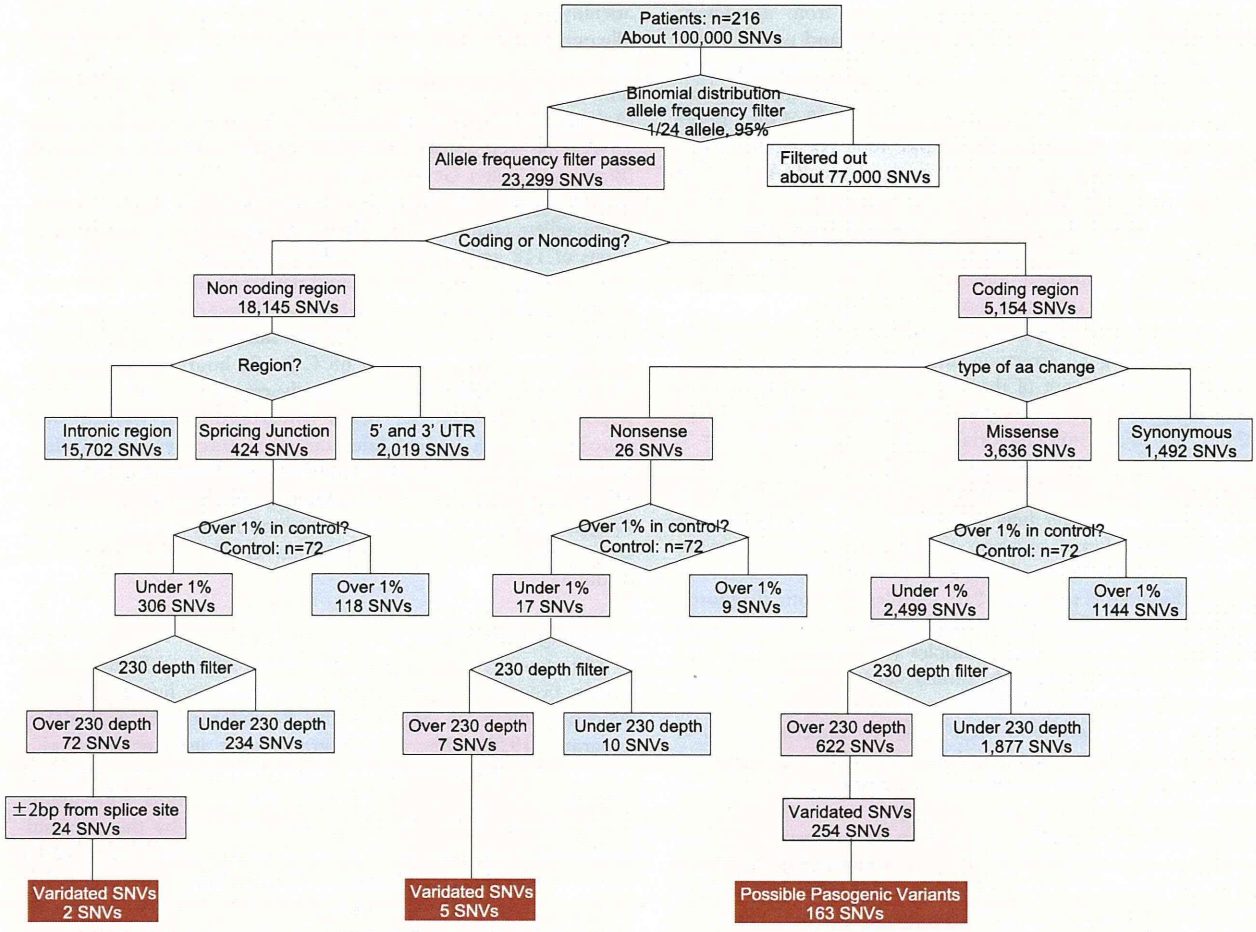


Figure 1. Algorithm applied in this study. Nonsense mutations, splice-site mutations, and missense mutations were chosen according to this algorithm.
doi:10.1371/journal.pone.0071381.g001

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**).

