Ⅳ. 研究成果の刊行に関する一覧表

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1. Yoshimura H, Iwasaki S, Kanda Y, Nakanishi H, Murata T, Iwasa YI, Nishio SY, Takumi Y, Usami SI. An Usher syndrome type 1 patient diagnosed before the appearance of visual symptoms by *MYO7A* mutation analysis. Int J Pediatr Otorhinolaryngol. 2013. 77:298-302.
2. Iwasaki S, Nishio S, Moteki H, Takumi Y, Fukushima K, Kasai N, Usami S. Language development in Japanese children who receive cochlear implant and/or hearing aid. Int J Pediatr Otorhinolaryngol. 76: 433-438. 2012
3. Iwasaki S, Suzuki H, Moteki H, Miyagawa M, Takumi Y, Usami S. Expeprience with the Vibrant Soundbridge RW-Coupler for round window Vibroplasty with tympanosclerosis Acta Otolaryngol. 132: 676-682. 2012
4. Usami S, Miyagawa M, Nishio S, Moteki H, Takumi Y, Suzuki M, Kitano Y, Iwasaki S. Patients with CDH23 mutations and the 1555A>G mitochondrial mutations are good candidates for electric acoustic stimulation (EAS). Acta Otolaryngol. 132: 377-384. 2012
5. Miyagawa M, Nishio S, Usami S. Prevalence and Clinical Features of Hearing Loss Patients with CDH23 Mutations: A Large Cohort Study. PLOS ONE. 7: e40366. 2012
6. 宇佐美真一編　「きこえと遺伝子２—難聴の遺伝子診断　ケーススタディ集—」　金原出版（東京都）　2012
7. 吉村豪兼、他：Usher 症候群の全国アンケート調査結果の検討. Otology Japan　22：40—46、2012
8. 岩崎　聡、他：Usher 症候群の臨床的タイプ分類の問題点. 日耳鼻会報　115：894～901、2012.
9. 岩崎　聡、他：Usher 症候群. 新生児・幼小児の耳音響放射と ABR. 編集　加我君孝　診断と治療社、東京、118-122、2012

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Case report

An Usher syndrome type 1 patient diagnosed before the appearance of visual symptoms by *MYO7A* mutation analysis

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ABSTRACT

Usher syndrome type 1 (USH1) appears to have only profound non-syndromic hearing loss in childhood and retinitis pigmentosa develops in later years. This study examined the frequency of USH1 before the appearance of visual symptoms in Japanese deaf children by *MYO7A* mutation analysis. We report the case of 6-year-old male with profound hearing loss, who did not have visual symptoms. The frequency of *MYO7A* mutations in profound hearing loss children is also discussed. We sequenced all exons of the *MYO7A* gene in 80 Japanese children with severe to profound non-syndromic HL not due to mutations of the *GJB2* gene (ages 0–14 years). A total of nine DNA variants were found and six of them were presumed to be non-pathogenic variants. In addition, three variants of them were found in two patients (2.5%) with deafness and were classified as possible pathogenic variants. Among them, at least one nonsense mutation and one missense mutation from the patient were confirmed to be responsible for deafness. After *MYO7A* mutation analysis, the patient was diagnosed with RP, and therefore, also diagnosed with USH1. This is the first case report to show the advantage of *MYO7A* mutation analysis to diagnose USH1 before the appearance of visual symptoms. We believed that *MYO7A* mutation analysis is valid for the early diagnosis of USH1.

1. Introduction

Usher syndrome (USH) is an autosomal recessive disorder characterized by hearing loss (HL), retinitis pigmentosa (RP) and vestibular dysfunction. Three clinical subtypes can be distinguished [1] and USH type 1 (USH1) is the most severe among them because of profound HL, absent vestibular responses, and prepubertal onset RP [2]. For USH1, early diagnosis has many immediate and several long-term advantages for patients and their families [3]. However, diagnosis in childhood, based on a clinical phenotype, can be difficult because patients appear to have only non-syndromic HL in childhood and RP develops in later years.

Early diagnosis is now possible through DNA testing [3]. To date, seven genetic loci for USH1 have been mapped to chromosomes 11q13.5, 11p15.1, 10q22.1, 21q21, 10q21–q22, 17q24–q25, and 15q22–q23. Five of the corresponding genes have been identified (*MYO7A* [4], *USH1C* [5], *CDH23* [6], *PCDH15* [7], and *USH1G* [8]). USH1B in the most common USH1 genetic subtype, encoding the actin-based motor protein myosin VIIa (*MYO7A*), accounts for 30–50% of USH1 cases in the UK and the USA [9]. In Japanese, little is known about the most common cause of USH1, but mutation screening for *MYO7A* and *CDH23* is also expected to be a highly sensitive method for diagnosis [10]. Mutations in *MYO7A* are known to be responsible for dominant non-syndromic HL (DFNA11) [11] and infrequently, recessive non-syndromic HL (DFNB2) [12]. However, there was no obvious correlation between mutation in *MYO7A* and the resulting phenotype [13], unlike mutations in *CDH23* [14]. Therefore, we thought that young deaf children with *MYO7A* mutations, should undergo ophthalmologic examination to determine whether they will develop RP.

To examine whether USH1 patients before the appearance of visual symptoms exist among the Japanese non-syndromic severe to profound HL children, mutation analysis of the *MYO7A* gene was performed.

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2. Materials and methods

2.1. Subjects

We screened 80 Japanese children with severe to profound non-syndromic HL (ages 0–14 years): 10 from autosomal recessive families (normal hearing parents and two or more affected siblings), and 70 with sporadic deafness compatible with recessive inheritance or non-genetic hearing loss. There were 35 males and 45 females. None of the subjects had any other associated neurological symptoms including vestibular or visual dysfunction. Severity of hearing loss was classified by a puretone average over 500, 1000, 2000 and 4000 Hz in the better hearing ear as follows: severe hearing loss, 71–95 dB; and profound hearing loss, greater than 95 dB. All probands had congenital or early onset hearing loss, and families were too small for linkage analysis. Patients with *GJB2* mutations were previously excluded from this study. The control group was composed of 190 unrelated Japanese individuals who had normal hearing shown by auditory testing. All subjects gave prior informed consent for participation in the project and the Ethical Committee of Shinshu University approved the study.

2.2. Mutation analysis

Polymerase chain reaction (PCR) was used to amplify all 49 exons and flanking intronic sequences of the *MYO7A* gene. Each genomic DNA sample (40 ng) was amplified, using the primers described by Kumar et al. [15] with a slight modification and KOD DNA polymerase (Toyobo, Osaka, Japan), for 8.5 min at 95 °C, followed by 30 three-step cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min, ending with a holding period at 4 °C in PCR thermal cycler (Takara, Shiga, Japan). PCR products were treated with ExoSAP-IT® (GE Healthcare Bio, Buckinghamshire, UK) 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 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 *MYO7A* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).

Gene accession number: NM_000260.3 Homo sapiens myosin VIIA (MYO7A), transcript variant 1, mRNA3.

3. Results

3.1. Mutation screening

A total of nine sequence variants were found in the present study (Table 1). Of these, three variants found in two patients with deafness were classified as possible pathogenic variants (Table 1). Of these, a nonsense mutation (p.Gln18X) has been previously reported [16] and two mutations (p.Cys1201Ser and p.Phe1774-Leu) were novel. A patient with compound heterozygotes; with a nonsense mutation (p.Gln18X) [16] and a missense mutation (p.Phe1774Leu) was confirmed by the segregation analysis (Fig. 1). This patient without any visual symptoms was then diagnosed with USH1 after detailed opthalmological examinations as described below. The remaining possible pathologic variant (p.Cys1201Ser) was found to be in heterozygous state without second mutation, and did not fulfill the criteria for recessive inheritance mode. The patient carrying the *MYO7A* mutation (heterozygous case) was too young (2 years old) to receive additional testing (ophthalmological testing such as ERG), but had no visual complaint.

The remaining six out of nine DNA variants were presumed to be non-pathogenic variants because they had high allele carrier rates (>2%) and/or because of the result on prediction software for evaluation of the pathogenicity of missense variants (Table 1). Of these possible polymorphisms, two variants (p.Glu1641Lys and p.Ala1950Thr) were novel. One variant (p.Thr1566Met), previously reported as a pathogenic mutation by Najera et al. [17], was indicated by the prediction software score to probably be a non-pathologic polymorphism. Other variants have already been described as non-pathologic polymorphisms [18,19].

3.2. Case

The patient visited an ENT clinic at the age of 14 months because the parents noticed no response to sound. Auditory steady-state evoked responses (ASSR), one of an objective audiometry, showed profound hearing loss for all frequencies. In addition, caloric testing showed no response bilaterally. The patient received a cochlear implantation (CI) in the right ear at the age of 31 months and in the left ear at the age of 6. To distinguish between non-syndromic HL USH1, we recommended that an ophthalmologist be consulted. Although the proband showed no apparent nyctalopia or dark adaptation problems, a fundus examination revealed attenuated retinal vessels in the midperiphery without apparent pigmentary clumps (Fig. 1). Goldman visual field examination showed mild constriction of visual fields.

Table 1
MYO7A variations found in Japanese non-syndromic hearing loss children and controls.

