Table 2. Gene expression levels of corresponding genes for non syndromic hearing loss in each cochlea turn.

Gene Symbol	Gene Name [Mus musculus]	Deafness causing Locus	Microarray						Quantitative RT-PCR		
			Signal Intensity Averages			Fold Change			Fold Change		
			apex	middle	base	middle/base	apex/middle	apex/base	apex/base	middle/base	apex/middle
Pou4f3	POU domain, class 4, transcription factor 3	DFNA15	200.1	167.3	77.9	2.33	1.09	2.52	3.19	2.59	1.23
Slc17a8	solute carrier family 17, member 8	DFNA25	152.3	104.8	47.7	2.36	1.33	3.15	5.07	2.49	2.03
Tmc1	transmembrane channel-like gene family 1	DFNA36, DFNB7	90.4	58.5	35.9	1.78	1.42	2.52	2.01	1.97	1.02
Crym	crystallin, mu	DFNA40	450.5	301.5	140.1	2.29	1.37	3.14	3.83	2.35	1.63
Otof	otoferlin	DFNB9	287.3	210.6	108.8	2.07	1.26	2.61	3.33	2.83	1.18
Strc	stereocilin	DFNB16	134.0	80.8	36.0	2.39	1.52	3.65	N/A	N/A	N/A
Ush1c	Usher syndrome 1C homolog (human)	DFNB18	211.3	146.7	102.5	1.54	1.33	2.04	1.48	1.39	1.07
Pcdh15	protocadherin 15	DFNB23	80.0	50.2	28.6	1.88	1.47	2.76	4.32	1.44	3.00
Grxcr1	glutaredoxin, cysteine rich 1	DFNB25	50.9	31.4	12.7	2.65	1.48	3.93	N/A	N/A	N/A
Dfnb59	deafness, autosomal recessive 59 (human)	DFNB59	216.5	187.2	86.7	2.34	1.07	2.50	N/A	N/A	N/A
Slc26a5	solute carrier family 26, member 5	DFNB61	358.4	201.6	47.0	4.70	1.63	7.65	8.41	5.87	1.86
Lhfpl5	lipoma HMGIC fusion partner-like 5	DFNB67	1228.7	902.1	402.9	2.43	1.26	3.07	4.90	2.63	1.86
Ptprq	protein tyrosine phosphatase, receptor type, Q	DFNB84	125.0	63.2	30.8	2.18	1.83	3.99	N/A	N/A	N/A

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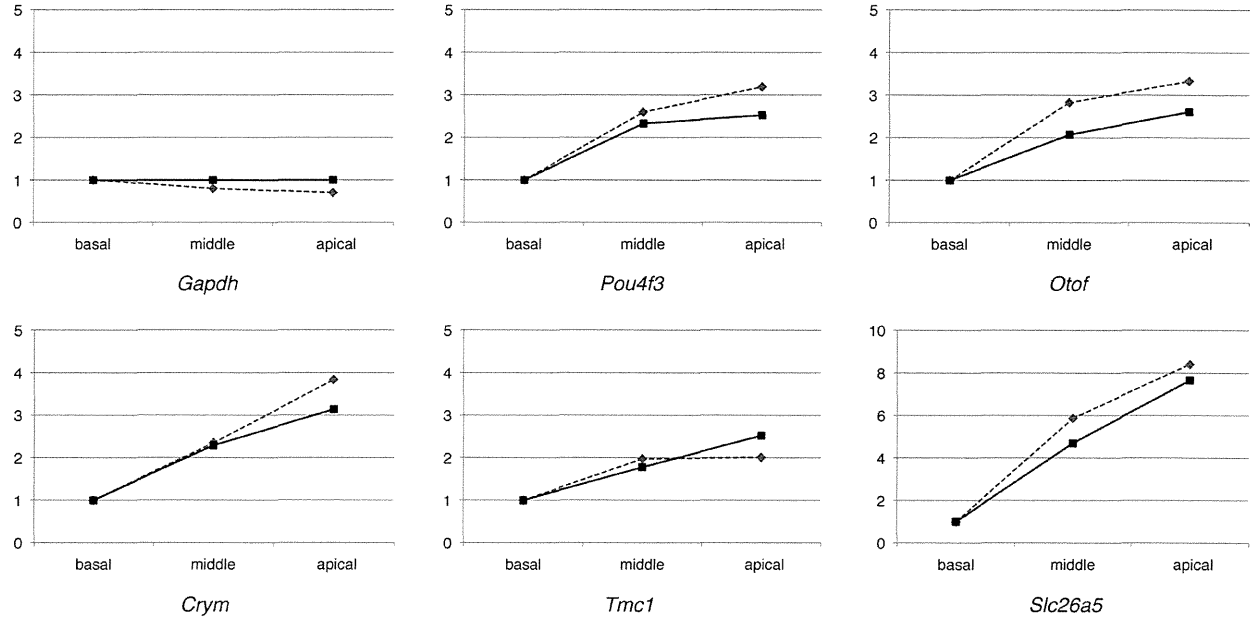

Figure 3. Gene expression patterns found by microarray analysis and quantitative RT-PCR. Values of each gene expression are indicated as a relative value to the basal turn. The expression level of each gene measured by microarray analysis (solid lines) was comparable with the level measured by quantitative RT-PCR (dotted lines).
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those was greater in the apex than in the base. However, there were no significant differences in *WFS1* gene expression.

