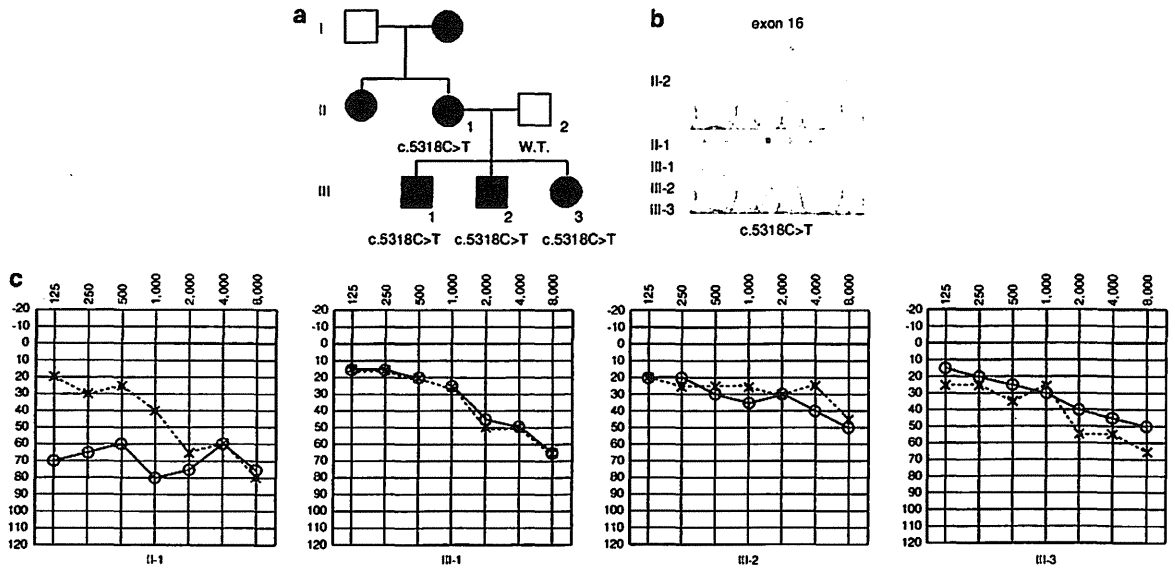

Figure 1 (a) Pedigree of the family F818 and audiograms of four different patients. Black and white symbols indicate the affected and the unaffected subjects, respectively. (b) Electropherograms for unaffected (wt) and affected family members showing the heterozygous c.5318C>T mutation of *TECTA* co-segregating with hearing loss in this family. (c) Audiograms of four different affected patients show high frequency hearing loss. Patient II-1 suffered decreasing hearing level in the right ear with cholesteatoma and postoperative change.

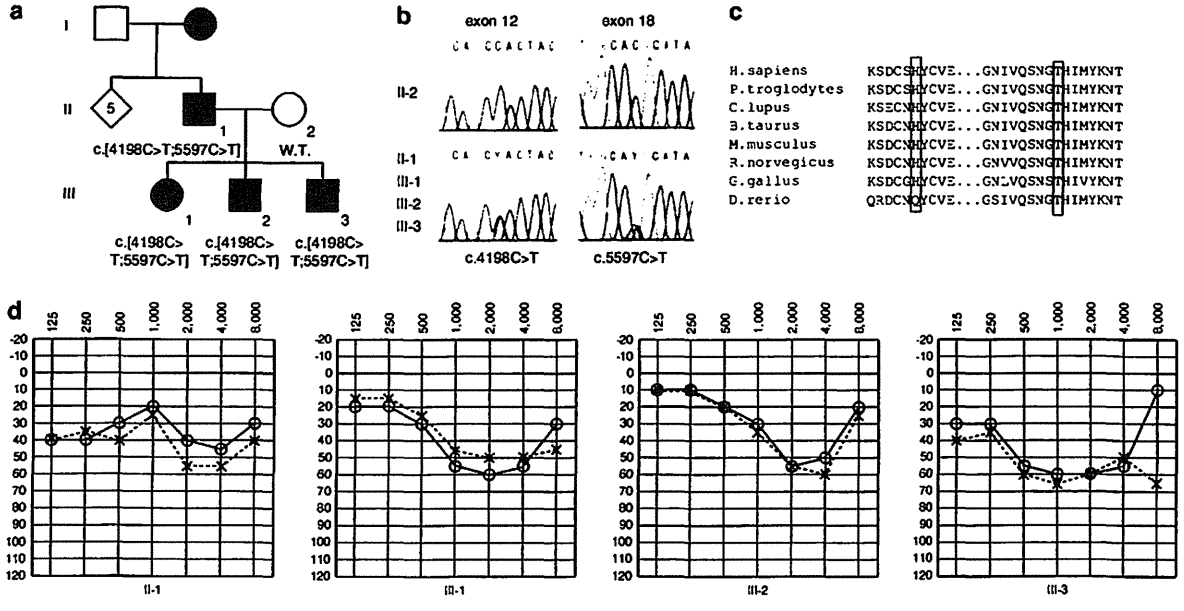

Figure 2 (a) Pedigree of the family F237 and audiograms of four different patients. The marks and symbols are as described in Figure 1. (b) Electropherograms for unaffected (wt) and affected family members showing the heterozygous c.4198C>T and c.5597C>T mutations of *TECTA*. (c) Multiple amino-acid alignment of proteins homologous to the alfa-tectorin ZP domain containing these mutated positions. Amino-acid residues that are identical among all of the homologs are enclosed. (d) Audiograms of four different affected patients showing deterioration in mid-frequency as a U-shaped audiogram.

Localization of ZP domain mutants

The inherent fluorescence of GFP determined the intracellular localization of the recombinant fusion proteins (Figure 4). Transfected GFP—ZP domains of α-tectorin wt (wild type) were found to be localized as labeled puncta, which may be secreted along the plasma membrane. In contrast, GFP–ZP domains of α-tectorin mutants, (GFP–ZP mut) C1837G, Y1870C and R2021H, were not recognized at the plasma membrane but were retained within the cytoplasm where they formed vesicles.

