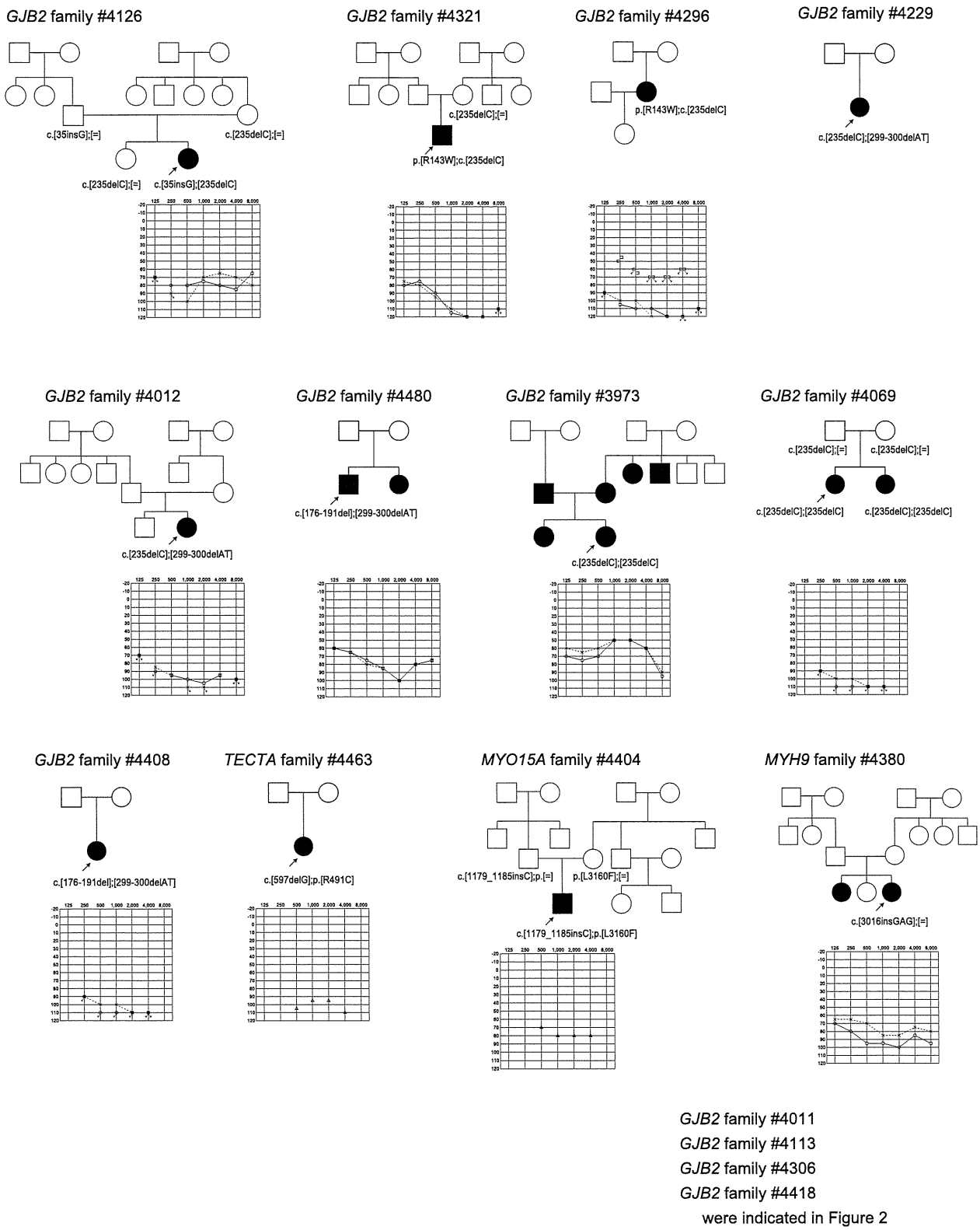


GJB2 family #4011
GJB2 family #4113
GJB2 family #4306
GJB2 family #4418
were indicated in Figure 2

