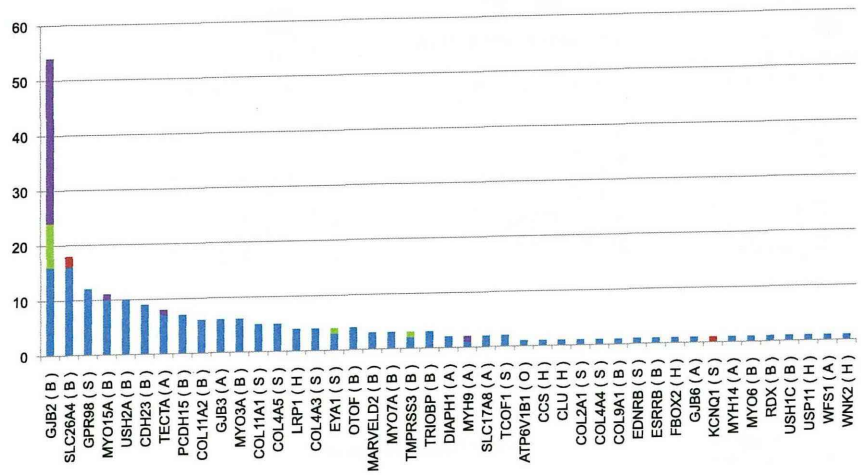


Figure 6. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g006

Of the 187 patients, in 69 the etiology of the hearing loss was completely explained (biallelic probably pathogenic mutations in autosomal recessive or sporadic cases, or one probably pathogenic mutation in autosomal dominant cases), and in 12 was possibly explained (two mutations with one probably pathogenic mutation and an unknown variant in the same gene in autosomal recessive or sporadic cases, or one unknown mutation in autosomal dominant cases).

A noteworthy result obtained in this study was that the data clarified the molecular epidemiology for deafness in our population. For two decades, there have been extensive efforts to identify the etiology of deafness and those studies have determined that genetic causes are commonly involved in congenital/early-onset