DISCUSSION

We have identified four independent AD families associated with four different *TECTA* mutations. Before this study, one Japanese family with 6063G>A (R2021H) mutation had been reported.[5] Including

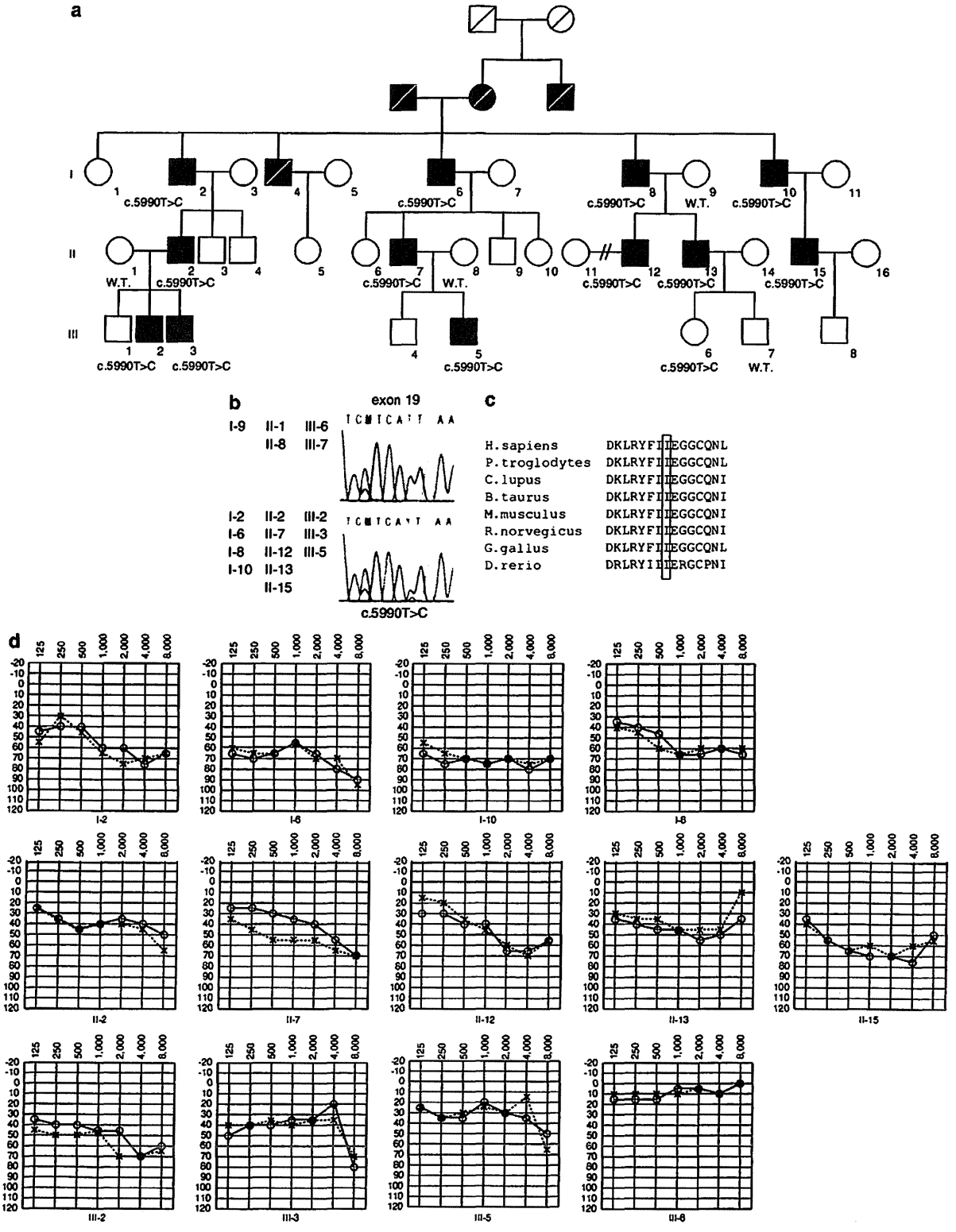

Figure 3 (a) Pedigree of the family F652, (b) electropherograms for unaffected (wt) and affected family members showing the heterozygous c.5990T>C mutation of *TECTA*. (c) c.5990T>C is predicted to substitute isoleucine for threonine acid at amino acid position 1997. Multiple amino-acid alignment of protein homologs was conserved. (d) Audiograms of affected patients were flat to U-shaped, and there was a tendency to decreased hearing level associated with age.

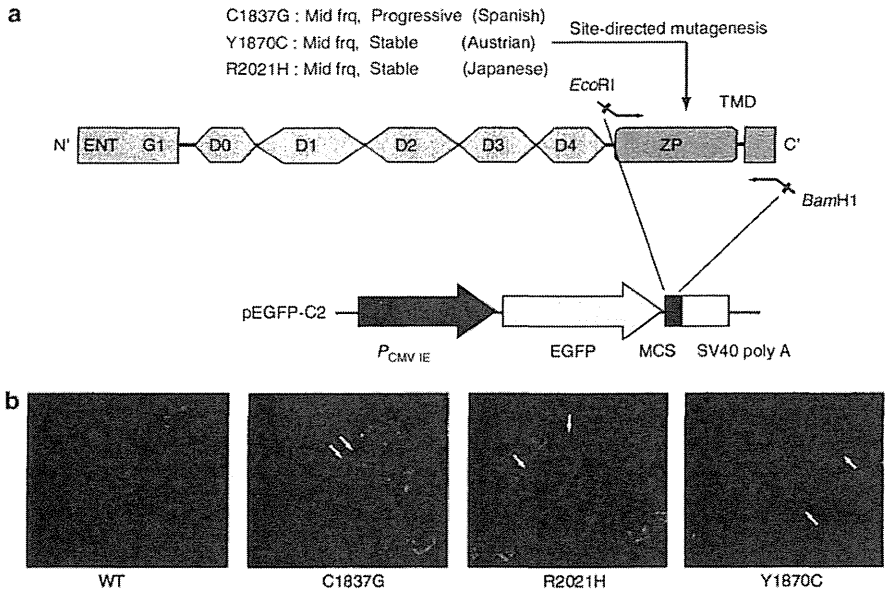

Figure 4 (a) Domain structure of the human alfa-tectorin protein. Three mutants in ZP domain protein including Transmembrane domain were generated by site-directed mutagenesis. Resulting cDNAs digested with *EcoRI*/ *Bam*HI and cloned into the site of pEGFP-C2 plasmid are shown below. (b) Protein expression in COS-7 cells transfected with GFP–ZP wt showing a characteristic puncta along the plasma membrane. In contrast, GFP–ZP mutants (mut) C1837G, R2021H and Y1870C, which were associated with mid-frequency hearing loss phenotypes, were not recognized at the plasma membrane but were retained within the cytoplasm as white arrows indicated.

that family, prevalence of ADNSHL with *TECTA* mutation was 2.9% (4/139 families), which may be a relatively high incidence. Hildebrand *et al.*[8] reported that its prevalence was about 4% in Spanish ADNSHL families (17/374 families). In our results, when limited to moderate hearing loss patients there was a higher rate of detection (7.7%; 4/52 families).

