

**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
						compound homozygote	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.S141S	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.

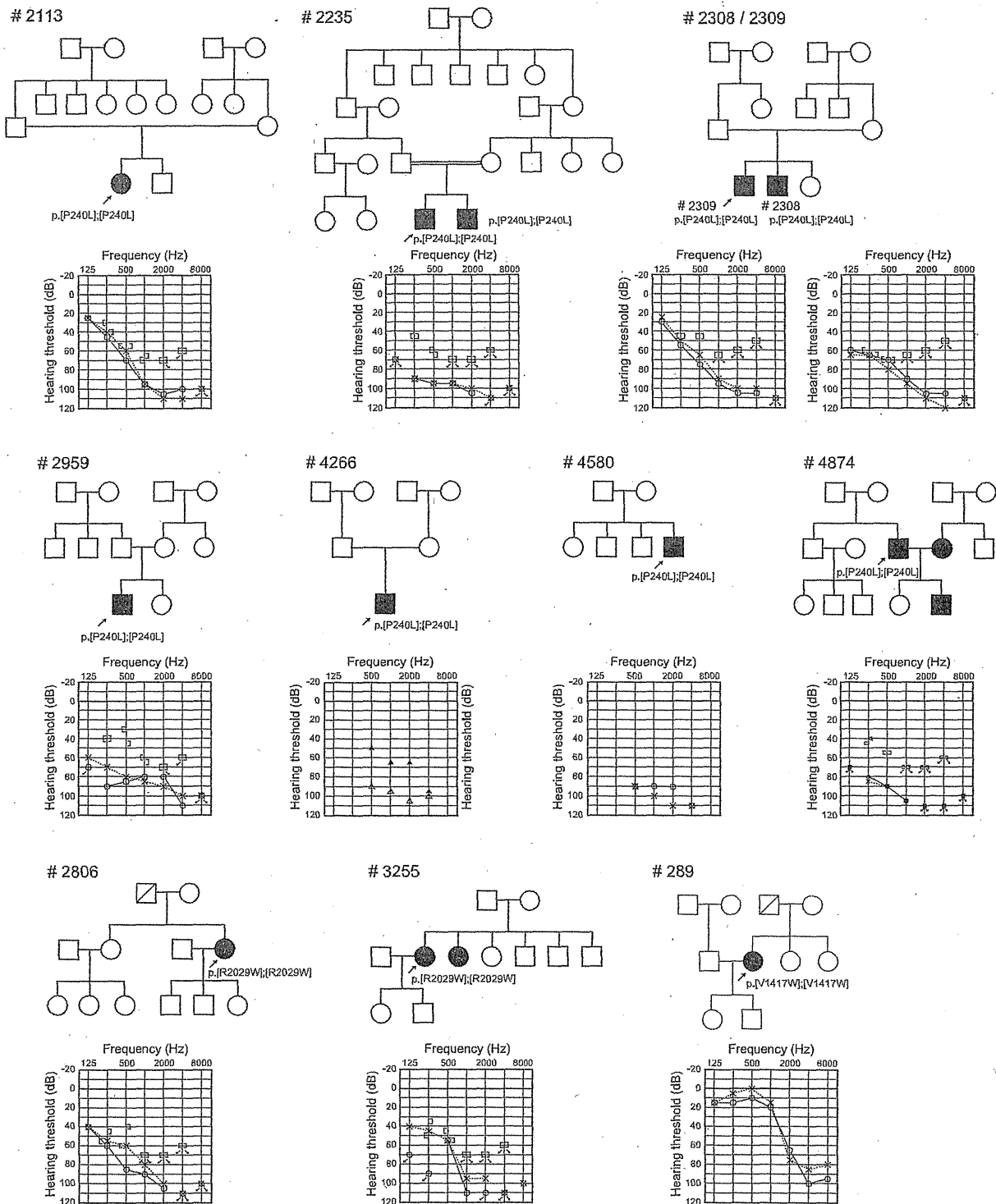
\*not confirmed by segregation study.

\*\*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.