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

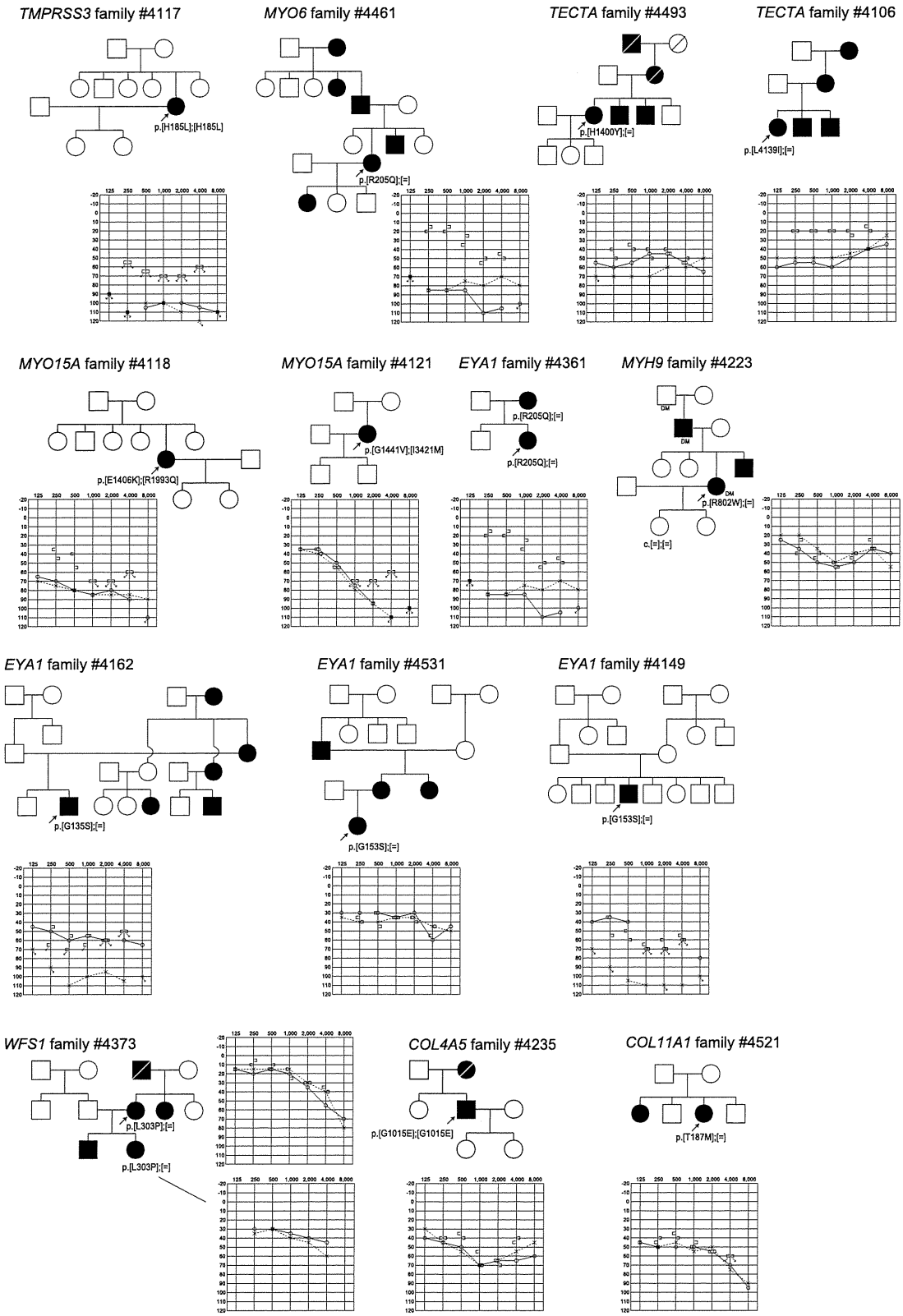


Figure 5. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g005

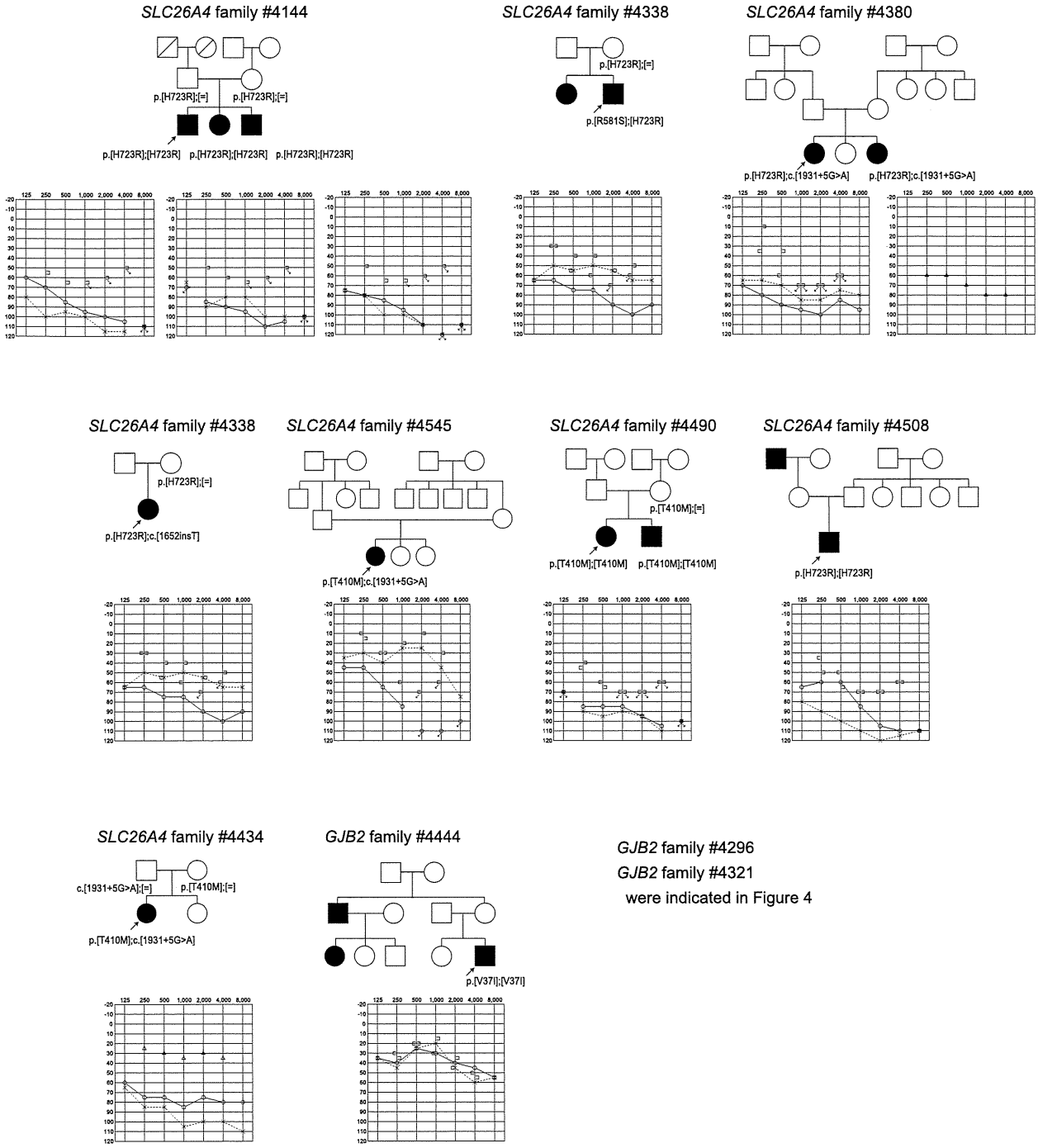
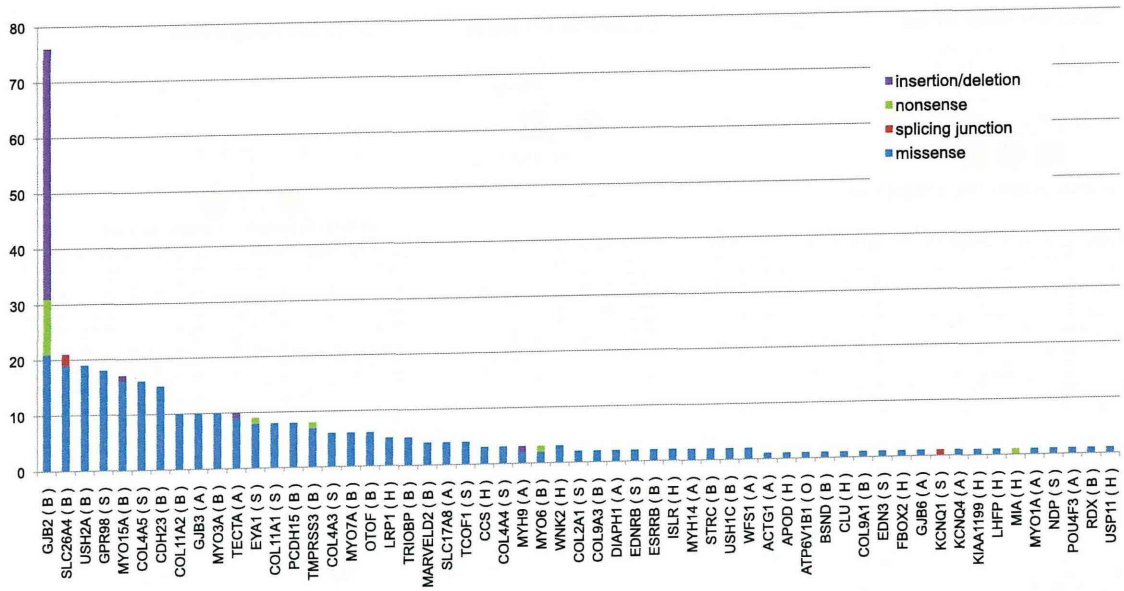