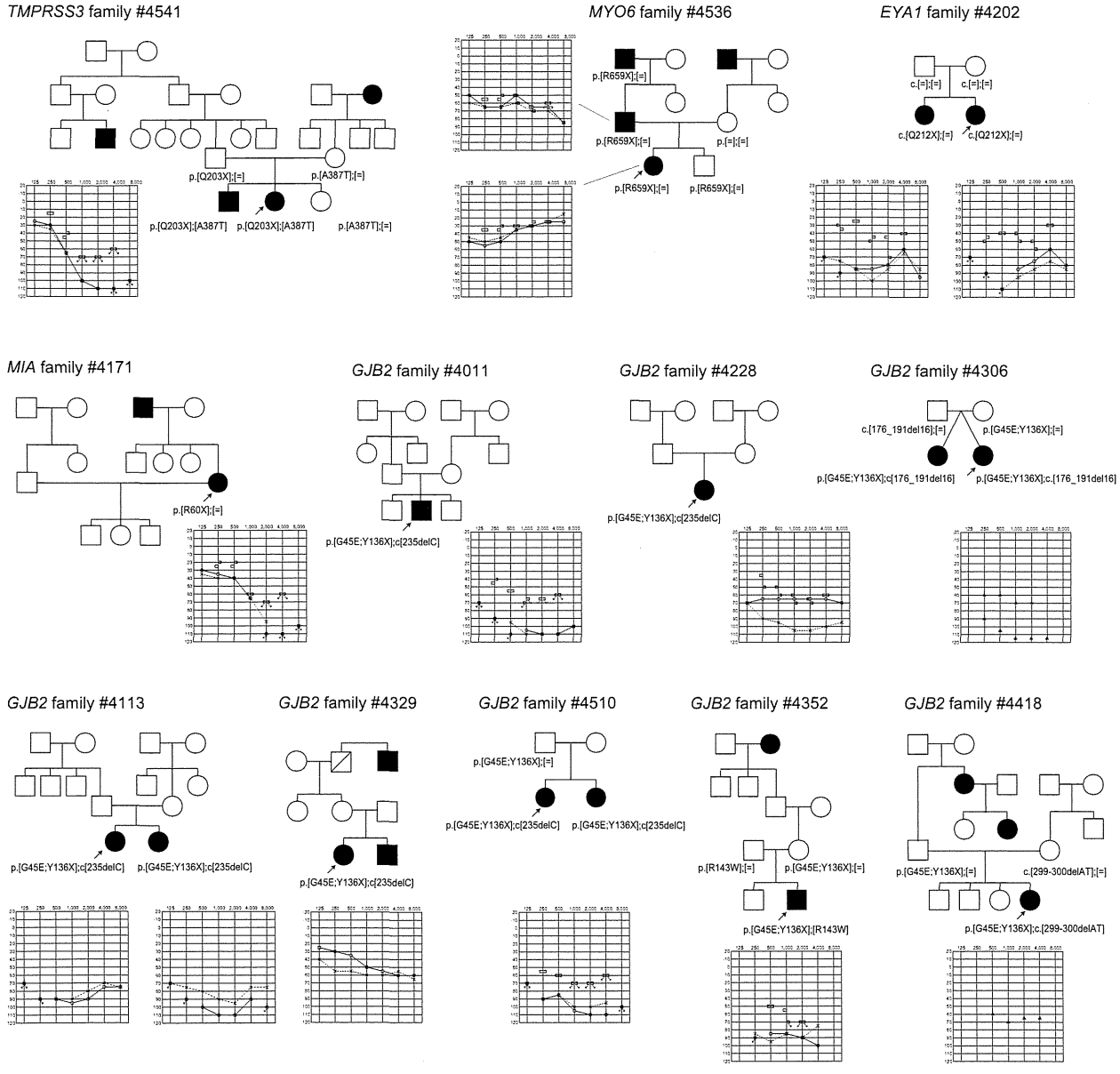


Figure 2. The pedigrees and audiograms of the patients with nonsense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g002

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

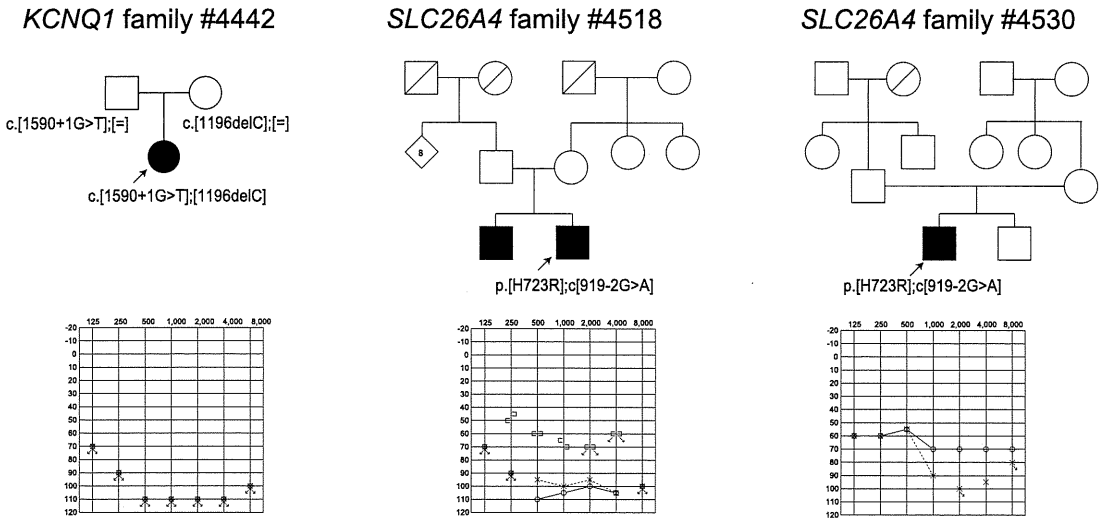


Figure 3. The pedigrees and audiograms of the patients with splice-site mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g003

