Acta Oto-Laryngologica, 2012; 132: 1160–1167

informa
healthcare

ORIGINAL ARTICLE

Unilateral cochlear nerve hypoplasia in children with mild to moderate hearing loss

HIDENOBU TAIJI[1], NORIKO MORIMOTO[1] & TATSUO MATSUNAGA[2]

[1]*Department of Otorhinolaryngology, National Center for Child Health and Development and* [2]*Laboratory of Auditory Disorders, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan*

Abstract

Conclusion: Even if hearing loss is mild to moderate, the presence of cochlear nerve (CN) hypoplasia associated with retrocochlear disorders should be considered. *Objectives:* CN hypoplasia is a term that refers to an absent cochlear nerve on high-resolution magnetic resonance imaging (MRI). Most cases of CN hypoplasia are associated with profound hearing loss. The present study reports six pediatric cases of unilateral CN hypoplasia with mild to moderate hearing loss. *Methods:* Between May 2008 and April 2011, pure-tone hearing tests were performed in 17 patients who were diagnosed with CN hypoplasia on high resolution for evaluation of unilateral sensorineural hearing loss at the National Center for Child Health and Development. Of these, six patients had average hearing levels in the affected ears of < 60 dB and were therefore included in this study. *Results:* All six ears with CN hypoplasia were associated with CN canal stenosis. DPOAEs were present in one (17%) of the six affected ears. The ABR thresholds of the ears with CN hypoplasia were significantly elevated compared with 1–4 kHz pure-tone hearing levels in one of three cases. In two of five cases, the maximum word recognition scores of the affected ears were poor compared with pure-tone hearing levels.

Keywords: *3-D constructive interference, steady-state magnetic resonance imaging, word recognition score, sensorineural hearing loss*

Introduction

Cochlear nerve (CN) hypoplasia refers to the absence of a visible CN on oblique sagittal magnetic resonance (MR) images of the lateral aspect of the inner auditory canal (IAC). CN hypoplasia is not an uncommon cause of congenital hearing loss as previously thought [1,2]. Although it is believed that most cases of CN hypoplasia are associated with profound hearing loss [1], a recent report presented a case of CN hypoplasia with moderate hearing loss limited to high frequencies [3]. The present study reports six pediatric cases of unilateral CN hypoplasia with mild to moderate hearing loss, and demonstrates that retrocochlear hearing loss is the predominant audiologic characteristic in these children.

Material and methods

Patient population

Between May 2008 and April 2011, 25 children who presented for evaluation of unilateral sensorineural hearing loss (SNHL) at the National Center for Child Health and Development were diagnosed with CN hypoplasia on high-resolution MR imaging (MRI).

Pure-tone hearing tests could be performed in 17 of these 25 children. Pure-tone audiometry was evaluated based on the three-tone average formulated by (a+b+c)/3, where a, b, and c are hearing levels at 0.5, 1, and 2 kHz, respectively. Eleven cases had profound hearing loss in the affected ears, with average hearing levels of >90 dB. The average hearing levels

Correspondence: Hidenobu Taiji MD, Department of Otorhinolaryngology, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan. Tel: +81 3 3416 0181. Fax: +81 3 5494 7909. E-mail: taijih5@gmail.com

(Received 20 April 2012; accepted 12 May 2012)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.3109/00016489.2012.696781

Table I. Audiologic and radiologic findings in children with unilateral cochlear nerve (CN) hypoplasia.

			Audiologic findings in affected ear				CNC diameter (mm)	
Case no.	Age (years)/sex	Side	Pure-tone hearing level (dB)	ABR thresholds (dB nHL)	Maximum speech discrimination (%)	DPOAE	Affected ear	Healthy ear
1	6/F	R	41	80	25	Absent	1.2	1.7
2	5/F	L	35	90	60	Absent	0.7	1.9
3	8/F	R	59	–	70	Absent	0.5	1.7
4	8/M	R	38	–	90	Normal	1.3	2.0
5	13/F	L	59	–	50	Absent	1.0	2.1
6	4/F	R	40	60	–	Absent	0.3	1.8

–, not evaluated; ABR, auditory brainstem response; CNC, cochlear nerve canal; DPOAE, distortion product otoacoustic emission; F, female; M, male.

of the affected ears in the remaining six (24%) cases were <60 dB (Table I). These six patients were therefore included in this study for further analysis.

The six pediatric cases consisted of one boy and five girls with a mean age of 7.3 years (range, 4–13 years). None of the children had known syndromes that could cause hearing loss or risk factors of hearing loss such as a history of prematurity, hypoxia, and hyperbilirubinemia.

Imaging

High-resolution computed tomography (HRCT) of the temporal bone in all patients was performed with

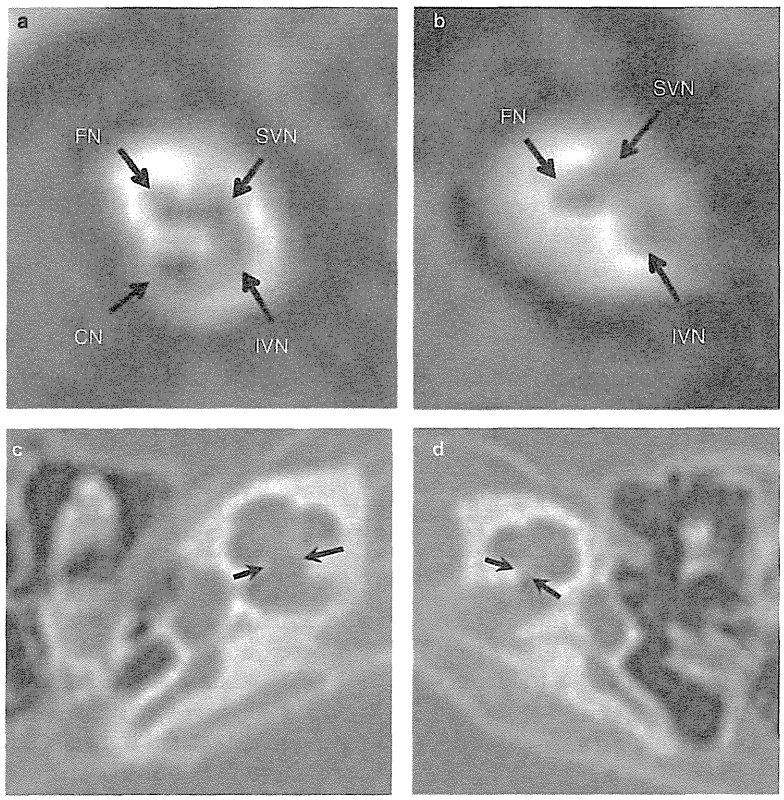

Figure 1. Cochlear nerve hypoplasia in a 5-year-old girl with left sensorineural hearing loss (SNHL) (case 2). (a, b) Oblique sagittal magnetic resonance (MR) images of the inner auditory canal (IAC). Four nerves were detected in the right (a), while the left cochlear nerve is not visible (b). CN, cochlear nerve; FN, facial nerve; IVN, inferior vestibular nerve; SVN, superior vestibular nerve. (c, d) Axial images of temporal bone high-resolution computed tomography (HRCT) show narrowing of left cochlear nerve canal (CNC) (d, 0.7 mm) compared with the right CNC (c, 1.9 mm).