Figure 6. Examples of the families and audiograms of the patients with missense mutations after confirmation by Sanger sequencing.
doi:10.1371/journal.pone.0071381.g006

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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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)
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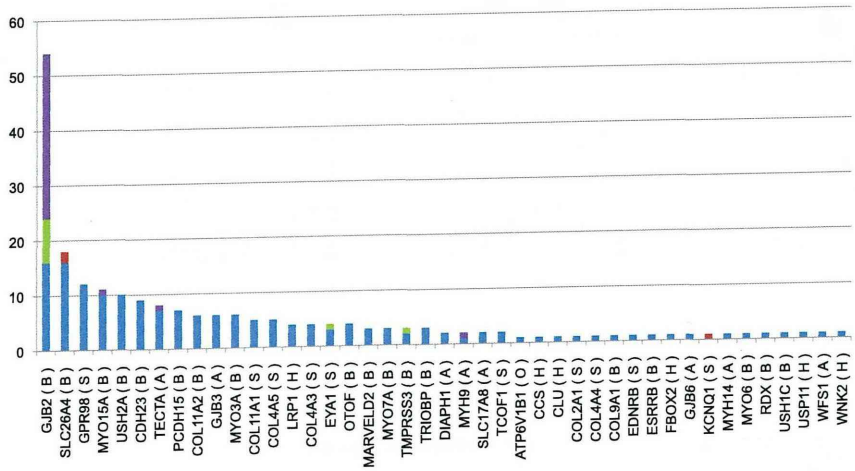
MIA (H)
MYO1A (A)
NDP (S)
POU4F3 (A)
RDX (B)
USP11 (H)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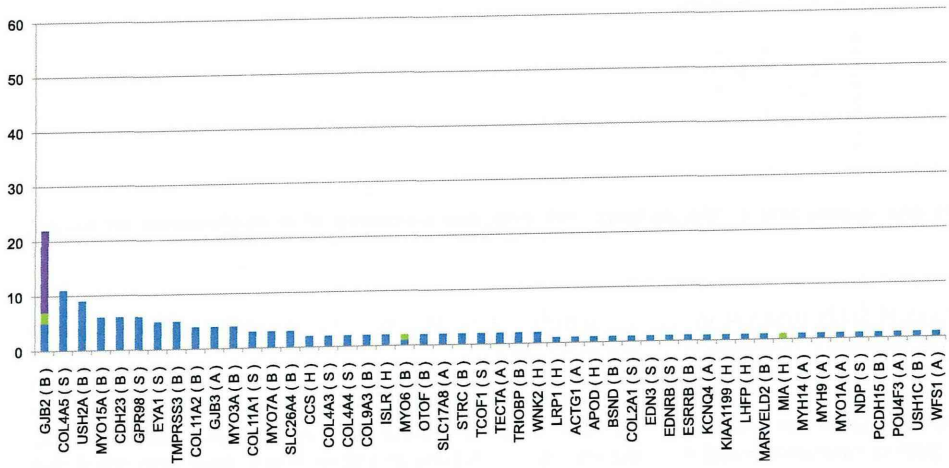
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ESRRB (B)
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KIAA1199 (H)
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MIA (H)
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MYH9 (A)
MYO1A (A)
NDP (S)
PCDH15 (B)
POU4F3 (A)
USH1C (B)
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)

Acknowledgments

We thank the participants of the Deafness Gene Study Consortium: Drs. Norihito Takeichi and Satoshi Fukuda (Hokkaido University), Drs. Atsushi Namba and Hideichi Shinkawa (Hirosaki University), Drs. Yumiko Kobayashi and Hiroaki Sato (Iwate Medical University), Drs. Tetsuaki Kawase and Toshimitsu Kobayashi (Tohoku University), Drs. Tomoo Watanabe, Tsukasa Ito and Masaru Aoyagi (Yamagata University), Drs. Hiroshi Ogawa and Koichi Omori (Fukushima Medical University), Drs. Kotaro Ishikawa and Keiichi Ichimura (Jichi Medical University), Drs. Kyoko Nagai and Nobuhiko Furuya (Gunma University), Drs. Shuntaro Shigihara, Yasuyuki Nomura and Minoru Ikeda (Nihon University School), Drs. Tetsuo Ikezono and Toshiaki Yagi (Nippon Medical School), Dr. Shunichi Tomiyama (Nippon Medical School Tama Nagayama Hospital), Drs. Hiromi Kojima, Yuika Sakurai and Hiroshi Moriyama (Jikei University), Dr. Kozo Kumakawa (Toranomon Hospital), Drs. Hajime Sano and Makito Okamoto (Kitasato University), Dr. Satoshi Iwasaki (Hamamatsu Medical University), Dr. Kazuhiko Takeuchi (Mie University), Dr. Masako Nakai (Shiga Medical Center for Children), Drs. Masahiko Higashikawa and Hiroshi Takenaka (Osaka Medical College), Drs. Yuko Saito, Masafumi Sakagami (Hyogo College of Medicine), Dr. Yasushi Naito (Kobe City Medical Center General Hospital), Drs. Keiji Fujihara, Akihiro Sakai and Noboru Yamanaka (Wakayama Medical University), Drs. Kunihiro Fukushima, and Kazunori Nishizaki (Okayama University), Drs. Kazuma Sugahara and Hiroshi Yamashita (Yamaguchi University), Drs. Naoto Hato and Kiyofumi Gyo (Ehime University), Drs. Yasuhiro Kakazu and Shizuo Komune (Kyushu University), Drs. Mayumi Sugamura and Takashi Nakagawa (Fukuoka University), Dr. Haruo Takahashi (Nagasaki University), Dr. Yukihiko Kanda (Kanda ENT

Clinic), Drs. Hirokazu Kawano and Tetsuya Tono (Miyazaki Medical College), Drs. Ikuyo Miyanohara andYuichi Kurono (Kagoshima University), and Drs. Akira Ganaha and Mikio Suzuki (Ryukyus University), for providing samples from their patients. We also thank Kazuyuki Nakazono for assistance with the statistical analysis, Richard J. Smith for valuable comments and A. C. Apple-Mathews for help in preparing the manuscript.

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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Iwasa et al. BMC Medical Genetics 2013, 14:95
http://www.biomedcentral.com/1471-2350/14/95

BMC Medical Genetics

RESEARCH ARTICLE Open Access

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.