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.

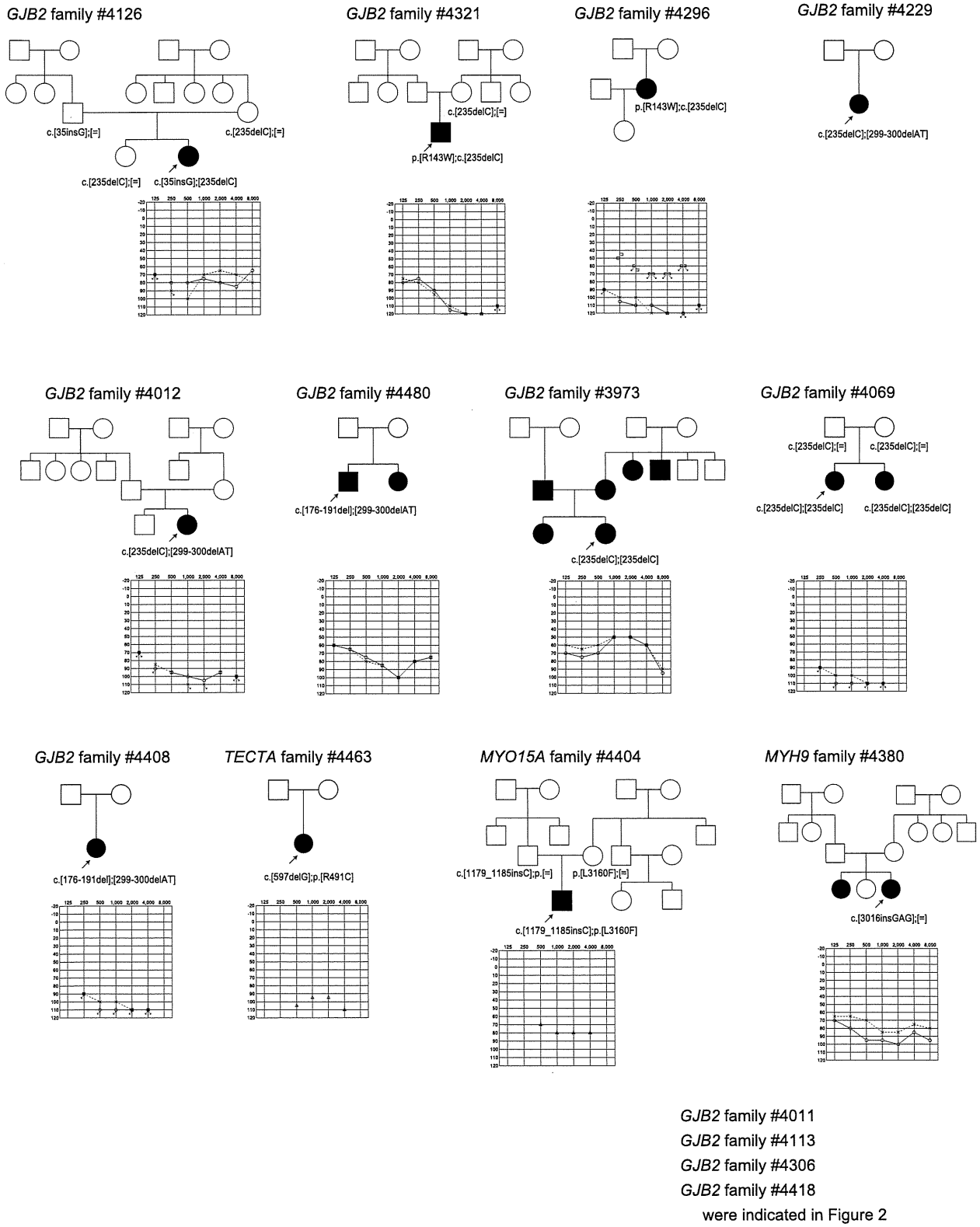
On the other hand, it is also true that one problem of the present algorithm is low specificity (high false positive rates: 16% for nonsense, 90% for splice-site, 75% for insertion-deletion mutations and 67% for missense mutations) necessitating time-consuming direct sequencing confirmation afterwards 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