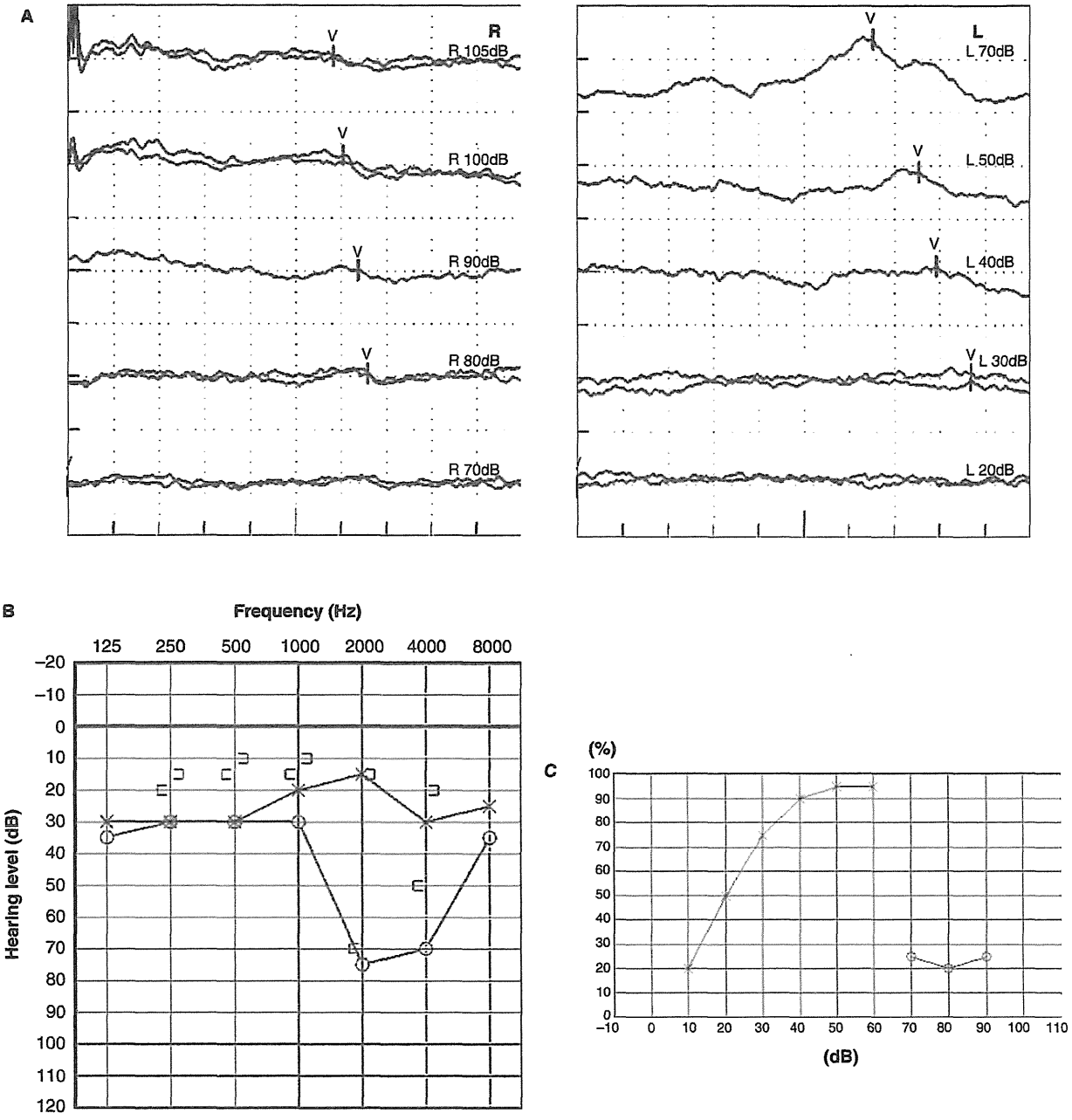

Figure 2. Audiologic findings in a child with right-sided cochlear nerve hypoplasia (case 1). (A) Auditory brainstem responses (ABRs), (B) pure-tone audiogram, (C) word recognition curve.

a multidetector-row CT scanner (8-detector, Lightspeed Ultra, GE, Milwaukee, USA). Images were acquired in the direct axial planes using a 0.652 mm slice thickness. The diameter of the CN canal (CNC) was measured along the inner margin of its bony walls at its middle portion on the axial image of the base of the modiolus.

MR images were obtained using a 1.5 Tesla system (Intera 1.5T; Philips, Belgium) according to a protocol described previously in detail [4]. The MRI scans included 3-D T2-weighted fast spin-echo sequences in axial and oblique sagittal images of the IAC with a 0.7 mm slice thickness. The 3-D constructive interference in steady-state (CISS) images was then reconstructed by traversing the IAC in a perpendicular orientation, producing images that visualized the four nerves (facial, superior vestibular, inferior vestibular, and cochlear). The