In this study, all patients showed typical genotype–phenotype correlations of SNHL with *TECTA* mutation as previously described.[8,12,15] The family F818 in which the R1773X (c.5318C>T) mutation in the ZA domain was detected, showed high frequency hearing loss that was slowly progressive. The affected proband (Figure 1) noticed bilateral hearing impairment when she was around age 20, and her right hearing level was worse than the left because of cholesteatoma in her right ear. Hearing impairment was detected in her sons in school health checks but they had never suffered vertigo and no inner ear abnormality was seen in CT scans.

In the family F237, two missense mutations, H1400W (c.4198C>T) and T1866M (c.5597C>T), were detected in α-tectorin. The mutation H1400W in exon 12 was in the ZA domain of α-tectorin, whereas T1866M was in the ZP domain. Both amino-acid residues were conserved among another species. It had been suggested in a previous report that *TECTA*-affected mid-frequency hearing impairment appeared to be related to the position of the mutations in the ZP domain of α-tectorin. Considering the phenotype and position of the mutation, T1866M was likely to be causative for hearing impairment in this family. The influence of the nucleotide change of c.4198C>T on apparent effect of splicing of the *TECTA* mRNA cannot be predicted. However, because this change was not present in the controls, it cannot be ruled out that it has an effect on the phenotype of these patients or it may even act synergistically with the T1866M (c.5597C>T) mutation. The similar results with two changes in one family were reported by Plantinga[12] in 2006. The T1866M mutation that we detected in this study was previously reported in one family each in Korea, Spain and the USA.[7,8] Hildebrand reported that the Spanish and American cases do not suggest a founder effect for this mutation.[8] Therefore, this T1866M mutation, now known to be existent in four independent families from four different countries, is suggested to be a possible mutational site hot spot.

In the family F652, we detected a novel mutation, 11997T (c.5990T>C), in exon 19 located in the ZP domain of α-tectorin. The audiograms of affected patients indicated U-shaped mid-frequency hearing loss, associated with a ZP domain mutation previously reported. Regarding progression of hearing loss, these audiograms showed that the thresholds depend on age among these generations. Accordingly, this mutation would lead to slowly progressive mid-frequency hearing loss. Interestingly, an affected female (Figure 3a(III-6)) exhibited normal hearing at the age of 12. The other affected male members had been diagnosed with hearing loss between the ages of 10 and 15. Pfister had reported that there was gender difference in the severity of hearing loss in affected family members bearing the same *TECTA* mutations, with males being significantly more affected than females.[16] Therefore, there is a need for more detailed audiologic analysis and follow-up in the other families to see whether they also show the same phenomenon in hearing impairment.

The present study further investigated whether the molecular mechanisms of hearing loss associated with *TECTA* mutations could be explained by protein expression. In contrast to COS-7 cells transfected with GFP–ZP wt, which were found to be localized in punctate spots along the plasma membrane (Figure 4b), the localization of GFP–ZP mutation proteins were not seen on the cellular membrane but mainly aggregated in the cytoplasm (Figure 4b). These mutations were located in the ZP domain of α-tectorin, this domain is responsible for secretion and polymerization of extracellular proteins into supramolecular structure.[17–19] The results of these

findings suggest that each missense mutation may lead to the lack of assembly of secretion, and may reduce the incorporation of α-tectorin into the tectorial membrane.

In this study, we have reported the prevalence of *TECTA* mutations in Japanese ADNSHL patients detected by genetic screening, and confirmed the genotype–phenotype correlations. We also elucidated how mutation in the ZP domain of α-tectorin causes hearing loss through protein expression study of ZP domain proteins. *TECTA* mutation screening should be considered for patients with mild to moderate inherited AD hearing loss because of its higher incidence. Further investigation of this gene is necessary to identify the function in the cochlea responsible for the distinct phenotype.

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

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Abstract

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

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Introduction

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

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

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

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

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

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

Table 1. Possible pathologic variants found in this study.

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

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

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

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

*not confirmed by segregation study.
**one normal hearing subject with homozygotes.
***Computer analysis to predict the effect of missense variants on *CDH23* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2;http://genetics.bwh.harvard.edu/pph2/).
N/A: TaqMan probe not available.
doi:10.1371/journal.pone.0040366.t002

