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# IV.　研究成果の刊行物・別刷

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

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## Abstract

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

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

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

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

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

## Background

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

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

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

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

## Methods

### Subjects

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

### Mutation analysis

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

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

## Results

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

## Discussion

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

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

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

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

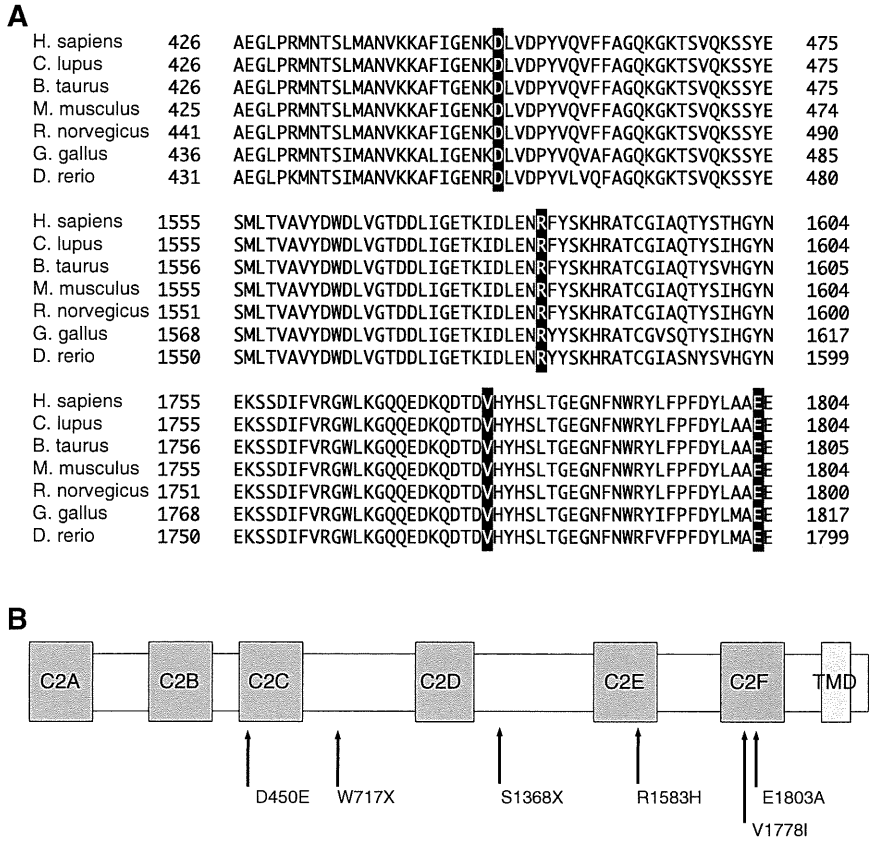


**Figure 1 The location of mutations in otoferlin protein and the evolutionary conservation of the amino acids. (A)** Evolutionary conservation. The locations of mutations are boxed. **(B)** Novel pathogenic *OTOF* mutations found in this work and relation to the functional domains of otoferlin. C2A-F: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

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

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

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

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

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

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

**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.

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# Massively Parallel DNA Sequencing Successfully Identifies New Causative Mutations in Deafness Genes in Patients with Cochlear Implantation and EAS

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## Abstract

Genetic factors, the most common etiology in severe to profound hearing loss, are one of the key determinants of Cochlear Implantation (CI) and Electric Acoustic Stimulation (EAS) outcomes. Satisfactory auditory performance after receiving a CI/EAS in patients with certain deafness gene mutations indicates that genetic testing would be helpful in predicting CI/EAS outcomes and deciding treatment choices. However, because of the extreme genetic heterogeneity of deafness, clinical application of genetic information still entails difficulties. Target exon sequencing using massively parallel DNA sequencing is a new powerful strategy to discover rare causative genes in Mendelian disorders such as deafness. We used massive sequencing of the exons of 58 target candidate genes to analyze 8 (4 early-onset, 4 late-onset) Japanese CI/EAS patients, who did not have mutations in commonly found genes including *GJB2, SLC26A4*, or mitochondrial 1555A>G or 3243A>G mutations. We successfully identified four rare causative mutations in the *MYO15A, TECTA, TMPRSS3,* and *ACTG1* genes in four patients who showed relatively good auditory performance with CI including EAS, suggesting that genetic testing may be able to predict the performance after implantation.