Exon	Nucleotide change	Amino acid change	Evolutionary conservation	Alleles in control chromosomes	Hereditary	PolyPhen2 score	SIFT score	Pathogenicity	Reference
3	c.47T>C	p.Leu16Ser	No	71/160	Sporadic	0	1		Janecke et al. [18]
3	c.52C>T	p.Gln18X	–	0/380	Sporadic	–	–	Pathogenic	Cremer et al. [16] This study
28	c.3602G>C	p.Cys1201Ser	Yes	1/382	Sporadic	0.999	0.02	Pathogenic	This study
35	c.4697C>T	p.Thr1566Met	No	0/380	Sporadic	0.011	0.15		Najera [17] This study
36	c.4921G>A	p.Glu1641Lys	No	0/380	Sporadic	0.671	0.15		This study
36	c.4996A>T	p.Ser1666Cys	No	71/150	Sporadic	0	1		Janecke et al. [18]
38	c.5320T>C	p.Phe1774Leu	Yes	0/384	Sporadic	0.987	0.01	Pathogenic	This study
42	c.5848G>A	p.Ala1950Thr	No	0/380	Sporadic	0.003	0.18		This study
43	c.5860C>A	p.Leu1954Ile	No	49/160	Sporadic	0	1		Bharadwaj [19]

Computer analysis to predict the effect of missense variants on *MYO7A* protein function was performed with sorting intolerant from tolerant (SIFT; http://sift.jcvi.org/), and polymorphism phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).

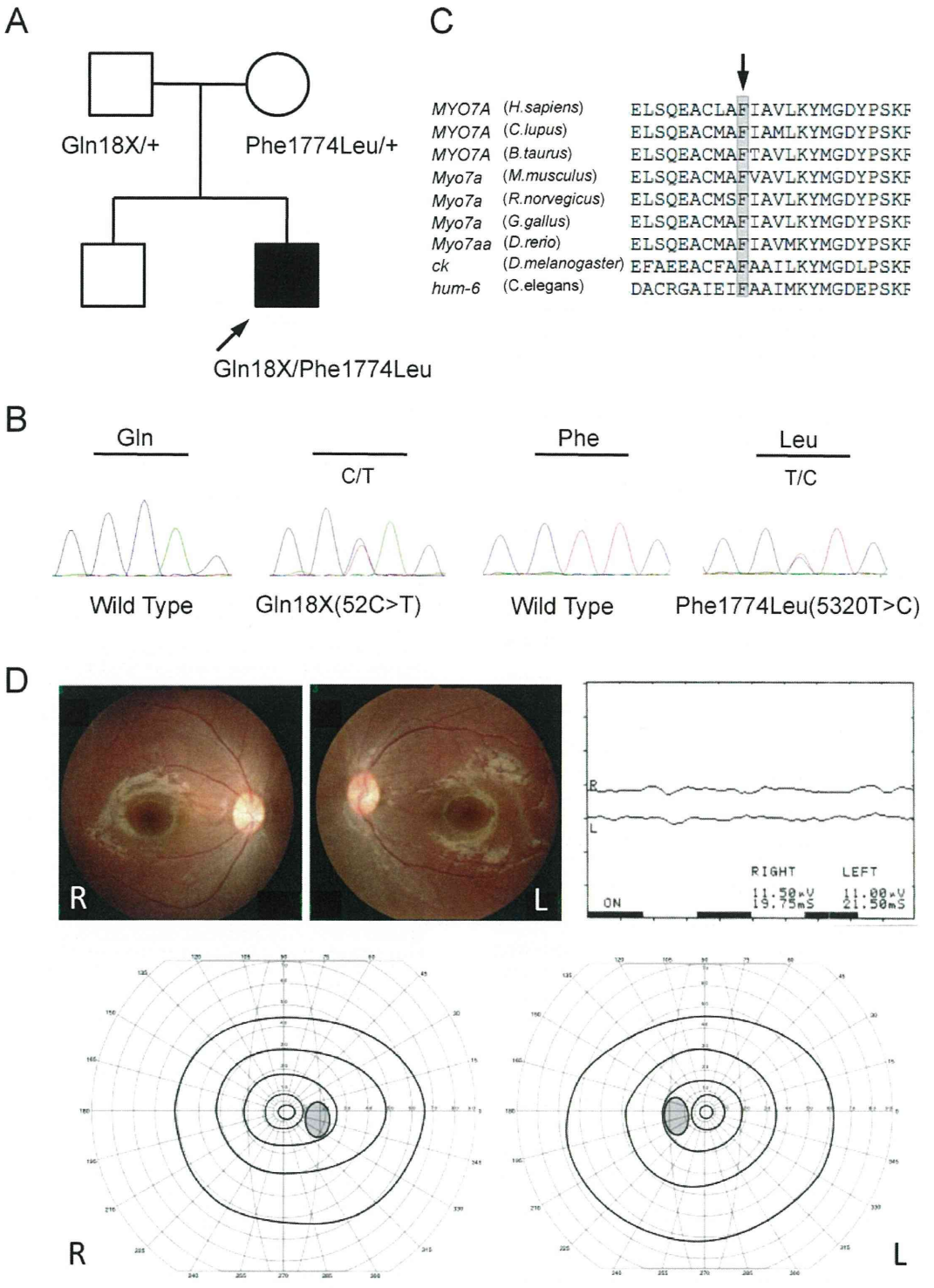

Fig. 1. Pedigree, sequence chromatograms, and ophthalmological findings of the patient with the Gln18X and Phe1774Leu mutations. (A) The pedigree and sequence results for the proband and family. (B) Sequence chromatograms from wild-type and mutations. The proband and his father carried a heterozygous 52C>T transition in exon 3, which results in an arginine to a stop codon (Gln18X). Another variation, 5320T>C (Phe1774Leu), was derived from the proband and his mother. (C) Evolutionary conservation of *MYO7A* gene in nine species. Arrow indicates mutation point found in the present study. (D) The proband had ophthalmologic test at the age of 7. Fundus examination revealed attenuated retinal vessels in the midperiphery without apparent pigmentary clumps. Goldman visual field examination showed mild constriction of visual fields. Full-field electroretiongram of the proband showed complete absence bilaterally.

A full-field electroretiongram showed complete absence bilaterally. Therefore, the proband was diagnosed with RP. On the basis of the results of ASSR, caloric test and ERG, the patient was then diagnosed with USH1. We provided genetic counseling to the family about (1) the risk of future vision loss and (2) the benefits of CI (in this case, however, the patient had already received bilateral CI).

4. Discussion

The present study reported nine *MYO7A* sequence variants in Japanese children with non-syndromic hearing loss. Among them, six were polymorphisms, and the remaining three variants are possible disease-causing mutations (Table 1). These three variants were found in none (or very few) of the controls, suggesting these are possible pathologic mutations. Of these three mutations, however, two mutations (p.Cys1201Ser and p.Phe1774Leu) have not been included in the USHbases (http://grenada.lumc.nl/LOVD2/Usher_montpellier/USHbases.html). We have previously reported genes responsible for deafness in Japanese patients and observed differences in mutation spectrum between Japanese (who are probably representative of other Asian populations) and populations with European ancestry [20]. Ethnic background is important and should be noted when genetic testing is performed.

Kimberling et al. showed that in deaf or hard of hearing children, negative for *GJB2/6* mutations, 12.7% (7/55) carried ≥1 Usher mutations [3]. In particular, *MYO7A* mutations were found in two of them (3.6%). In the present study, we found 2 *MYO7A* mutations in 80 Japanese children with severe to profound non-syndromic HL (2.5%), a not greatly different frequency.

The present case is the first to demonstrate that *MYO7A* mutation analysis could diagnose USH1 in a deaf child before the appearance of the visual symptoms. This result indicates the importance of *MYO7A* mutation screening in non-syndromic HL children. Anomalies of light-evoked electrical response of the retina can be detected by ERG at 2–3 years of age, which allows for early clinical confirmation of the disease [21]. In young children, ERG is not widely used because it is likely to involve the use of a general anesthetic and may be fraught with technical difficulties [21]. However, the mutations analysis of the *MYO7A* gene could replace the ERG testing.

Early detection of USH1 has mainly two benefits. First, we can provide the parents with the opportunity to choose CI, including bilateral, at an early stage. There is a need to provide USH children with the best hearing amplification available, with a preference for CI if possible, accompanied by intensive training and habilitation before the development of RP [22]. Especially, bilateral CI is believed to be essential for these children. In general, for implanted children, the best speech results are directly associated with cochlear implantation before the age of 5 years and with emphasis on pre- and post-implantation oral communication therapy [21]. Moreover, the children with USH1 do not differ from the rest of the implanted children; the best results are obtained with the young children [23]. In the current case, the patient has already received bilateral CI. We thought that this choice was appropriate. Secondly, ophthalmologic therapy to delay the progression of the RP may become available in the near future. Therapeutic strategies aim to treat retinal degeneration by targeting the specific genetic disorder (gene therapy) (http://www.oxfordbiomedica.co.uk/), slowing or stopping photoreceptor degeneration or apoptosis [24], or the use of blue and ultraviolet light filtering glasses [25].

In conclusion, we reported a case in which *MYO7A* mutation analysis diagnosed USH1 in a proband before the appearance of the visual symptoms. As shown in this case, *MYO7A* mutation analysis as a valid tool for the early diagnosis of USH1, however a new time- and cost-saving analytic procedure is necessary for a routine clinical testing. We are currently setting a new platform using massive parallel sequencing of all exons of USH1 related genes in deaf children by using next-generation sequencing.

Conflict of interest statement

We, the authors, declare that there were no conflicts of interest in conjunction with this paper.