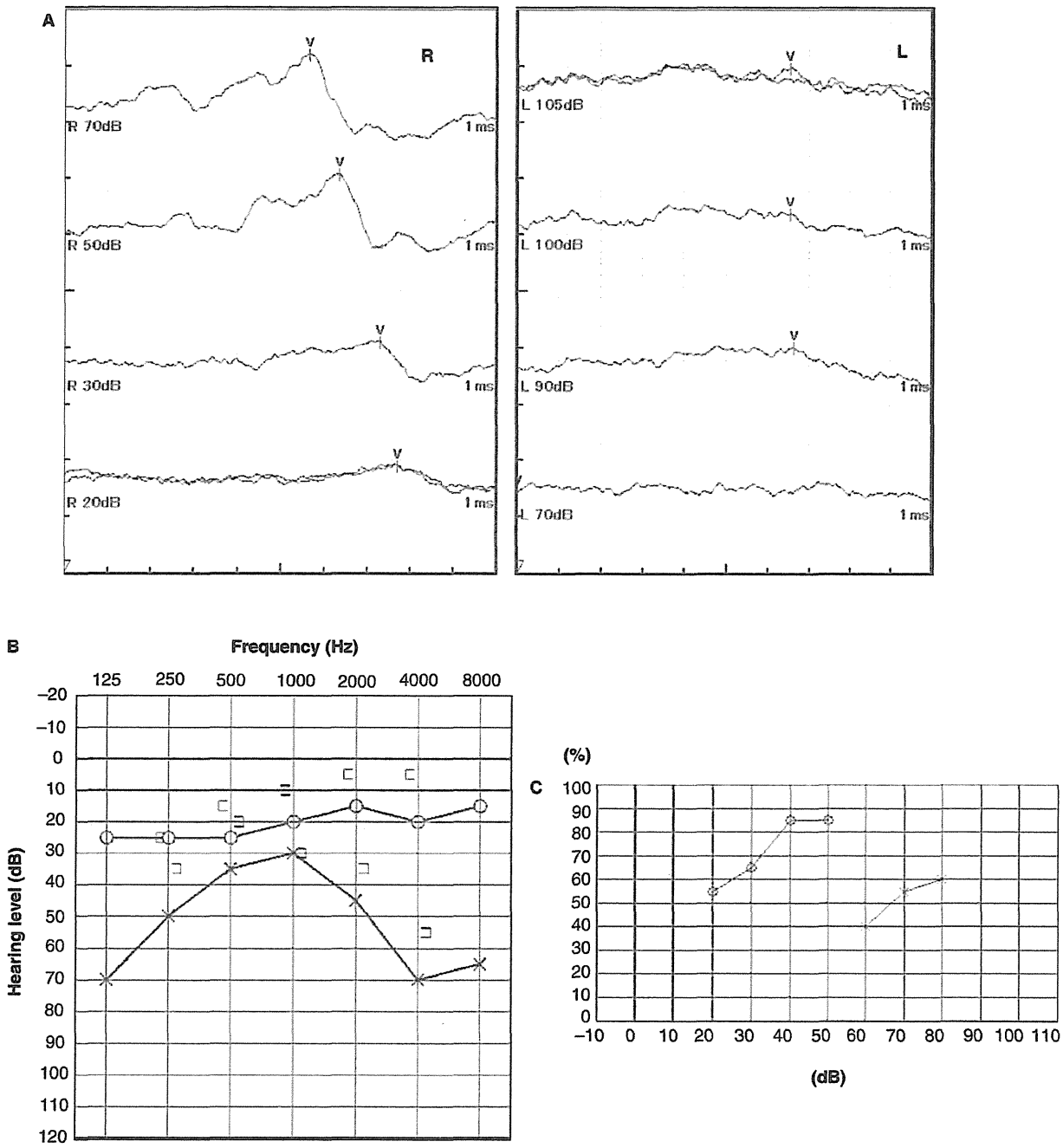

Figure 3. Audiologic findings in a child with left-sided cochlear nerve hypoplasia (case 2). (A) Auditory brainstem responses (ABRs), (B) pure-tone audiogram, (C) word recognition curve.

findings of a normal ear are shown in Figure 1a, b, (case 2, right ear).

Audiologic assessment

In addition to the pure-tone hearing test, distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR) testing, as well as speech audiometry were performed. DPOAEs were measured in all subjects for pairs of primary tones (f1 and f2), with a fixed ratio of f2/f1 = 1.2, and fixed levels of 65 dB SPL (L1) and 55 dB SPL (L2) using the ILO292 system (Otodynamics, UK). The frequency of f2 was stepped through a range of 1–6 kHz to yield a nine-point DPGram.

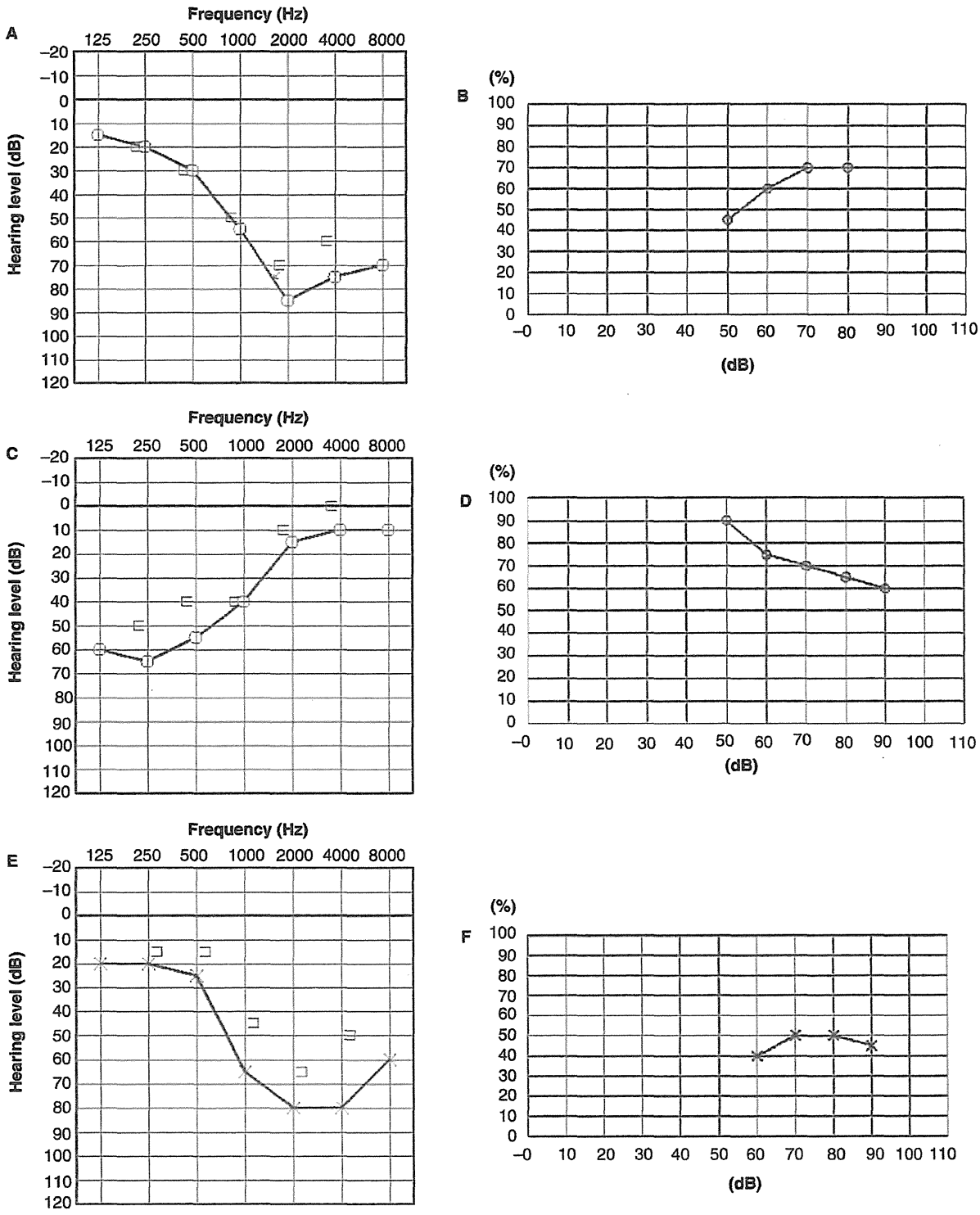

Figure 4. Audiologic findings in ears with cochlear nerve hypoplasia (cases 3–5). (a, c, e) Pure-tone audiograms: (a) case 3, (c) case 4, (e) case 5. (b, d, f) Word recognition curves: (b) case 3, (d) case 4, (f) case 5.