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## Introduction

Cochlear Implantation (CI) has been established as a standardized therapy for severe to profound hearing loss [1]. Electric Acoustic Stimulation (EAS) is a hearing implant system combining a cochlear implant and acoustic amplification technology in one device, and has recently become a standard intervention for the patients with partial deafness, defined as a mild to moderate low-frequency sensorineural hearing loss sloping to a profound hearing loss in the higher frequencies [1]. One difficult point is that outcomes of CI/EAS are variable and many factors are thought to be involved in post-implantation performance. Satisfactory auditory performance in the patients with various deafness gene mutations indicates that genetic background would be helpful in predicting performance after CI [2]. When genetic background is involved in intra-cochlear etiology, there is potential for good performance. Therefore, it is important to identify the involved region inside/outside of the cochlea by identifying the responsible gene. Decisions as to whether to undergo EAS surgery and the timing of the surgery, as well as prediction of outcome after EAS is sometimes difficult because of individual differences in progression, which is sometimes of a rather rapid nature but sometimes rather stable. One advantage of genetic testing is that the possible prognosis for hearing, i.e., progressive or not, can be predicted for individual patients.

Etiological studies have shown genetic disorders to be a common cause of deafness, but difficulty lies in the fact that deafness is an extremely heterogenous disorder.

Invader-based multi-gene screening for 13 genes/46 mutations commonly found in Japanese, identified the responsible mutations in approximately 30% of deafness patients [3], accelerating the clinical application of gene screening. However, the etiology of the rest of the patients is still unknown. In addition, the involvement of at least 58 distinct genes sometimes makes the precise diagnosis difficult.

Targeted exon sequencing of selected genes using the Massively Parallel DNA Sequencing (MPS) technology will potentially enable us to systematically tackle previously intractable monogenic disorders and improve molecular diagnosis. We have recently reported that target exon sequencing using MPS is a powerful tool to identify rare gene mutations for deafness patients [4].

In this study, we have chosen 58 deafness-causative genes, and conducted genetic analysis using MPS-based genetic screening to find the rare genes responsible for the patients who received CI or EAS.

