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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

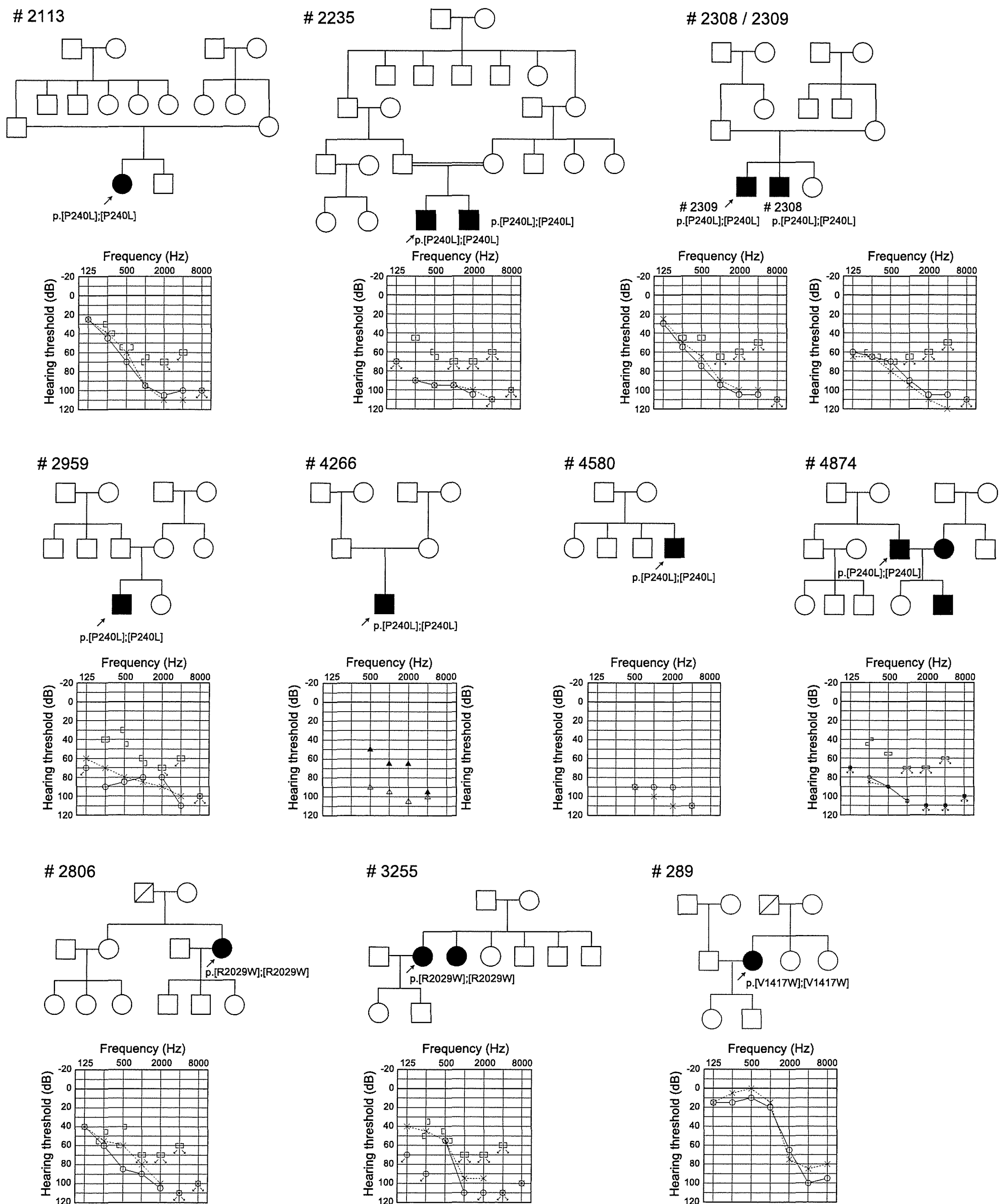

Figure 1. Pedigrees, mutations, and audiograms of the patients with homozygous *CDH23* mutations.
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Results

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.