80
70
60
50
40
30
20
10
0
insertion/deletion
nonsense
splicing junction
missense
GJB2 (B)
SLC26A4 (B)
USH2A (B)
GPR98 (S)
MYO15A (B)
COL4A5 (S)
CDH23 (B)
COL11A2 (B)
GJB3 (A)
MYO3A (B)
TECTA (A)
EYA1 (S)
COL11A1 (S)
PCDH15 (B)
TMPRSS3 (B)
COL4A3 (S)
MYO7A (B)
OTOF (B)
LRP1 (H)
TRIOBP (B)
MARVELD2 (B)
SLC17A8 (A)
TCOF1 (S)
CCS (H)
COL4A4 (S)
MYH9 (A)
MYO6 (B)
WNK2 (H)
COL2A1 (S)
COL9A3 (B)
DIAPH1 (A)
EDNRB (S)
ESRRB (B)
ISLR (H)
MYH14 (A)
STRC (B)
USH1C (B)
WFS1 (A)
ACTG1 (A)
APOD (H)
ATP6V1B1 (O)
BSND (B)
CLU (H)
COL9A1 (B)
EDN3 (S)
FBOX2 (H)
GJB6 (A)
KCNQ1 (S)
KCNQ4 (A)
KIAA1199 (H)
LHFP (H)
MIA (H)
MYO1A (A)
NDP (S)
POU4F3 (A)
RDX (B)
USP11 (H)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GJB2 (B)
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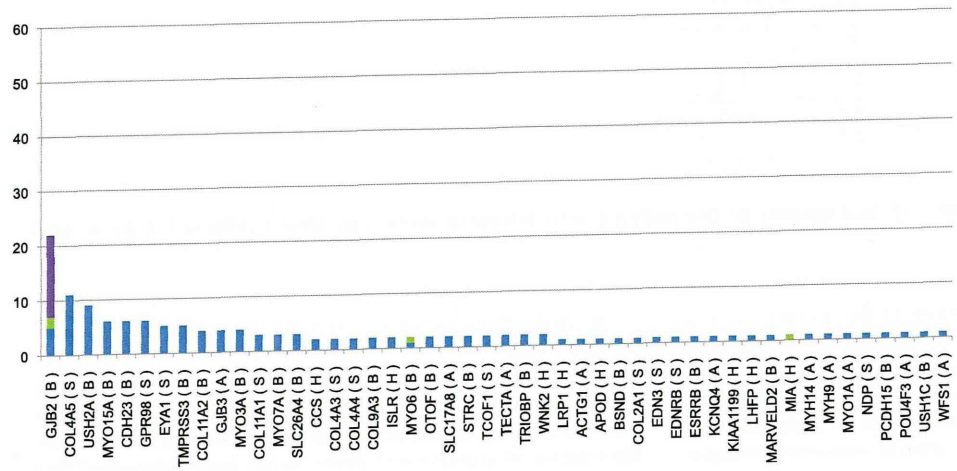
PCDH15 (B)
COL11A2 (B)
GJB3 (A)
MYO3A (B)
COL11A1 (S)
COL4A5 (S)
LRP1 (H)
COL4A3 (S)
EYA1 (S)
OTOF (B)
MARVELD2 (B)
MYO7A (B)
TMPRSS3 (B)
TRIOBP (B)
DIAPH1 (A)
MYH9 (A)
SLC17A8 (A)
TCOF1 (S)
ATP6V1B1 (O)
CCS (H)
CLU (H)
COL2A1 (S)
COL4A4 (S)
COL9A1 (B)
EDNRB (S)
ESRRB (B)
FBOX2 (H)
GJB6 (A)
KCNQ1 (S)
MYH14 (A)
MYO6 (B)
RDX (B)
USH1C (B)
USP11 (H)
WFS1 (A)
WNK2 (H)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GJB2 (B)
COL4A5 (S)
USH2A (B)
MYO15A (B)
CDH23 (B)
GPR98 (S)
EYA1 (S)
TMPRSS3 (B)
COL11A2 (B)
GJB3 (A)
MYO3A (B)
COL11A1 (S)
MYO7A (B)
SLC26A4 (B)
CCS (H)
COL4A3 (S)
COL4A4 (S)
COL9A3 (B)
ISLR (H)
MYO6 (B)
OTOF (B)
SLC17A8 (A)
STRC (B)
TCOF1 (S)
TECTA (A)
TRIOBP (B)
WNK2 (H)
LRP1 (H)
ACTG1 (A)
APOD (H)
BSND (B)
COL2A1 (S)
EDN3 (S)
EDNRB (S)
ESRRB (B)
KCNQ4 (A)
KIAA1199 (H)
LHFP (H)
MARVELD2 (B)
MIA (H)
MYH14 (A)
MYH9 (A)
MYO1A (A)
NDP (S)
PCDH15 (B)
POU4F3 (A)
USH1C (B)
WFS1 (A)

Figure 7. A: The number of mutations/mutation candidates indicating that the majority of the responsible gene mutations are accumulated in particular major causative genes. B: The number of mutations/mutation candidates in the early-onset group. C: The number of mutations/mutation candidates in the late-detected group.
doi:10.1371/journal.pone.0071381.g007

sensorineural hearing loss, but there has been no etiological data on a genetic basis using a large number of patients. It has been reported that more than 100 loci and 46 causative genes are causing deafness [25]. To evaluate which genes have an impact on deafness epidemiology, the number of mutations/mutation candidates was counted. Among the identified mutations, the number of *GJB2* mutations was exceptionally higher at 80 alleles, followed by those in *SLC26A4, USH2A, GPR98, MYO15A, COL4A5,* and *CDH23* (**Fig. 7**). Regarding the number of possible mutations in each gene, *GJB2* (54:22), *PCDH15* (7:1), *SLC26A4* (18:3), *TECTA* (8:2) were frequent in the early-onset group. In contrast, *TMPRSS3* (3:5) was predominantly found in the late-detected (based on the age of awareness) group. Such tendency is in line with reported phenotypes.