## Subjects and Methods

### Subjects

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

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

### Amplicon Library Preparation

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

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

### Emulsion PCR and Sequencing

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

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

### Base Call and Data Analysis

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

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

### Algorithm

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

### Direct Sequence Analysis

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

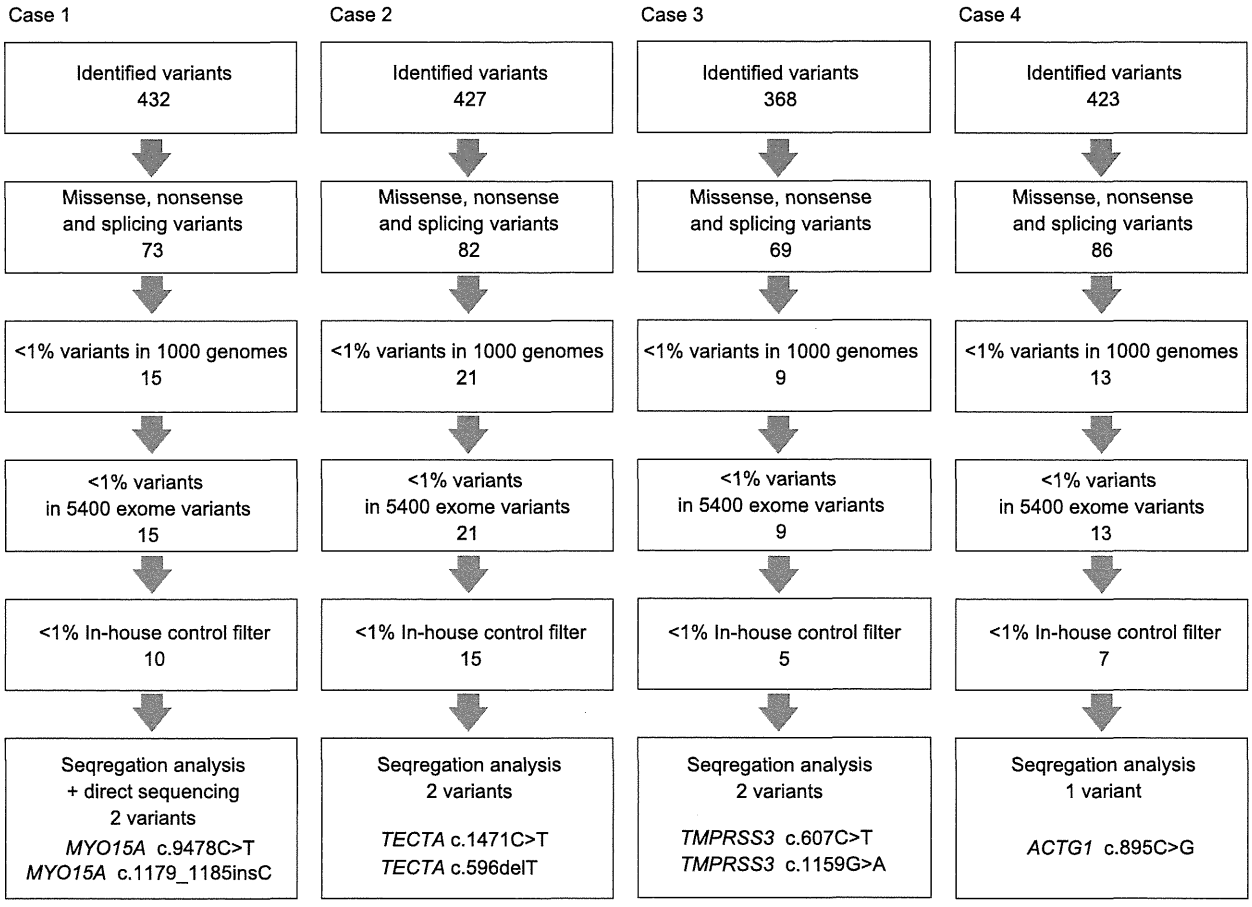


**Figure 1. Flow of informatics analysis.** Selected missense, nonsense, and splicing variants were filtered with 1) the 1000 genomes, 2) the 5400 exome variants, and 3) the in-house control. Responsible mutations were confirmed by segregation analysis.
doi:10.1371/journal.pone.0075793.g001

## Results

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

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

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

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

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

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

**Table 1.** Missense mutations found in this study.

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

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

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

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

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

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

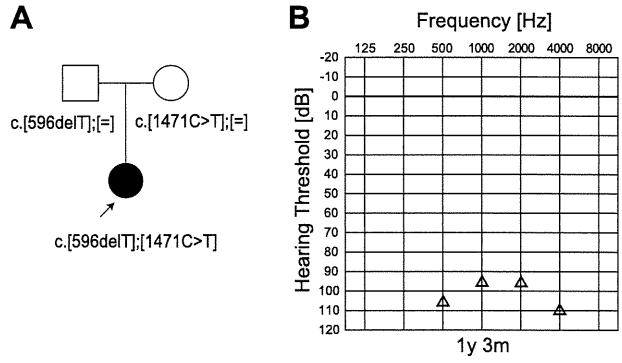


**Figure 3. The CI patient with *TECTA* mutations.** A: The patient has compound heterozygous *TECTA* mutations (c.[596delT];[1471C>T]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 9 m).
doi:10.1371/journal.pone.0075793.g003