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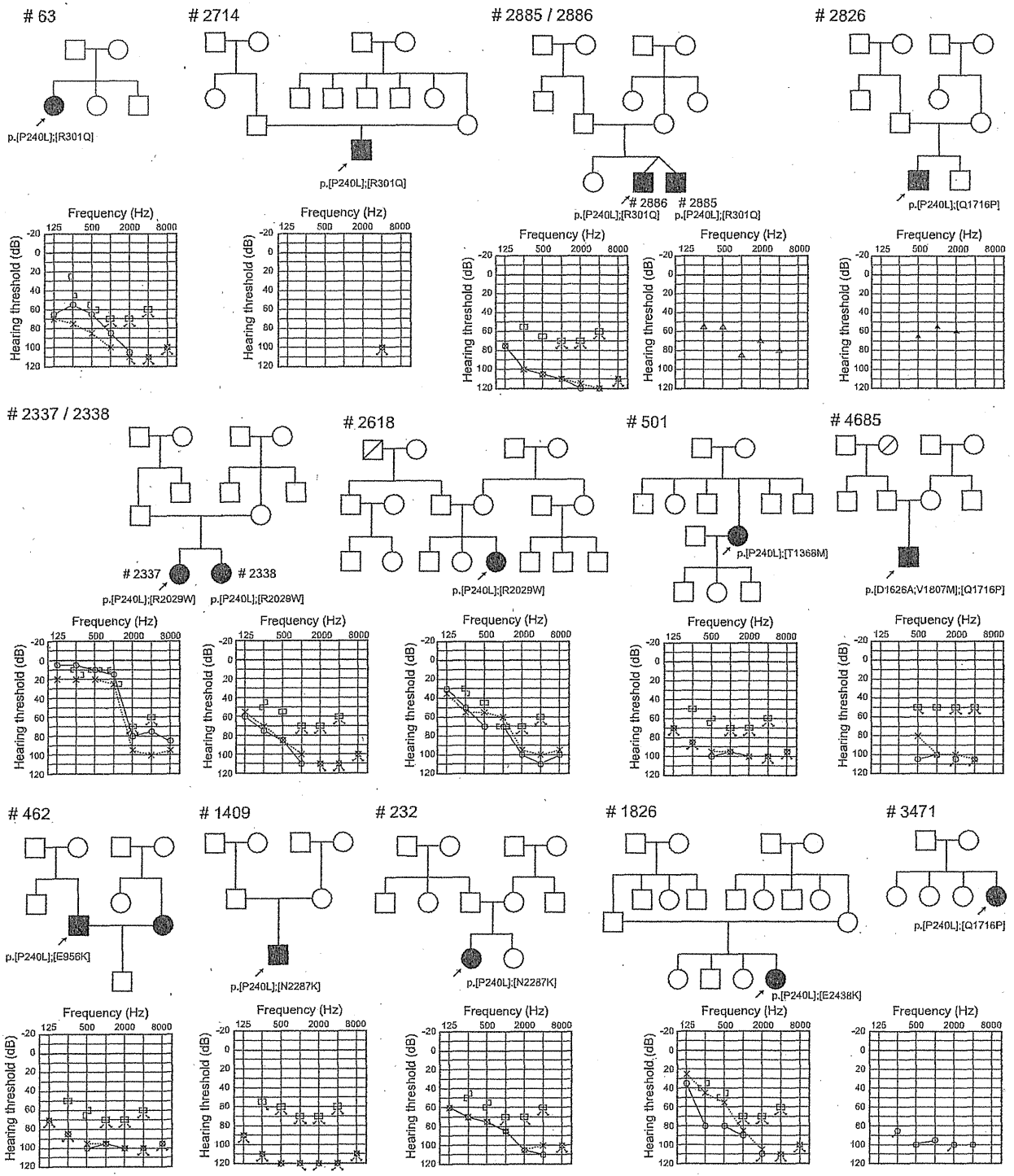


**Figure 1. Pedigrees, mutations, and audiograms of the patients with homozygous *CDH23* mutations.**  
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**Results**

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	Progressiveness	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.

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**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	Progressiveness	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.

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**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	+	-

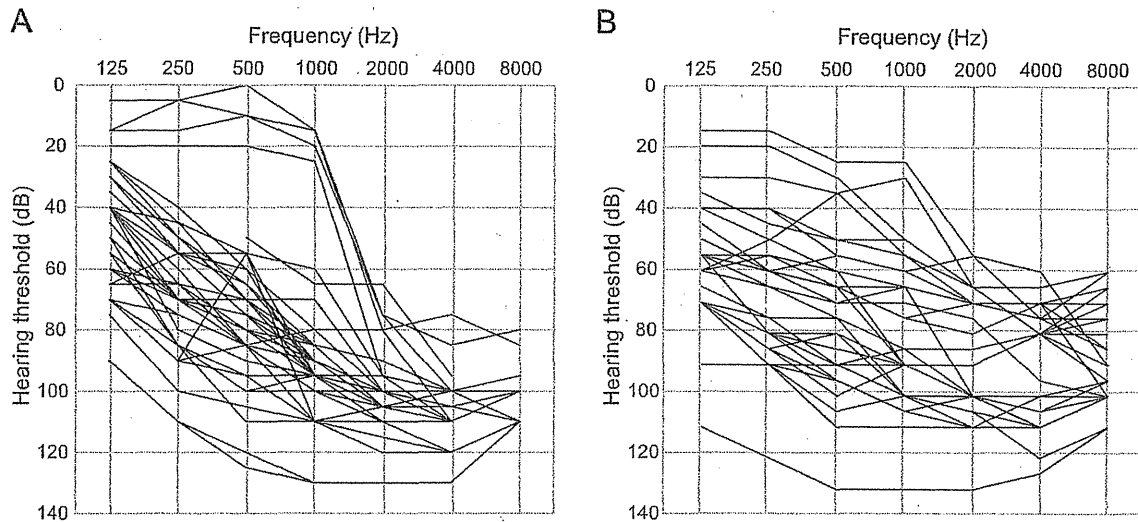
\*average of 500, 1000, 2000 and 4000 Hz.