Actually, detected mutations were confirmed to be pathogenic in selected families (**Fig. 2–6**). Although *USH2A* and *GPR98* (which underly Usher syndrome type 2) mutations were great in number, this is to be expected based on the extremely large size of the gene.

An important fact is that the samples we used were collected randomly from 33 different hospitals distributed throughout Japan, therefore we believe them to be a representative cohort of Japanese patients and suitable for epidemiological evaluation. We have developed an advanced screening strategy focusing on frequently recurring mutations that are most likely to be encountered in the clinical setting that identifies approximately 40% of deafness patients [5]. This indicates that 30–40% of patients have deafness due to recurrent mutations in particular genes, such as *GJB2* or *SLC26A4.* In fact, 25% (53/216 overall), and 42% (50/120 for early-onset) of the patients were diagnosed by those recurrent mutations. *GJB2* has been known as the most prevalent responsible gene for deafness worldwide and 14–16% (25–26% for congenital cases) of Japanese hearing loss patients have *GJB2* mutations [5,26]. Mutations in *SLC26A4, MYO15A,* and *CDH23* are also reported to be frequent and important causes of deafness [5,25]. The number of mutations of *GJB2* is actually the highest among the genes in the mutation database (**Fig. 7**), supporting the view that the majority of the responsible gene mutations are such commonly found ones with the remainder being various rare genes/mutations. Those 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 to identify such rare genes/mutations.

In conclusion, MPS enabled us to discover rare causative genes for a highly heterogeneous monogenic disease and revealed the genetic epidemiology of deafness. This epidemiologic data will shed light on gene evolution and provide the basis for future genetic screening strategies.

Supporting Information

Figure S1 The validity of the binomial distribution filter used in this study. The horizontal axis indicates depth of coverage of each SNV detected by MPS analysis and the vertical axis indicates calculated allele frequency in each 12-patient pool (calculated by alternative base read number divided by total (alternative+reference) base read number for each SNV). Mutations of the known three genes, *GJB2, KCNQ4,* and *CDH23* either by MPS (circle) or Sanger sequencing (dot). Red: *CDH23,* Blue: *GJB2,* Green: *KCNQ4.* The cut-off line using first filtering algorithm is indicated by a black line. Most of the SNVs detected by Sanger sequencing were distributed above the threshold indicating that mutations selected are effectively identified. *GJB2* (Blue) had a deeper depth which means MPS data is more reliable whereas *KCNQ4* (Green) had shallow depth, which is less reliable. Actually Sanger sequencing (dot) showed reasonable data.
(PDF)

Figure S2 A: The ROC curve for the optimal cut-off value of the allele frequency at each nucleotide position using the data obtained for all exons of the *GJB2, CDH23,* and *KCNQ4* genes by Sanger sequencing. **B:** The ROC curve for the optimal cut-off value of the depth at each nucleotide position using the data obtained for all exons of the *GJB2, CDH23,* and *KCNQ4* genes by Sanger sequencing.
(PDF)

Table S1 One hundred twelve potentially deafness-causative genes, including 54 reported causative non-syndromic hearing loss genes, 22 reported causative syndromic hearing loss genes, and 36 genes that are highly expressed in the inner ear.
(PDF)

Table S2 Mutations/mutation candidates confirmed by Sanger sequencing. Nonsense mutations, splice-site mutations, or missense mutations were found in 57 out of 112 genes.
(PDF)

Table S3 Comparison of data between the current algorithm and VIPR. 93.5% (87/93) and 84.1% (37/44) of the mutations was detected in *GJB2* and *SLC26A4* genes already fully sequenced by Sanger sequencing, respectively.
(PDF)

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: MM TN SN. Analyzed the data: MM TN SN. Wrote the paper: SU. Jointly supervised research: NK.