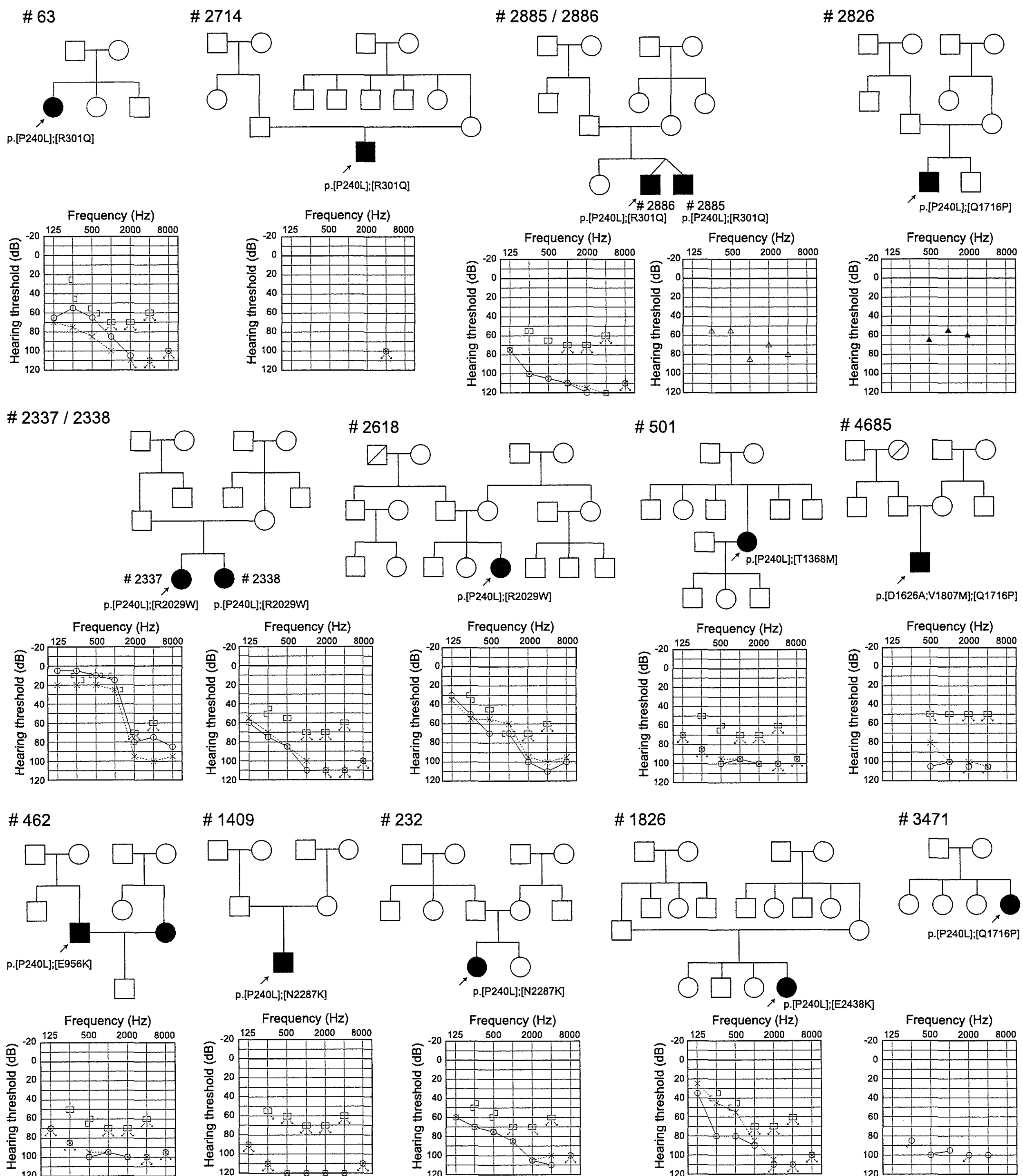

Figure 2. Pedigrees, mutations, and audiograms of the patients with compound heterozygous *CDH23* mutations.
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The following second screening based on TaqMan assay followed by Sanger sequencing confirmed 10 "possibly pathologic" mutations (Table 1) and 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.
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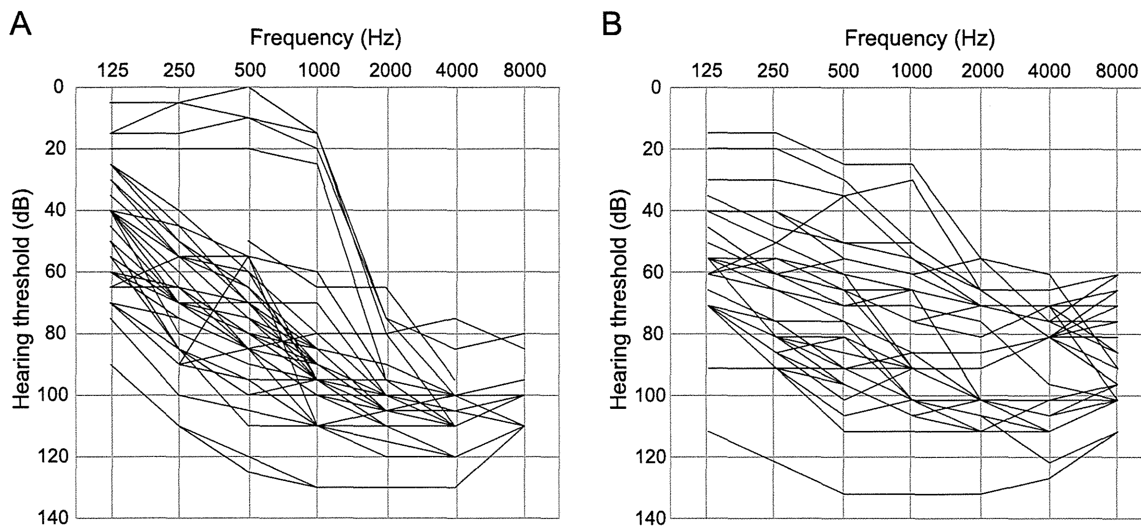

Figure 3. Overlapping audiograms of the patients with *CDH 23* mutations. A: patients with hearing loss caused by the *CDH23* mutations (homozygous or compound heterozygous cases), B: patients potentially caused by the *CDH23* mutations (heterozygous cases).
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of 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. traurus, 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