\*\*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 *CDH23* 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. rerio*. 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

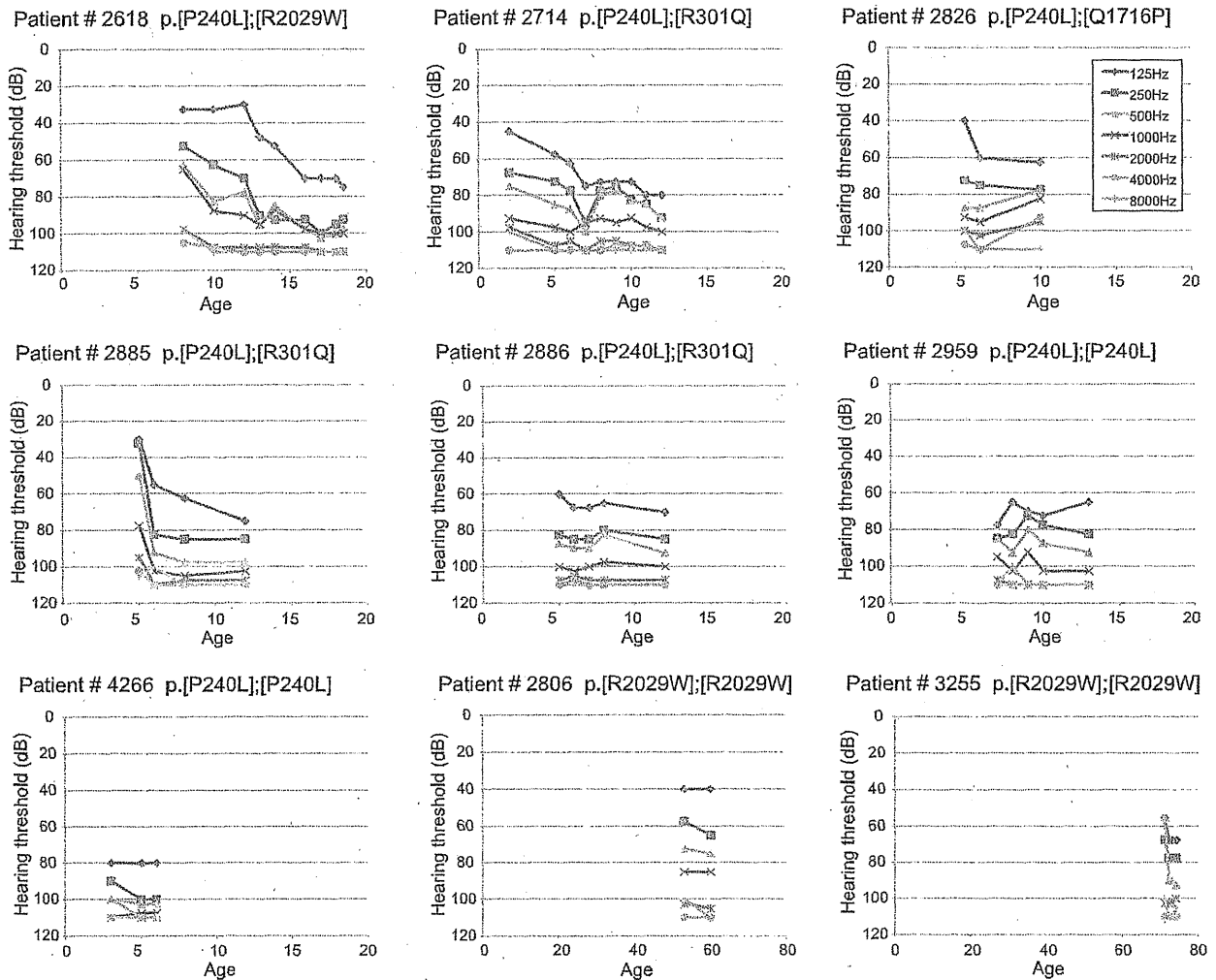
awareness), progressiveness of hearing loss, use of hearing aid/cochlear implantation, visual impairment, and vestibular symptoms. The ages of these patients were from 1 to 71 years. Age of onset (awareness of hearing loss) ranged from congenital to 60 years old, though the majority was congenital or 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. doi:10.1371/journal.pone.0040366.g004

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.[P240I];[P240I] 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<sup>®</sup> 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 GAIx 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].

## References

1. Bolz H, von Brederlow B, Ramirez 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 JM, 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 nonsyndromic 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. Augst BD, Marozzi 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, Shaikat 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, Moteiki H, Nishio S, et al. (2010) Genetic background of candidates for EAS (Electric-Acoustic Stimulation). *Audiological 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 I, Ramensky VF, Gerasimova A, et al. (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7: 248–249.