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

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

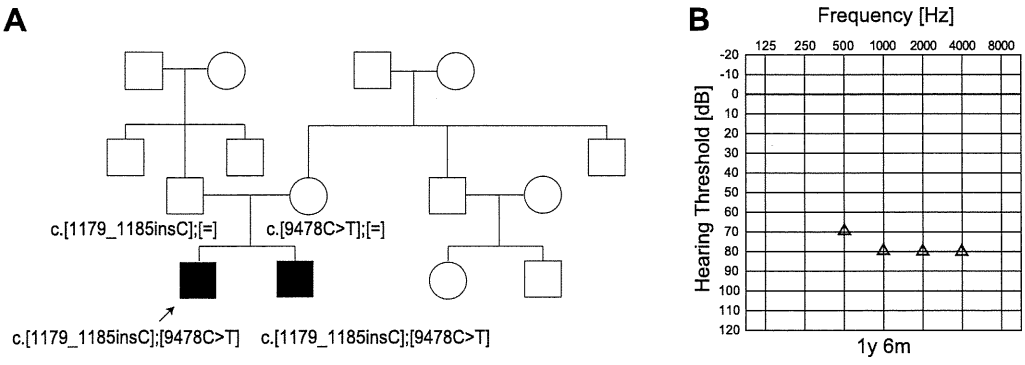


**Figure 2. The CI patient with *MYO15A* mutations.** A: The patient has compound heterozygous *MYO15A* mutations (c.[9478C>T];[1179_1185insC]), and the parents were found to be carriers for these mutations. B: COR audiogram finding (1y 6 m).
doi:10.1371/journal.pone.0075793.g002