ABRs were recorded in three subjects using the MEB-2204 system (Nihon Koden, Japan). The 0.1 ms clicks with alternating polarity were presented monaurally at a repetition rate of 10 Hz and a maximum intensity of 105 dB nHL.

Speech audiometry was performed in five subjects, but not in case 6 where the test could not be done. The maximum word recognition scores were evaluated based on the percentage of correct answers out of 20 words, using Japanese word list 67-S. In normal-hearing subjects, the maximum word recognition scores using the word list are usually ≥90%.

Results

A summary of the findings for the six children with unilateral CN hypoplasia diagnosed by MRI is shown in Table I. On the oblique sagittal MRI image of case 2, the left CN is undetectable, while the right CN is normal (Figure 1a, b). The hearing levels of the affected ears in the six cases ranged from 35 to 59 dB (Table I). The audiogram shapes (Figures 2, 3, and 4) were high-frequency sloping (cases 3, 5, and 6), rising (case 4), 2–4 kHz notch (case 1), and inverted scoop shape (case 2). DPOAEs were present in only one (17%) of the six affected ears, and the shape of the hearing loss curve for that ear was rising.

In one of the three cases in which ABR testing was performed, the ABR threshold of the ear with CN hypoplasia was significantly elevated compared with that expected from 1000–4000 Hz pure-tone hearing levels (case 2). Speech discrimination tests were performed in five cases (Figures 2c, 3c, and 4b, d, f) but not in the 4-year-old patient (case 6). In two of these five cases (cases 1 and 2), the maximum word recognition scores of the ears with CN hypoplasia were poor compared with those of pure-tone hearing levels. In one case (case 4), the maximum word recognition score of the affected ear was 90%, but the results for 50 dB and 90 dB were 90% and 60%, respectively. Therefore, the word recognition curve in case 4 showed marked roll-over.

The click-evoked ABR tracing, pure-tone audiogram, and word recognition curves for case 1 are shown in Figure 2. The pure-tone audiogram of the right ear (affected side) showed a 2–4 kHz notch configuration. The wave V threshold of the right ear was elevated (80 dB nHL). The ABR threshold compared with the 1–4 kHz pure-tone hearing level of the right ear was slightly higher than expected. The maximum word recognition score of the right ear was 25%, which was lower than expected for pure-tone hearing.

The click-evoked ABR tracing, pure-tone audiogram, and word recognition curves for case 2 are shown in Figure 3. The pure-tone audiogram of the left ear (affected side) showed an inverted scoop shape. The ABR threshold of the left ear was 90 dB nHL and the maximum word recognition score of the left ear was 60%. These values were smaller than expected for pure-tone hearing.

All six ears with CN hypoplasia were associated with CNC stenosis (CNC diameter, <1.5 mm; mean, 0.83 mm). In contrast, the unaffected ears of the six children had CNC diameters of >1.5 mm (mean, 1.9 mm). Axial HRCT images of a representative case (case 2) of severe CNC stenosis are shown in Figure 1c and d. No cochlear malformations were seen in the six children.

Discussion

We defined CN hypoplasia as an undetectable CN on axial, coronal, or reconstructed oblique sagittal MR images. An extremely small nerve, below the limits of resolution of MRI, could appear absent and therefore should not be disregarded. Therefore, we avoid the terms deficiency, aplasia, and agenesis.

The mechanism of CN hypoplasia in children remains speculative. Both congenital deficiency and acquired degeneration of the CN might be seen in children with SNHL [5]. In pediatric cases, it is possible that a vascular insult during critical periods in development may result in isolated CN agenesis or degeneration [1]. CN hypoplasia is often associated with cochlear anomalies [4] or various coexisting syndromes such as CHARGE association [1]. Neither cochlear malformations nor known syndromes were recognized in the patients presented in this report.

CN hypoplasia is not as uncommon as previously thought [1]. Recent studies suggest that CN dysfunction accounts for up to 10% of diagnosed cases of pediatric SNHL [2]. Miyasaka et al. [4] reported CN hypoplasia in 8 of 42 (19%) ears on MRI. Of these, four ears had inner ear malformations. In the present study, no cochlear malformations were recognized in the six ears with CN hypoplasia. In addition, a relationship between CNC stenosis and CN hypoplasia was previously reported [2,6,7]. CNC diameter measurements of <1.8 mm were considered moderate stenosis, while measurements of <1.0 mm were defined as critical stenosis [8]. A CNC diameter of <1.5 mm on CT suggested CN hypoplasia [4,6,7]. All six ears with CN hypoplasia in this study were associated with CNC stenosis (CNC diameter, <1.5 mm). And also 11 ears with CN hypoplasia with profound hearing loss were associated with CNC stenosis. The mean (SD) CNC diameter was 0.83 (0.40) mm in the affected side of 6 cases with mild to moderate hearing loss, and 0.72 (0.32) mm in the affected side of 11 cases with profound hearing

loss. The CNC diameters in the group with profound hearing loss were slightly narrower than in the group with mild to moderate hearing loss, but there was no statistically significant difference ($p = 0.28$, t test). CNC may require stimulation by its contents for normal development, meaning that CNC stenosis may occur secondary to CN hypoplasia [6]. However, it was previously reported that CNC stenosis can occur without CN hypoplasia [4]. CNC stenosis on CT may therefore be indicative of the diagnosis of CN hypoplasia, but MRI should be performed to confirm the diagnosis.

In the past, it was thought that CN hypoplasia was always associated with profound SNHL [1], but a case of CN hypoplasia without profound hearing loss was reported recently [3]. In that case, an extremely small, preserved, and partially functional CN was believed to be present in the affected ear [3]. A minimal number of residual CN fibers, which were too small to be detected by MRI, may be enough to deliver sound information without threshold elevation [8].

CN hypoplasia may present as auditory neuropathy spectrum disorder (ANSD) [1,9]. In this study, DPOAEs were detected in one of six cases, which indicated normal outer hair cell function. In the 11 cases of CN hypoplasia with profound hearing loss, DPOAEs at the affected ears were detected in four cases (36%). The presence rates of DPOAEs were supposed not to relate to hearing levels. The reason for absent DPOAEs in cases of mild hearing loss is unclear, but malformations of inner ear microstructures associated with congenital CN hypoplasia are considered to be the cause of absent DPOAEs. The shape of the hearing loss curve in the case with normal DPOAE was rising (Figure 4c), and the pure-tone thresholds at 2–8 kHz were ≤15 dB. The normal DPOAE response in this case is assumed to indicate the preservation of inner ear function at high frequencies.

In case 2, who was one of three cases in which ABR testing was performed, the ABR threshold of the affected ear was significantly elevated compared with that expected from 1–4 kHz pure-tone hearing levels. The elevated ABR threshold in the case suggests disorders of CN synchrony at high frequencies.