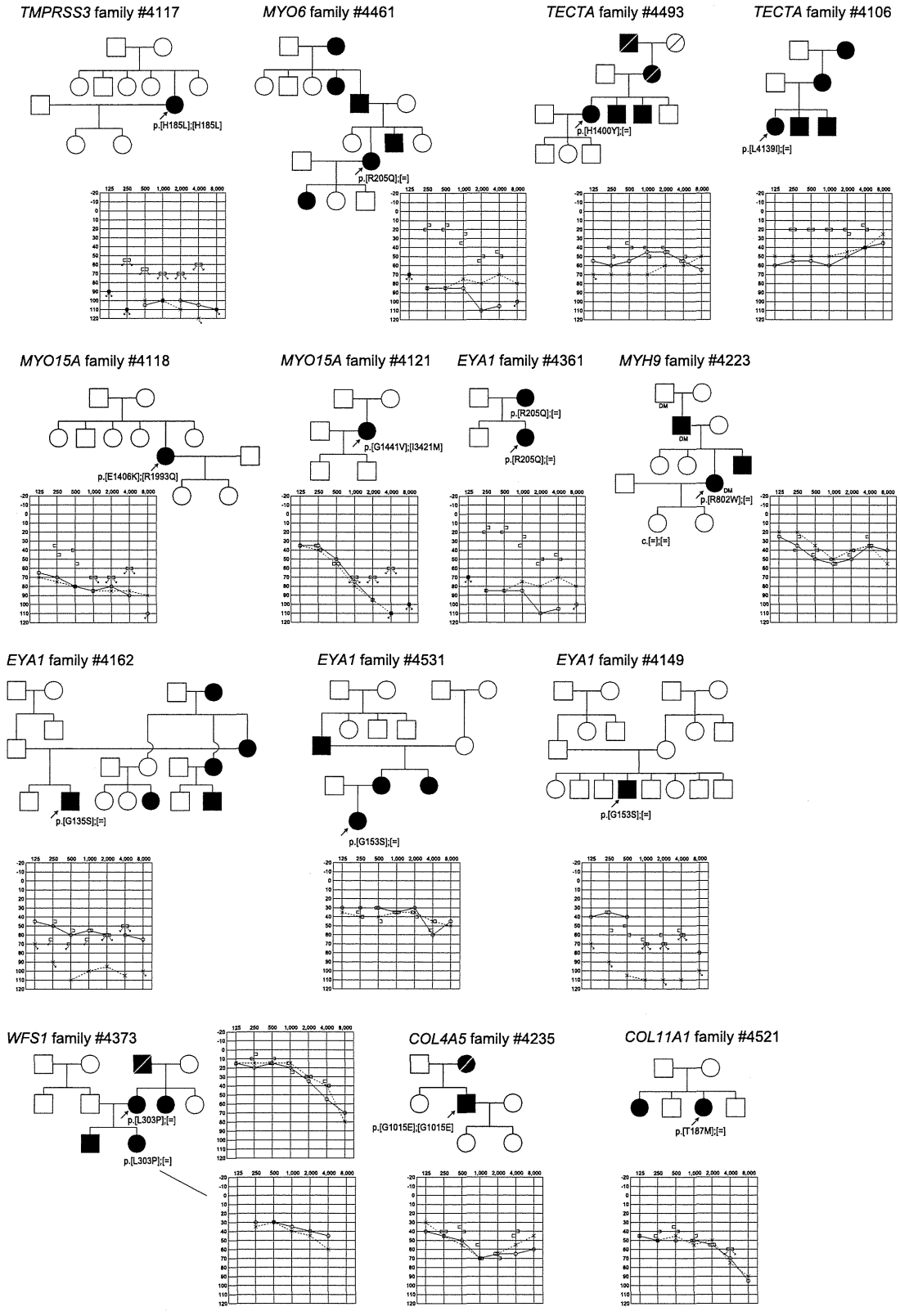


Figure 5. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g005

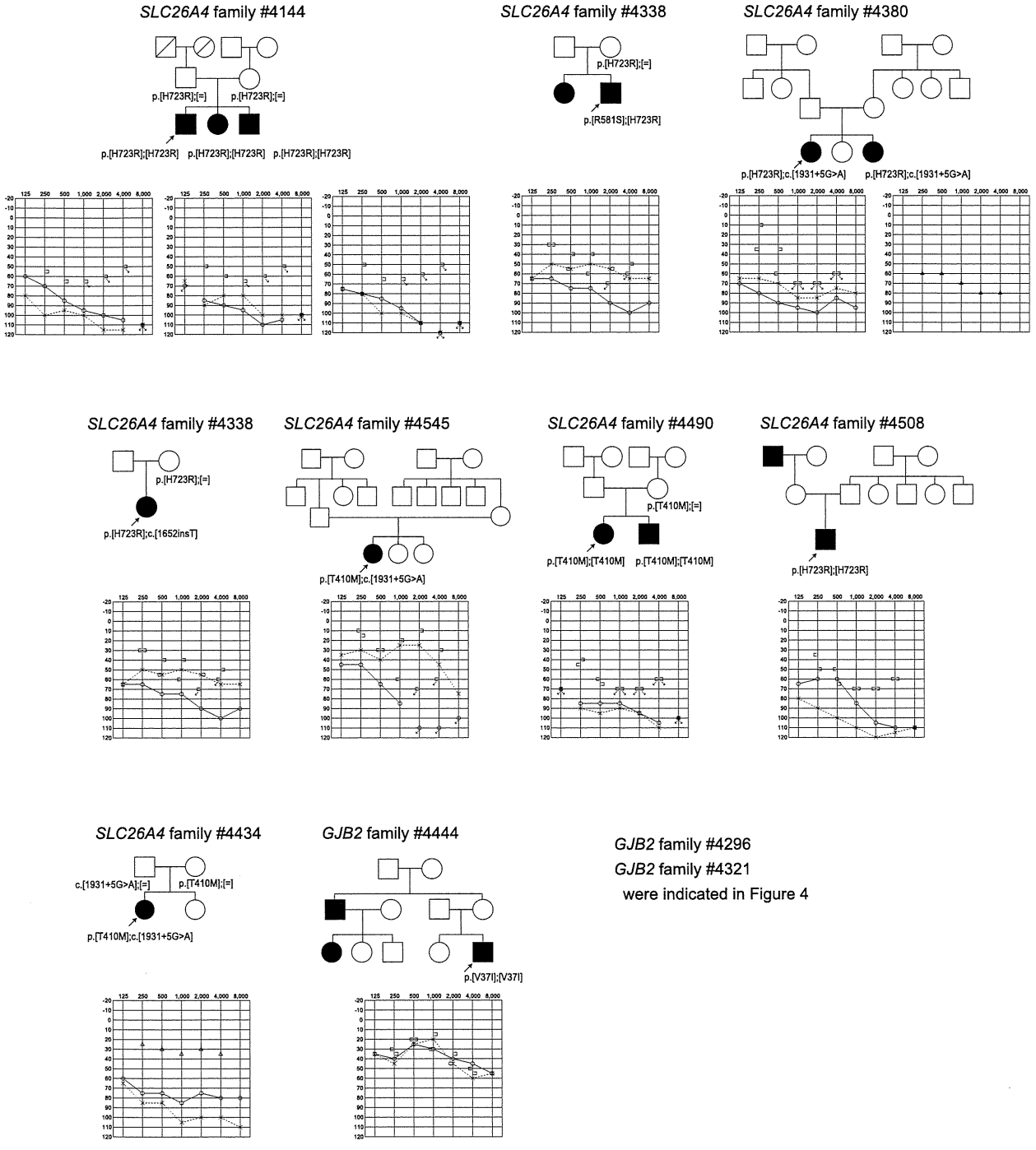
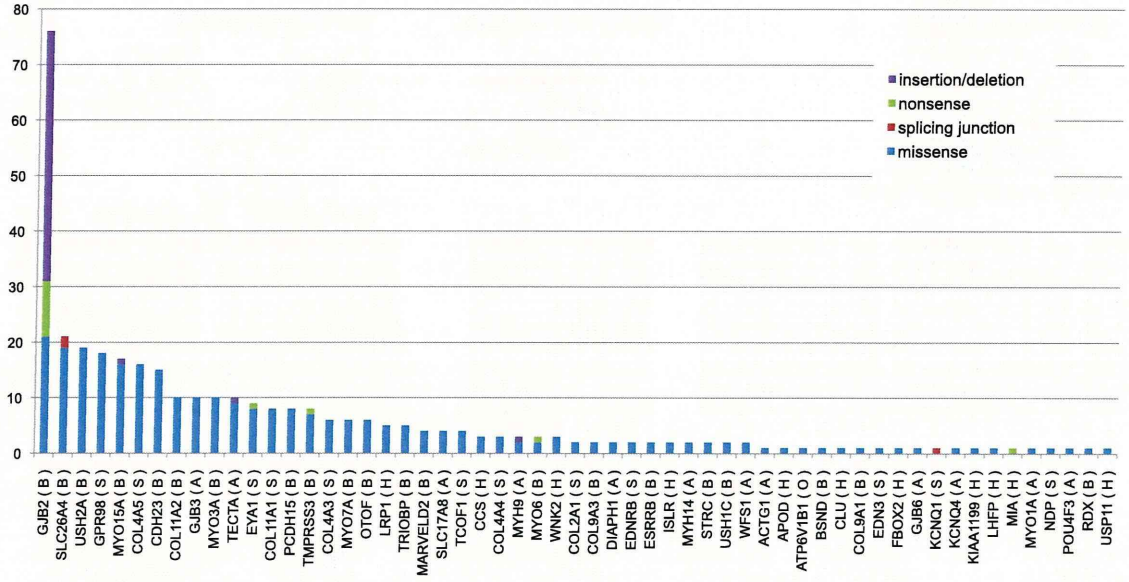