References

1. Morton CC, Nance WE (2006) Newborn hearing screening: a silent revolution. N Engl J Med 354: 2151–2164.
2. Kothiyal P, Cox S, Ebert J, Husami A, Kenna MA, et al. (2010) High-throughput detection of mutations responsible for childhood hearing loss using resequencing microarrays. BMC Biotechnol. 10: 10.
3. Rodriguez-Paris J, Pique L, Colen T, Roberson J, Gardner P, et al. (2010) Genotyping with a 198 mutation arrayed primer extension array for hereditary hearing loss: assessment of its diagnostic value for medical practice. PLoS One. 5: e11804.
4. Abe S, Yamaguchi T, Usami S (2007) Application of deafness diagnostic screening panel based on deafness mutation/gene database using invader assay. Genet Test. 11: 333–340.
5. Usami S, Nishio SY, Nagano M, Abe S, Yamaguchi T, et al. (2012) Simultaneous screening of multiple mutations by invader assay improves molecular diagnosis of hereditary hearing loss: a multicenter study. PLoS One. 7: e31276.
6. Robinson PN, Krawitz P, Mundlos S (2011) Strategies for exome and genome sequence data analysis in disease-gene discovery projects. Clin Genet 80: 127–132.
7. Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, et al. (2011) Exome sequencing as a tool for Mendelian disease gene discovery. Nat Rev Genet 12: 745–755.
8. Majewski J, Schwartzentruber J, Lalonde E, Montpetit A, Jabado N (2011) What can exome sequencing do for you? J Med Genet 48: 580–589.
9. Walsh T, Shahin H, Elkan-Miller T, Lee MK, Thornton AM, et al. (2010) Whole exome sequencing and homozygosity mapping identify mutation in the cell polarity protein *GPSM2* as the cause of nonsyndromic hearing loss DFNB82. Am J Hum Genet. 87: 90–94.
10. Rehman AU, Morell RJ, Belyantseva IA, Khan SY, Boger ET, et al. (2010) Targeted capture and next-generation sequencing identifies *C9orf75*, encoding taperin, as the mutated gene in nonsyndromic deafness DFNB79. Am J Hum Genet. 86: 378–388.
11. Shearer AE, DeLuca AP, Hildebrand MS, Taylor KR, Gurrola J 2nd, et al. (2010) Comprehensive genetic testing for hereditary hearing loss using massively parallel sequencing. Proc Natl Acad Sci USA 107: 21104–21109.
12. Brownstein Z, Friedman LM, Shahin H, Oron-Karni V, Kol N, et al. (2011) Targeted genomic capture and massively parallel sequencing to identify genes for hereditary hearing loss in middle eastern families. Genome Biol 12: R89. doi:10.1186/gb-2011-12-9-r89.
13. Schraders M, Haas SA, Weegerink NJ, Oostrik J, Hu H, et al. (2011) Next-generation sequencing identifies mutations of *SMPX*, which encodes the small muscle protein, X-linked, as a cause of progressive hearing impairment. Am J Hum Genet. 88: 628–634.
14. Bonnet C, Grati M, Marlin S, Levilliers J, Hardelin JP, et al. (2011) Complete exon sequencing of all known Usher syndrome genes greatly improves molecular diagnosis. Orphanet J Rare Dis. 6: 21.
15. De Keulenaer S, Hellemans J, Lefever S, Renard JP, De Schrijver J, et al. (2012) Molecular diagnostics for congenital hearing loss including 15 deafness genes using a next generation sequencing platform. BMC Med Genomics. 5: 17.
16. Tang W, Qian D, Ahmad S, Mattox D, Todd NW, et al. (2012) A low-cost exon capture method suitable for large-scale screening of genetic deafness by the massively-parallel sequencing approach. Genet Test Mol Biomarkers. 16: 536–542.
17. Lin X, Tang W, Ahmad S, Lu J, Colby CC, et al. (2012) Applications of targeted gene capture and next-generation sequencing technologies in studies of human deafness and other genetic disabilities. Hear Res. 288: 67–76.
18. Abe S, Katagiri T, Saito-Hisaminato A, Usami S, Inoue Y, et al. (2003) Identification of *CRYM* as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissues. Am J Hum Genet 72: 73–82.
19. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25. doi:10.1186/gb-2009-10-3-r25.
20. Li H, Durbin R (2009) Fast and accurate short-read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754–1760.
21. Reed R (2000) Mechanisms of fidelity in pre-mrna splicing. Curr Opin Cell Biol, 12: 340–345.
22. Krainer AR, Sachidanandam R (2006) Comprehensive splice-site analysis using comparative genomics. Nucleic Acids Res 34: 3955–3967.
23. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, et al. (2010) A method and server for predicting damaging missense mutations. Nat Methods. 7: 248–249.
24. Altmann A, Weber P, Quast C, Rex-Haffner M, Binder EB, et al. (2011) vipR: variant identification in pooled DNA using R. Bioinformatics 27: i77–i84.
25. Hilgert N, Smith RJ, Van Camp G (2009) Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics? Mutat Res 681: 189–196.
26. Tsukada K, Nishio S, Usami S, Deafness Gene Study Consortium (2010) A large cohort study of *GJB2* mutations in Japanese hearing loss patients. Clin Genet 78: 464–470.