Speech discrimination assessments showed poor maximum word recognition scores compared with that expected from pure-tone hearing levels in two affected ears. In addition, the word recognition curve of an affected ear had marked roll-over. The results of the speech discrimination tests suggested retrocochlear disorders in the affected ears. Some of the findings of the ABR and the word recognition tests in CN hypoplasia are consistent with audiologic characteristics of ANSD, which has been reported as retrocochlear hearing loss in CN hypoplasia [1]. It is believed that 6–28% of ANSD cases are due to CN hypoplasia [1,10,11]. CT is recommended for the initial screening of children with SNHL [4]. For children with ANSD, high-resolution MRI of the CN should be performed as the initial imaging study [12]. The results of the present study suggest that the imaging study for the screening of CN hypoplasia is desirable for even mild to moderate hearing loss.

Conclusion

Here, we presented six pediatric cases of CN hypoplasia with mild to moderate hearing loss. Audiologic characteristics of some ears with CN hypoplasia in this study suggested retrocochlear disorders. Even if hearing loss is mild to moderate, the presence of CN hypoplasia associated with retrocochlear disorders should be considered.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Buchman CA, Roush PA, Teagle FB, Brown CJ, Zdanski CJ, Grose JH. Auditory neuropathy characteristics in children with cochlear nerve deficiency. Ear Hear 2006; 27:399–408.

[2] Adunka OF, Roush PA, Teagle HF, Brown CJ, Zdanski CJ, Jewells V, et al. Internal auditory canal morphology in children with cochlear nerve deficiency. Otol Neurotol 2006;27: 793–801.

[3] Miyanohara I, Miyashita K, Takumi K, Nakajo M, Kurono Y. A case of cochlear nerve deficiency without profound sensorineural hearing loss. Otol Neurotol 2011; 32:529–32.

[4] Miyasaka M, Nosaka S, Morimoto N, Taiji H, Masaki H. CT and MR imaging for pediatric cochlear implantation: emphasis on the relationship between the cochlear nerve canal and the cochlear nerve. Pediatr Radiol 2010;40: 1509–16.

[5] Glastonbury CM, Davidson HC, Harnsberger HR, Butker J, Kertesz TR, Shelton C. Imaging findings of cochlear nerve deficiency. AJNR 2002;23:635–43.

[6] Kono T. Computed tomographic features of the bony canal of the cochlear nerve in pediatric patients with unilateral sensorineural hearing loss. Radiat Med 2008;26:115–19.

[7] Komatsubara S, Haruta A, Nagano Y, Kodama T. Evaluation of cochlear nerve imaging in severe congenital sensorineural hearing loss. ORL J Otorhinolaryngol Relat Spec 2007;69:198–202.

[8] Valero J, Blaser S, Papsin BC, James AL, Gordon KA. Electrophysiologic and behavioral outcomes of cochlear implantation in children with auditory nerve hypoplasia. Ear Hear 2012;33:3–18.

[9] O'Leary SJ, Gibson WP. Surviving cochlear function in the presence of auditory nerve agenesis. J Laryngol Otol 1999; 113:1008–10.

[10] Walton J, Gibson WP, Sanli H, Prelog K. Predicting cochlear implant outcomes in children with auditory neuropathy. Otol Neurotol 2008;29:302–9.

[11] Teagle HF, Roush PA, Woodard JS, Hatch DR, Zdanski CJ, Buss E, et al. Cochlear implantation in children with auditory neuropathy spectrum disorder. Ear Hear 2010;31:325–35.

[12] Roche JP, Huang BY, Castillo M, Bassim MK, Adunka OF, Buchman CA. Imaging characteristics of children with auditory neuropathy spectrum disorder. Otol Neurotol 2010;31:780–8.

OPEN ACCESS Freely available online

PLOS | ONE

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

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

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

Abstract

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

Citation: Naito T, Nishio S-y, Iwasa Y-i, Yano T, Kumakawa K, et al. (2013) Comprehensive Genetic Screening of *KCNQ4* in a Large Autosomal Dominant Nonsyndromic Hearing Loss Cohort: Genotype-Phenotype Correlations and a Founder Mutation. PLoS ONE 8(5): e63231. doi:10.1371/journal.pone.0063231

Editor: Klaus Brusgaard, Odense University hospital, Denmark

Received October 22, 2012; **Accepted** April 2, 2013; **Published** May 23, 2013

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 (http://www.mhlw.go.jp/english/) (S.U.), by the Acute Profound Deafness Research Committee of the Ministry of Health, Labour and Welfare of Japan (http://www.mhlw.go.jp/english/) (S.U.), by a Health and Labour Sciences Research Grant for Research on Specific Diseases (Vestibular Disorders) from the Japanese Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/english/) (S.U.), and by a Grant-in-Aid for Scientific Research from the 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.

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

Introduction

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

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

Materials and Methods

Subjects and clinical evaluation

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

Ethics Statement

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

Mutation analysis

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

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

Haplotype analysis

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

Statistical analysis of progression of hearing loss

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

Results

Mutation analysis

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

Frequency of KCNQ4 mutations

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

Haplotype analysis

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

Clinical characteristics

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

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

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

SIFT, Polyphen-2, PhyloP, LRT, Mutation Taster, and GERP++ are functional prediction scores in which increasing values indicate a probable mutation.
Abbreviations: Chr, chromosomes; P2, PolyPhen2; MutTaser, Mutation Taser; Fm, family; cyto, cytoplasmic; trans, transmembrane; PR, Pore region; (P), P-loop; T, tolerated; D, damaging or deleterious; B, benign; Pr, probably damaging; Po, possibly damaging; C, conserved; N, neutral. Neth, Netherlands; *, Novel mutations found in this study.
doi:10.1371/journal.pone.0063231.t001