Figure 6. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g006

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
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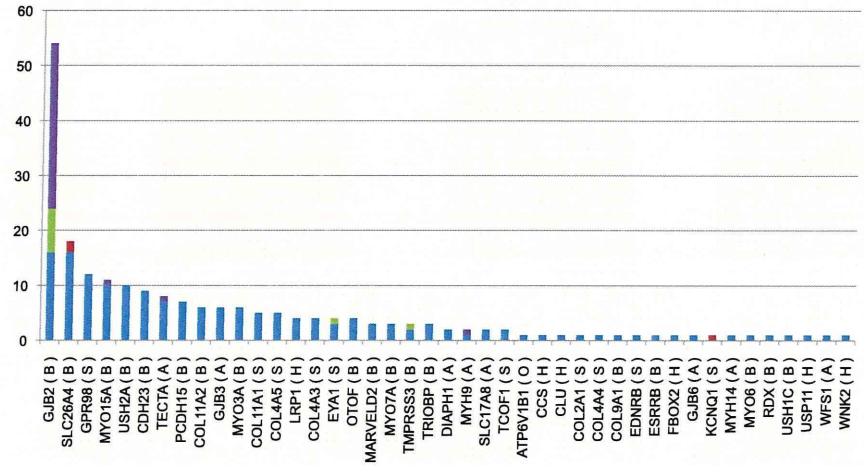
insertion/deletion
nonsense
splicing junction
missense
GJB2 (B)
SLC26A4 (B)
USH2A (B)
GPR98 (S)
MYO15A (B)
COL4A5 (S)
CDH23 (B)
COL11A2 (B)
GJB3 (A)
MYO3A (B)
TECTA (A)
EYA1 (S)
COL11A1 (S)
PCDH15 (B)
TMPRSS3 (B)
COL4A3 (S)
MYO7A (B)
OTOF (B)
LRP1 (H)
TRIOBP (B)
MARVELD2 (B)
SLC17A8 (A)
TCOF1 (S)
CCS (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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COL4A3 (S)
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TMPRSS3 (B)
TRIOBP (B)
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MYH9 (A)
SLC17A8 (A)
TCOF1 (S)
ATP6V1B1 (O)
CCS (H)
CLU (H)
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COL9A1 (B)
EDNRB (S)
ESRRB (B)
FBOX2 (H)
GJB6 (A)
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USH1C (B)
USP11 (H)
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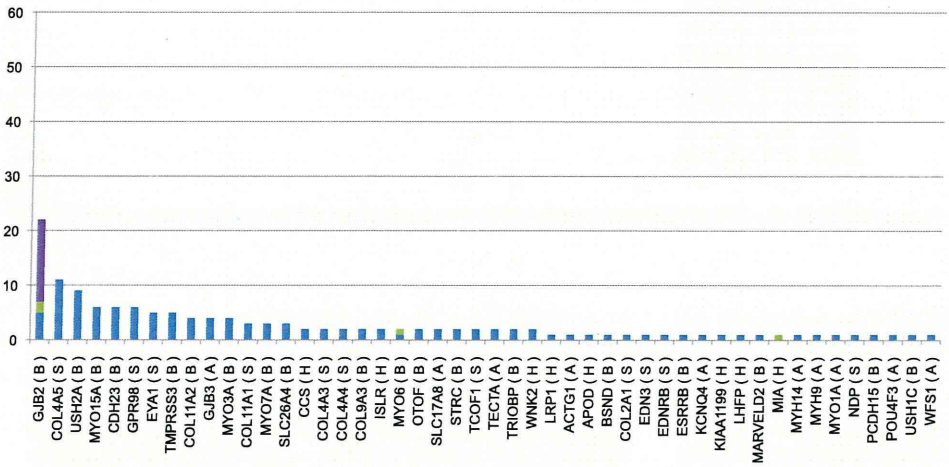
WNK2 (H)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GJB2 (B)
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USH2A (B)
MYO15A (B)
CDH23 (B)
GPR98 (S)
EYA1 (S)
TMPRSS3 (B)
COL11A2 (B)
GJB3 (A)
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COL11A1 (S)
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SLC26A4 (B)
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SLC17A8 (A)
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TRIOBP (B)
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MIA (H)
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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.

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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

Takehiko Naito[1], Shin-ya Nishio[1], Yoh-ichiro Iwasa[1], Takuya Yano[1], Kozo Kumakawa[2], Satoko Abe[2], Kotaro Ishikawa[3], Hiromi Kojima[4], Atsushi Namba[5], Chie Oshikawa[6], Shin-ichi Usami[1]*

1 Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan, **2** Department of Otolaryngology, Toranomon Hospital, Tokyo, Japan, **3** Department of Otorhinolaryngology, Jichi Medical University, Tochigi, Japan, **4** Department of Otorhinolaryngology, Jikei University School of Medicine, Tokyo, Japan, **5** Department of Otorhinolaryngology, Hirosaki University School of Medicine, Hirosaki, Japan, **6** Department of Otorhinolaryngology, Kyushu University School of Medicine, Fukuoka, Japan

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

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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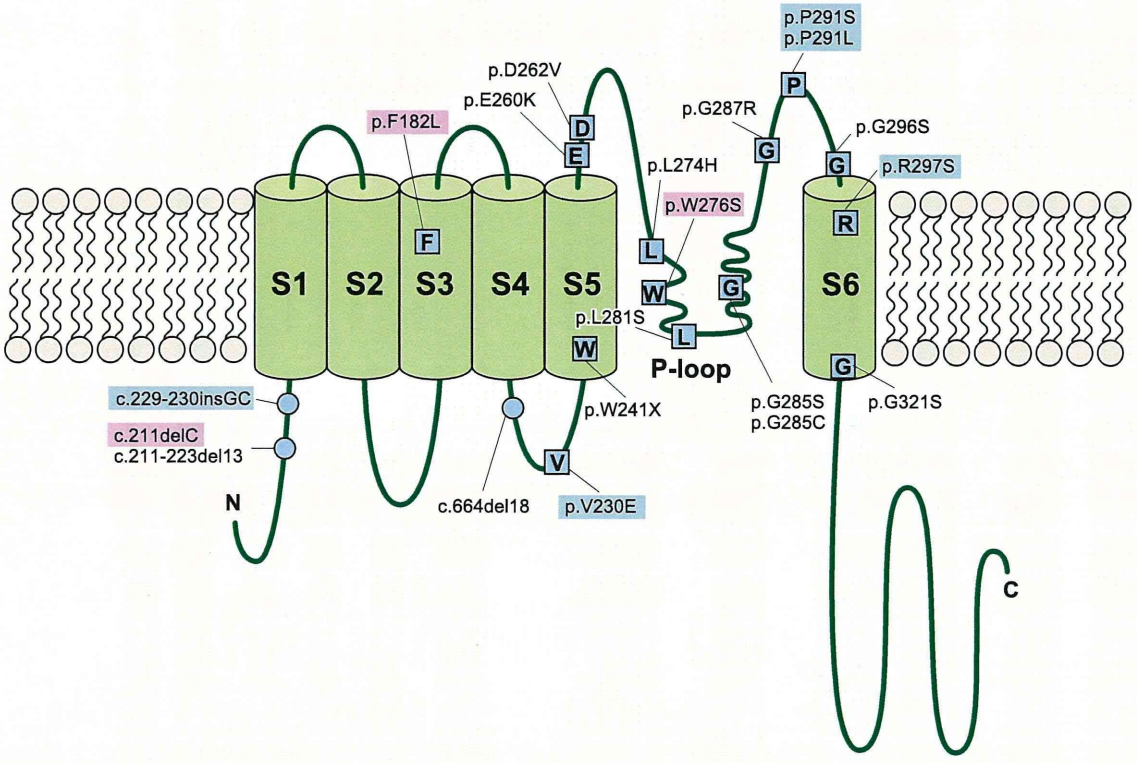

Figure 1. Localization of 20 *KCNQ4* mutations reported in previous studies in the protein. The 6 transmembrane domains (S1–S6) and the P-loop, located between S5 and S6, are shown. 5 mutations are concentrated in a narrow P-loop range. Mutations with pink and blue shadows; possible mutations detected 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