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References

[1] R.J. Smith, C.I. Berlin, J.F. Hejtmancik, B.J. Keats, W.J. Kimberling, R.A. Lewis, et al., Clinical diagnosis of the Usher syndromes, Am. J. Med. Genet. 50 (1994) 32–38.
[2] C.G. Moller, W.J. Kimberling, S.L. Davenport, I. Priluck, V. White, K. Biscone-Halterman, et al., Usher syndrome: an otoneurologic study, Laryngoscope 99 (1989) 73–79.
[3] W.J. Kimberling, M.S. Hildebrand, A.E. Shearer, M.L. Jensen, J.A. Halder, K. Trzupek, et al., Frequency of Usher syndrome in two pediatric populations: Implications for genetic screening of deaf and hard of hearing children, Genet. Med. 12 (2010) 512–516.
[4] D. Weil, S. Blanchard, J. Kaplan, P. Guilford, F. Gibson, J. Walsh, et al., Defective myosin VIIA gene responsible for Usher syndrome type 1B, Nature 374 (1995) 60–61.
[5] E. Verpy, M. Leibovici, I. Zwaenepoel, X.Z. Liu, A. Gal, N. Salem, et al., A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1C, Nat. Genet. 26 (2000) 51–55.
[6] J.M. Bork, L.M. Peters, S. Riazuddin, S.L. Bernstein, Z.M. Ahmed, S.L. Ness, et al., Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene CDH23, Am. J. Hum. Genet. 68 (2001) 26–37.
[7] Z.M. Ahmed, S. Riazuddin, S.L. Bernstein, Z. Ahmed, S. Khan, A.J. Griffith, et al., Mutations of the protocadherin gene PCDH15 cause Usher syndrome type 1F, Am. J. Hum. Genet. 69 (2001) 25–34.
[8] M. Mustapha, E. Chouery, D. Torchard-Pagnez, S. Nouaille, A. Khrais, F.N. Sayegh, et al., A novel locus for Usher syndrome type I, USH1G, maps to chromosome 17q24–25, Hum. Genet. 110 (2002) 348–350.
[9] D. Vozzi, A. Aaspollu, E. Athanasakis, A. Berto, A. Fabretto, D. Licastro, et al., Molecular epidemiology of Usher syndrome in Italy, Mol. Vis. 17 (2011) 1662–1668.
[10] H. Nakanishi, M. Ohtsubo, S. Iwasaki, Y. Hotta, Y. Takizawa, K. Hosono, et al., Mutation analysis of the MYO7A and CDH23 genes in Japanese patients with Usher syndrome type 1, J. Hum. Genet. 55 (2010) 796–800.
[11] X.Z. Liu, J. Walsh, Y. Tamagawa, K. Kitamura, M. Nishizawa, K.P. Steel, et al., Autosomal dominant non-syndromic deafness caused by a mutation in the myosin VIIA gene, Nat. Genet. 17 (1997) 268–269.
[12] S. Riazuddin, S. Nazli, Z.M. Ahmed, Y. Yang, F. Zulfiqar, R.S. Shaikh, et al., Mutation spectrum of MYO7A and evaluation of a novel nonsyndromic deafness DFNB2 allele with residual function, Hum. Mutat. 29 (2008) 502–511.
[13] D.X. Yan, Z. Liu, Genetics and pathological mechanisms of Usher syndrome, J. Hum. Genet. 55 (2010) 327–335.
[14] J.M. Schultz, R. Bhatti, A.C. Madeo, A. Turriff, J.A. Muskett, C.K. Zalewski, et al., Allelic hierarchy of CDH23 mutations causing non-syndromic deafness DFNB12 or Usher syndrome USH1D in compound heterozygotes, J. Med. Genet. 48 (2011) 767–775.
[15] A. Kumar, M. Babu, W.J. Kimberling, C.P. Venkatesh, Genetic analysis of a four generation Indian family with Usher syndrome: a novel insertion mutation in MYO7A, Mol. Vis. 10 (2004) 910–916.
[16] F.P. Cremers, W.J. Kimberling, M. Kulm, A.P. de Brouwer, E. van Wijk, H. te Brinke, et al., Development of a genotyping microarray for Usher syndrome, J. Med. Genet. 44 (2007) 153–160.
[17] C. Najera, M. Beneyto, J. Blanca, E. Aller, A. Fontcuberta, J.M. Millan, et al., Mutations in myosin VIIA (MYO7A) and usherin (USH2A) in Spanish patients with Usher syndrome types I and II, respectively, Hum. Mutat. 20 (2002) 76–77.
[18] A.R. Janecke, M. Meins, M. Sadeghi, K. Grundmann, E. Apfelstedt-Sylla, E. Zrenner, et al., Twelve novel myosin VIIA mutations in 34 patients with Usher syndrome type I: confirmation of genetic heterogeneity, Hum. Mutat. 13 (1999) 133–140.
[19] A.K. Bharadwaj, J.P. Kasztejna, S. Huq, E.L. Berson, T.P. Dryja, Evaluation of the myosin VIIA gene and visual function in patients with Usher syndrome type I, Exp. Eye Res. 71 (2000) 173–181.
[20] S. Usami, M. Wagatsuma, H. Fukuoka, H. Suzuki, K. Tsukada, S. Nishio, et al., The responsible genes in Japanese deafness patients and clinical application using Invader assay, Acta Otolaryngol. 128 (2008) 446–454.

[21] X.Z. Liu, S.I. Angeli, K. Rajput, D. Yan, A.V. Hodges, A. Eshraghi, et al., Cochlear implantation in individuals with Usher type 1 syndrome, Int. J. Pediatr. Otorhinolaryngol. 72 (2008) 841–847.
[22] Z. Brownstein, T. Ben-Yosef, O. Dagan, M. Frydman, D. Abeliovich, M. Sagi, et al., The R245X mutation of PCDH15 in Ashkenazi Jewish children diagnosed with nonsyndromic hearing loss foreshadows retinitis pigmentosa, Pediatr. Res. 55 (2004) 995–1000.
[23] A.F. Snik, M.J. Makhdoum, A.M. Vermeulen, J.P. Brokx, P. van den Broek, The relation between age at the time of cochlear implantation and long-term speech perception abilities in congenitally deaf subjects, Int. J. Pediatr. Otorhinolaryngol. 41 (1997) 121–131.
[24] J.M. Millan, E. Aller, T. Jaijo, F. Blanco-Kelly, A. Gimenez-Pardo, C. Ayuso, An update on the genetics of usher syndrome, J. Ophthalmol. 2011 (2011) 417217.
[25] A.V. Cideciyan, S.G. Jacobson, T.S. Aleman, D. Gu, S.E. Pearce-Kelling, A. Sumaroka, et al., In vivo dynamics of retinal injury and repair in the rhodopsin mutant dog model of human retinitis pigmentosa, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 5233–5238.

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Prevalence and Clinical Features of Hearing Loss Patients with *CDH23* Mutations: A Large Cohort Study

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Abstract

Screening for gene mutations in *CDH23*, which has many exons, has lagged even though it is likely to be an important cause for hearing loss patients. To assess the importance of *CDH23* mutations in non-syndromic hearing loss, two-step screening was applied and clinical characteristics of the patients with *CDH23* mutations were examined in this study. As a first screening, we performed Sanger sequencing using 304 probands compatible with recessive inheritance to find the pathologic mutations. Twenty-six possible mutations were detected to be pathologic in the first screening. For the second screening, using the probes for these 26 mutations, a large cohort of probands (n = 1396) was screened using Taqman amplification-based mutation analysis followed by Sanger sequencing. The hearing loss in a total of 52 families (10 homozygous, 13 compound heterogygous, and 29 heterozygous) was found to be caused by the *CDH23* mutations. The majority of the patients showed congenital, high frequency involved, progressive hearing loss. Interestingly, some particular mutations cause late onset moderate hearing loss. The present study is the first to demonstrate the prevalence of *CDH23* mutations among non-syndromic hearing loss patients and indicated that mutations of the *CDH23* gene are an important cause of non-syndromic hearing loss.

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Introduction

Mutations in the *CDH23* (NM_22124) gene are known to be responsible for both Usher syndrome type ID (USH1D) and non-syndromic hearing loss (DFNB12) [1,2]. Molecular confirmation of *CDH23* mutations has become important in the diagnosis of these conditions.

This gene encodes cadherin 23, a protein of 3354 amino acids with 27 extracellular (EC) domains, a single transmembrane domain and a short cytoplasmic domain. Cadherin-specific amino acid motifs such as DRE, DXNDN, and DXD, that are highly conserved in sequence and spacing and required for cadherin dimerization and calcium binding were found in each extracelluar domain [3].

The cadherin 23 protein is known to be an important composition of the tip link that maintains the arrangement of streocilia [4].

More than 50 mutations have been reported for the Usher phenotype (USH1D) and 24 mutations reported for the non-syndromic hearing loss phenotype (DFNB12) [1,2,5–7]. As suggested by genotype–phenotype correlation study, Usher 1D, which has congenital profound hearing impairment, vestibular dysfunction, and retinitis pigmentosa, is usually associated with nonsense mutations, whereas DFNB12, which has a milder phenotype, is associated with missense mutations [1,2,5–8].

We previously reported that four pathologic mutations were identified in 5 out of 64 Japanese families compatible with autosomal recessive inheritance, suggesting that *CDH23*-caused deafness may be commonly found among non-syndromic hearing loss patients [6]. *GJB2* has been shown to be a common gene involved in congenital hearing impairment. *SLC26A4* is also frequently involved among those patients. *GJB2* and *SLC26A4* are comparatively small making Sanger sequencing relatively easy. The latter is also associated with the typical inner ear anomaly, enlarged vestibular aqueduct. Therefore, screening is relatively easy and many studies have focused on just these two genes. Clinical molecular diagnosis has been dramatically improved for these genes. However, screening strategy of other hearing loss genes is difficult and Sanger sequencing of the candidate genes, such as *CDH23*, with many exons is time consuming. Consequently, only a few reports are available for the mutation spectrum of *CDH23*.

In the present study, we performed Sanger sequencing using 304 patients whose pedigrees are compatible with recessive inheritance to find additional pathologic mutations. Also, to find the novel pathologic mutations and to clarify the frequency and clinical characteristics of patients with *CDH23* mutations, a large cohort of probands from unrelated families (n = 1396) was screened using TaqMan amplification-based mutation analysis of the variants observed in the initial 304 patients.