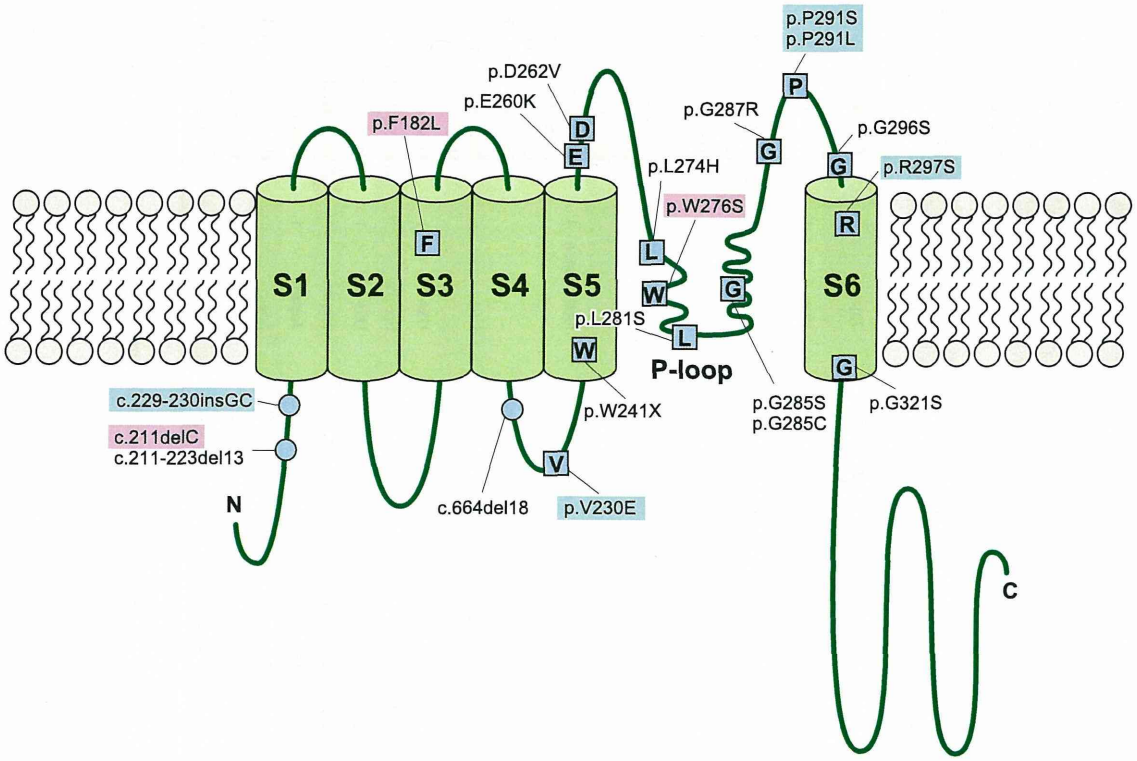

Figure 1. Localization of 20 *KCNQ4* mutations reported in previous studies in the protein. The 6 transmembrane domains (S1–S6) and the P-loop, located between S5 and S6, are shown. 5 mutations are concentrated in a narrow P-loop range. Mutations with pink and blue shadows; possible mutations detected in this study. Blue indicates novel mutations. Original schema is modified from Mencía A (2008) [14].
doi:10.1371/journal.pone.0063231.g001

from 3 to 40 years old, though the majority became aware when in their teens or younger. Most patients had associated tinnitus, but no vestibular symptoms except in a few cases.

Genotype/phenotype correlations

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

Therapeutic intervention

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

Progression analysis

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

Discussion

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

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

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

Figure 2. The haplotypes around c.211delC mutation of six families constructed using SNPs are shown. Each column shows an affected allele. Each base is defined by pure segregation analysis in the family. Allele frequencies of SNPs are derived from HapMap JPT+CHB samples. Families 2, 5, 10, and 13 shared a large common region of about more than 1 Mb in their haplotypes (blue). Abbreviation: Fm, Family.
doi:10.1371/journal.pone.0063231.g002

missense variants on *KCNQ4* protein function. A missense mutation (p.F182L) was found in one control patient with normal audiogram and the results showed that it is not likely to be a pathologic mutation.

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

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

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

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

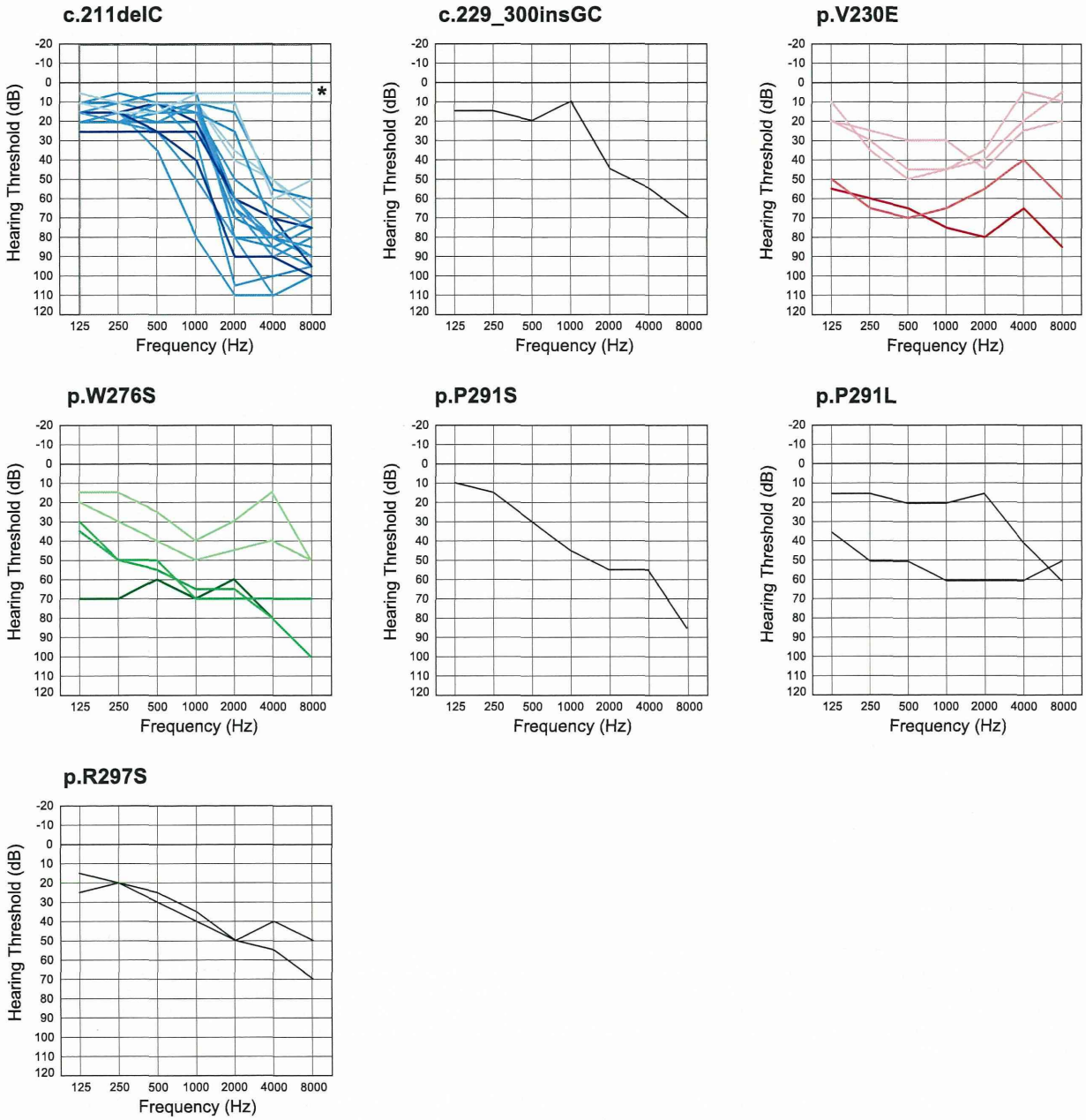

Figure 3. Overlapping audiograms from the better ear for each genotype. In cases of W276S, c.211delC, or V230E, light colored audiograms (green, blue, red) were from individuals aged 19 and under. Dark colored audiograms (green, blue, red) were from the patients aged 20–49 years old, and deep colored audiograms (green, blue, red) are from the patients in their 50 s and over. In family #13 with c.211delC, (*) a six-year-old boy's hearing is normal in spite of having the mutation.
doi:10.1371/journal.pone.0063231.g003

2.9%) of *TECTA* in Japanese ADNSHL families [4], therefore *KCNQ4* is found to be currently the most prevalent gene responsible for Japanese ADNSHL patients, and should be the first in line to be analyzed for ADNSHL patients.