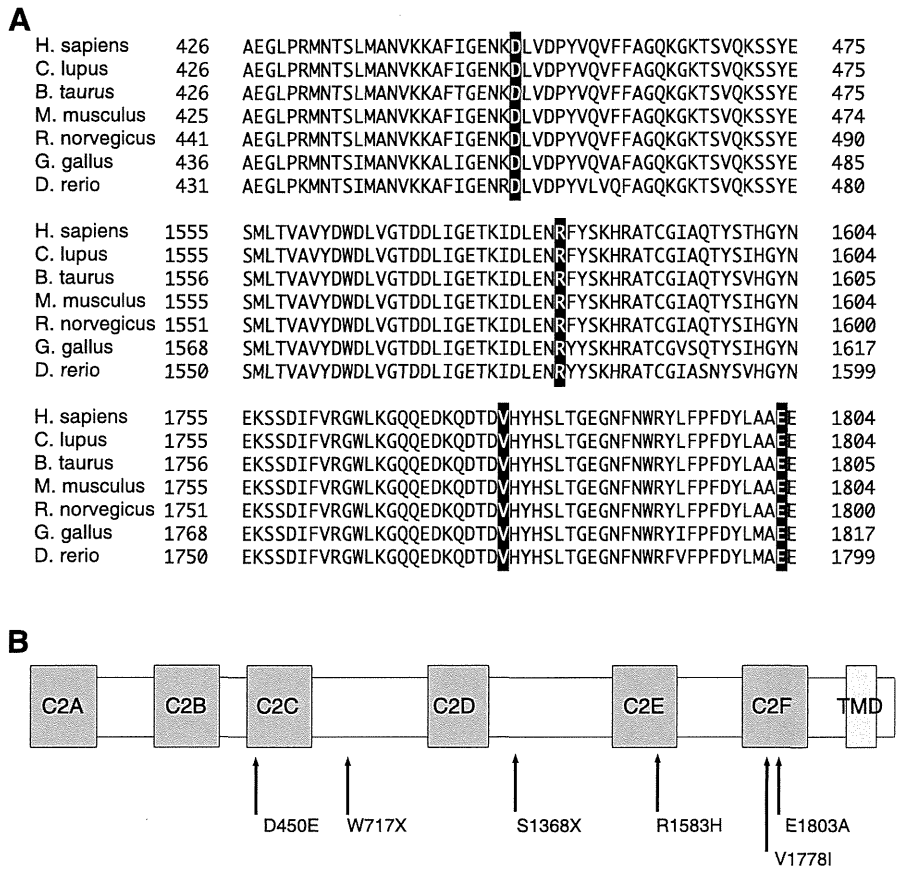

Figure 1 The location of mutations in otoferlin protein and the evolutionary conservation of the amino acids. (A) Evolutionary conservation. The locations of mutations are boxed. **(B)** Novel pathogenic *OTOF* mutations found in this work and relation to the functional domains of otoferlin. C2A-F: C2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

Table 3 Patients who have at least one pathogenic mutation identified in this study

Patient	DNA level	Protein level	Clinical diagnosis	OAE	Age at diagnosis	Hearing loss level
1	c.1422T>A / c.5567G>A	p.Y474X / p.R1856Q	ANSD	+	1y6m	Profound
2	c.1422T>A / c.5816G>A	p.Y474X / p.R1939Q	ANSD	+	NA	Profound
3	c.5816G>A / c.5816G>A	p.R1939Q / p.R1939Q	ANSD	+	4m	Profound
4	c.5816G>A / c.5816G>A	p.R1939Q / p.R1939Q	ANSD	+	10m	Profound
5	c.5816G>A / c.5816G>A	p.R1939Q / p.R1939Q	ANSD	+	NA	Profound
6	c.4748G>A / c.5816G>A	p.R1583H / p.R1939Q	NSHL	NA	6m	Profound
7	c.2151G>A / c.5816G>A	p.W717X / p.R1939Q	NSHL	-	1y4m	Profound
8	c.5816G>A / -	p.R1939Q /-	ANSD	+	1y5m	Profound
9	c.5816G>A / -	p.R1939Q /-	ANSD	+	7m	Profound
10	c.1194T>A / -	p.D398E / -	NSHL	NA	NA	Profound
11	c.1350C>G / -	p.D450E / -	NSHL	NA	2y	Severe
12	c.2180A>G / -	p.N727S / -	NSHL	NA	6m	Profound
13	c.2180A>G / -	p.N727S / -	NSHL	NA	1y	Severe
14	c.4103C>G / -	p.S1368X / -	NSHL	NA	7m	Profound
15	c.5332G>A / -	p.V1778I / -	NSHL	NA	NA	Profound
16	c.5408A>C / -	p.E1803A / -	NSHL	NA	4m	Profound

ANSD Auditory neuropathy spectrum disorder, *NSHL* Nonsyndromic sensorineural hearing loss.

As seen in previous mutation screening reports, including those for *OTOF* [12,23,30], there were a significant number of heterozygous cases without a second mutation even after direct sequencing of the coding region of the gene. Possible explanations are: 1) the existence of a second mutation in the intron or regulatory region of *OTOF*, which has not been explored, 2) the existence of a large deletion [31], 3) contribution to hearing loss by an additional modulatory gene, and 4) the existence of a mutation in another gene and just coincidental carrying of the *OTOF* mutation.

As seen in Table 3, two heterozygous patients (#8, 9) having the ANSD phenotype, are most likely to have *OTOF* related deafness.

It is assumed that *OTOF* mutations accounted for deafness in at least 7, and possibly 16, of the 160 patients (4.4-10.0%). As described in the subject section, we excluded the subjects carrying *GJB2* and *SLC26A4* mutations. We also excluded another responsible gene (*PJVK*), because no mutations in this gene were found. Since the frequencies of *GJB2* and *SLC26A4* gene mutations among the patients with nonsyndromic severe to profound congenital SNHL are 27.0% based on our database, mutation frequency of *OTOF* among the total of severe to profound recessive nonsyndromic SNHL is considered to be about 3.2-7.3% (which is calculated by ((7-16)/160×(100/73))×100%). Although simple comparison regarding frequency is difficult because of sampling bias, it is estimated that the frequency of OTOF mutations in Japanese may be almost equal to other populations, as mutation frequency of OTOF was reported at 2.3% (13/557) in Pakistanis [11], 5.0% in Turkish [32], 1.4% (1/73) in Chinese [23], and 18.2% (4/22) in Taiwanese [29], and 3.2% (23/708) in Spanish [12]. Although simple comparison regarding frequency is difficult because of sampling bias, it is estimated that the frequency of *OTOF* mutations in Japanese may be almost equal to other populations. In Japanese, *GJB2, SLC26A4, CDH23* and the 1555A>G mutation in the mitochondrial 12S rRNA are the major causes of hearing loss [33]. Considering the frequency, the *OTOF* gene may be one of the candidate genes to be screened for recessive severe to profound recessive SNHL.

The benefits of cochlear implantation for patients with ANSD has varied [34,35], but implantation has been shown to be effective for the patients with *OTOF* mutations [15,16,36], because their auditory nerves and spiral ganglions are preserved. Consequently, if an *OTOF* mutation is identified in a deaf patient, we can anticipate a good outcome of cochlear implantation, therefore, it is important and meaningful to identify genetic mutations in patients.

Most patients with *OTOF* mutations have a phenotype of stable prelingual and severe to profound nonsyndromic hearing loss. On the other hand, other phenotypes have also been reported. For example, a Taiwanese patient with an p.E1700Q mutation displayed moderate to profound progressive hearing loss [29]. Temperature sensitive ANSD, a particular form of ANSD, has also been reported in some populations [10,23,37].