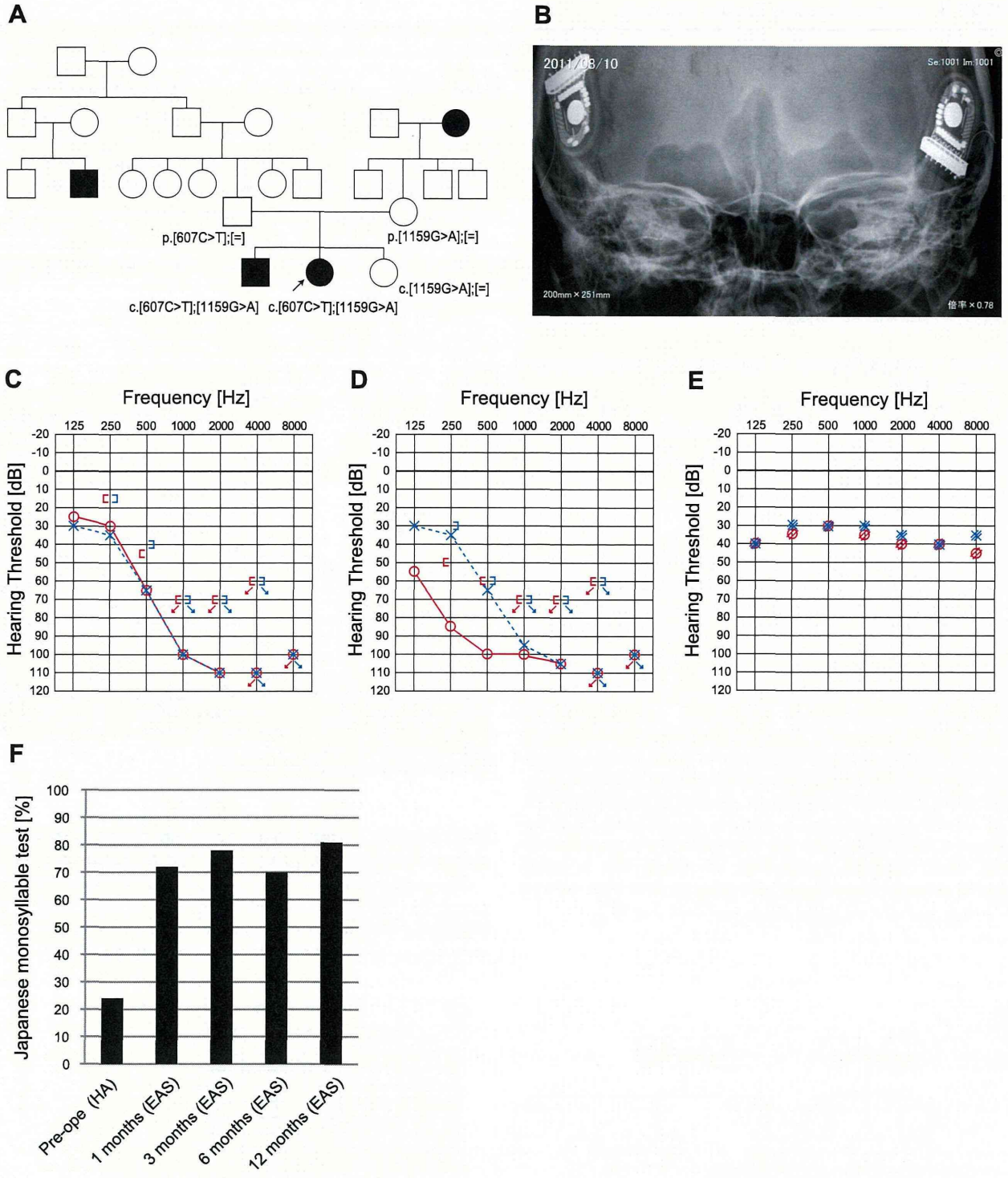


**Figure 4. The EAS patient with *TMPRSS3* mutations.** A: The patient has compound heterozygous *TMPRSS3* mutations, c.[607C>T];[1159G>A], and the parents were found to be carriers for these mutations. The patient's brother also has the same mutations. B: X-ray imaging after bilateral EAS. C: Pre-operative audiogram. D: Post-operative audiogram (left: 24 months after first EAS, right: 4 months after second EAS). E: Hearing threshold with bilateral EAS. F: Japanese monosyllable test (65dB SPL in quiet) showing dramatic improvement with bilateral EAS.
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software programs (Table 1). He noticed his hearing loss at around age 20. He received EAS due to progressive hearing loss. Residual hearing for acoustic amplification could be preserved, and hearing level with bilateral EAS was around 30dB (Fig. 5B, D, E). Japanese