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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.

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

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 FLEX <sub>cas</sub>	24
2	F	71	Late (30)	+	AD	N/I	PULSAR FLEX <sub>cas</sub>	24
3	F	45	Late (25-30)	+	Sporadic	N/I	PULSAR FLEX <sub>cas</sub>	24
4	F	38	Late (34)	+	Sporadic	N/I	PULSAR FLEX <sub>cas</sub>	24
5	F	46	Late (30)	+	AD	N/I	PULSAR FLEX <sub>cas</sub>	24
6	M	29	Late (10)	+	AD	N/I	PULSAR FLEX <sub>cas</sub>	24
7	M	39	Late (20)	+	AD	N/I	PULSAR FLEX <sub>cas</sub>	24
8	F	35	Late (25)	+	Sporadic	N/I	PULSAR FLEX <sub>cas</sub>	24
9	M	52	Late (25)	+	Mitochondrial	<i>Mit. 1555A&gt;G</i>	PULSAR FLEX <sub>cas</sub>	24
10	F	51	Late (30)	+	AD	N/I	PULSAR FLEX <sub>cas</sub>	24
11	M	39	Late (6)	+	Sporadic	N/I	PULSAR FLEX <sub>cas</sub>	24
12	F	45	Late (25)	+	Sporadic	N/I	PULSAR FLEX <sub>cas</sub>	24
13	F	38	Late (10)	+	AR	N/I	PULSAR FLEX <sub>cas</sub>	24
14	F	60	Late (40)	+	AD	N/I	Combi 40+ standard	31.5
15	M	68	Late (50)	+	Sporadic	N/I	PULSAR FLEX <sub>soft</sub>	31.5
16	M	12	Early (3)*	+	AR	<i>CDH23</i>	PULSAR FLEX <sub>soft</sub>	31.5
17	M	12	Early (1 year 8 months)*	+	AR	<i>CDH23</i>	PULSAR FLEX <sub>soft</sub>	31.5
18	M	1	Early (0) <sup>†</sup>	NA	Sporadic	<i>CDH23</i>	PULSAR FLEX <sub>soft</sub>	31.5

N/I, not identified within four genes.

\*Most probably congenital.

<sup>†</sup>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/FLEX<sub>soft</sub> 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)