In the very young child, electrophysiological testing may indicate that *OTOF*-related deafness is ANSD, but

by age two OAEs have generally disappeared and the test results are more in accord with the findings of cochlear lesions [14]. Therefore, if OAE is not tested at a very early age, patients with *OTOF* mutations are not deemed to have ANSD (i.e., hidden ANSD). In fact, 9 out of our 16 patients were diagnosed genetically as nonsyndromic sensorineural hearing loss (NSHL). According to the present data, screening for *OTOF* is necessary not only for the patients diagnosed with ANSD, but also should be extended to ARNSHL cases. The current data indicated that OAE testing must always be conducted in addition to ABR in infants. And we should bear in mind that there may be patients with *OTOF* mutations among the patients diagnosed as having ARNSHL.

Conclusions

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

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YI and SN carried out the molecular genetic studies and the sequence alignment, and participated in drafting the manuscript. SU conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank all the families that participated in the present study. We would also like to thank Ms. S. Matsuda for technical assistance, Ms. A. C. Apple-Mathews for help in preparing the manuscript, and Dr. T. Matsunaga for valuable comments. This study was supported by a Health Sciences Research Grant from the Ministry of Health and Welfare of Japan.

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Received: 7 November 2012 Accepted: 27 August 2013
Published: 22 September 2013

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doi:10.1186/1471-2350-14-95
Cite this article as: Iwasa *et al.*: *OTOF* mutation screening in Japanese severe to profound recessive hearing loss patients. *BMC Medical Genetics* 2013 **14**:95.

OPEN ACCESS Freely available online

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Massively Parallel DNA Sequencing Facilitates Diagnosis of Patients with Usher Syndrome Type 1

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Abstract

Usher syndrome is an autosomal recessive disorder manifesting hearing loss, retinitis pigmentosa and vestibular dysfunction, and having three clinical subtypes. Usher syndrome type 1 is the most severe subtype due to its profound hearing loss, lack of vestibular responses, and retinitis pigmentosa that appears in prepuberty. Six of the corresponding genes have been identified, making early diagnosis through DNA testing possible, with many immediate and several long-term advantages for patients and their families. However, the conventional genetic techniques, such as direct sequence analysis, are both time-consuming and expensive. Targeted exon sequencing of selected genes using the massively parallel DNA sequencing technology will potentially enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis. Using this technique combined with direct sequence analysis, we screened 17 unrelated Usher syndrome type 1 patients and detected probable pathogenic variants in the 16 of them (94.1%) who carried at least one mutation. Seven patients had the *MYO7A* mutation (41.2%), which is the most common type in Japanese. Most of the mutations were detected by only the massively parallel DNA sequencing. We report here four patients, who had probable pathogenic mutations in two different Usher syndrome type 1 genes, and one case of *MYO7A/PCDH15* digenic inheritance. This is the first report of Usher syndrome mutation analysis using massively parallel DNA sequencing and the frequency of Usher syndrome type 1 genes in Japanese. Mutation screening using this technique has the power to quickly identify mutations of many causative genes while maintaining cost-benefit performance. In addition, the simultaneous mutation analysis of large numbers of genes is useful for detecting mutations in different genes that are possibly disease modifiers or of digenic inheritance.

Citation: Yoshimura H, Iwasaki S, Nishio S-y, Kumakawa K, Tono T, et al. (2014) Massively Parallel DNA Sequencing Facilitates Diagnosis of Patients with Usher Syndrome Type 1. PLoS ONE 9(3): e90688. doi:10.1371/journal.pone.0090688

Editor: Klaus Brusgaard, Odense University hospital, Denmark

Received September 6, 2013; **Accepted** February 3, 2014; **Published** March 11, 2014

Funding: This study was supported by a Health and Labour Sciences Research Grant for Comprehensive Research on Disability Health and Welfare from the Ministry of Health, Labour and Welfare of Japan (S.U.), and by a Grant-in-Aid for Scientific Research from the (then) Ministry of Education, Science and Culture of Japan (http://www.mext.go.jp/english/) (S.U.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by hearing loss (HL), retinitis pigmentosa (RP) and vestibular dysfunction. Three clinical subtypes can be distinguished. USH type 1 (USH1) is the most severe among them because of profound HL, absent vestibular responses, and prepubertal onset RP. USH type 2 (USH2) is characterized by congenital moderate to severe HL, with a high-frequency sloping configuration. The vestibular function is normal and onset of RP is in the first or second decade. The onset of the visual symptoms such as night blindness in USH usually occurs several years later than in USH1. USH type 3 (USH3) is characterized by variable onset of progressive HL, variable onset of RP, and variable impairment of vestibular function (normal to absent) [1,2].

To date, nine genetic loci for USH1(*USH1B-H*, *J*, and *K*) have been mapped to chromosomes 11q13.5, 11p15.1, 10q22.1, 21q21, 10q21-q22, 17q24-q25, 15q22-q23 (*USH1H* and *J*), and 10p11.21–q21.1 [2,3,4]. Six of the corresponding genes have been identified: the actin-based motor protein myosin VIIa

(*MYO7A*, USH1B) [5]; two cadherin-related proteins, cadherin 23 (*CDH23*, USH1D) [6] and protocadherin 15 (*PCDH15*, USH1F) [7]; and two scaffold proteins, harmonin (*USH1C*) [8] and sans (*USH1G*) [9]; the Ca^{2+}- and integrin-binding protein (*CIB2*, USH1J) [4]. In Caucasian USH1 patients, previous studies showed that mutations in *MYO7A*, *USH1C*, *CDH23*, *PCDH15*, and *USH1G*, were found in 39–55%, 7–14%, 7–35%, 7–11%, and 0–7%, respectively (the frequency of *CIB2* is still unknown) [10,11,12]. In Japanese, Nakanishi et al. showed that *MYO7A* and *CDH23* mutations are present in USH1 patients [13], however, the frequency is not yet known. In addition, mutations in three corresponding genes (usherin *USH2A* [14], G protein-coupled receptor 98; *GPR98* [15], and deafness, autosomal recessive 31; *DFNB31* [16]) have been reported so far in USH2, and USH3 is caused by mutations in the clarin 1 (*CLRN1*) [17] gene.

Comprehensive molecular diagnosis of USH has been hampered both by genetic heterogeneity and the large number of exons for most of the USH genes. The six USH1 genes collectively contain 180 coding exons [4,9,10] the three USH2 genes comprise 175 coding exons [15,16,18], and the USH3 gene has five coding exons [17]. In addition some of these genes are alternatively spliced ([4,7,8,16,17] and NCBI database: http://www.ncbi.nlm.nih.gov/nuccore/). Thus far, large-scale mutation screening has been performed using direct sequence analysis, but that is both time-consuming and expensive. We thought that targeted exon sequencing of selected genes using the Massively Parallel DNA Sequencing (MPS) technology would enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis.

Therefore, in this study, we have conducted genetic analysis using MPS-based genetic screening to find mutations in nine causative USH genes (except *CIB2*) in Japanese USH1 patients.