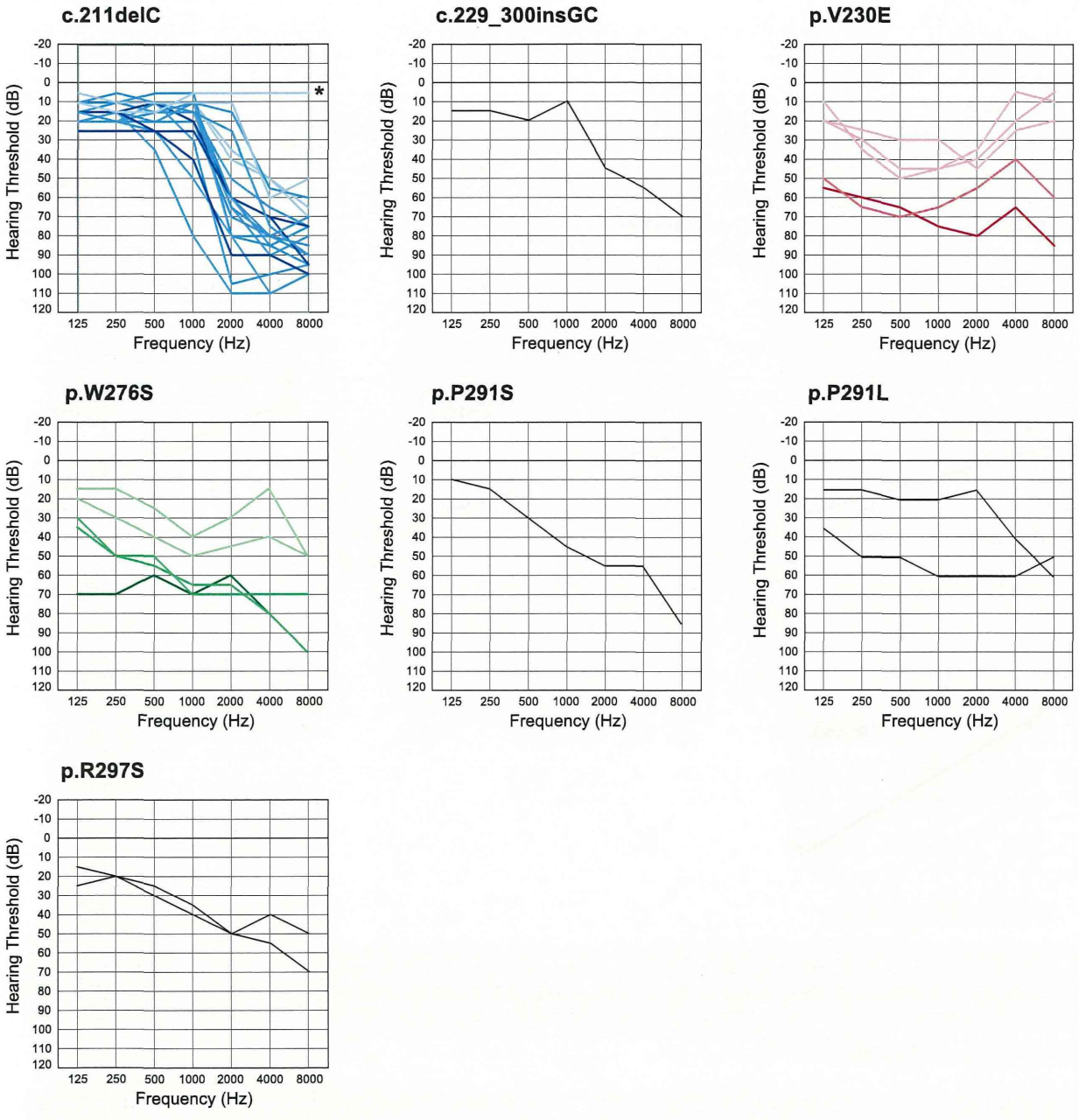

Figure 3. Overlapping audiograms from the better ear for each genotype. In cases of W276S, c.211delC, or V230E, light colored audiograms (green, blue, red) were from individuals aged 19 and under. Dark colored audiograms (green, blue, red) were from the patients aged 20–49 years old, and deep colored 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.
doi:10.1371/journal.pone.0063231.g003

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.