Table 1. Possible pathologic variants found in this study.

Amino acid change	Nucleotide change	EXON	Domain	Evolutionary conservation	The highly conserved calcium -binding elements	Number in probands (n = 1396)			Allele frequency in patients (in 2792 allele)	Allele frequency in control (in 384 allele)	Allele frequency in HL patients based on a Next generation sequencing database (in 432 allele)	Allele frequency in controls based on a Next generation sequencing database (in 144 allele)	PolyPhen 2 score*	SIFT Score*	Reference
						homozygote	compound heterozygote	heterozygote							
p.P240L	c.719C>T	7	EC3	7	-	7	12	19	1.612	0.260	0.63	0.67	0.999	0.06	Wagatsuma et al.
p.R301Q	c.902G>A	9	EC3	7	DRE	-	3	-	0.107	0.260	0	0	1.000	0	Wagatsuma et al.
p.E956K	c.2866G>A	25	EC9	7	DRE	-	1	2	0.107	0	0.21	0	1.000	0.04	this study
p.T1368M	c.4103C>T	32	EC13	7	-	-	1	-	0.036	0	0	0	1.000	0	this study
p.R1417W	c.4249C>T	35	EC13	5	-	1	-	2	0.143	0	0.25	0	0.998	0.19	Wagatsuma et al.
p.D1626A	c.4877A>C	39	EC15	7	DXNDN	-	1	-	0.036	0	0	0	0.999	0.01	this study
p.Q1716P	c.5147A>C	39	EC16	7	-	-	3	-	0.107	0	0	0	0.957	0.3	Wagatsuma et al.
p.R2029W	c.6085C>T	46	EC19	7	DRE	2	2	6	0.430	0	0	0	0.999	0.01	Wagatsuma et al.
p.N2287K	c.6861T>G	50	EC21	7	DXNDN	-	2	-	0.072	0	0	0	0.971	0	this study
p.E2438K	c.7312G>A	52	EC23	6	-	-	1	-	0.036	0	0	0	0.986	1	this study

*Computer analysis to predict the effect of missense variants on *CDH23* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2;http://genetics.bwh.harvard.edu/pph2/).

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Table 2. Variants with uncertain pathogenicity found in this study.

Amino acid change	Nucleotide change	EXON	Domain	Evolutionary conservation	The highly conserved calcium -binding elements	Number in probands (n = 1396)			Allele frequency in patients (in 2792 allele)	Allele frequency in control (in 384 allele)	Allele frequency in HL patients based on a Next generation sequencing database (in 432 allele)	Allele frequency in controls based on a Next generation sequencing database (in 144 allele)	PolyPhen 2 score***	SIFT Score***	Reference
						homozygote	compound heterozygote	heterozygote							
p.D160N	c.478G>A	4	EC2	7	DXD	-	-	2	0.072	0.260	0	0	1.000	0	this study
p.V803I	c.2407G>A	23	EC8	7	-	-	-	3	0.107	0	0	0	0.761	0.41	this study
p.S1415I	c.4244G>T	35	EC13	7	-	-	-	1	0.036	0	0	0	0.840	0.06	this study
p.A1443G *	c.4328C>G	35	EC14	7	-	1*	-	2	0.143	0	0.2	0	0.944	0.06	this study
p.R1588W **	c.4762C>T	38	EC15	7	-	4**	-	18	0.931	0.260	2.22	0	1.000	0.01	Wagatsuma et al.
p.V1711I	c.5131G>A	40	EC16	7	-	-	-	2	0.072	0	0	0	0.970	0.12	Wagatsuma et al.
p.V1807M	c.5419G>A	42	EC17	5	-	-	1	-	N/A	0.260	0	0	0.054	0.22	this study
p.S1876N	c.5627G>A	43	EC18	5	-	-	-	6	0.215	0	0	0	0.981	0.26	Wagatsuma et al.
p.V1908I	c.5722G>A	44	EC9	5	-	-	-	12	0.430	0.260	1.09	0.53	0.948	1	Wagatsuma et al.
p.A2130V	c.6389C>T	48	EC20	6	-	-	-	1	0.036	0	0	0	0.999	0.24	this study
p.R2171C	c.6511C>T	48	EC20	7	DXNDNR	-	-	1	0.036	0.521	0	0	0.999	0.11	Wagatsuma et al.
p.Q2227P	c.6680A>C	48	EC21	6	-	-	-	1	0.036	0.260	0	0	0.930	0.2	Wagatsuma et al.
p.L2473P	c.7418T>C	53	EC23	7	-	-	-	1	0.036	0	0	0	0.999	0	Wagatsuma et al.
p.I2669V	c.8005A>G	56	EC25	5	-	-	-	1	0.036	0	0	0	0.134	0.7	Wagatsuma et al.
p.F2801V	c.8401T>G	59	EC26	5	-	-	-	1	0.036	0.781	1.52	1.27	0.800	0.01	Wagatsuma et al.
p.G2912S	c.8734G>A	61	EC27	7	-	-	-	1	0.036	0	0.23	0	0.996	0	this study
p.R3175C	c.9523C>T	68	CYTO	7	-	-	-	1	0.036	0.260	0	0	0.886	0.01	Wagatsuma et al.

*not confirmed by segregation study.

**one normal hearing subject with homozygotes.

***Computer analysis to predict the effect of missense variants on *CDH23* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2;http://genetics.bwh.harvard.edu/pph2/).

N/A: TaqMan probe not available.

doi:10.1371/journal.pone.0040366.t002

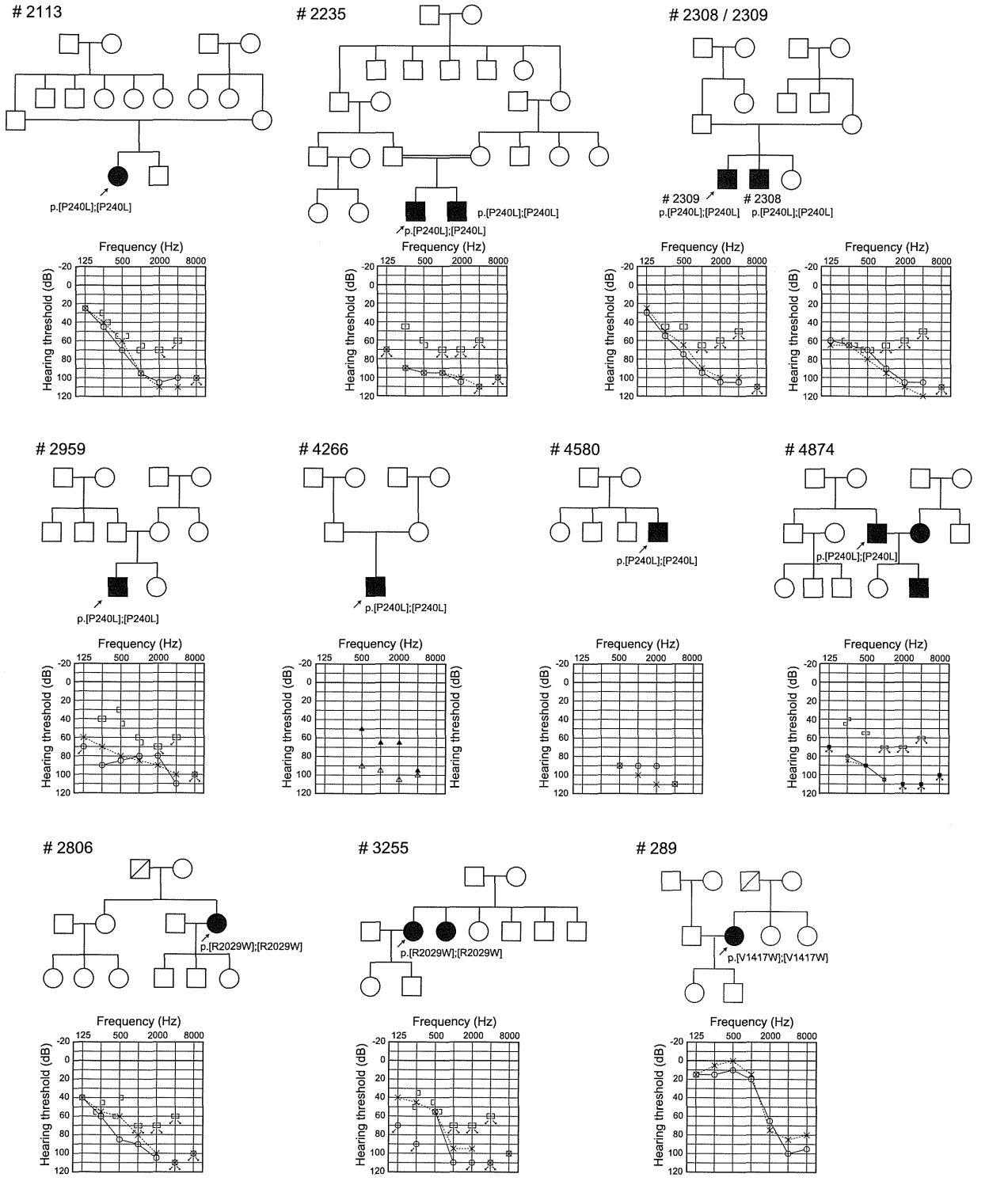

Figure 1. Pedigrees, mutations, and audiograms of the patients with homozygous *CDH23* mutations.
doi:10.1371/journal.pone.0040366.g001

Results

The first screening using 304 Japanese probands compatible with autosomal recessive inheritance identified 26 candidates for disease causing mutations. These include four previously reported pathologic mutations: p.P240L, p.R301Q, p.Q1716P, and p.R2029W, as well as 6 possible pathologic variants in the coding region of *CDH23*. All of the mutations were missense mutations.