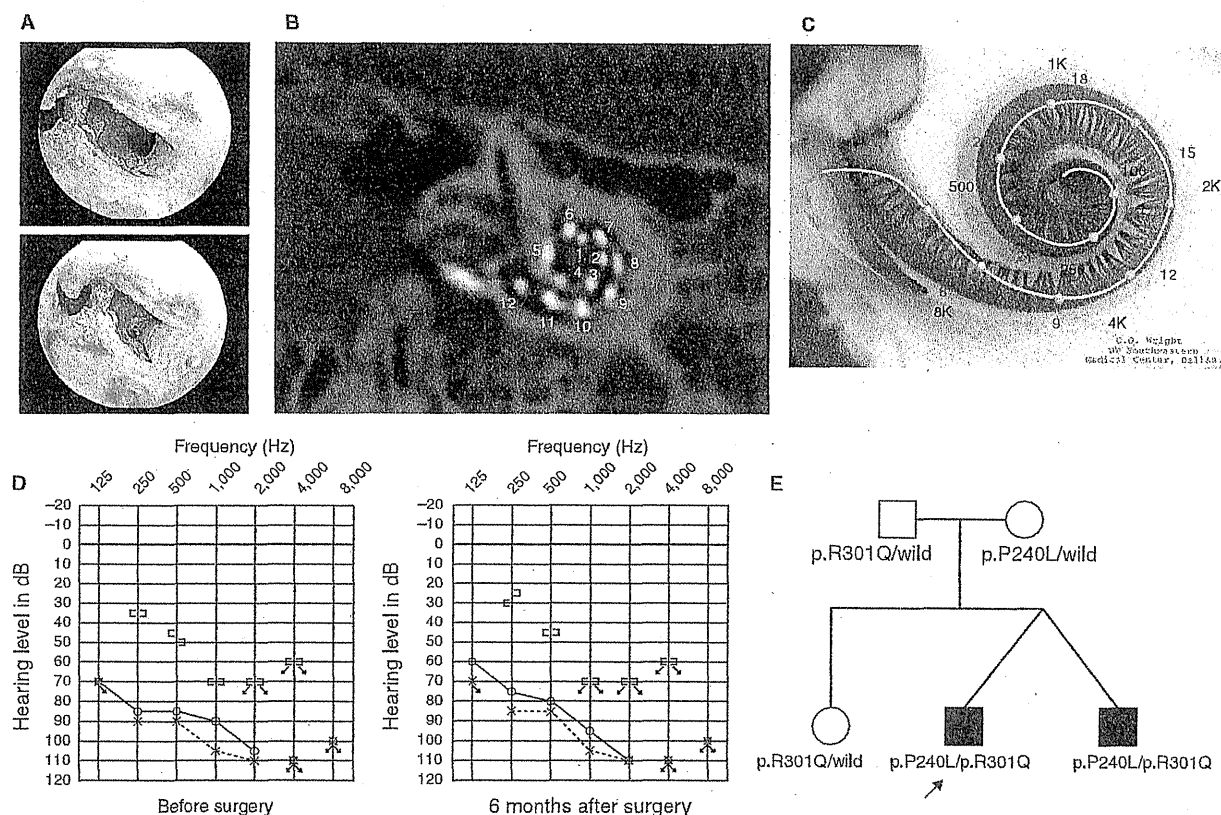


Figure 1. Case no. 16. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotopic map, (D) preoperative and postoperative audiograms. The image of human cochlea neural tissues stained by osmium tetroxide used in Figures 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,

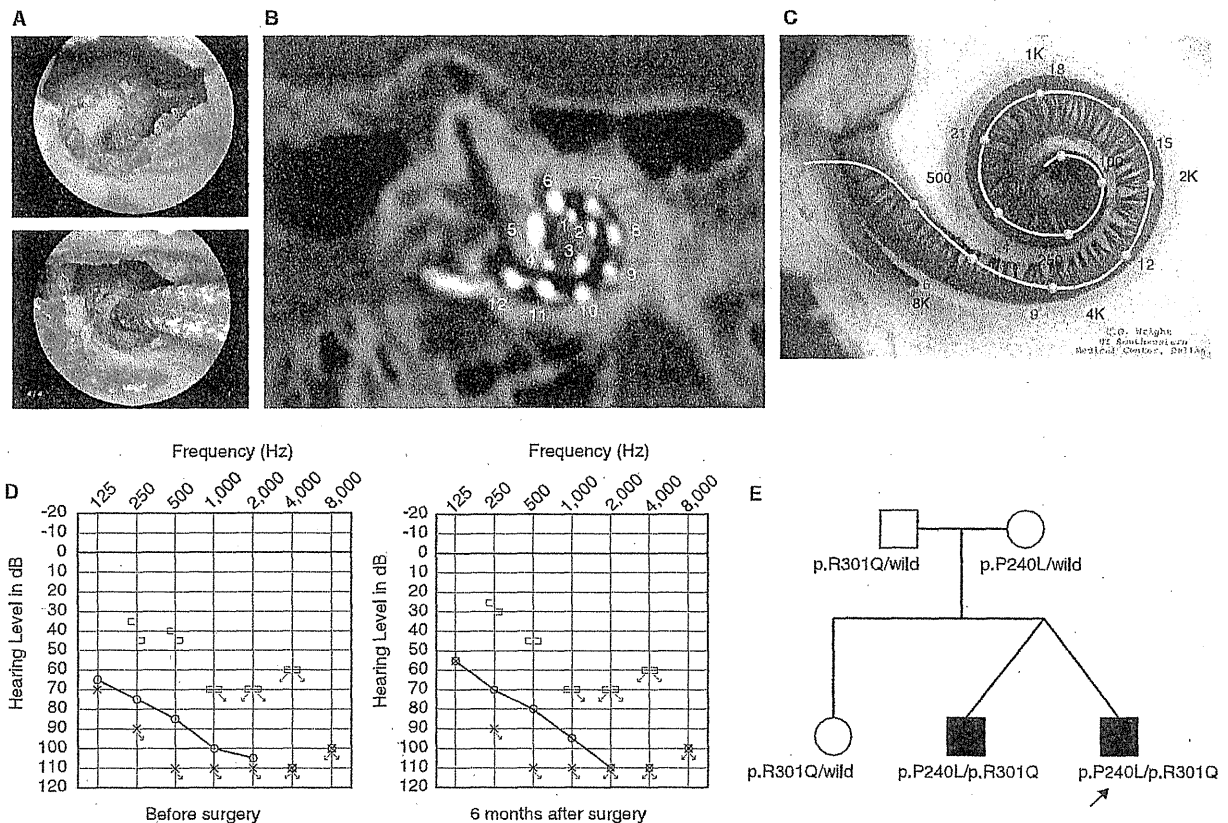


Figure 2. Case no. 17. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotopic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the mutations found in the *CDH23* gene.

an atraumatic electrode (MEDEL PULSAR CI100/FLEXeas electrode) was chosen (Figure 4A, B, C). Residual hearing was well preserved at 2 months post-operatively (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