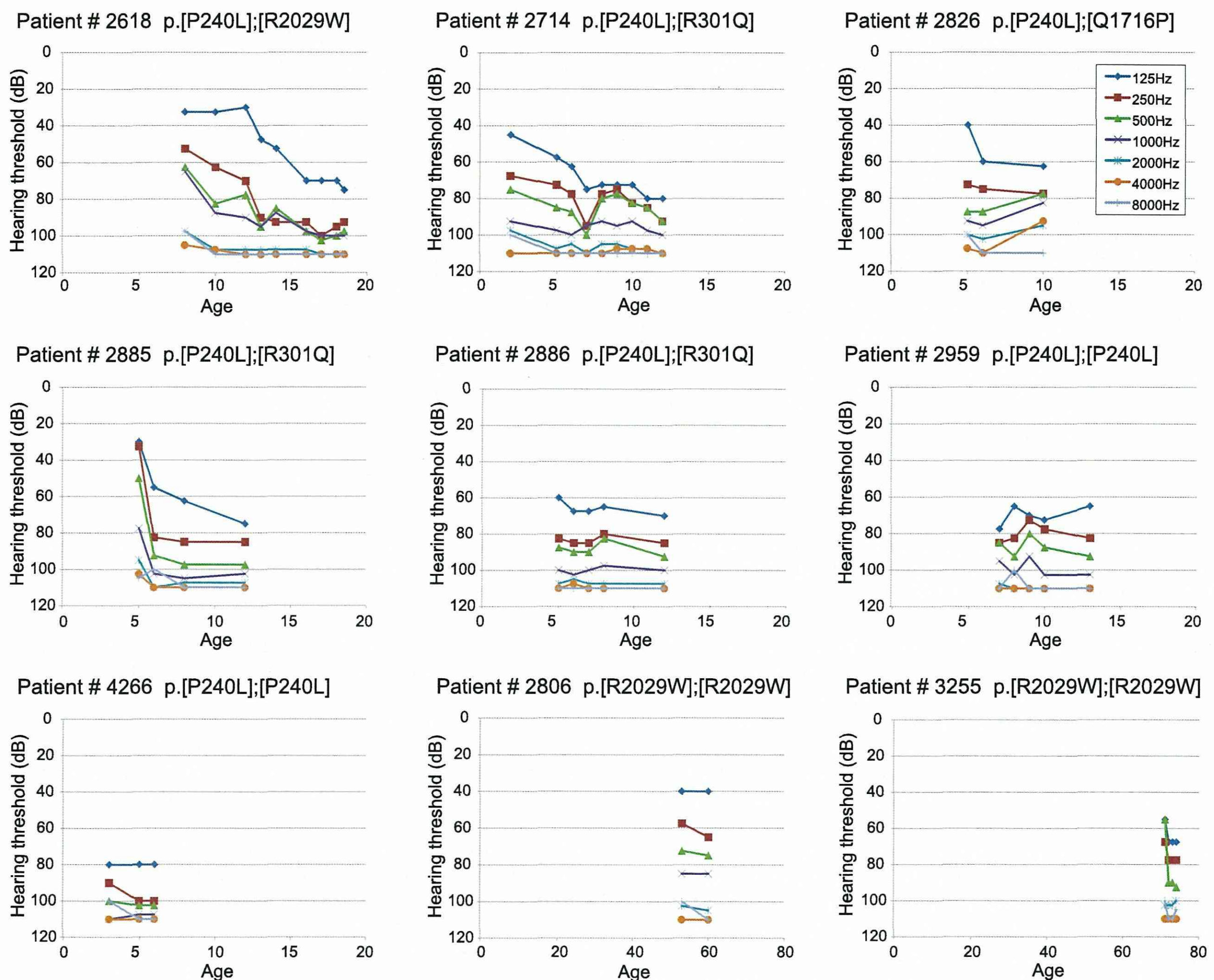

Figure 4. Hearing progression of the patients with *CDH23* mutations. Note that the high frequency portion was already worsened, and the low frequency portion was deteriorated by ages.
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mutations [2,5–7]. None had visual impairment, also supporting 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.

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ORIGINAL ARTICLE

Patients with *CDH23* mutations and the 1555A>G mitochondrial mutation are good candidates for electric acoustic stimulation (EAS)

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Abstract

Conclusions: CDH23 mutations and the 1555A>G mitochondrial mutation were identified among our series of electric acoustic stimulation (EAS) patients, confirming that these genes were important in hearing loss with involvement of high frequency. Successful hearing preservation as well as good outcomes from EAS indicated that patients with this combination of mutations are good candidates for EAS. *Objectives:* Screening for gene mutations that possibly cause hearing loss involving high frequency was performed to identify the responsible genes in patients with EAS. In addition to a review of the genetic background of the patients with residual hearing loss, the benefit of EAS for patients with particular gene mutations was evaluated. *Methods:* Eighteen patients (15 late-onset, 3 early-onset) with residual hearing who had received EAS were included in this study. Genetic analysis was performed to identify *GJB2, CDH23, SLC26A4,* and the 1555 mitochondrial mutations. *Results:* Three early-onset patients had *CDH23* mutations. One late-onset patient had the 1555 A>G mitochondrial mutation.

Keywords: *Residual hearing, hearing preservation, gene, mitochondria, 12S rRNA*

Introduction

Hearing loss in the majority of patients with residual hearing at lower frequencies is more or less progressive, although the speed of progression, i.e. rapid or rather stable, may be dependent on the etiology. An unresolved issue is the prediction of progressiveness based on the etiology of individual hearing loss. We have recently reported at least four genes that are responsible for the candidates for electric acoustic stimulation (EAS), and therefore there is not a single etiology but rather a great genetic heterogeneity involved in this particular type of hearing loss [1]. In this study, screening for mutations of four genes (*GJB2*, *CDH23*, *SLC26A4*, and the 1555 mitochondrial mutations), which possibly cause high frequency hearing loss, was performed to identify the responsible genes for 18 patients with EAS.