Results

Mutation analysis of the nine USH genes in 17 unrelated USH1 patients revealed 19 different probable pathogenic variants, of which 14 were novel (Table 1).

All mutations were detected in only one patient each and sixteen of the 17 patients (94.1%) carried at least one mutation, while one patient had no mutations. Thirteen of the 16 mutation carriers each had two pathogenic mutations (Table 2).

Nonsense, frame shift, and splice site mutations are all classified as pathogenic, whereas missense mutations are presumed to be probable pathogenic variants based on results of prediction software for evaluation of the pathogenicity of missense variants (Table 1).

Of the 19 probable pathogenic mutations that we found, 17 were detected by MPS. The remaining two (p.Lys542GlnfsX5 in *MYO7A* and c.5821-2A>G in *CDH23*) were sequenced by direct sequence analysis.

Of our 17 USH patients, seven had *MYO7A* mutations (41.2%), three had *CDH23* mutations (17.6%), and two had *PCDH15* mutations (11.8%). We did not find any probable pathogenic mutations in *USH1C*, *USH1G*, and USH2/3 genes.

Four USH1 patients (Cases #3, 5, 8, 15) had probable pathogenic mutations in two different USH genes, with one being a biallelic mutation (Table 3). The other heterozygous/homozygous mutations were missense variants. Three of these patients (Cases #3, 5, 8) presented with earlier RP onset (night blindness) than in the other patients with two pathogenic mutations (Cases #1, 6, 7, 9, 11, 16) ($p = 0.007$) (Fig. 1).

One patient (Case #4) had heterozygote mutations in two USH1 genes (p.Ala771Ser in *MYO7A* and c.158-1G>A in *PCDH15*). His parents and one brother were found to also be carriers for these mutations. Another brother had no variants (Fig. 2).

Discussion

For USH1, early diagnosis has many immediate and several long-term advantages for patients and their families [1]. However, diagnosis in childhood, based on a clinical phenotype, has been difficult because patients appear to have only non-syndromic HL in childhood and RP develops in later years. Although early diagnosis is now possible through DNA testing, performing large-scale mutation screening for USH genes in all non-syndromic HL children has been both time-consuming and expensive. Therefore, the availability of MPS, which facilitates comprehensive large-scale mutation screening [19] is a very welcome advance.

MPS technology enabled us to detect pathogenic mutations in USH1 patients efficiently, identifying one or two pathogenic/likely pathogenic mutations in 16 of 17 (94.1%) cases. This was comparable to previous direct sequence analysis results such as Bonnet et al. who detected one or two mutations in 24 out of 27 (89%) USH1 patients [11] and Le Quesne Stabej et al. who detected one or two mutations in 41 out of 47 (87.2%) USH1 patients [12].

In addition, MPS assists in the analysis of disease modifiers and digenic inheritance because it simultaneously investigates many causative genes for a specific disease, such as in our case, USH. Previous reports have described several USH cases with pathogenic mutations in two or three different USH genes [11,12,20]. In our study, four patients had two pathogenic mutations in one gene and missense variants in a different gene (Table 3). We considered the latter to possibly be a disease modifier. For example, *USH1C*:p.Tyr813Asp, which occurred in 0/384 control chromosomes and was predicted to be "probably damaging" by the Polyphen program, was found with a homozygous *CDH23* nonsense mutation (p.Arg2107X) (Case #15). As for what the variant "modifies", we speculate that for USH1 patients with a disease modifier, RP symptoms such as night blindness show an earlier onset. However, we think that profound HL and the absence of vestibular function in USH1 patients are not affected by modifiers as they are congenital and therefore not progressive.

Ebermann et al. described a USH2 patient with "digenic inheritance." a heterozygous truncating mutation in *GPR98*, and a truncating heterozygous mutation in PDZ domain-containing 7 (*PDZD7*), which is reported to be a cause of USH [20]. Our USH1 patient (Case #4) had segregated *MYO7A*:p.Ala771Ser and *PCDH15*:c.158-1G>A. Molecular analyses in mouse models have shown many interactions among the USH1 proteins [2]. In particular, *MYO7A* directly binds to *PCDH15* and both proteins are expressed in an overlapping pattern in hair bundles in a mouse model [21]. *PCDH15*:c.158-1G>A, predicted to alter the splice donor site of intron 3, has been classified as pathogenic. *MYO7A*:p.Ala771Ser is a non-truncating mutation, but was previously reported as disease-causing [13]. So, we consider the patient to be the first reported case of *MYO7A*/*PCDH15* digenic inheritance.

However, we should be aware of two limitations of MPS technology. First, the target region of MPS cannot cover all coding exons of USH genes. Actually, the coverage of the target exons was 97.0% in our study. So, it is impossible to detect a mutation in a region which is not covered using this system (Case #9: c.5821-2A>G). Secondarily, the MPS system used in this study, is not effective for detecting homo-polymer regions, for example poly C stretch [22] (Case #8: p.Lys542GlnfsX5). In addition, concerning

Table 1. Possible pathogenic variants found in this study.

Gene	Mutation type	Nucleotide change	Amino acid change	exon/intron number	Domain	control (in 384 alleles)	SIFT Score	PolyPhen Score	Reference
MYO7A	Frameshift	c.1623dup	p.Lys542GlnfsX5	Exon 14	-	N/A	-	-	Le Quesne Stabej et al. (2012)
		c.4482_4483insTG	p.Trp1495CysfsX55	Exon 34	-	N/A	-	-	This study
		c.6205_6206delAT	p.Ile2069ProfsX6	Exon 45	-	N/A	-	-	This study
	Nonsense	c.1477C>T	p.Gln493X	Exon 13	-	N/A	-	-	This study
		c.1708C>T	p.Arg570X	Exon 15	-	N/A	-	-	This study
		c.2115C>A	p.Cys705X	Exon 18	-	N/A	-	-	This study
		c.6321G>A	p.Trp2107X	Exon 46	-	N/A	-	-	This study
	Missense	c.2074G>A	p.Val692Met	Exon 17	Motor domain	0	0.09	0.982	This study
		c.2311G>T	p.Ala771Ser	Exon 20	IQ 2	0.0026	0.01	0.825	Nakanishi et al. (2010)
		c.6028G>A	p.Asp2010Asn	Exon 44	FERM 2	0	0	0.925	Jacobson et al. (2009)
CDH23	Frameshift	c.3567delG	p.Arg1189ArgfsX5	Exon 30	-	N/A	-	-	This study
		c.5780_5781delCT	p.Ser1927Cysfs16	Exon 44	-	N/A	-	-	This study
	Splicing	c.5821-2A>G	?	Intron 44	-	N/A	-	-	This study
	Nonsense	c.6319C>T	p.Arg2107X	Exon 48	-	N/A	-	-	Nakanishi et al. (2010)
PCDH15	Splicing	c.158-1G>A	?	Intron 3	-	N/A	-	-	This study
	Nonsense	c.1006C>T	p.Arg336X	Exon 10	-	N/A	-	-	This study
		c.2971C>T	p.Arg991X	Exon 22	-	N/A	-	-	Roux et al. (2006)
		c.3337G>T	p.Glu1113X	Exon 25	-	N/A	-	-	This study
	Missense	c.3724G>A	p.Val1242Met	Exon 28	Cadherin 11	0	0	1	This study

Computer analysis to predict the effect of missense variants on MYO7A protein function was performed with sorting intolerant from tolerant (SIFT; http://sift.jcvi.org/), and polymorphism phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).
N/A: not applicable.
doi:10.1371/journal.pone.0090688.t001

Table 2. Details of phenotype and genotype of 17 USH1 patients.