Tonotopic expression of sodium, potassium, and calcium channels

Many sodium, potassium, and calcium channels were differentially expressed between the basal and apical turns. Specifically, expression of most potassium voltage-gated channels (i.e., *Kcna1*, *Kcna2*, *Kcnab2*, *Kcnab3*, *Kcnb2*, *Kcnc1*, *Kcnc3*, *Kcnd2*, *Kcne4*, *Kcnh2*, *Kcnh5*, *Kcnq3*, and *Kcns3*) was greater in the apex. There was also differential expression of voltage-dependent calcium channels (i.e., *Cacna2d3* and *Cacng2* were higher up in the apex while *Cacng4* was higher up in the base basal). Additionally, sodium channels (i.e., *Scn1a*, *Scn4b*, and *Scn8a*) were differentially expressed between the base basal and apex, and expression of those was greater in the apex. These observations suggest important functional roles for some of these channels in the mouse inner ear.

Tonotopic expression of other genes important for cochlear function

Emilin-2, a major component of the cochlear basal membrane (BM), expressed more in the apex (12.58-fold). Additionally, *Tectb*, a glycoprotein that is localized to the tectorial membrane, also expressed more in the apex (23.85-fold).

Quantitative RT-PCR (qPCR) confirms microarray data

To validate the microarray data, qPCR primers were designed for of 15 selected genes. Of them, 9 deafness genes expressed more in the apical turn (*Pou4f3*, *Slc17a8*, *Tmc1*, *Crym*, *Otof*, *Ush1c*, *Pcdh15*, *Slc26a5*, and *Lhfpl5*) and six were internal controls (*Gapdh*, *Actb*, *Rps17*, *Rpl30*, *Atp6*, and *Ipo8*). In all genes, qPCR data was coincident to microarray data. Data of 9 genes together with the control (*Gapdh*) are shown in Figure 3.

Discussion

These data revealed the baseline of gene expression in each mouse cochlear turn. However, we identified only gene expressions in equal amounts of RNA at each cochlear turn rather than in specific tissue (e.g., the lateral wall, the organ of Corti, and hair cells). This data can be utilized as a tool for global gene analysis such as of the biological function of the genes expressed in the inner ear, or in the search for novel hearing loss causative genes. Sato et al. demonstrated differential gene expression profiles along the axis of the mouse cochlea by cDNA microarray [8]. However, some of our results were not consistent with their findings. This difference may be attributed to the number of microarray probes (165,984 exon probes used in our experiments compared to 20,289 gene probes in theirs). In addition, our microarray analysis results were confirmed by qPCR.

The most remarkable finding was gradients of gene expression, being greater in the apex than the base in ADNSHL genes (*Pou4f3*, *Slc17a8*, *Tmc1*, and *Crym*). There are two prevailing theories explaining autosomal dominant diseases [9]. One of these is haploinsufficiency, referring to a lack of sufficient gene function due to reduced wild-type gene copy number. Cook et al. proposed that haploinsufficiency diseases are caused when the gene expression that is essential to maintain biological function falls below some critical level due to a loss-of function mutation in one of the two homologous gene loci [10]. Many papers supported this theory by quantifying variability in gene expression [9]. If this theory is applied to genes such as *POU4F3*, *SLC17A8*, *TMC1*, and *CRYM*, mutations of these genes would cause reduction of gene products. In such a case, basal turn gene expression may fall below some critical level more rapidly compared with apical turn because of a gradient of gene expression greater in the apex than in the base, resulting in progressive high frequency hearing loss. This speculation is consistent with the reported hearing loss types (such as high frequency progressive) in patients with the

POU4F3 [11,12], *SLC17A8* [13], *TMC1* [14,15], and *CRYM* [16] mutations.

Emilin-2 is a major component of the cochlear BM. The considerably higher level of *Emilin-2* in the cochlea compared to kidney or other tissues suggests a specialized role in the development or biomechanical function of the cochlear BM [17]. Amma et al. considered that if *Emilin-2* confers elasticity on the BM, *Emilin-2* would decrease the rigidity [17] and our results that expression of *Emilin-2* was greater in the apex than in the base may help to explain increased stiffness in BM towards the base.

Tectb mRNA expression was 23-fold in the apical turn compared with the middle and basal turns. *Tectb* encodes β-tectorin, a glycoprotein that is localized to the TM and the absence of which leads to disruption of the TM's core structure [18]. Russell et al. reported that *Tectb*$^{-/-}$ mutant mice, in which exons 1–4 of the gene are deleted, had low frequency hearing loss [19]. Our data that *Tectb* was mainly expressed in the apex, which is sensitive to low frequencies, was consistent with theirs.

In summary, this study demonstrated the gene expression profiles in each mouse cochlear turn. Especially for ADNSHL genes (*Pou4f3*, *Slc17a8*, *Tmc1*, and *Crym*) and other genes important for cochlear function (*Emilin-2* and *Tectb*), gradual expression changes help to explain the findings obtained from previous studies.

Supporting Information

Table S1 Gene list showing at least two-fold change in expression in one turn compared to the other turn.
(XLSX)

Table S2 Gene list showing at least two-fold change in expression for apex vs. basal.
(XLSX)

Table S3 Gene list showing at least two-fold change in expression for apex vs. middle.
(XLSX)

Table S4 Gene list showing at least two-fold change in expression for middle vs. basal.
(XLSX)

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Author Contributions

Conceived and designed the experiments: SN SU. Performed the experiments: HY YT SN YI NS. Analyzed the data: HY YT SN SU. Wrote the paper: HY YT SN SU.