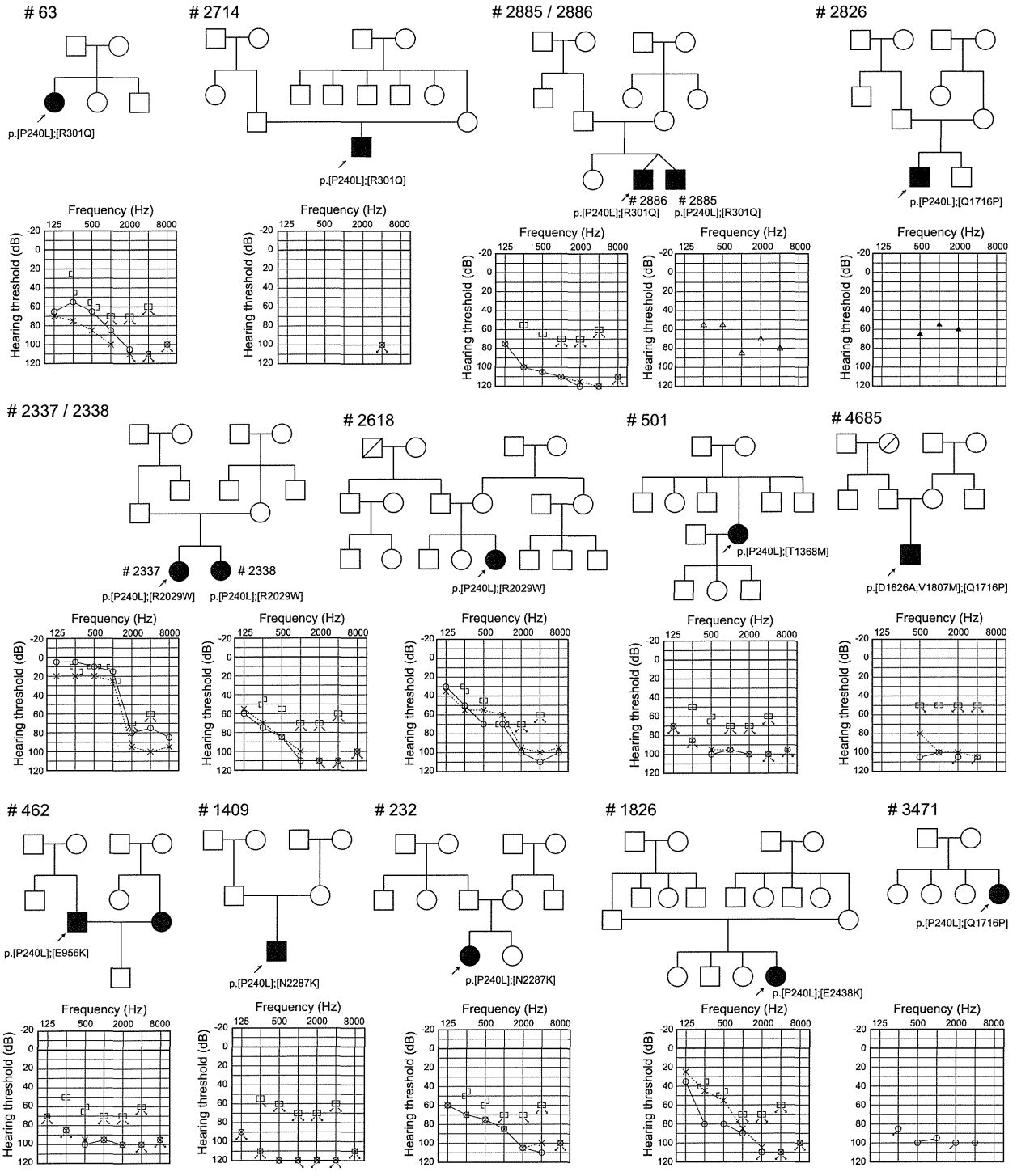

Figure 2. Pedigrees, mutations, and audiograms of the patients with compound heterozygous *CDH23* mutations.
doi:10.1371/journal.pone.0040366.g002

The following second screening based on TaqMan assay followed by Sanger sequencing confirmed 10 "possibly pathologic" mutations (Table 1) and 17 variants with uncertain pathogenicity (Table 2) in a large cohort of the patients. "Possible pathologic" mutations were defined as 1) mutations found to be homozygotes or compound heterozygotes (and determined by segregation study), 2) variants which were not found or were very few in 192 control subjects, 3) amino acids that were well-conserved among various species, 4) compatible with next generation sequencing database, and 5) compatible with the predicted effect

Table 3. Details of phenotype and genotype of 11 patients in 10 families with homozygous *CDH23* mutation.

Sample No	relationship	Amino acid Change	Hereditary form	Threshold* (Rt)(dB)	Threshold* (Lt)(dB)	severity	Residual hearing in the lower frequencies** (dB)	Hearing in the higher frequencies*** (dB)	Age	Age of awareness	Progres-siveness	Hearing aid/ cochlear implant	Vertigo	Tinnitus
#2113		p.[P240L]; [P240L]	sporadic	91.3	90	severe	44.2	104.2	12	6	+	HA	–	–
#2235		p.[P240L]; [P240L]	AR	97.5	96.3	profound	85.0	104.2	22	0	–	HA	–	–
#2308		p.[P240L]; [P240L]	AR	88.8	95	severe	67.5	110.0	11	0****	–	HA	–	–
#2309	sibling of #2308	p.[P240L]; [P240L]	AR	92.5	86.3	severe	50.0	105.0	9	0****	–	HA	–	–
#2959		p.[P240L]; [P240L]	sporadic	81.3	85	severe	75.8	96.7	8	0****	–	HA	–	–
#4266		p.[P240L]; [P240L]	sporadic	96.3	96.3	severe	70.0	91.3	3	0****	+	CI	–	–
#4580		p.[P240L]; [P240L]	sporadic	102.5	97.5	profound	88.3	106.7	1	0****	–	CI	–	N/A
#4874		p.[P240L]; [P240L]	sporadic	102.5	102.5	profound	80.8	106.7	38	2	+	HA	–	–
#2806		p.[R2029W]; [R2029W]	sporadic	92.5	80	severe	56.7	104.2	53	48	+	HA	–	+
#3255		p.[R2029W]; [R2029W]	AR	96.3	85	severe	59.2	104.2	71	60	+	HA	–	+
#289		p.[V1417W]; [V1417W]	sporadic	31.3	26.3	mild	10.0	85.0	34	14	+	HA	–	–

*average of 500, 1000, 2000 and 4000 Hz.
**average of 125, 250, and 500 Hz.
***average of 2000, 4000, and 8000 Hz.
****found by newborn hearing screening.
doi:10.1371/journal.pone.0040366.t003

Table 4. Details of phenotype and genotype of 15 patients in 13 families with compound heterozygous *CDH23* mutation.

Sample No	relationship	Amino acid Change	Hereditary form	Threshold* (Rt)(dB)	Threshold* (Lt)(dB)	severity	Residual hearing in the lower frequencies** (dB)	Hearing in the higher frequencies*** (dB)	Age	Age of awareness	Progres-siveness	Hearing aid/ cochlear implant	Vertigo	Tinnitus
#63		p.[P240L]; [R301Q]	sporadic	85	98.8	severe	69.2	105.8	27	0	−	HA	−	+
#2714		p.[P240L]; [R301Q]	sporadic	97.5	97.5	profound	71.7	105.0	2	0****	+	HA	−	−
#2885		p.[P240L]; [R301Q]	AR	90	108.7	profound	55.0	75.0	13	3	+	CI	−	−
#2886	sibling of #2885	p.[P240L]; [R301Q]	AR	115	110	profound	93.3	115.8	13	2	+	CI	−	−
#2337		p.[P240L]; [R2029W]	AR	30	41.3	mild	13.3	88.3	13	11	+	HA	−	+
#2338	sibling of #2337	p.[P240L]; [R2029W]	AR	103.8	98.8	profound	71.7	106.7	8	2	+	HA	−	−
#2618		p.[P240L]; [R2029W]	sporadic	77.5	67.5	moderate	49.2	100.0	8	3	+	CI	−	−
#2826		p.[P240L]; [Q1716P]	sporadic	91.3	95	profound	66.7	112.5	6	0	+	HA	−	−
#3471		p.[P240L]; [Q1716P]	sporadic	97.5	97.5	profound	92.5	100.0	4	0	−	CI	−	−
#462		p.[P240L]; [E956K]	sporadic	97.5	97.3	profound	84.2	98.3	38	10	−	HA	−	−
#501		p.[P240L]; [T1368M]	sporadic	>90	>90	profound	N/A	N/A	68	44	+	HA	+	+
#1409		p.[P240L]; [N2287K]	sporadic	120	120	profound	107.5	123.3	17	0	+	HA	−	−
#232		p.[P240L]; [N2287K]	sporadic	87.5	86.3	severe	67.5	104.2	15	0	−	HA	−	+
#1826		p.[P240L]; [E2438K]	sporadic	91.3	106.3	severe	70.8	105.8	11	3	+	HA	−	−
#4685		p.[D1626A; V1807M]; [Q1716P]	sporadic	97.5	103.8	severe	96.3	105.0	1	0*	−	CI	−	N/A

*average of 500, 1000, 2000 and 4000 Hz.
**average of 125, 250, and 500 Hz.
***average of 2000, 4000, and 8000 Hz.
****found by newborn hearing screening.
doi:10.1371/journal.pone.0040366.t004

Table 5. Details of phenotype and genotype of 29 patients with heterozygous *CDH23* mutation.