Material and methods

Eighteen patients (8 males and 10 females, aged 1–68 years) were included in this study. Clinical features of the subjects are summarized in Table I. As regards onset of hearing loss, 15 patients were late-onset (10–50 years old) and 3 patients were early-onset (most probably congenital). Anamnestic evaluation and/or serial audiogram indicated that all of the patients had progressive sensorineural hearing loss. No patients had any anomalies such as enlarged vestibular aqueduct. All patients had some residual hearing in the lower frequencies, and therefore received EAS. The round window approach was applied for all the patients, and intraoperative and postoperative intravenous administration of dexamethasone was used as described in a previous report [2]. For genetic analysis, direct sequencing for *GJB2, SLC26A4, CDH23*, and

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Table I. Clinical features of subjects in study.

Case no.	Gender	Age (EAS)	Onset (age)	Progressiveness	Inheritance mode	Responsible gene	Implant	Insertion depth (mm)
1	F	59	Late (43)	+	Sporadic	N/I	PULSAR FLEXeas	24
2	F	71	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
3	F	45	Late (25–30)	+	Sporadic	N/I	PULSAR FLEXeas	24
4	F	38	Late (34)	+	Sporadic	N/I	PULSAR FLEXeas	24
5	F	46	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
6	M	29	Late (10)	+	AD	N/I	PULSAR FLEXeas	24
7	M	39	Late (20)	+	AD	N/I	PULSAR FLEXeas	24
8	F	35	Late (25)	+	Sporadic	N/I	PULSAR FLEXeas	24
9	M	52	Late (25)	+	Mitochondrial	*Mit. 1555A>G*	PULSAR FLEXeas	24
10	F	51	Late (30)	+	AD	N/I	PULSAR FLEXeas	24
11	M	39	Late (6)	+	Sporadic	N/I	PULSAR FLEXeas	24
12	F	45	Late (25)	+	Sporadic	N/I	PULSAR FLEXeas	24
13	F	38	Late (10)	+	AR	N/I	PULSAR FLEXeas	24
14	F	60	Late (40)	+	AD	N/I	Combi 40+ standard	31.5
15	M	68	Late (50)	+	Sporadic	N/I	PULSAR FLEXsoft	31.5
16	M	12	Early (3)*	+	AR	*CDH23*	PULSAR FLEXsoft	31.5
17	M	12	Early (1 year 8 months)*	+	AR	*CDH23*	PULSAR FLEXsoft	31.5
18	M	1	Early (0)†	NA	Sporadic	*CDH23*	PULSAR FLEXsoft	31.5

N/I, not identified within four genes.
*Most probably congenital.
†Newborn hearing screening.

the 1555 mitochondrial mutation was performed. Detailed methods are described elsewhere [3-6].

Results

All three early-onset patients had *CDH23* mutations (case nos 16, 17, and 18; Figures 1,2,3). One post-lingual patient had the 1555 A>G mitochondrial mutation (case no. 9; Figure 4). Hearing in the low frequencies after cochlear implantation was well preserved in all 18 cases including these 4 cases.

Case nos 16 and 17 (Figures 1 and 2)

The patients were 12-year-old twins, had the same mutations in the *CDH23* gene, and showed similar audiograms and a slowly progressive nature confirmed by serial audiograms. Both had some residual hearing in the lower frequencies and used hearing aids, but due to the progression of their hearing loss, they received cochlear implants (Nucleus CI24M device, with complete insertion of a straight array through cochleostomy) for the left ear at the age of 5 (no. 16) and 6 (no. 17). In one of the twins (no. 16) residual hearing was successfully preserved (Figure 1D), but the other (no. 17) lost his air-conduction thresholds after cochlear implantation even though the bone-conduction threshold remained stable (Figure 2D). Their audiological performance was good with the cochlear implantation (electric stimulation only). They wanted to have cochlear implants on the other sides, considering their residual hearing and the progressive nature of the hearing loss, and we decided to use a longer atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) to cover the low frequencies (Figure 1A, B, C; Figure 2A, B, C). Hearing was well preserved 6 months postoperatively (Figures 1D and 2D). Both had compound heterozygous mutations (p.P240L/p.R301Q), and their parents were found to be carriers for these mutations (Figure 2E). After identification of the *CDH23* mutations, they were referred for ophthalmologic examination including electroretinography (ERG) and visual field analysis. Both had normal ERG response and no visual field deficits, confirming the nonsyndromic phenotype (DFNB12). Furthermore, they did not have any vestibular problems and showed normal responses in caloric testing. Their hearing thresholds improved to 30 dB and 35 dB (nos 16 and 17, respectively)