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

## Discussion

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

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

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

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

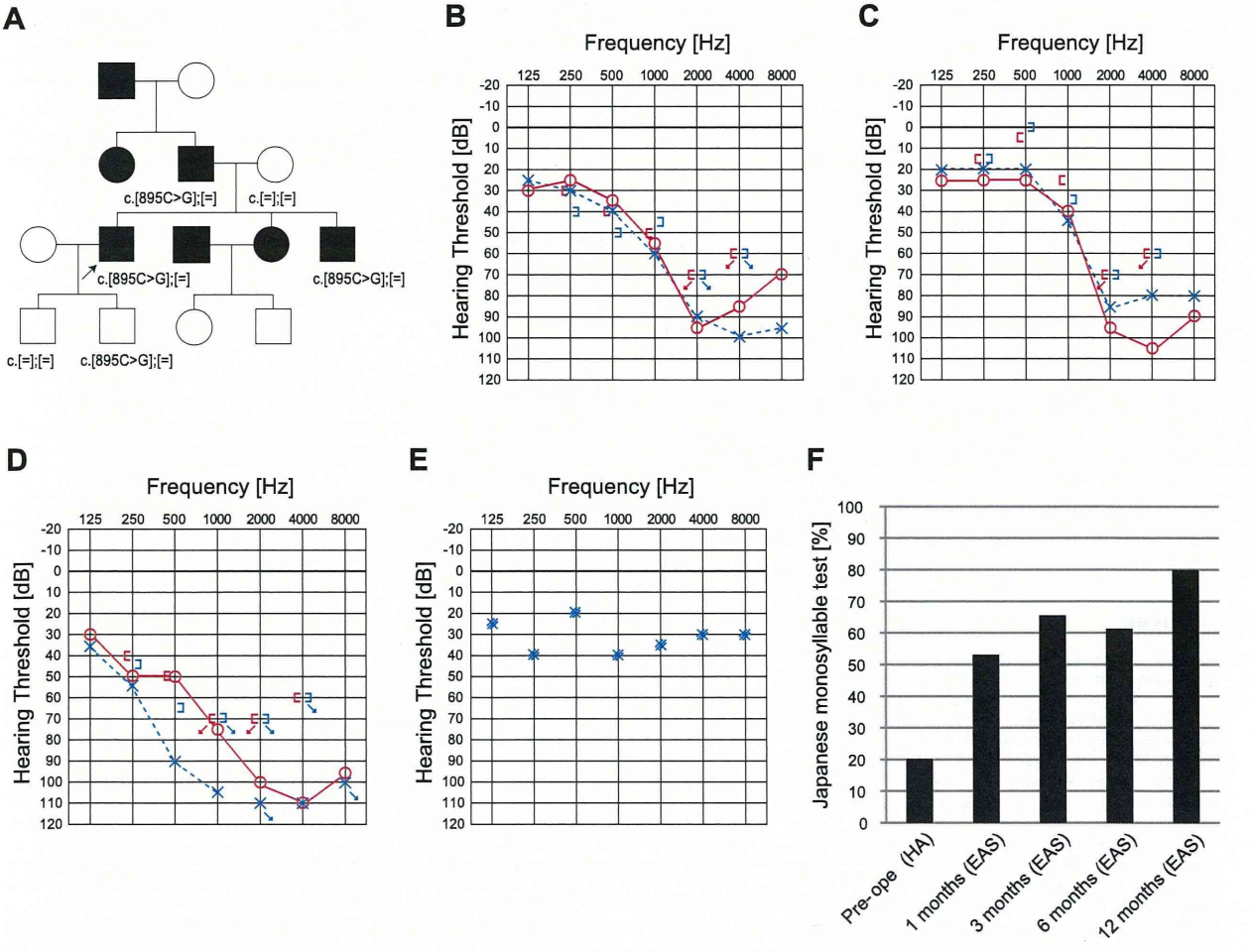


**Figure 5. The EAS patient with *ACTG1* mutation.** A: The patient has heterozygous *ACTG1* mutation, c.895C>G. Pedigree is compatible with autosomal dominant hearing loss. His father and brother carried the same mutation. B: Pre-operative audiogram. C: Audiogram of brother. D: Post-operative audiogram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

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

*TMPRSS3* has been reported to be responsible for DFNB8/10. Typically, the patients show ski-slope type audiograms and progressive HL [16], being compatible with the phenotype of the present patient. Outcome of CI for patients with *TMPRSS3* is controversial [2,16,17]. Two older papers reported good outcome of CI, while a recent report described poorer performance. Eppsteiner et al. [2] reported two cases of 58-year-old patients with a history of progressive hearing loss starting at the age of 5–6 years. Both of their outcomes were poorer compared with other patients, and the authors hypothesized that it was because the encoded protein is also expressed in the spiral ganglion. However, the present 40-year-old patient showed completely different performance after EAS, indicating that CI is not a contraindication and CI and/or EAS can be a recommended therapeutic option. Especially, the previously reported typical phenotype is high frequency involved hearing loss, which is a good indication for EAS. In the literature, there is also a severe phenotype with all frequencies affected [18]. Our 40-year-old patient did not have rapid progressive hearing loss (only 24 dB (125+250+500 Hz/3) during the 7-year follow-up period), supporting that this patient was a good candidate for EAS. Within this family, intra-familial variation was observed, i.e., an elder brother with the same mutations showed early onset (10 y.o.) profound hearing loss. Therefore, other factors may also potentially be involved in determining the phenotype (including severity and progression).

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

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

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

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

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

## Supporting Information

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

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## Author Contributions

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

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

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## Abstract

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

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## Introduction

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

## Materials and Methods

### Subjects

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

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

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

## Targeted Enrichment and DNA Sequencing

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

## Mapping and Filtering

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

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

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