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ORIGINAL ARTICLE

Frequency of mitochondrial mutations in non-syndromic hearing loss as well as possibly responsible variants found by whole mitochondrial genome screening

Takuya Yano, Shin-ya Nishio, Shin-ichi Usami and the Deafness Gene Study Consortium[1]

Mutations in mitochondrial DNA (mtDNA) are reported to be responsible for the pathogenesis of maternally inherited hearing loss. Complete mtDNA sequencing may detect pathogenic mutations, but whether they are indeed pathogenic can be difficult to interpret because of normal ethnic-associated haplogroup variation and other rare variations existing among control populations. In this study, we performed systemic mutational analysis of mtDNA in 394 Japanese patients with hearing loss. Two different cohorts were analyzed in this study: Cohort 1, 254 maternally inherited patients; and Cohort 2, 140 patients with various inheritance modes. After screening of the entire mtDNA genome with direct sequencing, we evaluated the frequency of previously reported mutations and the frequency and pathogenicity of the novel variants. As a result, the 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%). 1555A>G ($n=23$) is the most common mutation, followed by the 3243A>G ($n=11$) mutations. On the basis of prediction analysis, we detected 10 novel homoplasmic mitochondrial variants. After further classification, the 3595A>G and 6204A>G variants were found to be new candidate mutations possibly associated with hearing loss.
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Keywords: mitochondrial mutation; non-syndromic hearing loss

INTRODUCTION

Hearing impairment is one of the most common sensory handicaps, with a frequency of at least 1/1000 at birth, and 50% of these cases can be attributed to genetic causes. Furthermore, causative mitochondrial DNA (mtDNA) mutations have been found in 5–10% of patients with postlingual non-syndromic hearing loss.[1]

Among mitochondrial mutations, 1555A>G mutations in the mitochondrial *12S rRNA* are found frequently (0.6–5.3%, depending on the ethnic group) in aminoglycoside-induced and late-onset non-syndromic hearing loss.[2–4] A 1494C>T mutation in *12S rRNA* is also associated with aminoglycoside-induced and non-syndromic hearing loss.[5] A 3243A>G mutation in the $tRNA^{Leu(UUR)}$ is associated with maternally inherited diabetes combined with deafness,[6] and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), which frequently present with hearing loss. 7445A>C/G/T,[7,8] 7472insC and 7510T>C[9] mutations in the $tRNA^{Ser(UCN)}$ are also associated with aminoglycoside-induced or non-syndromic hearing loss.

Moreover, additional mutations in *12S rRNA* (827A>G,[10] 961T>C, 961delT+Cn, 1005T>C and 1095T>C[11]) have been reported as mitochondrial hearing loss mutations. Although there were growing numbers of reports of various novel mtDNA mutations associated with hearing loss, most focused on a few limited nucleotide positions or only the *12SrRNA* region.[12] Therefore, we conducted a whole mitochondrial genome mutational analysis by direct sequencing using samples from 254 maternally inherited and 140 non-syndromic Japanese hearing loss probands with various inheritance modes, and summarized the frequencies of the mutations, as well as the spectrum and phenotypes found in the hearing loss patients with mtDNA mutations.

MATERIALS AND METHODS

Subjects

Two cohorts were used in this study: Cohort 1, 254 Japanese maternally (or possibly autosomal dominant with affected mother and one or more affected children) inherited sensorineural hearing loss (SNHL) subjects; and Cohort 2, 140 Japanese SNHL subjects with various inheritance modes (14 autosomal dominant or mitochondrial inherited, 126 autosomal recessive inherited or sporadic cases), both collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the

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project, which was approved by the ethical committee of each hospital. The control group consisted of 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

Whole mtDNA from each patient was amplified into two long fragments, A and B, by LA Taq DNA polymerase (TaKaRa BIO, Shiga, Japan) as described elsewhere.[13] In brief, each genomic DNA sample was amplified by long PCR for 1 min at 94 °C, followed by 30 three-step cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 6 min, with a final extension at 72 °C for 5 min, ending with a holding period at 4 °C.

After the PCR amplification, resulting products were purified and direct sequenced with ABI Big Dye terminators and ABI 3130 autosequencer (Applied Biosystems, Carlsbad, CA, USA). Sequencing reaction was performed with 50 primers for the whole mitochondrial genome, designed with mitoSEQr Resequencing System (Applied Biosystems).

Sequencing data were analyzed by SeqScape ver2.6 and SeqAnalysis (Applied Biosystems). The sequencing result from each patient was compared with the rCRS (Reversed Cambridge Reference Sequence) to identify mtDNA mutations. Mitochondrial DNA mutations included in the mtSNP (http://mtsnp.tmig.or.jp/mtsnp/index.shtml), MITOMAP (http://www.mitomap.org/MITOMAP) or Uppsala mtDB (http://www.genpat.uu.se/mtDB/) databases were excluded as non-pathogenic variants when we search to novel variants.