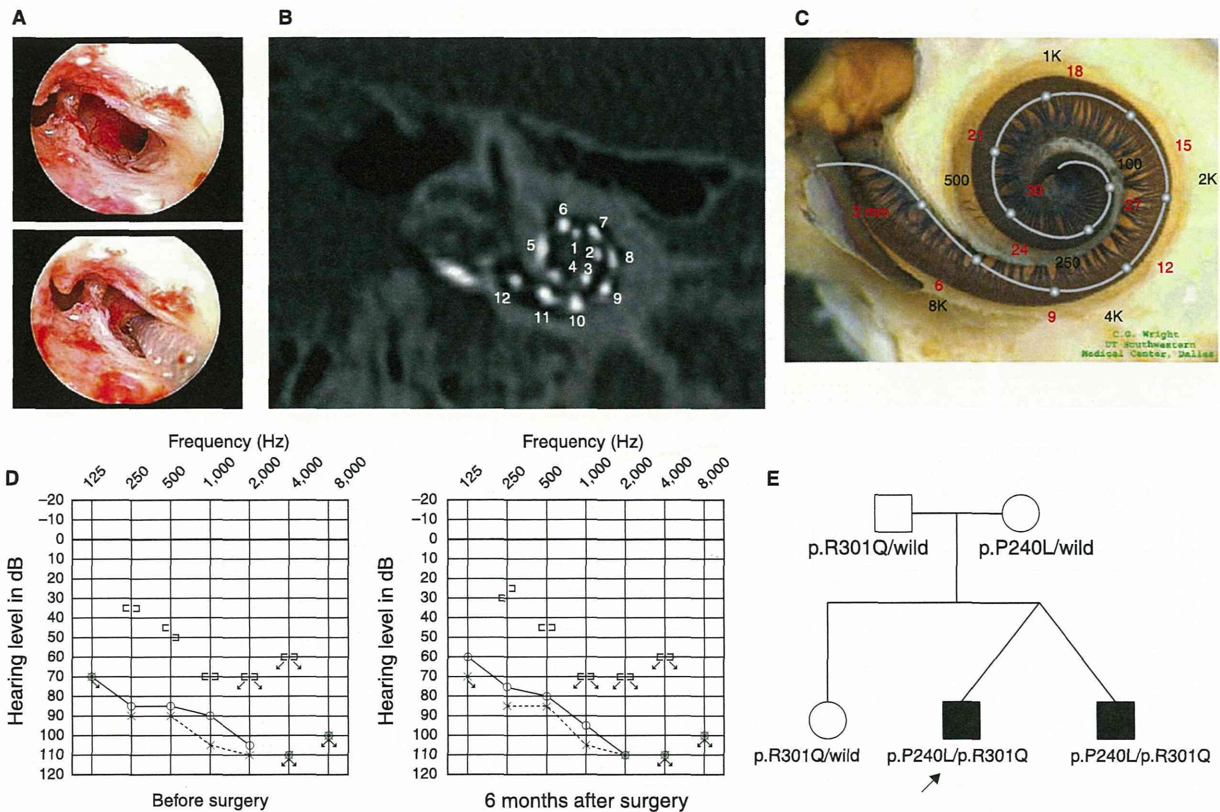

Figure 1. Case no. 16. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. The image of human cochlea neural tissues stained by osmium tetroxide used in Figures 1,2,3,4 was kindly provided by Dr C.G. Wright, USWT, Dallas, USA (red, mm from round window; black, corresponding frequency). (E) Pedigree and the mutations found in the CDH23 gene.

(average for all frequencies from 125 to 8000 Hz) 1 year after cochlear implantation. Their word recognition scores in quiet improved from 64% to 76% (no. 16) and from 60% to 76% (no. 17) at 1 year postoperatively.

Case no. 18 (Figure 3)

This case was a 1-year-old boy with the *CDH23* mutations. Auditory steady-state response (ASSR) evaluated at the age of 4 and 7 months showed some residual hearing at 500 Hz in the right ear (Figure 3D). He first received a left cochlear implant (MEDEL PULSAR CI100/standard electrode) at the age of 9 months. The parents wanted him to use a cochlear implant on the right side as well, and we decided to use a more atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) because of the possible residual hearing in the low frequencies (Figure 3A, B, C). The second cochlear implant surgery was performed at the age of 12 months. Residual hearing measured by conditioned orientation reflex (COR) audiometry [7] was well preserved 1 year after cochlear implantation (Figure 3D). This patient had compound heterozygous mutations (p.[D1216A; V1807M]/p.Q1716P) and the parents were found to be carriers for these mutations (Figure 3E). Although the patient was too young to undergo ophthalmologic examination, he did not have any problems in vision or any vestibular problems, and there is no indicative evidence for Usher syndrome at this time.

In this very young case, auditory behavioral development was assessed by using the LittlEARS® Auditory Questionnaire, which has been designed for children under the age of 2 years [8,9]. The development curve showed a rapid increase in auditory behavior and reached the score seen in normally developed children (c 3F).

Case no. 9 (Figure 4)

This case was a 52-year-old male with the 1555A>G mitochondrial mutation. He noticed hearing loss around age 38 and used hearing aids, but his hearing loss was slowly progressive as evaluated by serial audiograms. Due to residual hearing in the lower frequencies,