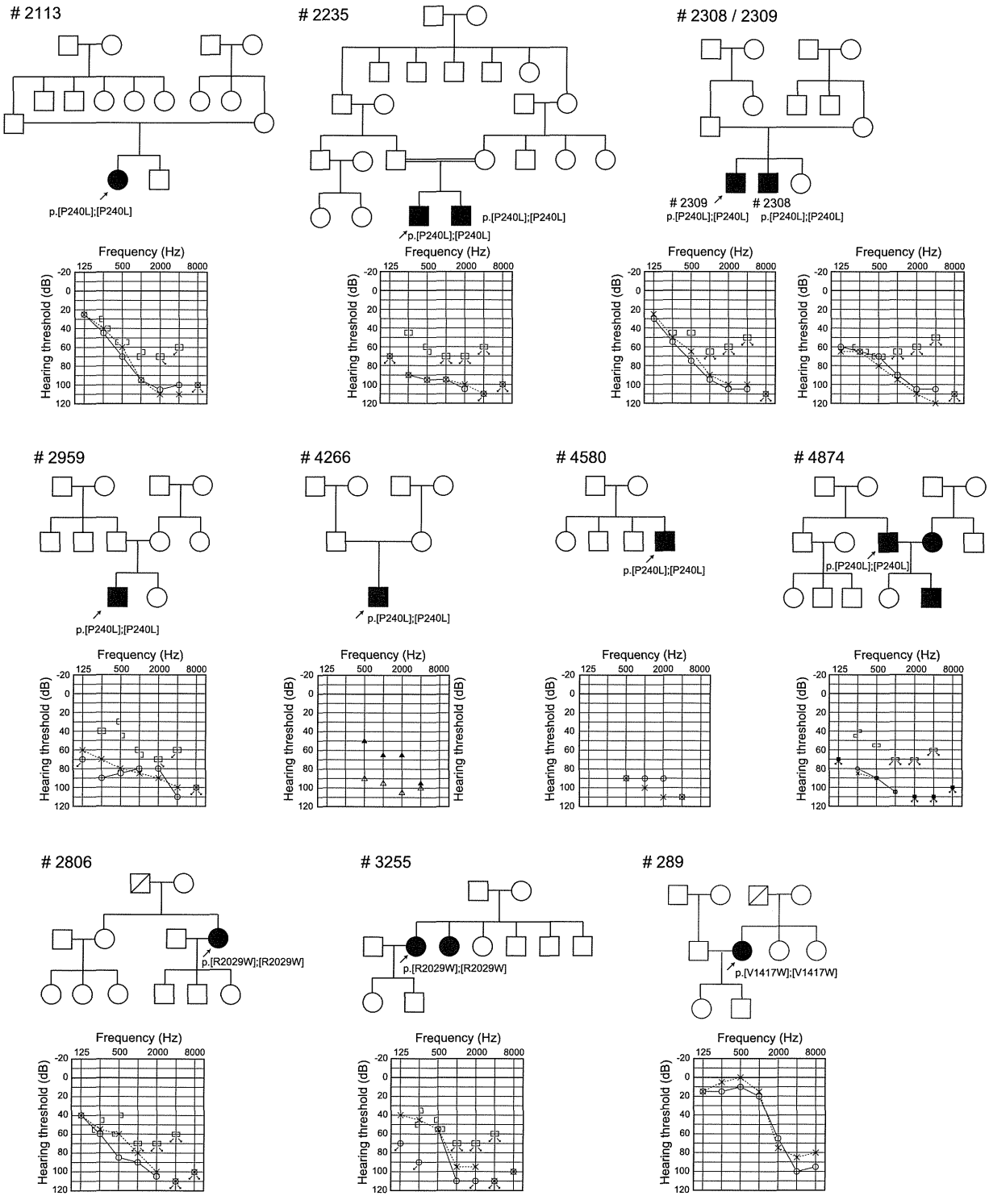

Figure 1. Pedigrees, mutations, and audiograms of the patients with homozygous *CDH23* mutations.
doi:10.1371/journal.pone.0040366.g001

Results

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

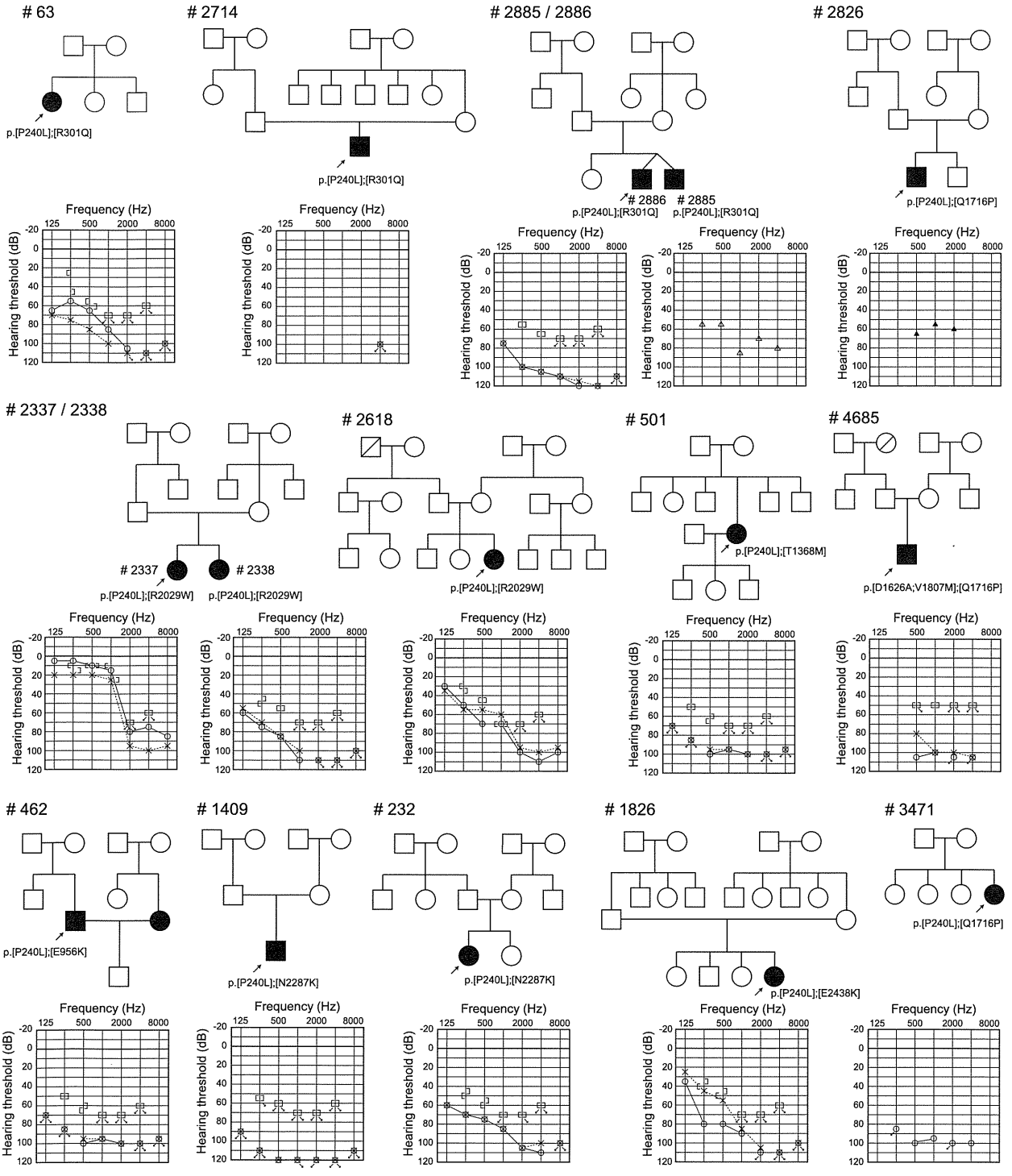

Figure 2. Pedigrees, mutations, and audiograms of the patients with compound heterozygous *CDH23* mutations.
doi:10.1371/journal.pone.0040366.g002

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

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

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

*average of 500, 1000, 2000 and 4000 Hz.
**average of 125, 250, and 500 Hz.
***average of 2000, 4000, and 8000 Hz.
****found by newborn hearing screening.
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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	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.
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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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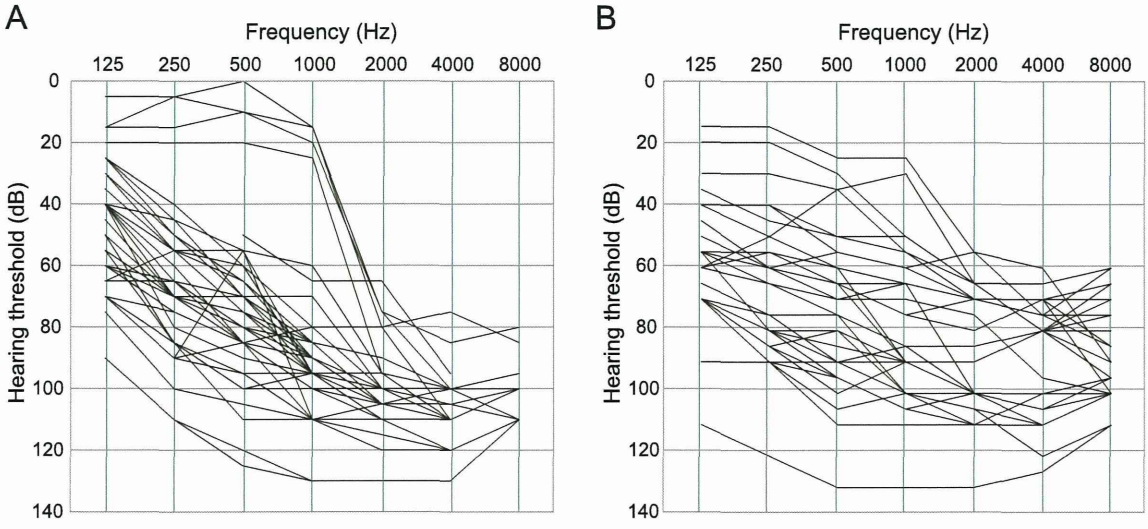