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

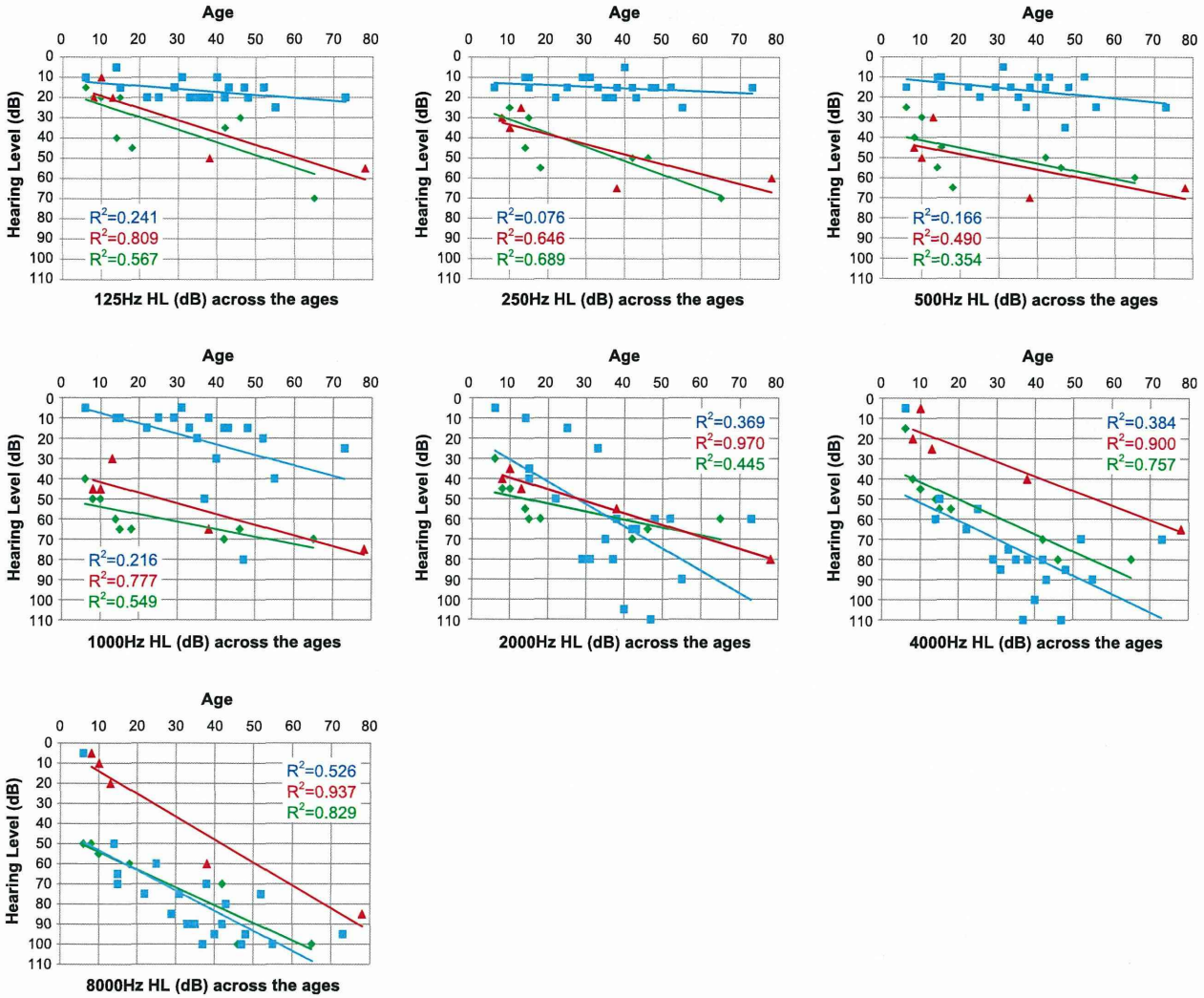

Figure 4. Detailed progression analysis in each frequency. A single audiogram (the better ear) from a single patient was plotted. Gradual progression is characterized regardless of frequency. Average progressive rates of hearing loss (db/year) for the patients with c.211delC, for 125 (0.15) and 250 Hz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

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

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

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

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

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

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

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

Supporting Information

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

Acknowledgments

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

Author Contributions

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

References

1. Hilgert N, Smith RJ, Van Camp G (2009) Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics? Mutat Res. 681: 189–196.
2. Kubisch C, Schroeder BC, Friedrich T, Lütjohann B, El-Amraoui A, et al. (1999) *KCNQ4*, a Novel Potassium Channel Expressed in Sensory Outer Hair Cells, Is Mutated in Dominant Deafness. Cell 96: 437–446.
3. Dominguez LM, Dodson KM (2012) Genetics of hearing loss: focus on DFNA2. The Application of Clinical Genetics 5: 97–104.
4. Moteki H, Nishio SY, Hashimoto S, Takumi Y, Iwasaki S, et al. (2012) *TECTA* mutations in Japanese with mid-frequency hearing loss affected by zona pellucida domain protein secretion. J Hum Genet 21 (In press).
5. Van Camp G, Coucke PJ, Akita J, Fransen E, Abe S, et al. (2002) A mutational hot spot in the *KCNQ4* gene responsible for autosomal dominant hearing impairment. Hum Mutat 20: 15–19.
6. Van Laer L, Coucke P, Mueller RF, Caethoven G, Flothmann K, et al. (2001) A common founder for the 35delG *GJB2* gene mutation in connexin 26 hearing impairment. J Med Genet 38: 515–518.
7. Yan D, Park HJ, Ouyang XM, Pandya A, Doi K, et al. (2003) Evidence of a founder effect for the 235delC mutation of *GJB2* (connexin 26) in east Asians. Hum Genet 114: 44–50.
8. Park HJ, Shaukat S, Liu XZ, Hahn SH, Naz S, et al. (2003) Origins and frequencies of *SLC26A4* (PDS) mutations in east and south Asians: global implications for the epidemiology of deafness. J Med Genet 40: 242–248.
9. Miyagawa M, Nishio SY, Usami S (2012) Prevalence and clinical features of hearing loss patients with *CDH23* mutation: a large cohort study. Plos one (In press).
10. Park HJ, Cho HJ, Baek JI, Ben-Yosef T, Kwon TJ, et al. (2010) Evidence for a founder mutation causing *DFNA5* hearing loss in East Asians. J Hum Genet 55: 59–62.
11. Akita J, Abe S, Shinkawa H, Kimberling WJ, Usami S (2001) Clinical and genetic features of nonsyndromic autosomal dominant sensorineural hearing loss: *KCNQ4* is a gene responsible in Japanese. J Hum Genet 46: 355–361.
12. Arnett J, Emery SB, Kim TB, Boerst AK, Lee K, et al. (2011) Autosomal dominant progressive sensorineural hearing loss due to a novel mutation in the *KCNQ4* gene. Arch Otol Head Neck Surg 137: 54–59.
13. Hildebrand MS, Tack D, McMordie SJ, DeLuca A, Hur IA, et al. (2008) Audioprofile-directed screening identifies novel mutations in *KCNQ4* causing hearing loss at the DFNA2 locus. Genet Med 10: 797–804.
14. Mencía A, González-Nieto D, Modamio-Høybjør S, Etxeberría A, Aránguez G, et al. (2008) A novel *KCNQ4* pore-region mutation (p.G296S) causes deafness by impairing cell-surface channel expression. Hum Genet 123: 41–53.
15. Baek JI, Park HJ, Park K, Choi SJ, Lee KY, et al. (2010) Pathogenic effects of a novel mutation (c.664_681del) in *KCNQ4* channels associated with auditory pathology. Biochimica et Biophysica Acta 536–543.
16. Kamada F, Kure S, Kudo T, Suzuki Y, Oshima T, et al. (2006) A novel *KCNQ4* one-base deletion in a large pedigree with hearing loss: implication for the genotype-phenotype correlation. J Hum Genet 51: 455–460.
17. Coucke PJ, Van Hauwe P, Kelley PM, Kunst H, Schatteman I, et al. (1999) Mutations in the KCNQ4 gene are responsible for autosomal dominant deafness in four DFNA2 families. Hum Mole Genet 8: 1321–1328.
18. von Ilberg CA, Baumann U, Kiefer J, Tillein J, Adunka OF (2011) Electric-acoustic stimulation of the auditory system: a review of the first decade. Audiol Neurootol 16: 1–30.