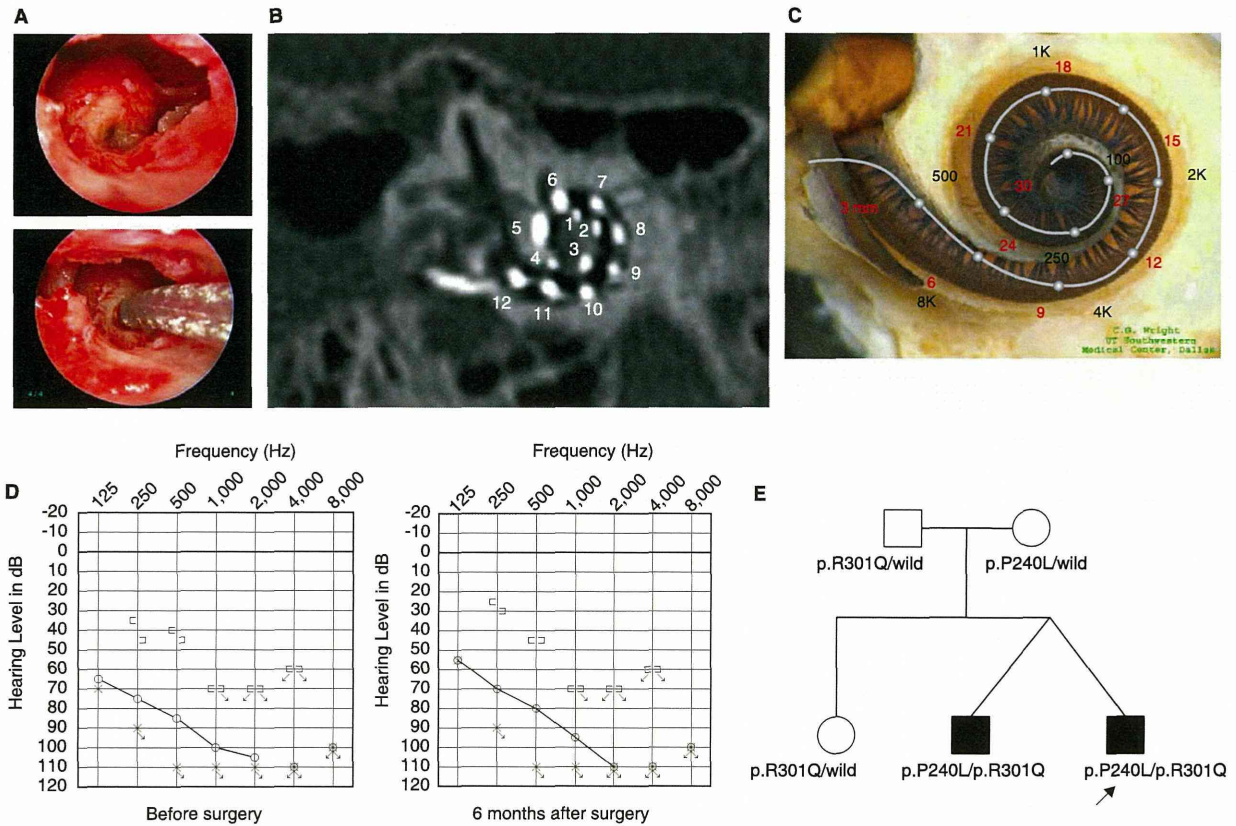

Figure 2. Case no. 17. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the mutations found in the *CDH23* gene.

an atraumatic electrode (MEDEL PULSAR CI100/ FLEXeas electrode) was chosen (Figure 4A, B, C). Residual hearing was well preserved at 2 months postoperatively (Figure 4D). His parents had hearing loss, and the pedigree was consistent with mitochondrial inheritance (as well as autosomal dominant inheritance) (Figure 4E). Genetic screening detected the 1555 mitochondrial mutation in the patient and his mother. He had no history of exposure to aminoglycoside antibiotics. No vestibular symptoms were noted, and no abnormal findings were seen in vestibular testing including caloric response and vestibular evoked myogenic potential (VEMP). His hearing threshold improved to 30 dB (average for all frequencies from 125 to 8000 Hz) 2 months after cochlear implantation. Due to an insufficient follow-up period, his speech recognition score has not yet been evaluated.

Discussion

As predicted from our previous study [1] using patients who fulfilled the criteria for EAS, the *CDH23* mutations and the 1555A>G mitochondrial mutation were in fact found among our series of EAS patients.

Our previous study indicated that the *CDH23* mutations were frequently found in patients with recessive inheritance and the presence of residual hearing is one particular phenotypic feature of the patients with *CDH23* mutations [5], and actually all of the early-onset patients had the mutations in this gene.

The *CDH23* gene encodes cadherin 23, a protein thought to be a molecule that forms the lateral links between the stereocilia of hair cells [10]. One remarkable result in this study is that function of the lateral links remained stable even after deep insertion of the electrode of the cochlear implant. Such functional preservation enabled hearing preservation even in the presence of an electrode covering the corresponding frequency region.

As suggested by genotype–phenotype correlation study, USH1D, which has a more severe phenotype including severe to profound hearing loss, vestibular dysfunction, and retinitis pigmentosa, is usually associated with nonsense, splicing-site, and frameshift