Sample No.	Age	Sex	Allele1	Allele2	Hereditary form	Onset of night blindness	Cataract	Hearing Aid	Cochlear Implant
MYO7A									
1	37	M	p.Gln493X	p.Trp1495CysfsX55	sporadic	13	no	unilateral	unilateral
2	41	W	p.I2069fsX6	p.I2069fsX6	AR	unknown	both eyes	bilateral	no
5	54	M	p.Val692Met	p.Val692Met	AR	5	both eyes	no	no
6	54	W	p.Arg570X	p.Arg570X	sporadic	6	no	no	no
8	14	M	p.Lys542GlnfsX5	p.Lys542GlnfsX5	sporadic	6	no	unilateral	unilateral
11	54	M	p.Asp2010Asn	p.Trp2107X	sporadic	13	no	no	no
17	56	W	p.Cys705X	p.Cys705X	sporadic	unknown	no	no	no
CDH23									
7	12	W	p.Arg1189ArglfsX5	p.Arg1189ArglfsX5	sporadic	12	both eyes	no	bilateral
9	9	M	p.Ser1927Cysfs16	c.5821-2A>G	sporadic	8	no	unilateral	unilateral
15	16	W	p.Arg2107X	p.Arg2107X	sporadic	unknown	no	no	no
PCDH15									
3	47	W	p.Glu1113X	p.Glu1113X	sporadic	5	both eyes	no	no
16	28	W	p.Arg991X	p.Arg991X	AR	10	no	no	no
10	62	M	p.Arg962Cys	unknown	sporadic	9	both eyes	no	no
12	52	M	p.Arg336X	unknown	sporadic	3	no	no	no
13	51	M	p.Val1242Met	unknown	sporadic	10	no	no	no
MYO7A[*1]*/PCDH15*[*2]									
4	21	M	p.Ala771Ser[*1]	c.158-1G>A[*2]	sporadic	10	no	unilateral	unilateral
unknown									
14	64	W	unknown	unknown	sporadic	15	both eyes	unilateral	no

*All subjects have congenital deafness and RP.

doi:10.1371/journal.pone.0090688.t002

pathogenecity of mutations identified, functional analysis will be necessary to draw the final conclusion in the future.

In UK and US Caucasian USH1 patients, USH1B (*MYO7A*) has been reported as the most common USH1 genetic subtype [11,12], while USH1F (*PCDH15*) has been reported as the most common USH1 genetic subtype in North American Ashkenazi Jews [23]. In Japanese, our study revealed that the most common type was *MYO7A* (41.7%), which was similar to the frequency in the above Caucasian patients (46.8~55%) [11,12]. However, the small number of USH1 patients in our study might have biased the frequency and further large cohort study will be needed in the future.

In addition, most of our detected mutations were novel. We have previously reported genes responsible for deafness in Japanese patients and observed differences in mutation spectrum between Japanese (who are probably representative of other Asian populations) and populations with European ancestry [24].

In conclusion, our study was the first report of USH mutation analysis using MPS and the frequency of USH1 genes in Japanese. Mutation screening using MPS has the potential power to quickly identify mutations of many causative genes such as USH while maintaining cost-benefit performance. In addition, the simultaneous mutation analysis of large numbers of genes was useful for detecting mutations in different genes that are possibly disease modifiers or of digenic inheritance.

Materials and Methods

Subjects

We screened 17 Japanese USH1 patients (aged 9 to 64 years): three from autosomal recessive families (non-affected parents and two or more affected siblings), and 14 from sporadic families. There were 9 males and 8 females. None of the subjects had any other noteworthy symptoms. All subjects or next of kin 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.

Amplicon Library Preparation

An Amplicon library of the target exons was prepared with an Ion AmpliSeq Custom Panel (Applied Biosystems, Life Technologies, Carlsbad, CA) designed with Ion AmpliSeq Designer (https://www.ampliseq.com/browse.action) for nine USH genes by using Ion AmpliSeq Library Kit 2.0 (Applied Biosystems, Life Technologies) and Ion Xpress Barcode Adapter 1–16 Kit (Applied Biosystems, Life Technologies) according to the manufacturers' procedures.

In brief, DNA concentration was measured with Quant-iT dsDNA HS Assay (Invitrogen, Life Technologies) and Qubit Fluorometer (Invitrogen, Life Technologies) and DNA quality was confirmed by agarose gel electrophoresis. 10 ng of each genomic DNA sample was amplified, using Ion AmpliSeq HiFi Master Mix (Applied Biosystems, Life Technologies) and AmpliSeq Custom primer pools, for 2 min at 99°C, followed by 15 two-step cycles of

Table 3. The patients with mutations in two different genes.

Sample	Genes with two pathogenic mutations	Gene with one heterozygous mutation	Nucleotide change	Amino acid change	control	SIFT score	PolyPhen score	Referense
5	*MYO7A*	*CDH23*	c.C719T	p.P240L*	0.26	0.06	0.999	Wagatsuma et al. (2007)
8	*MYO7A*	*CDH23*	c.2568C>G	p.Ile856Met	0	0.08	1	This study
15	*CDH15*	*USH1C*	c.2437T>G	p.Tyr813Asp	0	0.19	0.932	This study
3	*PCDH15*	*USH1G*	c.28C>T	p.Arg10Trp	0	0.19	1	This study

*homozygotes.
doi:10.1371/journal.pone.0090688.t003

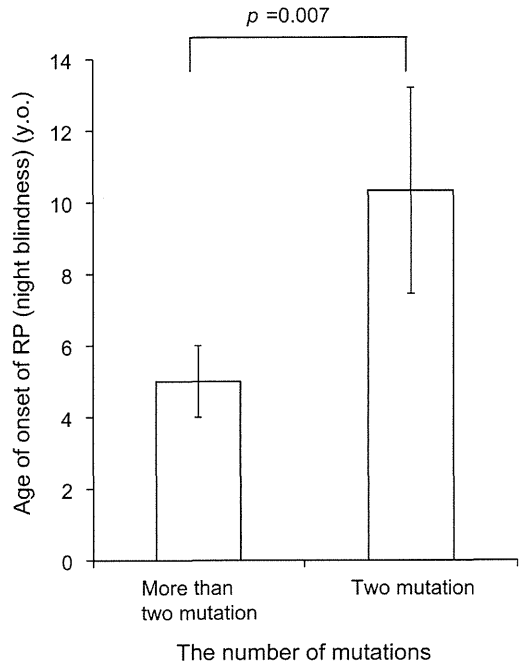

Figure 1. The number of mutations and the age of RP onset in Usher syndrome type 1 patients. The age of RP onset is earlier in the patients with more than two pathogenic mutations. RP: retinitis pigmentosa.
doi:10.1371/journal.pone.0090688.g001