We evaluated mutations according to evaluation criteria derived from a previous report by Zaragoza *et al.*[14]

Prediction of pathogenicity of mtDNA mutations

Initially, we measured the frequencies of each mutation found in healthy controls in our study ($n=192$) and in the mtSNP database ($n=2153$, including: centenarians in Gifu and Tokyo, type 2 diabetes mellitus patients (with or without vascular disorders), overweight and non-overweight young adult males, Parkinson's disease patients and Alzheimer's disease patients in Japan). The nucleotide conservation in each gene from humans and 60 mammalian species (*Artibeus jamaicensis*, NC_002009; *Balaenoptera musculus*, NC_001601; *Balaenoptera physalus*, NC_001321; *Bos taurus*, NC_006853; *Canis familiaris*, NC_002008; *Cavia porcellus*, NC_000884; *Cebus albifrons*, NC_002763; *Ceratotherium simum*, NC_001808; *Chalinolobus tuberculatus*, NC_002626; *Dasypus novemcinctus*, NC_001821; *Didelphis virginiana*, NC_001610; *Dugong dugon*, NC_003314; *Echinops telfairi*, NC_002631; *Echinosorex gymnura*, NC_002808; *Equus asinus*, NC_001788; *Equus caballus*, NC_001640; *Erinaceus europaeus*, NC_002080; *Felis catus*, NC_001700; *Gorilla gorilla*, NC_001645; *Halichoerus grypus*, NC_001602; *Hippopotamus amphibious*, NC_000889; *Hylobates lar*, NC_002082; *Isoodon macrourus*, NC_002746; *Lama pacos*, NC_002504; *Loxodonta africana*, NC_000934; *Macaca sylvanus*, NC_002764; *Macropus robustus*, NC_001794; *Mus musculus*, NC_005089; *Myoxus glis*, NC_001892; *Nycticebus coucang*, NC_002765; *Ochotona collaris*, NC_003033; *Ornithorhynchus anatinus*, NC_000891; *Orycteropus afer*, NC_002078; *Oryctolagus cuniculus*, NC_001913; *Ovis aries*, NC_001941; *Pan paniscus*, NC_001644; *Pan troglodytes*, NC_001643; *Papio hamadryas*, NC_001992; *Phoca vitulina*, NC_001325; *Physeter catodon*, NC_002503; *Pongo pygmaeus*, NC_002083; *Pongo pygmaeus* abelii, NC_002083; *Pteropus dasymallus*, NC_002612; *Pteropus scapulatus*, NC_002619; *Rattus norvegicus*, NC_001665; *Rhinoceros unicornis*, NC_001779; *Sciurus vulgaris*, NC_002369; *Soriculus fumidus*, NC_003040; *Sus scrofa*, NC_000845; *Tachyglossus aculeatus*, NC_003321; *Talpa europaea*, NC_002391; *Tarsius bancanus*, NC_002811; *Thryonomys swinderianus*, NC_002658; *Trichosurus vulpecula*, NC_003039; *Tupaia belangeri*, NC_002521; *Ursus americanus*, NC_003426; *Ursus arctos*, NC_003427; *Ursus maritimus*, NC_003428; *Volemys kikuchii*, NC_003041; *Vombatus ursinus*, NC_003322) was evaluated by the ClustalW method or the mtSNP database (mtSAP Evaluation; http://mtsnp.tmig.or.jp/mtsnp/search_mtSAP_evaluation.html). The mutations were considered to be possibly pathogenic if the original amino acid or base was conserved in >50% of the species (31 or more of 61 species).[15]

RESULTS

Direct sequence screening of the 254 probands of Japanese maternally inherited SNHL families and 140 non-syndromic hearing loss probands with various severities of hearing loss revealed 634 single-nucleotide polymorphisms in whole mitochondrial genome. Among those single-nucleotide polymorphisms, 19 were previously reported as associated with hearing loss: 792C>T ($n=1$), 827A>G ($n=10$), 856A>G ($n=3$), 961T>C ($n=3$), 1005T>C ($n=2$), 1095T>C ($n=1$), 1310C>T ($n=3$), 1494C>T ($n=1$), 1555A>G ($n=23$), 3243A>G ($n=11$), 3398T>C ($n=1$), 3421G>A ($n=2$), 5628T>C ($n=1$), 7511T>C ($n=3$), 8108A>G ($n=1$), 8348A>G ($n=1$), 11696G>A ($n=4$), 14693A>G ($n=1$) and 15927G>A ($n=4$) (Tables 1 and 2). In this study, based on the MITOMAP database, status was considered to be 'Confirmed' if at least two or more independent laboratories had published reports on the pathogenicity of a specific mutation (Table 1). More ambiguous substitutions were categorized as 'Unclear,' 'Reported' or 'Point mutation/polymorphism' (Table 2). 'Reported' status indicates that one or more reports have considered the mutation as possibly pathologic. 'Point mutation/

Table 1 'Comfirmed' mitochondrial mutations associated with sensorineural hearing loss found in this study

								Case				
Allele	Locus	Status[a]	Disease	Total (/394)	Cohort 1 (/254)	Cohort 2 (/140)	Control (/192)	Hearing characteristics	Progression of hearing loss	Tinnitus	Vertigo	Associated symptom	Reference
C1494T	12S rRNA	Confirmed	SNHL	1	0	1	0	High frequency	1/1	1/1	0/1	0	5
A1555G	12S rRNA	Confirmed	SNHL	23	23	0	0	High frequency	15/21	13/16	6/16	0	2
A3243G	tRNA ^{Leu} (UUR)	Confirmed	SNHL/DM/FSGS/ Cardiac dysfunction	11	11	0	0	Flat	10/10	6/10	6/10	Diabetes mellitus (8/10)	6
T7511C	tRNA ^{Ser} (UCN)	Confirmed	SNHL	3	3	0	0	High frequency	1/2	3/4	0/4	0	23
Total					37/254 (14.6%)	1/140 (0.7%)			27/34	23/31	12/31		

Abbreviations: DM, diabetes mellitus; FSGS, focal segmental glomerulosclerosis; SNHL, sensorineural hearing loss.
[a]Based on the MITOMAP database; 'Confirmed' status indicates that at least two or more independent laboratories have published reports on the pathogenicity of a specific mutation.