Figure 3. Overlapping audiograms of the patients with *CDH 23* mutations. A: patients with hearing loss caused by the *CDH23* mutations (homozygous or compound heterozygous cases), B: patients potentially caused by the *CDH23* mutations (heterozygous cases).
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of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

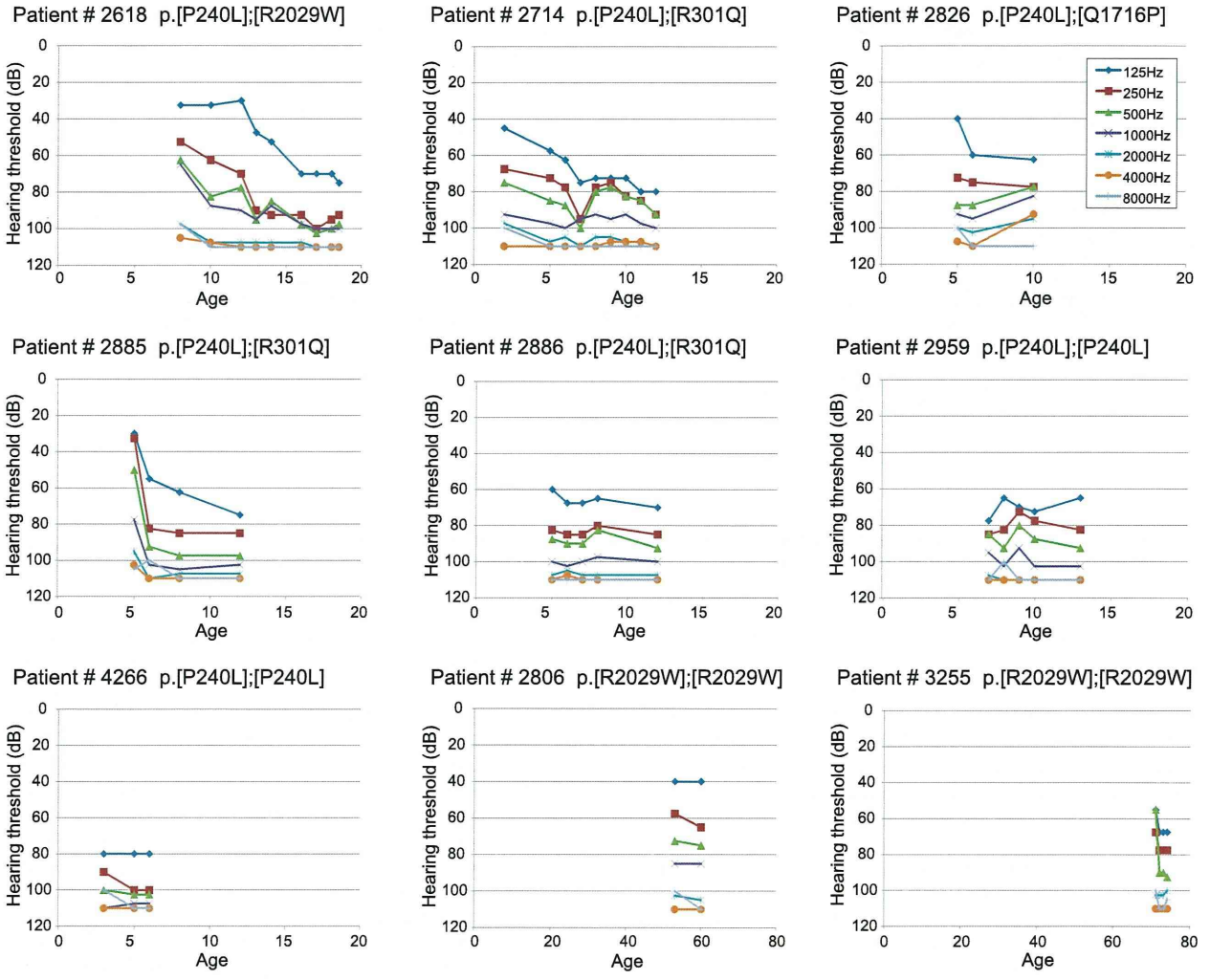

Figure 4. Hearing progression of the patients with *CDH23* mutations. Note that the high frequency portion was already worsened, and the low frequency portion was deteriorated by ages.
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mutations [2,5–7]. None had visual impairment, also supporting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

Inner hair cells of mice express the glutamine transporter SAT1

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ABSTRACT

Glutamate has been implicated in signal transmission between inner hair cells and afferent fibers of the organ of Corti. The inner hair cells are enriched in glutamate and the postsynaptic membranes express AMPA glutamate receptors. However, it is not known whether inner hair cells contain a mechanism for glutamate replenishment. Such a mechanism must be in place to sustain glutamate neurotransmission. Here we provide RT-PCR and immunofluorescence data indicating that system A transporter 1 (SLC38A1), which is associated with neuronal glutamine transport and synthesis of the neurotransmitters GABA and glutamate in CNS, is expressed in inner hair cells. It was previously shown that inner hair cells contain glutaminase that converts glutamine to glutamate. Thus, our finding that inner hair cells express a glutamine transporter and the key glutamine metabolizing enzyme glutaminase, provides a mechanism for glutamate replenishment and bolsters the idea that glutamate serves as a transmitter in the peripheral synapse of the auditory system.