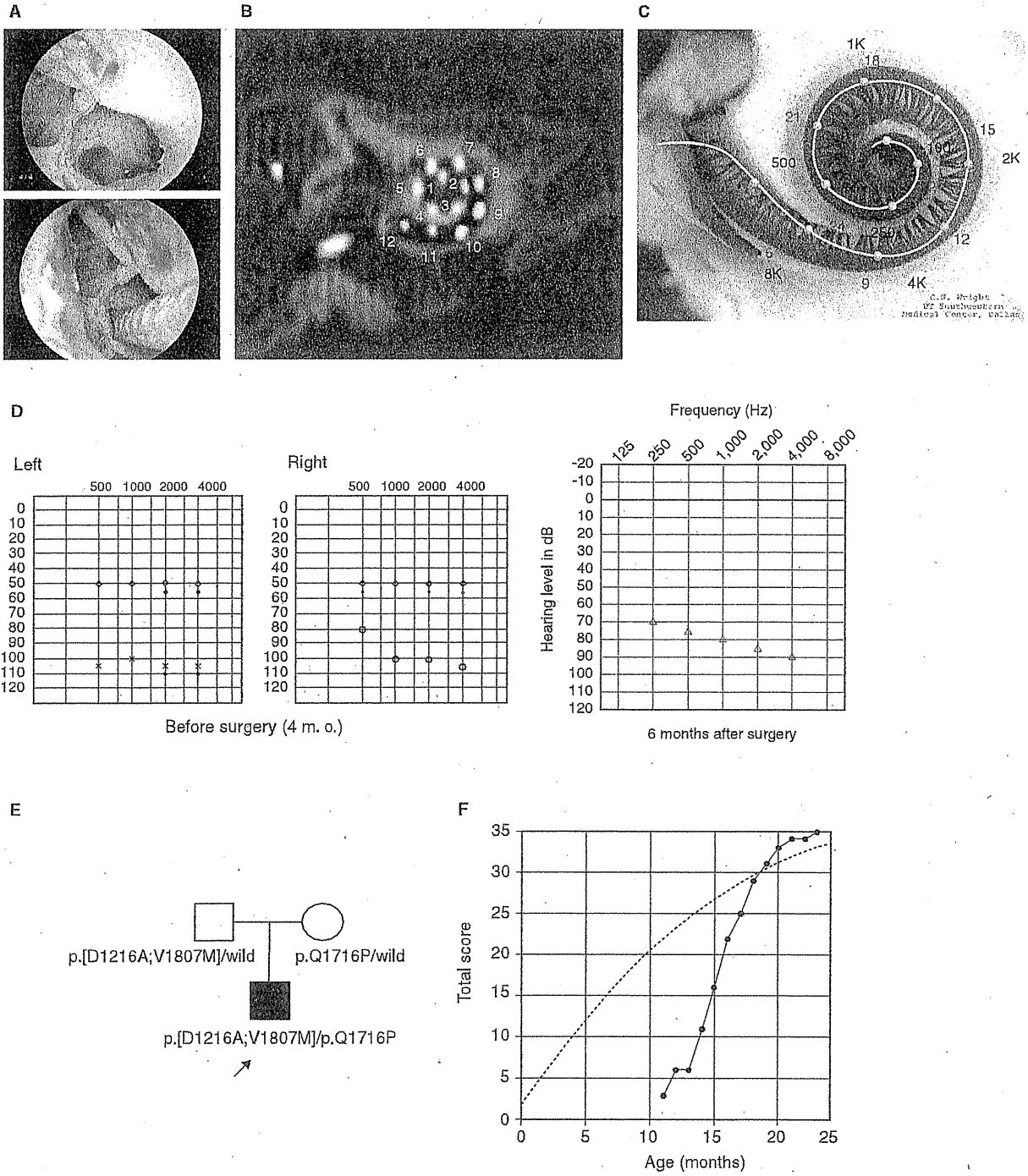


Figure 3. Case no. 18. (A) Endoscopic view of round window insertion, (B) montage CT image, (C) imaging with putative location of electrode and the referential tonotopic map, (D) preoperative ASSR findings (blue, left; red, right) and postoperative COR audiogram finding. (E) Pedigree and the mutations found in the *CDH23* gene. (F) Auditory behavioral development assessed by LittEARS® 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