Table 2 Ambiguous-status mitochondrial substitutions associated with sensorineural hearing loss found in this study

								Case					
Allele	*Locus*	*Status*[a]	*Disease*	*Total (/394)*	*Cohort 1 (/254)*	*Cohort 2 (/140)*	*Control (/96)*	*Hearing characteristics*	*Progression of hearing loss*	*Tinnitus*	*Vertigo*	*Associated symptom*	*Reference*
C792T	12S rRNA	Reported	SNHL	1	1	0	0	Flat	1/1	1/1	1/1	0	24
A827G	12S rRNA	Conflicting reports	SNHL	10	5	5	1	High frequency	4/11	6/11	2/11	0	10
A856G	12S rRNA	Reported	SNHL/LHON/AD	3	3	0	0	Flat	1/1	1/1	1/1	0	25
T961C	12S rRNA	Unclear	SNHL/LVNC	3	3	0	2	Profound	1/1	1/1	1/1	0	26
T1005C	12S rRNA	Unclear	SNHL	2	1	1	1	Low frequency	2/2	1/1	1/1	0	26
T1095C	12S rRNA	Unclear	SNHL	1	1	0	0	Flat	1/1	1/1	1/1	0	11
C1310T	12S rRNA	Reported	SNHL	3	0	3	0	unknown	1/3	0/3	0/3	0	24
T3398C	ND1	Reported	SNHL/DM/HCM/ GDM/LVNC/ Cardiomyopathy	1	1	0	0	Profound	1/1	1/1	0/1	0	27
G3421A	ND2	Reported	SNHL	2	1	1	0	Profound	1/1	1/1	0/1	0	28
T5628C	$tRNA^{Ala}$	Reported	SNHL/CPEO	1	1	0	1	Profound	1/1	0/1	1/1	0	29
A8108G	CO2	Reported	SNHL	1	1	0	0	Low frequency	1/1	1/1	1/1	0	30
A8348G	$tRNA^{Lys}$	Reported	SNHL/Cardiomyopathy/HT	1	0	1	0	Low frequency	1/1	0/1	1/1	0	31
G11696A	ND4	Reported	SNHL/LHON/LDYT/ HT	4	0	4	2	Profound	1/4	1/4	0/4	0	32
A14693G	$tRNA^{Glu}$	Reported	SNHL/MELAS/LHON/ HT	1	0	1	1	Profound	0/1	0/1	0/1	0	33
G15927A	$tRNA^{Thr}$	Point mutation/ Polymorphism	SNHL/MS	4	1	3	4	High frequency	3/4	0/4	0/4	0	34
Total					19/254 (7.5%)	19/140 (13.6%)			20/34	15/33	10/33		

Abbreviations: AD, Alzheimer's disease; DM, diabetes mellitus; FSGS, focal segmental glomerulosclerosis; HT, hypertension; LDYT, Leber's hereditary optic neuropathy and dystonia; LHON, Leber hereditary optic neuropathy; LVNC, left ventricular non-compaction; MELAS, mitochondrial encephalomyopathy lactic acidosis, and stroke-like episodes; MIDD, maternally inherited diabetes and deafness; MS, multiple sclerosis; SNHL, sensorineural hearing loss.
'Point mutation/Polymorphism' status indicates that some published reports have determined the mutation to be a non-pathogenic polymorphism.
[a]Based on the MITOMAP database; 'Reported' status indicates that one or more reports have considered the mutation as possibly pathologic.

Table 3 Ten novel mitochondrial SNPs

Location	*Mutation*	*Conservation in 61 species (base) (/61)*	*Conservation rate (base) (%)*	*Amino-acid change*	*Conservation in 61 species (amino acid) (/61)*	*Conservation rate (amino acid) (%)*	*Amino-acid number/all amino acid of locus*	*Control (/192)*	*Mode of inheritance*	*Type of hearing loss*
16S rRNA	2069T>C	16	31.4	—	—	—	—	0	AD or Mit[a]	High frequency
16S rRNA	2285T>C	22	43.1	—	—	—	—	0	AD or Mit[a]	High frequency
16S rRNA	2285T>G	22	43.1	—	—	—	—	0	Sporadic	Dish shaped
16S rRNA	2634T>C	34	66.7	—	—	—	—	0	Sporadic	Profound
ND1	3595A>G	54	88.5	Asn>Asp	54	88.5	97/318	0	AD or Mit[a]	High frequency
COI	6204A>G	61	100	Ser>Gly	61	100	101/513	0	AD or Mit[a]	High frequency
ATPase6	9124A>G	60	98.4	Thr>Ala	59	96.7	200/226	0	Sporadic	Unilateral
ND4L	10680G>A	59	96.7	Ala >Thr	59	96.7	71/98	0	Sporadic	Unknown
ND5	13153A>G	44	72.1	Ile >Val	35	57.4	273/603	0	Sporadic	High frequency
Cytb	15003G>C	61	100	Gly >Ala	61	100	86/380	0	Sporadic	Profound

Abbreviation: SNPs, single-nucleotide polymorphisms
[a]AD or Mit; autosomal dominant inheritance or maternal inheritance.

polymorphism' status indicates that some reports have determined the mutation to be a non-pathogenic polymorphism. In all, 14.6% (37/254) of the patients in Cohort 1 (maternally inherited patients) were associated with the 'Confirmed' mutations. Only 0.7% (1/140) of the patients had the 'Confirmed' mutations in Cohort 2 (patients with various inherited modes) (Table 1). Ambiguous-status substitutions were associated in 7.5% (19/254) of Cohort 1, in contrast to 13.6% (19/140) of Cohort 2 (Table 2).