Sample No relationship	Amino acid Change	Hereditary form	Threshold* (Rt)(dB)	Threshold* (Lt)(dB)	severity	Residual hearing in the lower frequencies** (dB)	Hearing in the higher frequencies*** (dB)	Age	Age of awareness	Progressiveness	Hearing aid/ cochlear implant	Vertigo	Tinnitus
#334	p.[P240L];[-]	AD	96.25	83.75	severe	63.3	96.7	23	0	+	HA	N/A	+
#340	p.[P240L];[-]	sporadic	>90	>90	profound	N/A	N/A	54	14	+	HA	N/A	N/A
#569	p.[P240L];[-]	sporadic	86.25	90	severe	75.0	98.3	26	3	+	HA	−	−
#653	p.[P240L];[-]	sporadic	53.75	57.5	moderate	44.2	71.7	36	33	+	HA	−	+
#754	p.[P240L];[-]	sporadic	110	101.25	profound	87.5	104.2	57	0	+	HA	N/A	N/A
#1039	p.[P240L];[-]	sporadic	48.75	56.25	moderate	33.3	74.2	76	76	−	HA	+	−
#1598	p.[P240L];[-]	sporadic	56.25	10	unilateral	34.2	41.7	60	49	−	−	+	+
#1807	p.[P240L];[-]	sporadic	110	8.75	unilateral	50.8	60.0	50	9	−	−	−	−
#1846	p.[P240L];[-]	AD	100	96.25	profound	83.3	98.3	62	6	+	HA	+	+
#2159	p.[P240L];[-]	AR	67.5	66.25	moderate	60.0	69.2	10	65	+	HA	−	−
#2374	p.[P240L];[-]	AR	86.25	90	severe	78.3	78.3	5	0	−	HA	−	−
#2835	p.[P240L];[-]	sporadic	85	91.25	severe	65.8	101.7	12	3	+	HA	+	−
#3492	p.[P240L];[-]	AD	103.75	103.75	profound	88.8	107.5	1	0	−	HA	−	−
#3499	p.[P240L];[-]	AD	96.25	110	severe	84.2	105.8	57	50	−	CI	−	+
#3761	p.[P240L];[-]	AR	32.5	40	mild	43.3	75.8	71	0	−	−	−	+
#4040	p.[P240L];[-]	AR	S/O	S/O	profound	S/O	S/O	2	0	+	HA	−	−
#4159	p.[P240L];[-]	AR	97.5	71.25	severe	71.7	95.0	38	38	+	HA	+	+
#4313	p.[P240L];[-]	AD/Mit	130	102.5	profound	107.5	116.7	6	0	−	CI	−	−
#4615	p.[P240L];[-]	sporadic	90	90	profound	90.0	90.0	0	0****	−	CI	−	−
#265	p.[E956K];[-]	sporadic	110	6.25	unilateral	57.5	59.2	16	0	−	−	−	−
#3116	p.[E956K];[-]	AD	47.5	53.75	moderate	58.3	40.8	63	N/A	+	HA	−	+
#280	p.[R1417W];[-]	sporadic	110	6.25	unilateral	50.0	55.8	8	3	−	−	N/A	N/A
#2649	p.[R1417W];[-]	sporadic	95	110	profound	87.5	105.0	11	0	+	CI	−	N/A
#1131	p.[R2029W];[-]	sporadic	73.75	72.5	severe	55.0	93.3	24	17	+	HA	−	−
#1539	p.[R2029W];[-]	AD	53.75	110	moderate	70.0	83.3	71	60	+	HA	−	+
#1618	p.[R2029W];[-]	sporadic	26.25	61.25	mild	31.7	60.8	67	N/A	−	−	−	+
#1919	p.[R2029W];[-]	AD	38.75	36.25	mild	20.8	75.0	25	3	+	−	N/A	N/A
#2271	p.[R2029W];[-]	AD	58.75	62.5	moderate	41.7	50.0	6	N/A	N/A	HA	N/A	N/A
#4138	p.[R2029W];[-]	AR	71.25	53.75	moderate	50.8	65.8	10	3	+	HA	+	−

*average of 500, 1000, 2000 and 4000 Hz.
**average of 125, 250, and 500 Hz.
***average of 2000, 4000, and 8000 Hz.
****found by newborn hearing screening.
doi:10.1371/journal.pone.0040366.t005

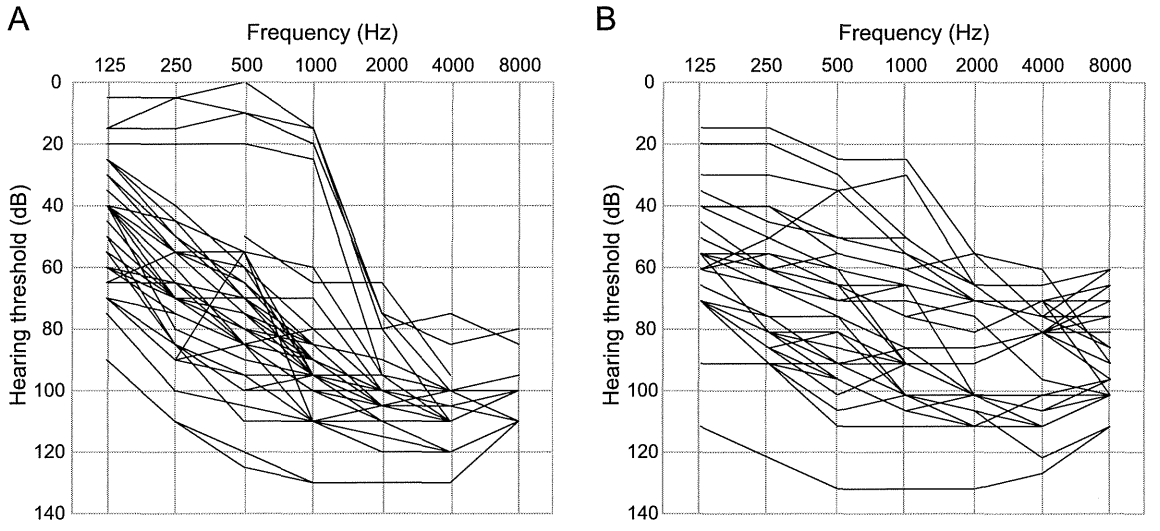

Figure 3. Overlapping audiograms of the patients with *CDH 23* mutations. A: patients with hearing loss caused by the *CDH23* mutations (homozygous or compound heterozygous cases), B: patients potentially caused by the *CDH23* mutations (heterozygous cases).
doi:10.1371/journal.pone.0040366.g003

of missense mutations on *CDH23* protein function. Results of the compatibility of the next generation sequence database, the SIFT and PolyPhen2 score for prediction are shown in Tables 1 and 2.

The 17 variants found as heterozygous and therefore with uncertain pathogenicity did not fulfill all the above criteria. For example, p.A1443G was uncertain because DNA samples from family members were not available and we could not confirm its pathogenicity by segregation study. p.R1588W was found to be homozygous in 4 patients and heterozygous in 16 patients, but only 1 was found in 384 control alleles. However, a member of the patient's family (#2841) showed normal hearing instead of being homozygous. Also p.V803I, p.V1807M and p.I2669V are obscure from the functional prediction analysis.

In one family (#4685), three mutation were found in proband and two of them were found in same allele p.[D16126A;V1807M] confirmed by segregation analysis.

As p.V1807M predicted to have no effect on *CDH23* structure, p.D1626A might be a pathogenic mutation.

For 10 possible pathologic mutations, amino acids were well-conserved among various species, including *Homo sapiens, P. troglodytes, B. taurus, M. musculus, R. norvegicus, G. gallus*, and *D. rario*. Many mutations (5 out of 10 possible pathologic mutations, 2 out of 17 uncertain variants) were found in DRE, DXNDN, and DXD motif (Table 1 and 2). Ten possible pathologic mutations were found to be either homozygotes (n = 11, Table 3, Fig. 1) or compound heterozygotes (n = 15) (Table 4, Fig. 2). Twenty-nine patients were found to be heterozygous without a second mutation (Table 5).

Tables 3 and 4 summarize 23 families with hearing loss caused by the *CDH23* mutations (homozygous or compound heterozygous cases) and Table 5 summarizes 29 families with hearing loss potentially caused by the *CDH23* mutations (heterozygous cases). The frequency was 1.6% (23/1396) or 2.1% (29/1396) of the overall hearing loss population. When restricted to patients compatible with recessive inheritance, the frequency was increased to 2.5% (23/919) or 3.2% (29/919). Table 3, 4 and 5 also summarize clinical characteristics including hereditary form, hearing threshold, severity, residual hearing in the lower frequencies, hearing in the higher frequencies, onset age (age of awareness), progressiveness of hearing loss, use of hearing aid/cochlear implantation, visual impairment, and vestibular symptoms. The ages of these patients were from 1 to 71 years. Age of onset (awareness of hearing loss) ranged from congenital to 60 years old, though the majority was congenital or early onset. There were some correlations between genotype and phenotype (onset age). The patients associated with p.P240L showed congenital and severe hearing loss regardless of whether associated with one more mutation, whereas the patients with p.R2029W or p.T1368M showed late-onset moderate hearing loss (Tables 3 and 4). Concerning type of hearing loss, the majority of the patients had some residual hearing in the lower frequencies, and overlapping audiograms showed characteristic high frequency involved hearing loss (Fig. 3). The majority of the patients showed progressive nature of hearing loss evaluated by serial audiogram (Fig. 4). No patients had associated visual impairment or vestibular symptoms (Tables 3, 4 and 5). Seven patients received cochlear implantation due to the insufficient amplification of hearing aids (Tables 3, 4 and 5).

Discussion

Mutations in the *CDH23* gene are known to be responsible for both Usher syndrome type ID (USH1D) as well as non-syndromic hearing loss (DFNB12), and molecular confirmation of *CDH23* mutations is clinically important for diagnosis of these conditions. However, clinical application of the detection of *CDH23* mutations has lagged because of the size of the gene. Especially for DFNB12, which is not associated with visual impairment, screening is comparatively difficult, and therefore, little is known about frequencies among the hearing loss population as well as clinical characteristics.