99°C for 15 sec and 60°C for 4 min, ending with a holding period at 10°C in a PCR thermal cycler (Takara, Shiga, Japan). After the Multiplex PCR amplification, amplified DNA samples were digested with FuPa enzyme at 50°C for 10 min and 55°C for 10 min and the enzyme was successively inactivated for 60°C for 20 min incubation. After digestion, diluted barcode adapter mix including Ion Xpress Barcode Adapter and Ion P1 adaptor were ligated to the end of the digested amplicons with ligase in the kit for 30 min at 22°C and the ligase was successively inactivated at 60°C for 20 min incubation. Adaptor ligated amplicon libraries were purified with the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA). The amplicon libraries were quantified by using Ion Library Quantitation Kit (Applied Biosystems, Life Technologies) and the StepOne plus realtime PCR system (Applied Biosystems, Life Technologies) according to the manufacturers' procedures. After quantification, each amplicon library was diluted to 20 pM and the same amount of the 12 libraries for 12 patients were pooled for one sequence reaction.

Emulsion PCR and Sequencing

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

After the Ion Sphere Particle preparation, MPS was performed with an Ion Torrent Personal Genome Machine (PGM) system

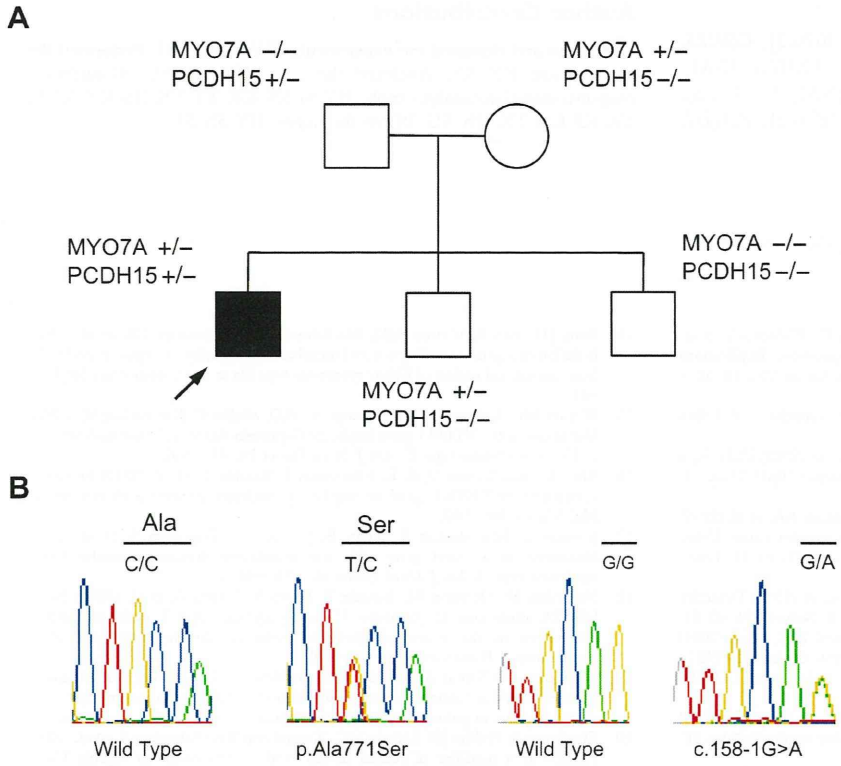

Figure 2. Pedigree and sequence chromatograms of the patient with the p.Ala771Ser in *MYO7A* and c.158-1G>A in *PCDH15* mutations. (A) The pedigree and sequence results of the proband and family. (B) Sequence chromatograms from wild-type and mutations. The proband, his mothor and one brother carried a heterozygous 2311G>T transition in exon 20, which results in an alanine to a serine (Ala771Ser) in *MYO7A*. Another variation, 158-1G>A in intron 3 of *PCDH15*, was derived from the proband and his father. Another brother had no variants.
doi:10.1371/journal.pone.0090688.g002

using the Ion PGM 200 Sequencing Kit and Ion 318 Chip (Life Technologies) according to the manufacturer's procedures.

Base Call and Data Analysis

The sequence data were processed with standard Ion Torrent Suite Software and Torrent Server successively mapped to human genome sequence (build GRCh37/hg19) with Torrent Mapping Alignment Program optimized to Ion Torrent data. The average of 562.33 Mb sequences with about 4,300,000 reads was obtained by one Ion 318 chip. The 98.0% sequences were mapped to the human genome and 94% of them were on the target region. Average coverage of depth in the target region was 314.2 and 93.8% of them were over 20 coverage.

After the sequence mapping, the DNA variant regions were piled up with Torrent Variant Caller plug-in software. Selected variant candidates were filtered with the average base QV (minimum average base quality 25), variant frequency (40–60% for heterozygous mutations and 80–100% for homozygous mutations) and coverage of depth (minimum coverage of depth 10). After the filtrations, variant effects were analyzed with the wANNOVAR web site [25,26] (http://wannovar.usc.edu) including the functional prediction software for missense variants: Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/). The sequencing data was available in the DNA databank of Japan (Accession number: DRA001273).

Algorithm

Missense, nonsense, and splicing variants were selected among the identified variants. Variants were further selected as less than 1% of: 1) the 1000 genome database (http://www.1000genomes.org/), 2) the 5400 exome variants (http://evs.gs.washington.edu/EVS/), and 3) the in-house control. Candidate mutations were confirmed by Sanger sequencing and the responsible mutations were identified by segregation analysis using samples from family members of the patients. In addition, the cases with heterozygous or no causative mutation were fully sequenced by Sanger sequencing for USH1 genes in order to verify the MPS results.

Direct Sequence Analysis

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

Accession numbers

MYO7A, [NM_000260.3]; *USH1C*, [NM_ 153676.3]; *CDH23*, [NM_ 022124.5]; *PCDH15*, [NM_ 033056.3]; *USH1G*, [NM_ 173477.2]; *USH2A*, [NM_206933.2]; *GPR98*, [NM_ 032119.3]; *DFNB31*, [NM_ 015404.3]; *CLRN1*, [NM_ 174878.2]; *PDZD7*, [NM_ 001195263.1].

Acknowledgments

We thank A. C. Apple-Mathews for help in preparing the manuscript.

Author Contributions

Conceived and designed the experiments: HY SI SN SU. Performed the experiments: HY SN. Analyzed the data: HY SN SU. Contributed reagents/materials/analysis tools: HY SI SN KK TT YK HS KN KI TI YN KF CO TK HN SU. Wrote the paper: HY SN SU.

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