With regard to the audiogram configuration, various types were found. In all, 69% (79% in Cohort 1 and 59% in Cohort 2) of the patients had progressive hearing loss and 59% (74% in Cohort 1 and 45% in Cohort 2) had tinnitus, while 34% (39% in Cohort 1 and 30% in Cohort 2) of the patients were associated with vertigo (Tables 1 and 2). Concerning clinical symptoms other than hearing loss, 80% (8/10) of the patients with the 3243A>G mutation had diabetes mellitus, but no other clinical symptoms were noticed (Table 1).

Ten novel variants that were not included in the public mtDNA databases were found in this study and they were located in the *16S rRNA*, *ND1*, *COI*, *ATPase6*, *ND4L*, *ND5*, and *Cytb* regions (Table 3). All new variants were found in only one different family each.

Four of the novel variants were found in the *16S rRNA* gene: 2069T>C, 2285T>G, 2285T>C and 2634T>C. Although the 2634T>C variant had a high conservation rate (66.7%), the 2069T>C, 2285T>G and 2285T>C variants had low conservation rates: 31.4, 43.1 and 43.1%, respectively.

Table 4 Conservation rate of 'Confirmed' mitochondrial mutations

Location	*Mutation*	*Conservation in 61 species (base) (/61)*	*Conservation rate (base) (%)*
12S rRNA	1494A>G	61	100.0
12S rRNA	1555A>G	56	91.8
$tRNA^{Leu\ (UUR)}$	3243A>G	60	98.4
$tRNA^{Leu\ (UUR)}$	3291T>C	58	95.0
$tRNA^{Ser\ (UCN)}$	7445A>G	42	68.9
$tRNA^{Ser\ (UCN)}$	7511T>C	60	98.4
$tRNA^{Lys}$	8363G>A	49	80.3
$tRNA^{His}$	12147G>A	61	100.0
$tRNA^{Glu}$	14709T>C	58	95.0

The remaining six novel variants were located in the protein coding regions: 3595A>G in *NADH dehydrogenase 1* gene (MTND1 (MIM 516000), 6204A>G in *cytochrome oxydase I* gene (MTCOI (MIM 516030)), 9124A>G in *ATPase 6* gene (MTATP6 (MIM 516060)), 10680G>A in *NADH dehydrogenase 4L* gene (MTND4L (MIM 516004)), 13153A>G in *NADH dehydrogenase 5* gene (MTND5 (MIM 516005)) and 15003G>C in *cytochrome b* gene (MTCYB (MIM 516020)).

These variants are found in very well-conserved gene positions (57.4–100%).

The conservation rates in all 'Confirmed' mtDNA mutations were high (Table 4).

However, as in Table 3, the 9124A>G, 10680G>A, 13153A>G and 15003G>C variants were found in sporadic cases which are not generally compatible with mitochondrial deafness. On the basis of the above evaluations, we categorized 3595A>G, and 6204A>G as possibly pathogenic mutants, and the remaining eight others as uncertain pathogenic mutants.

The homoplasmic mutation 3595A>G in the *ND1* was found in a 4-year-old male patient with prelingual, severe hearing loss of high frequencies (Figure 1). He was suspected to have hearing impairment when he was about 1 year old, but ABR testing and Computed Tomography resulted in a diagnosis of normal hearing. However, when he was 3 years old, his mother again suspected that he had hearing impairment and testing confirmed it. The mother, who had the same mutation, also had hearing impairment as well as progressive bilateral tinnitus and occasional vertigo from childhood.

The homoplasmic mutation 6204A>G in the *COI* gene was found in a 62-year-old male with mild hearing loss of high frequencies (Figure 2). He noticed his hearing loss at the age of 50 and suffered

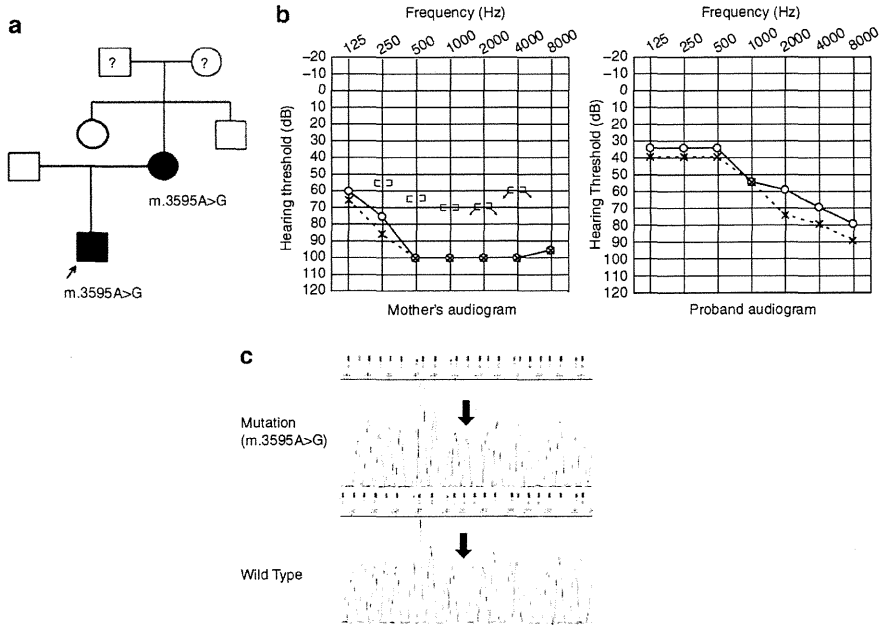

Figure 1 Clinical features of the proband carrying the homoplasmic 3595A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiograms of the proband and mother. (**c**) Electropherogram depicting the 3595A>G sequence and its flanks. Arrow indicates the position of the 3595A>G variant.