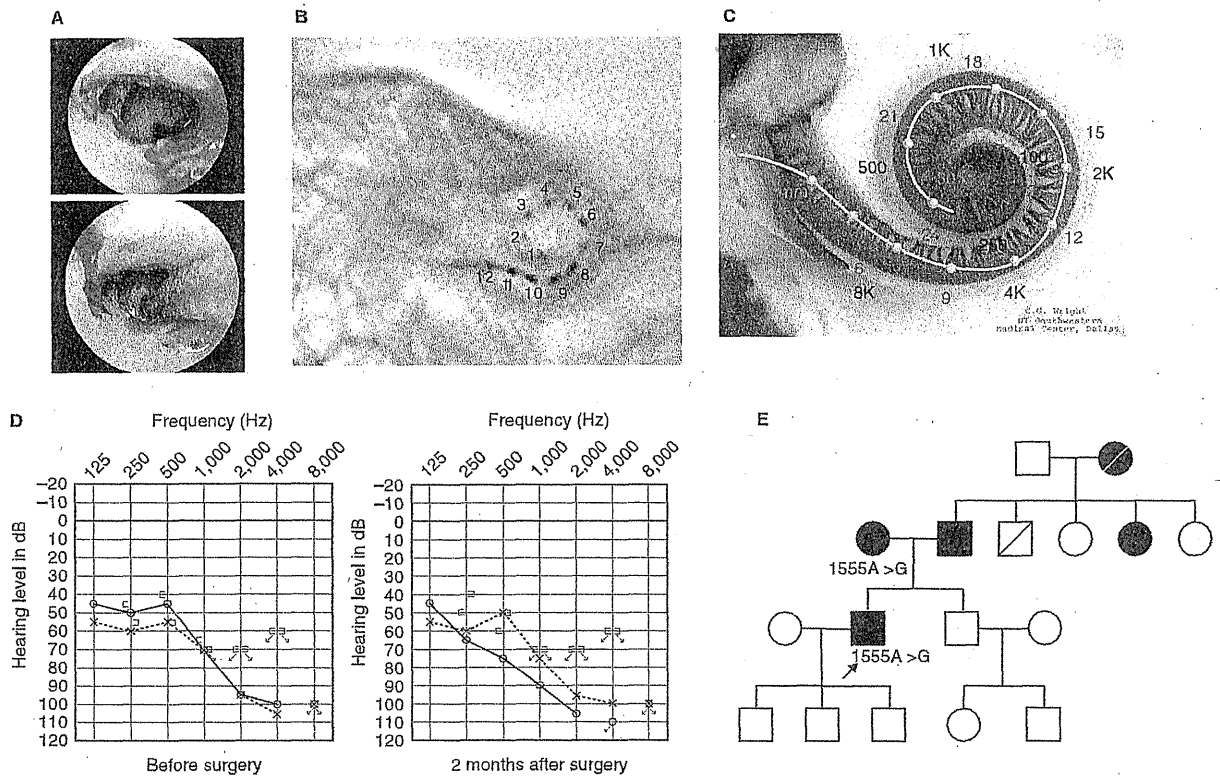


Figure 4. Case no. 9. (A) Endoscopic view of round window insertion, (B) postoperative X-ray finding, (C) imaging with putative location of electrode and the referential tonotopic map, (D) preoperative and postoperative audiograms. (E) Pedigree and the subjects with the mitochondrial 1555 mutations.

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

#### References

- [1] Usami S, Miyagawa M, Suzuki N, Moteki H, Nishio S, Takumi Y, et al. Genetic background of candidates for EAS (Electric-Acoustic Stimulation). *Audiol Med* 2010;8:28–32.
- [2] Usami S, Moteki H, Suzuki N, Fukuoka H, Miyagawa M, Nishio SY, et al. Achievement of hearing preservation in the presence of an electrode covering the residual hearing region. *Acta Otolaryngol* 2011;131:405–12.
- [3] Abe S, Usami S, Shinkawa H, Kelley PM, Kimberling WJ. Prevalent connexin 26 gene (*GJB2*) mutations in Japanese. *J Med Genet* 2000;37:41–3.
- [4] Usami S, Abe S, Weston MD, Shinkawa H, Van Camp G, Kimberling WJ. Non-syndromic hearing loss associated with enlarged vestibular aqueduct is caused by PDS mutations. *Hum Genet* 1999;104:188–92.
- [5] Wagatsuma M, Kitoh R, Suzuki H, Fukuoka H, Takumi Y, Usami S. Distribution and frequencies of *CDH23* mutations in Japanese patients with non-syndromic hearing loss. *Clin Genet* 2007;72:339–44.
- [6] Usami S, Abe S, Kasai M, Shinkawa H, Moeller B, Kenyon JB, et al. Genetic and clinical features of sensorineural hearing loss associated with the 1555 mitochondrial mutation. *Laryngoscope* 1997;107:483–90.
- [7] Suzuki T, Ogiba Y. Conditioned orientation reflex audiometry. *Arch Otolaryngol* 1961;74:192–8.
- [8] Tsiakpini L, Weichbold V, Kuehn-Inacker H, Coninx F, D'Haese P, Almadin S. 2004. LittEARS Auditory Questionnaire. Innsbruck: MED-EL.
- [9] Coninx F, Weichbold V, Tsiakpini L, Autrique E, Bescond G, Tamas L, et al. Validation of the LittEARS ((R)) auditory questionnaire in children with normal hearing. *Int J Pediatr Otorhinolaryngol* 2009;73:1761–8.
- [10] Siemens J, Lillo C, Dumont RA, Reynolds A, Williams DS, Gillespie PG, et al. Cadherin 23 is a component of the tip link in hair-cell stereocilia. *Nature* 2004;428:950–5.
- [11] Bork JM, Peters LM, Riazuddin S, Bernstein SL, Ahmed ZM, Ness SL, et al. Usher syndrome 1D and non-syndromic autosomal recessive deafness DFNB12 are caused by allelic mutations of the novel cadherin-like gene *CDH23*. *Am J Hum Genet* 2001;68:26–37.
- [12] Astuto LM, Bork JM, Weston MD, Askew JW, Fields RR, Orten DJ, et al. *CDH23* mutation and phenotype heterogeneity: a profile of 107 diverse families with Usher syndrome and nonsyndromic deafness. *Am J Hum Genet* 2002;71:262–75.
- [13] Liu XZ, Angeli SL, Rajput K, Yan D, Hodges AV, Eshraghi A, et al. Cochlear implantation in individuals with Usher type 1 syndrome. *Int J Pediatr Otorhinolaryngol* 2008;72:841–7.