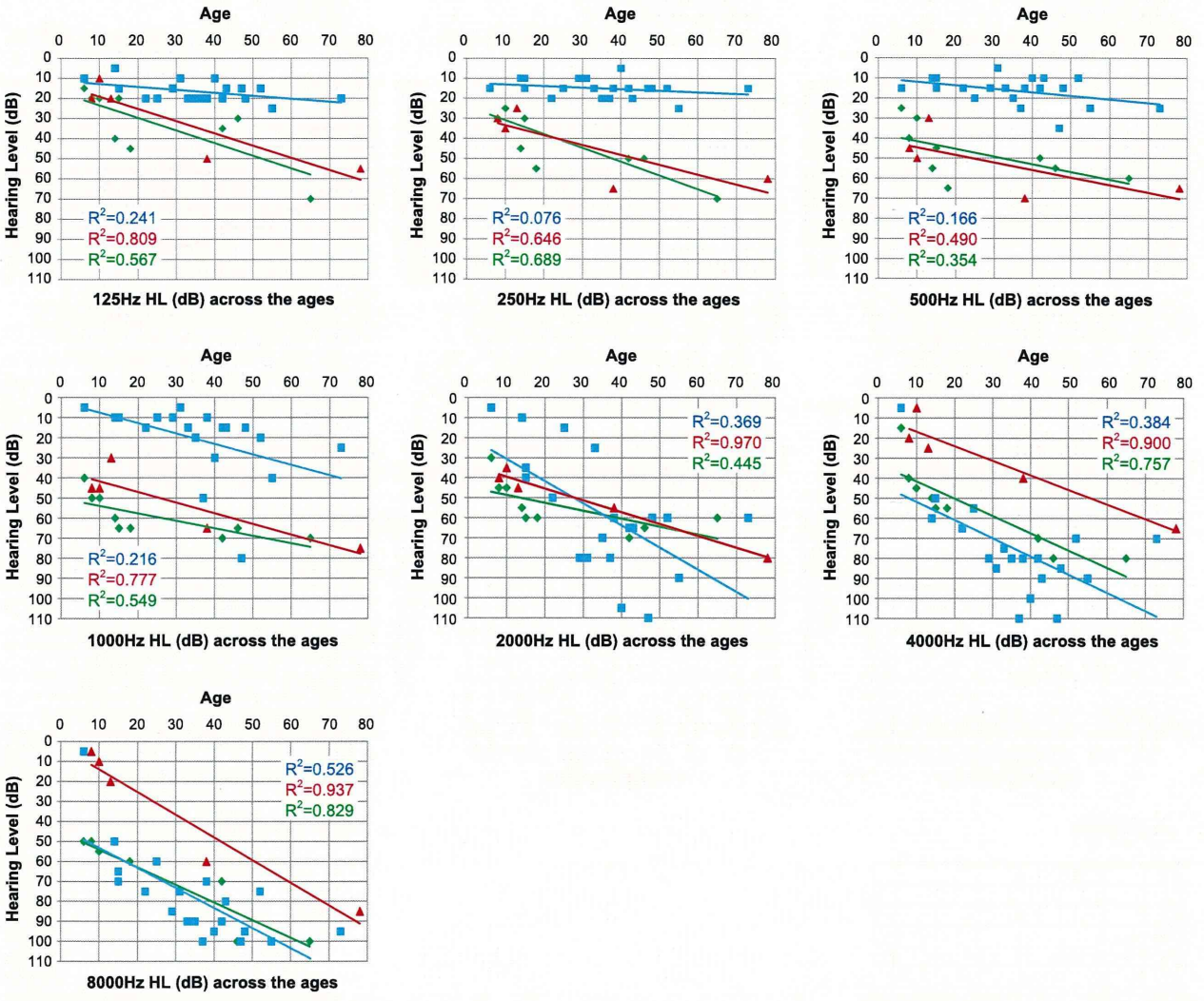

Figure 4. Detailed progression analysis in each frequency. A single audiogram (the better ear) from a single patient was plotted. Gradual progression is characterized regardless of frequency. Average progressive rates of hearing loss (db/year) for the patients with c.211delC, for 125 (0.15) and 250 Hz (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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ORIGINAL ARTICLE

Frequency of mitochondrial mutations in non-syndromic hearing loss as well as possibly responsible variants found by whole mitochondrial genome screening

Takuya Yano, Shin-ya Nishio, Shin-ichi Usami and the Deafness Gene Study Consortium[1]

Mutations in mitochondrial DNA (mtDNA) are reported to be responsible for the pathogenesis of maternally inherited hearing loss. Complete mtDNA sequencing may detect pathogenic mutations, but whether they are indeed pathogenic can be difficult to interpret because of normal ethnic-associated haplogroup variation and other rare variations existing among control populations. In this study, we performed systemic mutational analysis of mtDNA in 394 Japanese patients with hearing loss. Two different cohorts were analyzed in this study: Cohort 1, 254 maternally inherited patients; and Cohort 2, 140 patients with various inheritance modes. After screening of the entire mtDNA genome with direct sequencing, we evaluated the frequency of previously reported mutations and the frequency and pathogenicity of the novel variants. As a result, the 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%). 1555A>G ($n=23$) is the most common mutation, followed by the 3243A>G ($n=11$) mutations. On the basis of prediction analysis, we detected 10 novel homoplasmic mitochondrial variants. After further classification, the 3595A>G and 6204A>G variants were found to be new candidate mutations possibly associated with hearing loss.
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Keywords: mitochondrial mutation; non-syndromic hearing loss

INTRODUCTION

Hearing impairment is one of the most common sensory handicaps, with a frequency of at least 1/1000 at birth, and 50% of these cases can be attributed to genetic causes. Furthermore, causative mitochondrial DNA (mtDNA) mutations have been found in 5–10% of patients with postlingual non-syndromic hearing loss.[1]

Among mitochondrial mutations, 1555A>G mutations in the mitochondrial *12S rRNA* are found frequently (0.6–5.3%, depending on the ethnic group) in aminoglycoside-induced and late-onset non-syndromic hearing loss.[2–4] A 1494C>T mutation in *12S rRNA* is also associated with aminoglycoside-induced and non-syndromic hearing loss.[5] A 3243A>G mutation in the $tRNA^{Leu(UUR)}$ is associated with maternally inherited diabetes combined with deafness,[6] and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), which frequently present with hearing loss. 7445A>C/G/T,[7,8] 7472insC and 7510T>C[9] mutations in the $tRNA^{Ser(UCN)}$ are also associated with aminoglycoside-induced or non-syndromic hearing loss.

Moreover, additional mutations in *12S rRNA* (827A>G,[10] 961T>C, 961delT+Cn, 1005T>C and 1095T>C[11]) have been reported as mitochondrial hearing loss mutations. Although there were growing numbers of reports of various novel mtDNA mutations associated with hearing loss, most focused on a few limited nucleotide positions or only the *12SrRNA* region.[12] Therefore, we conducted a whole mitochondrial genome mutational analysis by direct sequencing using samples from 254 maternally inherited and 140 non-syndromic Japanese hearing loss probands with various inheritance modes, and summarized the frequencies of the mutations, as well as the spectrum and phenotypes found in the hearing loss patients with mtDNA mutations.

MATERIALS AND METHODS

Subjects

Two cohorts were used in this study: Cohort 1, 254 Japanese maternally (or possibly autosomal dominant with affected mother and one or more affected children) inherited sensorineural hearing loss (SNHL) subjects; and Cohort 2, 140 Japanese SNHL subjects with various inheritance modes (14 autosomal dominant or mitochondrial inherited, 126 autosomal recessive inherited or sporadic cases), both collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the

Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan
[1]Participating institutions: see Acknowledgments.
Correspondence: Professor S Usami, Department of Otorhinolaryngology, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.
E-mail: usami@shinshu-u.ac.jp
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project, which was approved by the ethical committee of each hospital. The control group consisted of 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

Whole mtDNA from each patient was amplified into two long fragments, A and B, by LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan) as described elsewhere.[13] In brief, each genomic DNA sample was amplified by long PCR for 1 min at 94 °C, followed by 30 three-step cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 6 min, with a final extension at 72 °C for 5 min, ending with a holding period at 4 °C.

After the PCR amplification, resulting products were purified and direct sequenced with ABI Big Dye terminators and ABI 3130 autosequencer (Applied Biosystems, Carlsbad, CA, USA). Sequencing reaction was performed with 50 primers for the whole mitochondrial genome, designed with mitoSEQr Resequencing System (Applied Biosystems).

Sequencing data were analyzed by SeqScape ver2.6 and SeqAnalysis (Applied Biosystems). The sequencing result from each patient was compared with the rCRS (Reversed Cambridge Reference Sequence) to identify mtDNA mutations. Mitochondrial DNA mutations included in the mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index.shtml), MITOMAP (http://www.mitomap.org/MITOMAP) or Uppsala mtDB (http://www.genpat.uu.se/mtDB/) databases were excluded as non-pathogenic variants when we search to novel variants.