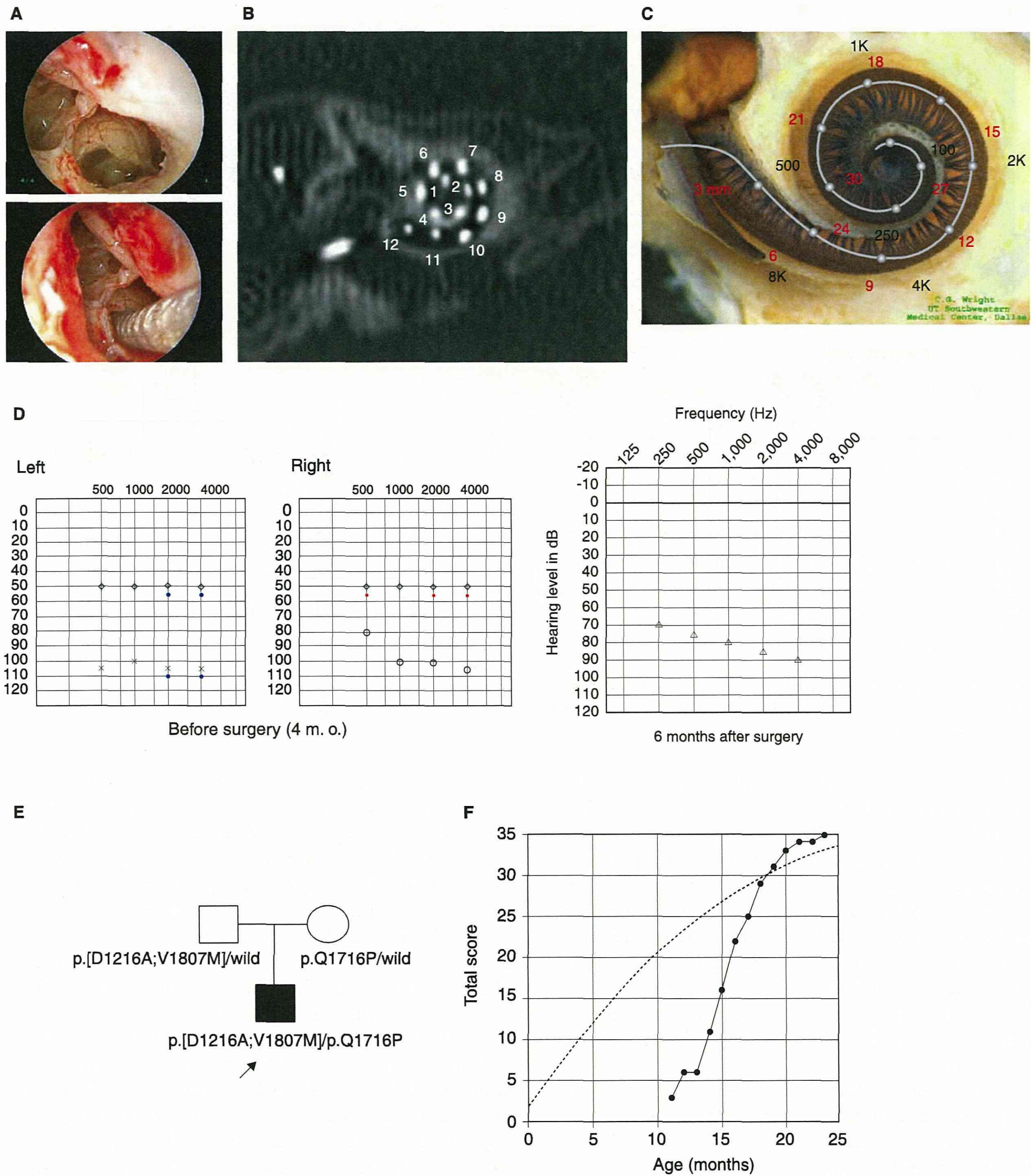

Figure 3. Case no. 18. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative ASSR findings (blue, left; red, right) and postoperative COR audiogram finding. (E) Pedigree and the mutations found in the *CDH23* gene. (F) Auditory behavioral development assessed by LittlEARS® Auditory Questionnaire. The development curve shows rapid improvement in auditory behavior reaching the curve of normally developed children.

mutations. In contrast, DFNB12, which has a milder phenotype, is associated with missense mutations [11,12]. The mutations found in the present three cases (we previously reported case nos 16 and 17 as family no. 3 [5]) are consistent with the general genotype–phenotype correlation rule.

In Usher type I patients, known to have the same etiology, improvement in sound detection as well as