1. Introduction

Several lines of evidence point to glutamate as the likely transmitter of inner hair cells (IHCs) of the organ of Corti (OC) (Eybalin and Altschuler, 1990; Hakuba et al., 2000; Ottersen et al., 1998; Takumi et al., 1997). These cells are enriched with glutamate and glutaminase (GLNase) – a glutamate synthesizing enzyme – and are presynaptic to dendrites that express high densities of AMPA glutamate receptors (Matsubara et al., 1996; Takumi et al., 1999; Usami et al., 1992). However, before conclusions can be drawn on transmitter identity it needs to be shown how the transmitter pool can be sustained. In the central nervous system, transmitter glutamate is replenished by recycling of the glutamate carbon skeleton. Essential steps in this "glutamate–glutamine cycle" are uptake of released glutamate in glial cells, conversion of glutamate to glutamine by glial glutamine synthetase, and shuttling of glutamine from glia to neurons for conversion to glutamate by neuronal GLNase. Several of these steps appear to be in place in the OC, with supporting cells substituting for CNS glia. What is missing is evidence for glutamine uptake in IHCs. Specifically, there is a need to show that IHCs are equipped with a glutamine transporter. Recently, several glutamine transporters have been described (Chaudhry et al., 2008) but none of these have been investigated for their possible presence in the OC.

Here we show that the glutamine transporter SAT1 (Solbu et al., 2010) is strongly and specifically expressed in IHCs and that this transporter is concentrated at the apical aspect of these cells. In contrast, the vesicular glutamate transporter VGLUT3 is found in the basal part of the hair cells, at the site of glutamate release. Thus, IHCs show a clear functional polarization in regard to glutamine and glutamate transport.

The mechanisms that underlie release and handling of transmitters in the OC are fundamental to our understanding of auditory processing and also bear on a number of pathological conditions including acoustic trauma (Hakuba et al., 2000; Robertson, 1983; Saunders et al., 1985). Our data support the idea that IHCs and adjacent supporting cells form a glutamate processing unit similar to that formed by glutamate releasing terminals and adjacent astrocytes in the CNS.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BSA, bovine serum albumin; CNS, central nervous system; GP, Guinea Pig; GS, glutamine synthetase; HSA, human serum albumin; IHCs, inner hair cells; NGS, normal goat serum; OC, organ of Corti; GLNase, glutaminase; PB, phosphate buffer; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SAT1, system A transporter 1; TBNT, Tris buffer (5 mM) containing 0.9% NaCl and 0.1% Triton X-100; VGLUT, vesicular glutamate transporter.

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2. Materials and methods

2.1. Tissue preparation

C57BL/6 mice, 8 weeks old, were used in the present study. The mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.).

For the confocal microscopic analysis, 1 ml of fixative [4% formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4] was injected through the tympanic membrane. Subsequently, the mice were perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 15 s) followed by the fixative used for tympanic injection (room temperature, 5 min). After perfusion the temporal bones were removed and postfixed in the same fixative (4–8 h, 4 °C). The cochlear tissues were dissected from the temporal bone under an operation microscope, and rinsed in 0.1 M PB (pH 7.4, 4 °C, overnight). The specimens were immersed in 10% sucrose, 20% sucrose and 30% sucrose in 0.1 M PB (pH 7.4, 8 h each) and then sectioned at a cryostat at 14 μm.

For postembedding immunogold analysis, 1 ml of fixative consisting of 4% formaldehyde and 0.1% glutaraldehyde in 0.1% PB (pH 7.4, 4 °C) was injected into the labyrinth. The mice were then perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 15 s) followed by the fixative used for labyrinth injection (room temperature, 5 min). After perfusion the temporal bones were removed and postfixed in the same fixative (4–8 h, 4 °C). The cochlear tissues were dissected from the temporal bone under an operation microscope, rinsed in 0.1 M PB with 4% glucose (pH 7.4, 4 °C, overnight) and cryoprotected in 10% glycerol, 20% glycerol and 30% glycerol in 0.1 M PB (pH 7.4, 8 h each). Freeze substitution and low-temperature embedding in a methacrylate resin were carried out as described previously (Matsubara et al., 1996).

For reverse transcription-polymerase chain reaction (RT-PCR), the mice were perfused transcardially at 8 ml/min with 2% dextran in 0.1 M PB (pH 7.4, 4 °C, 5 min) and decapitated. Temporal bones were quickly removed and the cochlear tissues were dissected out from the temporal bone and then rapidly frozen in liquid nitrogen for storage at −80 °C.

2.2. Antibodies

Antibodies to rabbit anti-SAT1 were prepared and characterized as described. The specificity has already been demonstrated (Buntup et al., 2008; Solbu, et al., 2010). Other primary antibodies used were: Guinea Pig (GP) anti-VGLUT1 (1:200; Millipore, Bedford, MA, USA), GP anti-VGLUT2 (1:200; Millipore, Bedford, MA, USA) and GP anti-VGLUT3 (1:2000; Millipore, Bedford, MA, USA). Secondary antibodies were Cy3-conjugated donkey anti-rabbit IgG and Cy5-conjugated donkey anti-GP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA).

2.3. Immunofluorescence staining of cryostat sections

Immunocytochemical staining of SAT1 and VGLUTs was performed using an indirect immunofluorescence method. The sections were rinsed twice for 10 min in 0.01 M phosphate buffer saline (PBS) (pH 7.4) and incubated for 1 h in blocking buffer [10% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X100 in 0.01 M PBS (pH 7.4)]. The sections were rinsed three times for 10 min each in 0.01 M PBS (pH 7.4) and incubated 2 h at room temperature with the primary antibodies (anti-SAT1 2 μg/ml and VGLUTs) in primary antibody solution [3% NGS, 1% BSA, 0.5% Triton X-100, 0.05% NaN_3 in 0.01 M PBS (pH 7.4)]. After three 10-min rinses in PBS (pH 7.4), the sections were incubated for 1 h with secondary antibody diluted 1:1000 in 0.01 M PBS (pH 7.4) containing 3% NGS, 1% BSA, and 0.5% Triton X-100. The sections were rinsed three times for 10 min each in 0.01 M PBS (pH 7.4) and mounted in ProlongGold antifade reagent premixed with the nuclear stain, DAPI (4′, 6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA). The sections were viewed and photographed with a Zeiss LSM 5 Pascal confocal microscope (Carl Zeiss, Jena, Germany). For control, the primary antibodies were absorbed with the synthetic peptides used for immunization. As an additional control, parallel sections were incubated without primary antibodies (to test for possible spurious staining due to the detection system). These controls showed faint and homogeneous background fluorescence, attesting to the selectivity of the immunocytochemical procedure (data not shown).