In this study, we have applied two-step screening and identified a significant number of novel pathologic mutations of *CDH23* responsible for non-syndromic hearing loss in a large cohort of patients. All of the possible pathologic mutations identified in this study (Table 1) were missense mutations, being consistent with previous reports that DFNB12 patients associated with missense mutations have milder hearing impairment than in USH1D, which is associated with nonsense, splice-site, or frameshift

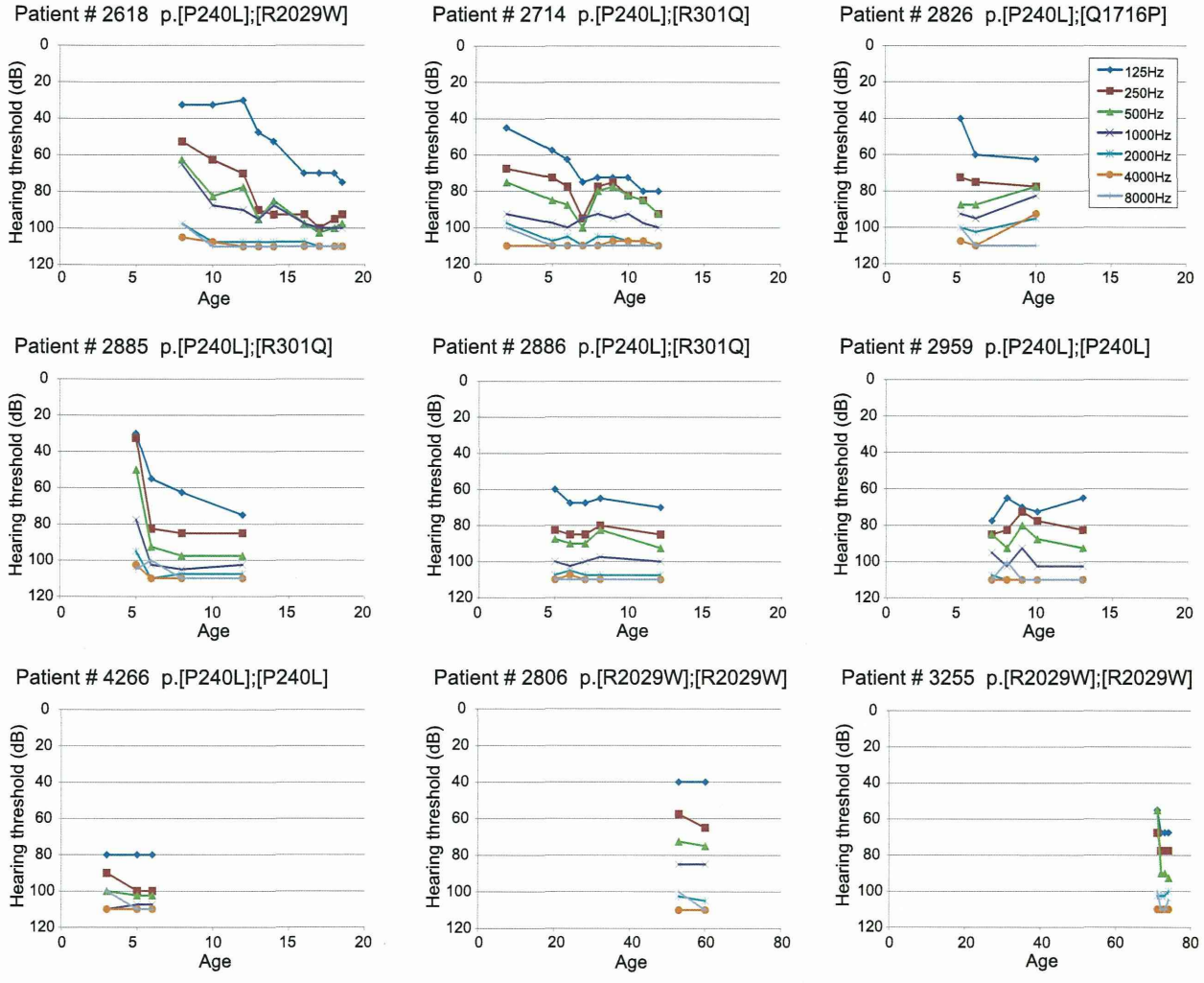

Figure 4. Hearing progression of the patients with *CDH23* mutations. Note that the high frequency portion was already worsened, and the low frequency portion was deteriorated by ages.
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mutations [2,5–7]. None had visual impairment, also supporting this rule. That the majority was found in the EC domain with only one exception found in the cytoplasmic domain, was also in line with the previous reports on DFNB12 [2,5–7]. Of these 26 mutations, five out of 10 possible pathologic mutations were found in DRE, DXNDN, and DXD motifs, which are thought to be important for calcium binding property. These highly conserved EC calcium binding motifs are thought to be essential for linearization, rigidification, and dimerization of the cadherin molecules [9,10]. And the results of computer analysis to predict the impact of amino acid change, all of 10 possible pathologic mutations predicted to cause a severe damage for protein function of *CDH23*.

As a result, 26 patients (from 23 families) had two mutations (in a homozygous or compound heterozygous state), and met criteria for recessive inheritance. A hallmark of recessive mutations is the detection of two mutations in the paternal and maternal alleles and the parents having normal hearing. As seen in previous mutation screening reports, including those for *CDH23* [6,7] as well as *GJB2* and *SLC26A4* [11,12], we encountered 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 *CDH23*, which has not been explored, 2) the observed mutations are rare polymorphisms, 3) the screening method fails to detect the second mutation, and 4) an additional modulatory gene may contribute to hearing loss (for example, *PCDH15*). Although we have not reached the final conclusion, it is most likely that these heterozygous cases are also related to *CDH23* mutations because: 1) allele frequencies are found to be higher in the hearing loss group (Table 2), and 2) the phenotype is similar to that of the patients with two mutations. As shown in Fig. 3, overlapping audiograms of the patients with only one mutation was similar to that with the patients with two mutations (high frequency involved sensorineural hearing loss with residual hearing at the lower frequencies).

Based on the frequencies of 3.7% (including heterozygous cases) of the hearing loss population and 5.7% (including heterozygous cases) of the recessive inherited cases in this study, we confirmed that mutations of *CDH23* are an important cause for non-

syndromic hearing loss and should be borne in mind next to *GJB2* or *SLC26A4* screening. This study revealed that p.P240L account for nearly 43.3%(45/104) of all *CDH23* mutated families in Japan. Common mutations, such as c.35delG or c.235delC in *GJB2* or p.H723R in the *SLC26A4* gene, have been reported in many recessive deafness genes, and usually they are population-specific [12–14]. It is an interesting question whether p.P240L is frequent because of a founder effect or mutational hot spot, but the existence of such a common mutation makes mutation screening easier. Additional frequent mutations found in this study together with TaqMan procedures will facilitate genetic testing for deafness patients.

Concerning mutation spectrum, as in our previous report [6], the *CDH23* mutation spectrum in Japanese is very different from that found in Caucasians and may be representative of those in Eastern Asian populations. Its elucidation is expected to facilitate the molecular diagnosis of DFNB12 and USH1D. It has also been known that prevalent *GJB2* mutations are highly ethnic-specific (see The connexin-deafness homepage; http://davinci.crg.es/deafness/): c.35delG is common in the Caucasoid population, c.167delT was reported as prevalent in Ashkenazi Jews, p.R143W in a restricted village in Africa, and c.235delC in East Asian populations. A series of studies proved a founder effect for these frequent mutations [11,15].

In the present study, using a large cohort of patients, clinical characteristics (onset age, progression, audiograms) of patients with *CDH23* mutations were clarified.

Concerning genotype/phenotype correlations, hearing of the patients with p.[P240L];[P240L] is worse than in those with the other mutations, and tends to be congenital and severe. In contrast, the patients with p.[R2029W];[R2029W] showed a milder phenotype of middle age onset. Overlapping audiograms showed typical high frequency involved sensorineural hearing loss with residual hearing at the lower frequencies.

Concerning age of onset (awareness of hearing loss), the majority was congenital or early onset. But rather later-onset was seen in three patients (#2806, 3255, 501), and they were associated with some particular mutations (p.R2029W and p.T1368M). Their phenotype was rather mild and gradually progressive. It is interesting to note that their phenotype was similar to presbycusis. Actually, *CDH23* mutations have been reported as responsible for age-related hearing loss in mice [16,17].

Progressive nature of hearing loss and the presence of residual hearing are particular phenotypic features of the patients with *CDH23* mutations. Our previous genetic analysis for the patients with high frequency involved hearing loss successfully identified *CDH23* mutations [18]. Seven patients received cochlear implantation and showed good performance after implantation. For the patients with residual hearing, newly developed cochlear implantation; EAS (Electric Acoustic Stimulation) is a good therapeutic option and therefore much attention should be paid to the etiology when considering individual intervention, i.e., regular cochlear implantation or EAS. Genetic testing will be very important prognostic information together with various hearing tests.

In conclusion, a large cohort study using Taqman amplification-based mutation analysis indicated that mutations of the *CDH23* gene are important causes of non-syndromic hearing loss. A mutation screening strategy using TaqMan assay based on the ethnic-specific frequent mutations is a powerful and effective method for such a large gene. Clinical characteristics of patients with *CDH23* mutations is that hearing loss is progressive, high frequency involved sensorineural hearing loss with residual hearing in the lower frequencies. Most cases are congenital but care is needed because some patients show presbycusis-like hearing loss. Cochlear implantation (including EAS) is a good therapeutic intervention for the patients with *CDH23* mutations.