OPEN ACCESS Freely available online

PLOS ONE

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

Maiko Miyagawa[1], Shin-ya Nishio[1], Takuo Ikeda[2], Kunihiro Fukushima[3], Shin-ichi Usami[1]*

1 Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Japan, **2** Department of Otolaryngology, Tsudumigaura Handicapped Children's Hospital, Shunan, Japan, **3** Department of Otorhinolaryngology, Okayama University School of Medicine, Okayama, Japan

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.

Citation: Miyagawa M, Nishio S-y, Ikeda T, Fukushima K, Usami S-i (2013) Massively Parallel DNA Sequencing Successfully Identifies New Causative Mutations in Deafness Genes in Patients with Cochlear Implantation and EAS. PLoS ONE 8(10): e75793. doi:10.1371/journal.pone.0075793

Editor: Akinori Kimura, Tokyo Medical and Dental University, Japan

Received July 16, 2013; **Accepted** August 21, 2013; **Published** October 9, 2013

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 (http://www.mhlw.go.jp/english/) (S.U.), and by a Grant-in-Aid for Scientific Research from the 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: All authors declare no competing interests.

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

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).

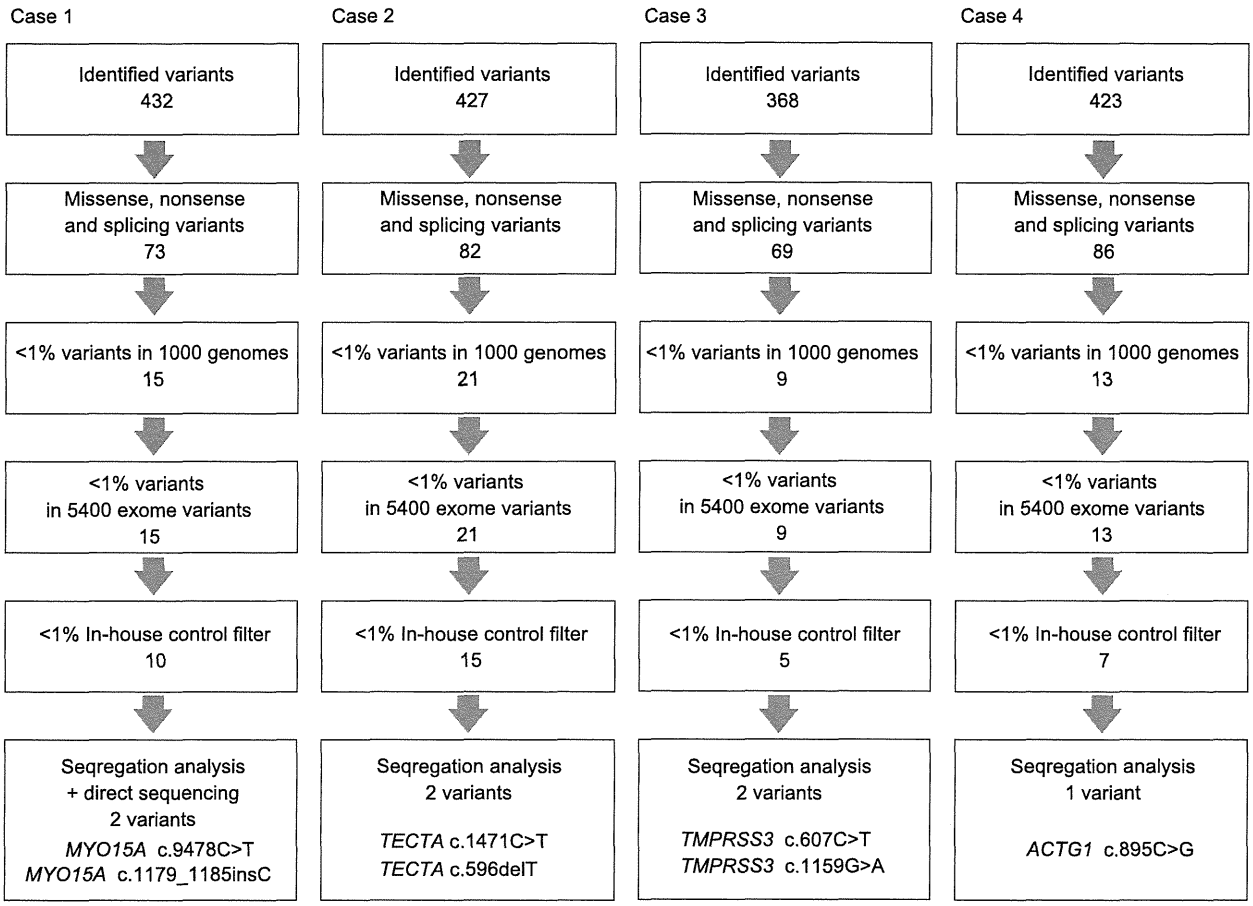

Figure 1. Flow of informatics analysis. Selected missense, nonsense, and splicing variants were filtered with 1) the 1000 genomes, 2) the 5400 exome variants, and 3) the in-house control. Responsible mutations were confirmed by segregation analysis.
doi:10.1371/journal.pone.0075793.g001

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