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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.

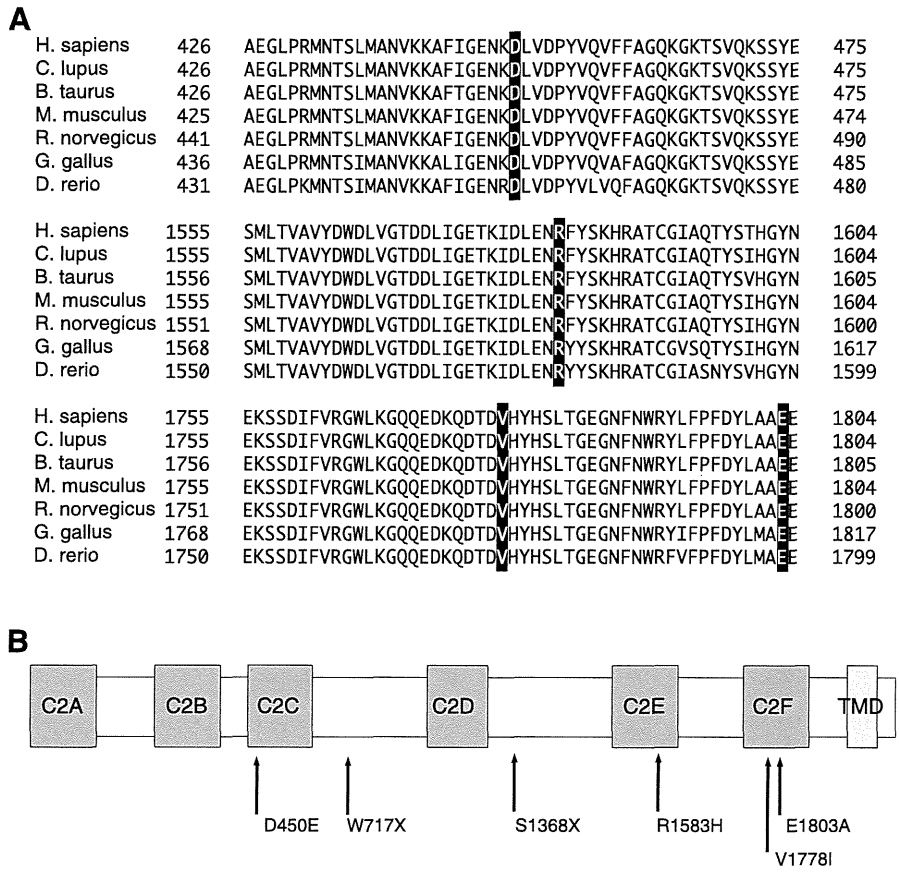

Figure 1 The location of mutations in otoferlin protein and the evolutionary conservation of the amino acids. (A) Evolutionary conservation. The locations of mutations are boxed. **(B)** Novel pathogenic *OTOF* mutations found in this work and relation to the functional domains of otoferlin. C2A-F: C2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