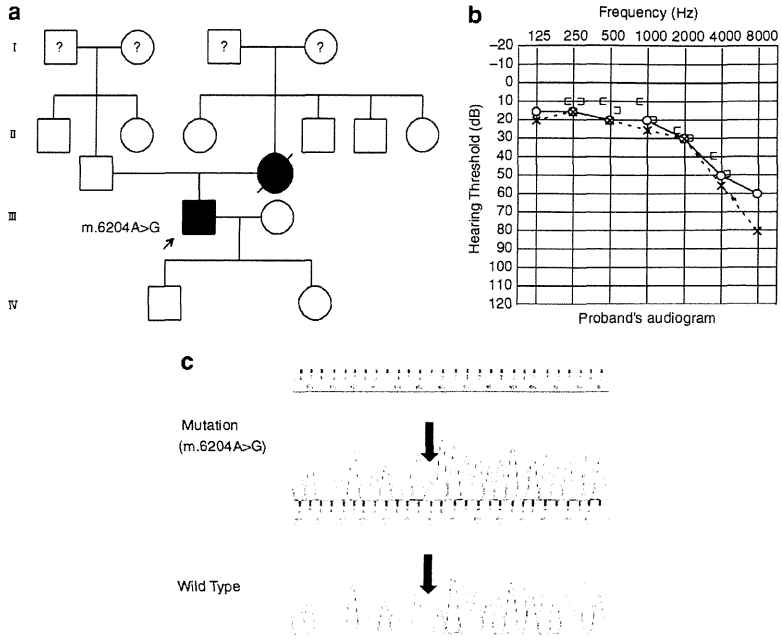

Figure 2 Clinical features of the proband carrying the homoplasmic 6204A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiogram of the proband. (**c**) Electropherogram depicting the 6204A>G sequence and its flanks. Arrow indicates the position of the 6204A>G variant.

from tinnitus, and mild diabetes mellitus. His mother also had hearing impairment that gradually progressed with age. DNA samples were not obtained from other family members.

DISCUSSION

Nineteen known mitochondrial mutations were found predominantly in the maternally inherited group (Tables 1 and 2). Clarification of pathogenecity of mitochondrial substitutions was hampered by low penetrance (probably due to heteroplasmy). Therefore, based on the MITOMAP database, they were classified as 'Confirmed' or 'Ambiguous-status' substitutions (Tables 1 and 2). The 'Confirmed' mitochondrial mutations were found predominantly in Cohort 1 rather than in Cohort 2 (14.6 vs 0.7%), supporting the pathogenicity of these mutations. Frequencies of 1555A>G and 3243A>G mutations were significantly high, indicating that these two mutations are important causes of maternally inherited hearing loss. In general, patients with these mitochondrial mutations showed more or less similar clinical characteristics, that is, progressive hearing loss with tinnitus (Table 1).

Among the 10 novel variants (Table 3), two, the *ND1* mutation 3595A>G and *COI* mutation 6204A>G, are thought to be possibly pathogenic, because (1) they are found in autosomal dominant or maternal inheritance (some of the others are found as sporadic cases); (2) the conservation rate of the variation at the position among mammals is at least over 50%, as is the conservation rate in all confirmed mtDNA mutations associated with phenotypes (Table 4) and (3) they are associated with high frequency hearing loss; the characteristic hearing type of mitochondrial hearing loss. These mutations affected a conserved nucleotide in the mitochondrial gene in primates and other species and had a conservation index of >50% (88.5 and 100%, respectively). None of these mutations were found in the controls or in the databases, further indicating that they are associated with hearing loss, however, no conclusion can be drawn without enzymatic analysis. Unfortunately, this study was a retrospective study using collected DNA samples from 1995 to 2012, so it was impossible to contact the patients and to get muscle or living samples from them. Therefore, enzymatic analysis of these mtDNA samples was not feasible.

In this study, we found one novel possibly pathogenic mutation in the ND1 hydrophobic arm region, in a patient with a homoplasmic 3595A>G mutation and hearing loss of the high frequencies from age 3 without complications. The family members of this patient did not have diabetes mellitus.

On the other hand, the novel possibly pathogenic mutation 6204A>G was located in the *COI* gene. The amino-acid conservation rate of this position was 100% (61/61 mammals). In previous reports, more than 20 pathogenic mutations in the *MT-ND1* gene were reported in patients with LHON (Leber's hereditary optic neuropathy) and MELAS. Also, *ND1* mutation-related hearing impairment has been reported: 3308T>C causing MELAS with deafness,[16] 3395A>G causing hypertrophic cardiomyopathy with profound SNHL,[17] and 3396T>C and 3421G>A causing maternally inherited diabetes and deafness.[18,19] Three COI mutations related to hearing loss have also been reported (7443A>G,[20] 7444G>A[21] and 7445A>G[7,8]). Our results taken with these previous reports support the possibility that mutations in the ND1 and COI regions are associated with hearing impairment.