- [14] Picton TW, Durieux-Smith A, Champagne SC, Whittingham J, Moran LM, Giguère C, et al. Objective evaluation of aided thresholds using auditory steady-state responses. *J Am Acad Audiol* 1998;9:315-31.
- [15] Prezant TR, Agopian JV, Bohman MC, Bu X, Oztas S, Qiu WQ, et al. Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat Genet* 1993;4:289-94.
- [16] Usami S, Abe S, Akita J, Namba A, Shinkawa H, Ishii M, et al. Prevalence of mitochondrial gene mutations among hearing impaired patients. *J Med Genet* 2000;37:38-40.
- [17] Lu SY, Nishio S, Tsukada K, Oguchi T, Kobayashi K, Abe S, et al. Factors that affect hearing level in individuals with the mitochondrial 1555A>G mutation. *Clin Genet* 2009;75:480-4.
- [18] Tono T, Ushisako Y, Kiyomizu K, Usami S, Abe S, Shinkawa H, et al. Cochlear implantation in a patient with profound hearing loss with the A1555G mitochondrial mutation. *Am J Otol* 1998;19:754-7.
- [19] Usami S, Abe S, Akita J, Shinkawa H, Kimberling WJ. Sensorineural hearing loss associated with the mitochondrial mutations. *Adv Otorhinolaryngol* 2000;56:203-11.

## 残存聴力活用型人工内耳 (EAS : electric acoustic stimulation)

～低侵襲手術, 聴力保存成績, 術後聴取能, 遺伝的背景について～

宇佐美 真 一

### Our Experience with EAS (Electric Acoustic Stimulation) in Japan

—Atraumatic Surgery, Hearing Preservation, Outcome, and Genetic Background of the Patients—

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Advances in developing new atraumatic concepts of electrode design as well as in the surgical technique have enabled hearing preservation after cochlear implantation surgery, and EAS (electric acoustic stimulation) accompanied with hearing preservation is a new trend for patients with residual hearing at the lower frequencies. Combined post-operative imaging with the referential tonotopic map clearly indicated that hearing preservation can be achieved even in the presence of a long electrode covering the residual hearing region. Achievement of atraumaticity of round window insertion in the present cases has been confirmed from the viewpoint of minimal drilling time as well as preserved vestibular function. Post-operative evaluation after full insertion of the electrodes showed hearing at low frequencies was well preserved in all 16 cases (16 ears) who were followed up for more than one month. As for outcome of EAS, together with successful hearing preservation, all patients obtained 25-40dB in their average hearing threshold after implantation. The word recognition score significantly improved post-operatively. With regard to the genetic background of the patients, *CDH23* mutations and the 1555A>G mitochondrial mutation were identified among our series of EAS patients, confirming that these genes were the important genes responsible for high frequency involved hearing loss. Successful hearing preservation as well as good outcomes from EAS in these patients indicated that those with this combination of mutations are good candidates for EAS.

**Keywords :** EAS (electric acoustic stimulation), atraumatic surgery, hearing preservation, *CDH23*, gene, mitochondria

#### はじめに

高音急墜あるいは漸傾型の聴力像を呈する難聴患者は低音部に残存聴力を有するものの高音部の聴取能がきわめて悪く, 補聴器のフィッティングが難しいために患者の望む補聴効果の得られない場合が多い。一方, 低音部の聴力が残存しているために従来の人工内耳の適応に当てはまらず, 有効な治療法がなかった。近年, 残存聴力のある低音部は音響刺激で, 重度難聴の高音部は電気刺激で音を送り込む「残存聴力活用型人工内耳 (electric acoustic stimulation : EAS)」が開発され, 人工内耳の適

応や可能性を広げるものとして注目されている (図 1, 2)。従来は内耳に電極を挿入することにより, もともと内耳機能 (基底板の振動による音響入力) は失われると考えられていたが, 1999年 von Ilberg ら<sup>1)</sup>が低音部に残存聴力を有する高音急墜型の聴力像を呈する難聴患者に対して, 低音部は音響刺激で, 高音部は人工内耳で聞き取るいわゆる EAS を臨床的に応用し報告した。これは通常の音の振動による情報と, 人工内耳により聴神経に入れた電氣的刺激による情報が聴覚中枢で融合可能であることを初めて実証したきわめて画期的な報告である。