Materials and Methods

To identify additional pathologic *CDH23* mutations, two-step screening was applied in this study. Subjects from independent families were collected from 33 ENT departments nationwide in Japan. All subjects gave prior informed consent for participation in the project, which was approved by the ethical committee of each hospital. Genomic DNA was isolated from peripheral blood by DNeasy Blood and Tissue Kit (QIAGEN, Düsseldorf, Germany) according to the manufacturer's procedure.

First screening (Direct sequencing)

First, we sequenced the *CDH23* gene in 304 Japanese non-syndromic sensorineural hearing loss probands (including our previously reported 64 samples [6]) compatible with autosomal recessive inheritance or sporadic cases. None of the subjects had any other associated neurological signs, vestibular or visual dysfunction. Sanger sequencing was applied to these samples to find mutations responsible for deafness. Detailed procedures were described in our previous report [6]. 26 candidates for disease causing mutations were collected according to the following criteria; 1) non-synonymous variants, and 2) allele carrier rates were less than 2% in control subjects.

Second screening (TaqMan genotyping assay based screening and Direct sequencing)

For the second screening, probes of these 26 mutations selected in the first screening was applied for a custom TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA) [19]. 1396 probands of sensorineural hearing loss patients including 304 probands used in the first screening were used for the second assay. Of them, 1347 had bilateral sensorineural hearing loss and 49 had unilateral sensorineural hearing loss. The inheritance composition of the subjects was as follows: 298 subjects from autosomal dominant or maternally inherited families (two or more generations affected); 919 subjects from autosomal recessive families (parents with normal hearing and two or more affected siblings) or subjects with sporadic deafness (compatible with recessive inheritance or non-genetic hearing loss); the rest had unknown inheritance mode. After TaqMan assay, Sanger sequencing was performed: 1) to confirm these mutations found in TaqMan genotyping assays, 2) to confirm whether mutations were homozygotes or heterozygote, and 3) in cases found in heterozygous state, direct sequencing of the coding region of the *CDH23* was performed.

Controls

The control group consisted of 192 unrelated Japanese individuals without any noticeable hearing loss evaluated by auditory testing.

Next generation sequencing and computer analysis

To elucidate the allele frequency of 26 mutations, comparison was made between allele frequency found in 216 deafness patients and 72 controls based on a next generation sequencing database that is currently being established at Shinshu University (unpublished). In brief, exome sequencing was performed with SureSelect target DNA enrichment (Agilent Technologies, Santa Clara, CA) and Illumina GAIIx sequencing (Illumina, San Diego, CA) according to the manufacturers' procedures. In the SureSelect

library, 76 already reported genes responsible for sensorineural hearing loss and syndromic hearing loss were contained. After base calling, sequence results were aligned with a bowtie program [20] and allele frequencies of each *CDH23* mutation in patients and the control population were calculated. Computer analysis to predict the effect of missense variants on *CDH23* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/) [21,22].

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Author Contributions

Conceived and designed the experiments: SU. Performed the experiments: MM SN. Analyzed the data: MM SN. Wrote the paper: MM SU.

References

1. Bolz H, von Brederlow B, Ramírez A, Bryda EC, Kutsche K, et al. (2001) Mutation of *CDH23*, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nat Genet 27: 108–112.
2. Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, et al. (2001) Usher syndrome 1D and nonsyndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene *CDH23*. Am J Hum Genet 68: 26–37.
3. Rowlands TM, Symonds JM, Farookhi R, Blaschuk OW (2000) Cadherins: crucial regulators of structure and function in reproductive tissues. Rev Reprod 5: 53–61.
4. Muller U (2008) Cadherins and mechanotransduction by hair cells. Curr Opin Cell Biol 20: 557–566.
5. Astuto LM, Bork JM, Weston MD, Askew JW, Fields RR, et al. (2002) *CDH23* mutation and phenotype heterogeneity: a profile of 107 diverse families with Usher syndrome and nonsyndromic deafness. Am J Hum Genet 71: 262–275.
6. Wagatsuma M, Kitoh R, Suzuki H, Fukuoka H, Takumi Y, et al. (2007) Distribution and frequencies of *CDH23* mutations in Japanese patients with non-syndromic hearing loss. Clin Genet 72: 339–344.
7. Oshima A, Jaijo T, Aller E, Millan JM, Carney C, et al. (2008) Mutation profile of the *CDH23* gene in 56 probands with Usher syndrome type I. Hum Mutat 29: E37–46.
8. McHugh RK, Friedman RA (2006) Genetics of hearing loss: Allelism and modifier genes produce a phenotypic continuum. Anat Rec A Discov Mol Cell Evol Biol 288: 370–381.
9. Nagar B, Overduin M, Ikura M, Rini JM (1996) Structural basis of calcium-induced E-cadherin rigidification and dimerization. Nature 380: 360–364.
10. Angst BD, Marcozzi C, Magee AI (2001) The cadherin superfamily: diversity in form and function. J Cell Sci 114: 629–641.
11. Tsukada K, Nishio S, Usami S (2010) A large cohort study of *GJB2* mutations in Japanese hearing loss patients. Clin Genet 78: 464–470.
12. Tsukamoto K, Suzuki H, Harada D, Namba A, Abe S, et al. (2003) Distribution and frequencies of PDS (*SLC26A4*) mutations in Pendred syndrome and nonsyndromic hearing loss associated with enlarged vestibular aqueduct: a unique spectrum of mutations in Japanese. Eur J Hum Genet 11: 916–922.
13. 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.
14. Usami S, Wagatsuma M, Fukuoka H, Suzuki H, Tsukada K, et al. (2008) The responsible genes in Japanese deafness patients and clinical application using Invader assay. Acta Otolaryngol 128: 446–454.
15. 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.
16. Johnson KR, Erway LC, Cook SA, Willott JF, Zheng QY (1997) A major gene affecting age-related hearing loss in C57BL/6J mice. Hear Res 114: 83–92.
17. Zheng QY, Yan D, Ouyang XM, Du LL, Yu H, et al. (2005) Digenic inheritance of deafness caused by mutations in genes encoding cadherin 23 and protocadherin 15 in mice and humans. Hum Mol Genet 14: 103–111.
18. Usami S, Miyagawa M, Suzuki N, Moteki H, Nishio S, et al. (2010) Genetic background of candidates for EAS (Electric-Acoustic Stimulation). Audiologidal Medicine 8: 28–32.
19. de Kok JB, Wiegerinck ET, Giesendorf BA, Swinkels DW (2002) Rapid genotyping of single nucleotide polymorphisms using novel minor groove binding DNA oligonucleotides (MGB probes). Hum Mutat 19: 554–559.
20. Langmead B, Trapnell C, Pop M, Salzberg SL (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10: R25.
21. Kumar P, Henikoff S, Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 4: 1073–1081.
22. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, et al. (2010) A method and server for predicting damaging missense mutations. Nat Methods 7: 248–249.

2. 内耳疾患(感音難聴)

6) Usher 症候群

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新生児・幼小児期に難聴と診断され，その後視覚障害を合併するとコミュニケーション手段の習得に大きな影響を及ぼす．その代表的な疾患が Usher 症候群であり，感音難聴に網膜色素変性症(retinitis pigementosa：RP)を伴い，von Graefe(1858 年)によって報告[1]された．遺伝性であること(常染色体劣性遺伝)が Charles Usher(1914 年)によって報告[2]され，Usher 症候群との名称が付けられた．また，Smith ら(1994 年)によって症状と症状のみられる時期によって 3 つのタイプに分類された[3]．それ以外に，感音難聴に虹彩色素異常を伴う Waardenburg 症候群，進行性感音難聴と腎障害および水晶体異常などを伴う Alport 症候群，進行性感音難聴に偽網膜膠腫や精神発達遅滞を伴う Norrie 病などが難聴に眼科異常を合併する症候群としてあげられる．視聴覚障害を伴う場合は，聴覚障害のみとは異なる介入方法が必要となるため，疾患の早期診断が重要となる．本項では Usher 症候群の症例を呈示し，具体的な検査の進め方を紹介する．

病態生理

A 臨床症状

1 頻度

Usher 症候群の頻度は海外では国により多少の差はあるが，人口 10 万人当たり 3.0 ～ 6.2 人と報告されている[4]．わが国における Usher 症候群の頻度はタイプ 1 の小児(19 歳以下)を対象にした大鳥ら(1978 年)の報告[5]では 10 万人当たり 0.6 人と極端に少ない結果であったが，岩崎ら(2006 年)の RP 患者を対象とした自覚症状に基づいたアンケート調査[4]では 10 万人当たり 6.7 人であり，諸外国と類似した頻度が報告されている．しかし，わが国においてはいまだ Usher 症候群の頻度を含めた実態はわかっていない．

2 タイプ分類(表 1)

Usher 症候群タイプ 1 は新生児・幼小児期より高度～重度難聴を呈し，前庭機能障害を伴う例が多く，視覚症状は思春期前から生じる．タイプ 2 も新生児・幼小児期より高音障害型の難聴を呈し，視覚症状は思春期以降に生じるが，前庭機能障害は伴わない例が多い．タイプ 3 は進行性の難聴を伴うのが特徴である．したがって，タイプ 1 は新生児・幼小児期に高度～重度難聴が診断されても Usher 症候群との鑑別は困難である．Usher 症候群は一般的に先に難聴が発症し，その後夜盲や視野狭窄などの視覚症状がみられる[6]．これまでの海外の報告では，タイプ 1 が 25 ～ 39.3 %，タイプ 2 が 12.2 ～ 75 %，タイプ 3 は 0 ～ 20 % の頻度であり，国によりばらつきがみられたが，わが国における調査結果[7](タイプ 1 は 25.4 %，タイプ 2 は 45.8 %，タイプ 3 は