2.4. Postembedding immunogold analysis

The Postembedding immunogold analysis procedure has been described (Matsubara et al., 1996). In brief, ultrathin sections were incubated in the following solutions at room temperature: (1) 0.1% sodium borohydride and 50 mM glycine in Tris buffer (5 mM) containing 0.9% NaCl and 0.1% Triton X-100 (TBNT; 10 min); (2) 2% human serum albumin (HSA) in TBNT (10 min); (3) rabbit polyclonal antibodies to SAT1 (2 μg/ml) in TBNT containing 2% HSA (8 h); (4) 2% HSA in TBNT (10 min); (5) goat anti-rabbit IgG coupled to 10 nm gold particles (GAR10, Abcam, United Kingdom) diluted 1:20 in TBNT containing 2% HSA and polyethyleneglycol (5 mg/ml, 2 h). The sections were counterstained by 0.7% uranyl acetate (90 s) and 0.3% lead citrate (90 s) and examined with a Philips Tecnai 12 transmission electron microscope at 60 kV.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cochlear tissues with the Catrimox-14 RNA Isolation Kit Ver. 2.11 (Iowa Biotechnology, Urbandale, IA, USA). The yield of total RNA was determined by Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). RT-PCR assay was performed with the aid of an RNA PCR Kit (Takara, Tokyo, Japan). Reactions without the reverse transcriptase enzyme (−RT) were performed as control, and reactions using the dissection medium as template were used to control for contamination from lysed cells. The +RT and −RT reaction products were used for RT-PCR. Primer sequences are as follows: SAT1 forward (agaagtagaaaacggccagataaat) and reverse (atacttacatactcgtcgcatttcc), 111 bp; VGLUT1 forward (cagtattcaggatggagttctgtct) and reverse (tgttaaacttcgtaacagggttcat), 190 bp; VGLUT2 forward (taggattcagtggatttgctatctc) and reverse (agtgcataaaatatgactccaccat), 229 bp; and VGLUT3 forward (actctgaacatgtttatccccttctg) and reverse (cccaaacataccgtagatgtaaaag), 230 bp. PCR steps were denatured at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and then processed with a final extension at 72 °C for 5 min. After amplification, expected sizes of PCR products were confirmed on 2% agarose gel, and the bands were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

All studies were carried out in accordance with Shinshu University School of Medicine and National Institutes of Health guidelines for the ethical treatment of experimental animals. Care was taken to minimize number of mice used as well as their suffering.

3. Results

By use of RT-PCR we could show that the cochlear tissues contains mRNA encoding SAT1. In addition we confirmed that the cochlea expresses the genes that code for the VGLUT1–3 (Fig. 1; cf. Seal et al., 2008).

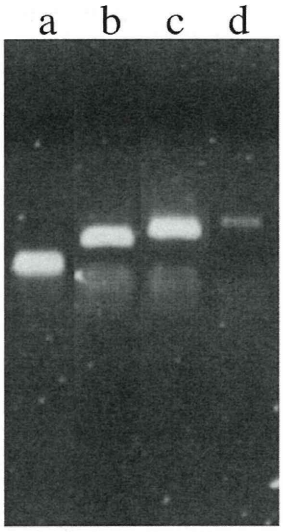

Fig. 1. RT-PCR of the cochlear tissues for SAT1 and three VGLUT isoforms. Specific transcripts for SAT1, VGLUT1, VGLUT2 and VGLUT3 were detected in the cochlea. (a) SAT1 111 bp, (b) VGLUT1 190 bp, (c) VGLUT2 229 bp, (d) VGLUT3 230 bp. No products were detected when PCR was performed in the absence of RT (data not shown).

An immunofluorescence analysis was performed to identify the exact sites of SAT1 expression. A careful examination of a series of sections covering the entire cochlea revealed that the SAT1 immunosignal is restricted to the IHCs (Fig. 2). No signal could be detected in the outer hair cells or in the supporting cells of the OC. Double labeling with antibodies to SAT1 and VGLUT3 showed that the two transporters are segregated into separate cellular compartments. While SAT1 is concentrated apical to the nucleus, VGLUT3 is expressed in the basal parts of the IHCs. Immunofluorescence signals for VGLUT1 and VGLUT2 were not detected.

Absorption with the immunizing peptide removed the SAT1 immunolabelling.

4. Discussion

The present findings indicate that SAT1 is expressed in IHCs but absent from outer hair cells and supporting cells. This highly specific expression pattern of SAT1 is consistent with the idea that SAT1 serves to accumulate glutamine from the extracellular space so as to replenish the transmitter pool of glutamate in IHCs (Fig. 3). Previously it has been shown that IHCs are enriched with GLNase, which catalyzes the conversion of glutamine to glutamate (Ottersen et al., 1998; Takumi et al., 1999; Wiet et al., 1986). Before exocytosis, glutamate thus formed must be transported into the synaptic vesicles that are clustered near the basal aspects of the IHCs. The glutamate transporter VGLUT3 is likely to be instrumental in this regard, as judged by its specific subcellular localization. Previous immunogold analyses have provided direct evidence of an accumulation of glutamate in the synaptic vesicles that cluster around the presynaptic dense body (Matsubara et al., 1996; Usami et al., 1992).

We did not detect any immunocytochemical signals for VGLUT1 or VGLUT2 in IHCs, consistent with the data of Seal et al. (2008). This requires an explanation, as our RT-PCR analysis of the cochlear tissues revealed expression of VGLUT1 through VGLUT3. The most probable cause of this apparent discrepancy is that the different mRNA species are translated at different rates and that the levels of VGLUT1 and VGLUT2 are too low to permit detection by the antibodies used. It is also possible that species differences are at play. Thus, using guinea pigs, Furness and Lawton (2003) reported a VGLUT1 immunosignal in IHCs, but failed to detect a signal for VGLUT2.

The finding of SAT1 in IHCs corroborates the idea that IHCs and adjacent supporting cells are engaged in recycling of the glutamate carbon skeleton (Fig. 3), analogous to the glutamate–glutamine cycle that is involved in glutamate handling in the CNS (Jenstad