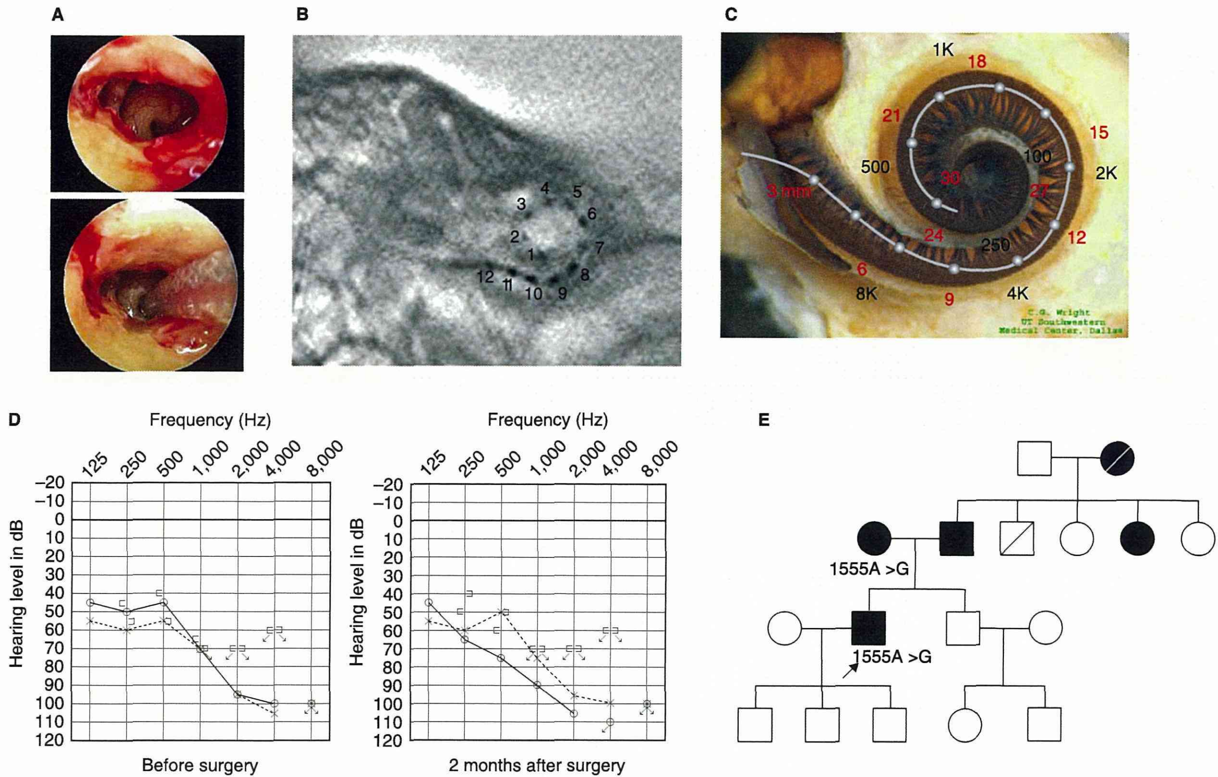

Figure 4. Case no. 9. (A) Endoscopic view of round window insertion, (B) postoperative X-ray finding, (C) imaging with putative location of electrode and the referential tonotoic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the subjects with the mitochondrial 1555 mutations.

speech perception was seen in all patients, especially younger ones [13]. The present study clearly indicates that patients with the *CDH23* mutations are good candidates for EAS. The previous report together with the present cases indicates that progressiveness of hearing loss is a characteristic feature of the patients with this mutation [5,12]. Therefore, deep insertion with longer electrodes is recommended to prevent future deterioration. Successful hearing preservation and prediction of future hearing level by genetic diagnosis may facilitate decision making for early intervention.

It is interesting that *GJB2*, the most prevalent causative gene among the prelingual patients, was not found in the present series of patients. This is probably due to their more or less flat audiograms [1] and therefore they may be good candidates for conventional cochlear implantation.

In very young children, pure tone audiograms are not available. Acoustic brainstem response (ABR) is usually used to evaluate their hearing, but it is difficult to measure residual hearing in the low frequencies. Recently, acoustic steady-state response (ASSR) has been clinically available to measure hearing levels of 500 Hz or 250 Hz, but sometimes the low frequency part is not reliable or convincing [14]. In addition to such hearing testing, genetic testing is useful to predict the residual hearing at low frequencies. Especially for cases with *CDH23* mutations, predicted audiograms can be obtained for the very young patients. Based on this concept, together with consideration of their expected long life (which includes a risk of progression), we chose a longer atraumatic electrode (MEDEL PULSAR CI100/FLEXsoft electrode) for three patients with *CDH23* mutations.

It is known that patients with the 1555A>G mitochondrial mutation are susceptible to aminoglycoside antibiotics [15]. The 1555A>G mutation is one of the most important mutations among the hearing loss population in Japan, and approximately 3% of patients with sensorineural hearing loss possess this mutation [16]. Their hearing loss is known to be slowly progressive [6,17]. This mutation is an important cause in the post-lingual cochlear implant patients, found in 10% of them [16]. It has been reported that a patient with cochlear implantation showed excellent auditory performance [18], indicating that cochlear implantation is a valuable choice of therapy for patients with profound hearing loss caused by this mutation. This mutation was also found in

patients without any aminoglycoside exposure and their hearing loss was usually milder than those with aminoglycoside exposure [19]. Environmental causative factors other than aminoglycoside antibiotics – such as noise or mechanical stress – have been speculated, although not confirmed. The present study provided an important clinical experience that EAS could be safely performed even if the patients have this mutation and therefore possible association of susceptibility for any mechanical stress.

For outcome of EAS, together with successful hearing preservation, all four patients obtained 25–35 dB in average hearing threshold after implantation. Since EAS was implanted as a second cochlear implant for three cases with *CDH23* mutations, it is difficult to evaluate the independent benefit of EAS. However, improvement of word recognition scores after EAS was observed in case nos 16 and 17, indicating that additive benefit was clearly obtained even after a rather long period following the first implants (at 7 years and 6 years, respectively). For case no. 18, although it is also difficult to evaluate the independent benefit of EAS because of the very young age, the auditory behavioral development as assessed by the LittlEARS® Auditory Questionnaire was significantly improved after two consecutive implantations. Since the *CDH23* mutation will be potentially found in rather young candidates, this genetic marker could be available for the existence of residual hearing. For those patients, it is strongly suggested that the surgeon keep in mind the option of performing atraumatic surgery.

In the present series, there are many families with autosomal dominant hearing loss (6 of 18), suggesting that many other genes responsible for dominant hearing loss may be involved. It is also important to note that all of the patients showed progressive hearing loss. We are currently searching for the responsible genes for the patients with high frequency hearing loss.

In conclusion, the *CDH23* mutations and the 1555A>G mitochondrial mutation were identified among our series of EAS patients, confirming that these genes were important in high frequency hearing loss. Successful hearing preservation in these patients as well as good outcomes of EAS indicated that those with these mutations are good candidates for EAS. The present study indicates that genetic testing provides useful information regarding residual hearing and consequent therapeutic options.

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