We evaluated mutations according to evaluation criteria derived from a previous report by Zaragoza *et al.*[14]

Prediction of pathogenicity of mtDNA mutations

Initially, we measured the frequencies of each mutation found in healthy controls in our study ($n=192$) and in the mtSNP database ($n=2153$, including: centenarians in Gifu and Tokyo, type 2 diabetes mellitus patients (with or without vascular disorders), overweight and non-overweight young adult males, Parkinson's disease patients and Alzheimer's disease patients in Japan). The nucleotide conservation in each gene from humans and 60 mammalian species (*Artibeus jamaicensis*, NC_002009; *Balaenoptera musculus*, NC_001601; *Balaenoptera physalus*, NC_001321; *Bos taurus*, NC_006853; *Canis familiaris*, NC_002008; *Cavia porcellus*, NC_000884; *Cebus albifrons*, NC_002763; *Ceratotherium simum*, NC_001808; *Chalinolobus tuberculatus*, NC_002626; *Dasypus novemcinctus*, NC_001821; *Didelphis virginiana*, NC_001610; *Dugong dugon*, NC_003314; *Echinops telfairi*, NC_002631; *Echinosorex gymnura*, NC_002808; *Equus asinus*, NC_001788; *Equus caballus*, NC_001640; *Erinaceus europaeus*, NC_002080; *Felis catus*, NC_001700; *Gorilla gorilla*, NC_001645; *Halichoerus grypus*, NC_001602; *Hippopotamus amphibious*, NC_000889; *Hylobates lar*, NC_002082; *Isoodon macrourus*, NC_002746; *Lama pacos*, NC_002504; *Loxodonta africana*, NC_000934; *Macaca sylvanus*, NC_002764; *Macropus robustus*, NC_001794; *Mus musculus*, NC_005089; *Myoxus glis*, NC_001892; *Nycticebus coucang*, NC_002765; *Ochotona collaris*, NC_003033; *Ornithorhynchus anatinus*, NC_000891; *Orycteropus afer*, NC_002078; *Oryctolagus cuniculus*, NC_001913; *Ovis aries*, NC_001941; *Pan paniscus*, NC_001644; *Pan troglodytes*, NC_001643; *Papio hamadryas*, NC_001992; *Phoca vitulina*, NC_001325; *Physeter catodon*, NC_002503; *Pongo pygmaeus*, NC_002083; *Pongo pygmaeus* abelii, NC_002083; *Pteropus dasymallus*, NC_002612; *Pteropus scapulatus*, NC_002619; *Rattus norvegicus*, NC_001665; *Rhinoceros unicornis*, NC_001779; *Sciurus vulgaris*, NC_002369; *Soriculus fumidus*, NC_003040; *Sus scrofa*, NC_000845; *Tachyglossus aculeatus*, NC_003321; *Talpa europaea*, NC_002391; *Tarsius bancanus*, NC_002811; *Thryonomys swinderianus*, NC_002658; *Trichosurus vulpecula*, NC_003039; *Tupaia belangeri*, NC_002521; *Ursus americanus*, NC_003426; *Ursus arctos*, NC_003427; *Ursus maritimus*, NC_003428; *Volemys kikuchii*, NC_003041; *Vombatus ursinus*, NC_003322) was evaluated by the ClustalW method or the mtSNP database (mtSAP Evaluation; http://mtsnp.tmig.or.jp/mtsnp/search_mtSAP_evaluation.html). The mutations were considered to be possibly pathogenic if the original amino acid or base was conserved in >50% of the species (31 or more of 61 species).[15]

RESULTS

Direct sequence screening of the 254 probands of Japanese maternally inherited SNHL families and 140 non-syndromic hearing loss probands with various severities of hearing loss revealed 634 single-nucleotide polymorphisms in whole mitochondrial genome. Among those single-nucleotide polymorphisms, 19 were previously reported as associated with hearing loss: 792C>T ($n=1$), 827A>G ($n=10$), 856A>G ($n=3$), 961T>C ($n=3$), 1005T>C ($n=2$), 1095T>C ($n=1$), 1310C>T ($n=3$), 1494C>T ($n=1$), 1555A>G ($n=23$), 3243A>G ($n=11$), 3398T>C ($n=1$), 3421G>A ($n=2$), 5628T>C ($n=1$), 7511T>C ($n=3$), 8108A>G ($n=1$), 8348A>G ($n=1$), 11696G>A ($n=4$), 14693A>G ($n=1$) and 15927G>A ($n=4$) (Tables 1 and 2). In this study, based on the MITOMAP database, status was considered to be 'Confirmed' if at least two or more independent laboratories had published reports on the pathogenicity of a specific mutation (Table 1). More ambiguous substitutions were categorized as 'Unclear,' 'Reported' or 'Point mutation/polymorphism' (Table 2). 'Reported' status indicates that one or more reports have considered the mutation as possibly pathologic. 'Point mutation/

Table 1 'Comfirmed' mitochondrial mutations associated with sensorineural hearing loss found in this study

								Case					
Allele	Locus	Status[a]	Disease	Total (/394)	Cohort 1 (/254)	Cohort 2 (/140)	Control (/192)	Hearing characteristics	Progression of hearing loss	Tinnitus	Vertigo	Associated symptom	Reference
C1494T	12S rRNA	Confirmed	SNHL	1	0	1	0	High frequency	1/1	1/1	0/1	0	5
A1555G	12S rRNA	Confirmed	SNHL	23	23	0	0	High frequency	15/21	13/16	6/16	0	2
A3243G	tRNA ^{Leu} (UUR)	Confirmed	SNHL/DM/FSGS/ Cardiac dysfunction	11	11	0	0	Flat	10/10	6/10	6/10	Diabetes mellitus (8/10)	6
T7511C	tRNA $^{Ser\ (UCN)}$	Confirmed	SNHL	3	3	0	0	High frequency	1/2	3/4	0/4	0	23
Total					37/254 (14.6%)	1/140 (0.7%)			27/34	23/31	12/31		

Abbreviations: DM, diabetes mellitus; FSGS, focal segmental glomerulosclerosis; SNHL, sensorineural hearing loss.
[a]Based on the MITOMAP database; 'Confirmed' status indicates that at least two or more independent laboratories have published reports on the pathogenicity of a specific mutation.