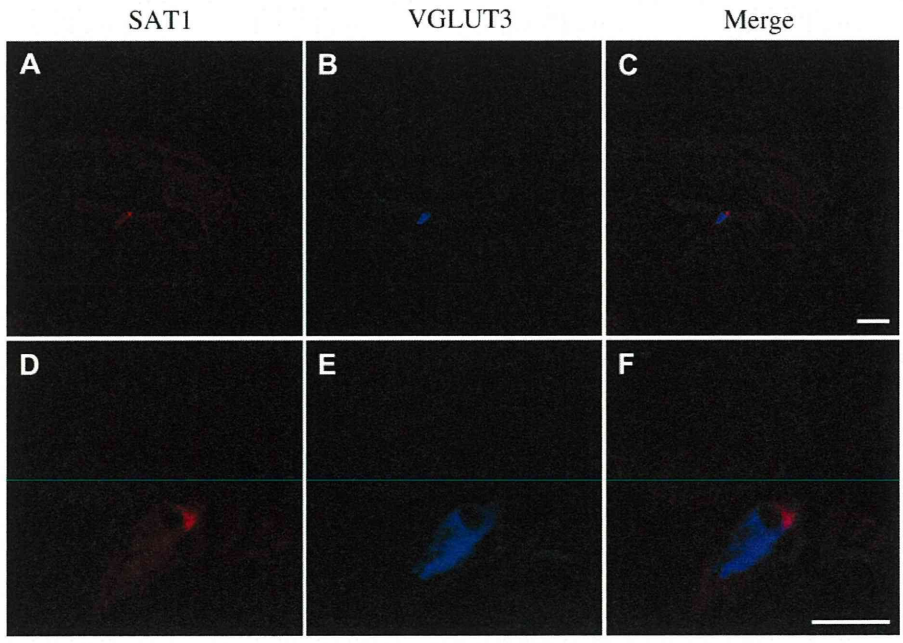

Fig. 2. Immunofluorescence for SAT1 (A, D, red) and VGLUT3 (B, E, blue) in cochlea, showing strong immunoreactivity only in the IHCs. SAT1 immunoreactivity (red) was localized to the apical parts of the IHCs. (A, B, C) Bar = 50 μm (D, E, F) Bar = 20 μm in higher magnification.

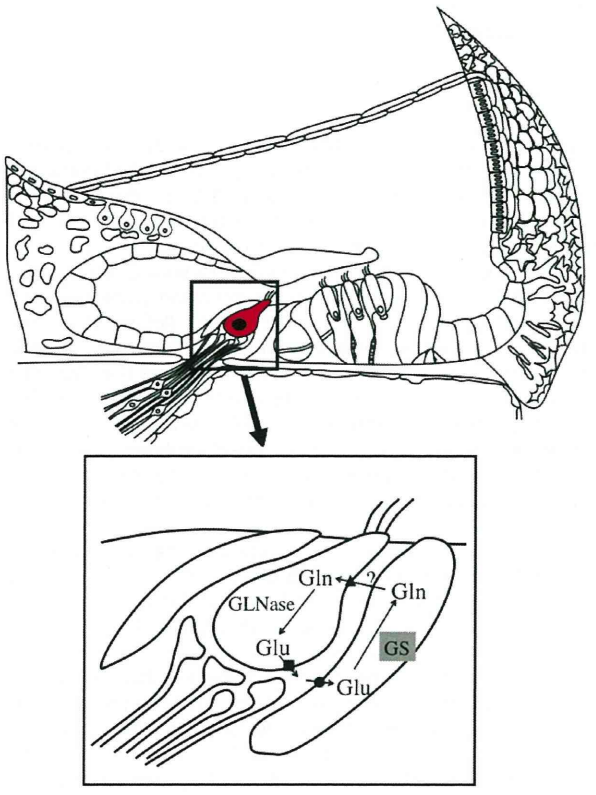

Fig. 3. Simplified diagram illustrating SAT1 localization and present concepts of the glutamate–glutamine cycle at afferent synapses in cochlear IHCs. Glutamate (Glu) is released from the site of the presynaptic body after having been accumulated in vesicles by vesicular glutamate transporter VGLUT3 (square). Released glutamate is taken up by supporting cells (inner phalangeal cells and border cells) through the glutamate transporter GLAST (circle). Glutamate is converted to glutamine (Gln) by glutamine synthetase (GS) (which has yet to be immunolocalized to those supporting cells that abut on the IHCs). Glutamine is released from supporting cells by a glutamine transporter of unknown identity (the most likely candidates being SN1 and SN2) and taken up into IHCs by glutamine transporter SAT1 (triangle). In the IHCs, glutamine is converted to glutamate by glutaminase (GLNase), which resides primarily in mitochondria. Glutamate is then accumulated in vesicles and ready for a new round of exocytosis.

et al., 2009). Previous reports have localized the glutamate transporter EAAT1 (GLAST) to supporting cells, including inner phalangeal cells (IPCs) and border cells (Furness and Lehre, 1997). These cells are located in close vicinity to IHC afferent synapses and are ideally positioned to remove transmitter glutamate from the extracellular space (Furness and Lehre, 1997; Furness and Lawton, 2003; Glowatzki et al., 2006; Hakuba et al., 2000). Once taken up, glutamate may be converted to glutamine by GS (Bak et al., 2006; Danbolt, 2001). This enzyme has been demonstrated in supporting cells of the inner ear, but has yet to be immunolocalized specifically to those supporting cells that abut on the IHCs (Eybalin et al., 1996; Takumi et al., 1997).

The molecular player of the glutamate–glutamine cycle that remains to be identified in the inner ear is the (hypothetical) transporter that mediates glutamine release from supporting cells. Based on data from the CNS, the most likely candidates are SN1 and SN2 (Bak, et al., 2006; Boulland, et al., 2003; Chaudhry, et al., 1999; Hamdani et al., 2012). The settling of this point has to await the generation of an appropriate antibody.

As to the subcellular compartmentation of SAT1 it is noteworthy that the SAT1 immunosignal is restricted to the apical parts of the IHCs. The distribution of this signal suggests that SAT1 is expressed in membranes facing cortilymph as well as endolymph. Unfortunately, none of the SAT1 antibodies worked at the EM level so the precise subcellular localization of SAT1 could not be established. The large absorptive surface represented by the apical membrane would help secure adequate transport capacity for glutamine. The concentration of glutamine in endolymph is rather low (53.9 ± 6.2 μM) (Thalmann et al., 1981; Thalmann, 1985). By comparison the Km of SAT1 has been estimated to be 0.3–0.49 mM in vitro (Chaudhry et al., 2002; Mackenzie, et al. 2003; Varoqui, et al. 2000).

VGLUT3 knockout mice show hearing loss and mutations in the gene encoding VGLUT3 have been associated with an autosomal-dominant form of progressive, high frequency non-syndromic deafness (Ruel et al., 2008; Seal et al., 2008). Studies will now be conducted to resolve whether mutations in the SAT1 gene similarly interfere with normal hearing.

5. Conclusion

Our RT-PCR and immunofluorescence observations show that IHCs express SAT1 as well as VGLUT3. These findings are consistent with the existence of a glutamate–glutamine cycle between IHCs and supporting cells in the cochlea. The gene encoding SAT1 may be a candidate gene for hearing loss, like the gene encoding VGLUT3. Further studies will be performed to explore this hypothesis.

Contributors

T.O., S.U., F.A.C., and O.P.O. designed and oversaw the execution of the study, T.O., N.S., S.H., and G.A.C. performed the experiments, T.O. analyzed the results, T.O. and O.P.O. wrote the paper, and all authors read and approved of the paper before submission.

Conflict of interest statement

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

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