Most of the mtDNA mutations associated with hearing loss indicate low penetrance explained as a mild biochemical defect indicating that the mutation itself is not sufficient to produce the clinical phenotype. Thus, other modifying factors including nuclear

backgrounds, environmental factors and mitochondrial haplotypes are necessary for the phenotypic manifestation of the mutation. The degree of hearing loss from mtDNA mutation can be similar within individual families but varied among different family groups, probably due to the modifier effect by nuclear genes.[22]

CONCLUSION

Nineteen known mitochondrial mutations were found predominantly in the maternally inherited group. Among them, frequencies of 1555A>G and 3243A>G mutations were significantly high, indicating that these two mutations are important causes of maternally inherited hearing loss. In addition to the previously reported mitochondrial mutations, we detected 10 novel homoplasmic mutations in the mitochondrial genes related to hearing loss by direct sequencing of whole mitochondrial genomes in Japanese patients. Two of them, 3595A>G and 6204A>G, are possibly associated with hearing loss.

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Clinical Case Reports

Case Report Open Access

A Novel Mutation of *MYO15A* Associated with Hearing Loss in a Japanese Family

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Abstract

Mutations in the *MYO15A* gene located on chromosome 17p11.2, are responsible for non-syndromic autosomal recessive profound hearing loss (DFNB3). Direct sequencing of 96 Japanese families with profound congenital hearing loss revealed one family with a novel homozygous mutation in *MYO15A*, a T to A transition at the nucleotide of 9413 (c.9413T>A) that encodes the MyTh4 domain of the protein (p. L3138Q). This is the first report of an East Asian hearing loss patient with a *MYO15A* mutation.

Keywords: DFNB3; MYO15A; Mutation; Hearing loss

Introduction

Hearing loss is one of the most common communication disorders in humans, affecting millions of individuals worldwide. To date, 95 loci for autosomal recessive sensorineural hearing loss (ARSNHL) have been reported and at 41 of these loci, the causative genes have been identified (Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org/). *MYO15A* is comprised of 66 exons distributed across 71 kbp of DNA on chromosome 17p11.2. The *MYO15A* mRNA transcript encodes a 3530 amino acid protein in its longest form. *MYO15A* has MyTh4 (Myosin-Tail like Homology region 4) domains, FERM (4.1 protein, Ezrin, Radixin, and Moesin) motifs, a SH3 (Src Homology 3) domain, and the PDZ domain.

In humans, 36 different *MYO15A* mutations have been reported and 35 of these cause congenital profound ARSNHL. The remaining *MYO15A* mutation was a heterozygous missense mutation detected in a Smith-Magenis syndrome patient who had moderate sensorineural hearing loss.

In this report, we describe the first identified novel missense *MYO15A* mutation in a Japanese ARSNHL patient together with a review of the previous literature. This mutation is located in a MyTh4 domain and is thought to disrupt normal *MYO15A* function, resulting in congenital hearing loss.

Subjects

DNA samples from 96 independent subjects who had profound congenital ARSNHL were collected from 33 ENT departments nationwide in Japan. All subjects gave prior written informed consent for participation in the project, which was approved by each hospital's ethical committee. Anamnestic and physical examinations were performed to exclude those with syndromic symptoms, outer and/or middle ear diseases, and environmental factors such as premature birth, or newborn meningitis. Controls were 192 Japanese healthy individuals with normal hearing confirmed by pure tone audiometry.

Mutation Analysis

All of the *MYO15A* exons were amplified using gene-specific primers described elsewhere [1]. PCR reactions were performed with 25 μl in 1.5 mM $MgCl_2$, 100 mM of each dNTP, 1U of Taq DNA polymerase, and 2 mM forward and reverse primers. After an initial denaturation at 95°C for 90 seconds, amplification was performed for 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. Then, a final extension was performed at 72°C for 5 minutes. Sequencing was performed with a BigDye™ v1.1 Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing products were analyzed by an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Computer analysis to predict the effect of missense variants on *MYO15A* protein function was performed with Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), and Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/).

Results

Direct sequencing revealed a novel homozygous mutation of *MYO15A* at exon 57 (c.9413T>A) in one patient (Figure 1). This mutation (p. L3138Q) is located in the MyTh4 domain of the myosin 15a protein, and is predicted to be pathologic by prediction programs (Table 1). We also confirmed that the patient's father and mother had heterozygous mutations and that the mutation was absent in the controls. The patient had no mutations in *GJB2*, the gene most frequently involved with hearing impairment in Japanese, nor in mitochondrial 1555A>G.

In detail, the patient was a female with congenital severe to profound sensorineural hearing loss. At age one, her mother became aware of her hearing impairment because she did not speak. The patient visited the hospital for genetic testing the age of 17 (Figure 1). Computed Tomography examination indicated that she did not have any malformations, such as ossicular anomalies, cochlear hypoplasia, vestibular dilation or enlarged vestibular aqueduct. In addition, she had no history of vertigo. Her sister also had severe congenital hearing loss, but her parents, brother, and other relatives did not have hearing impairment (Figure 1). DNA samples were not obtained from her siblings.

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Exon	Domain	Nucleotide change	Amino acid change	Frequency	Control	Hereditary	Age of onset	Hearing level	Functional prediction	
									Poly Phen2 score	SIFT score
3	N-terminal extension	3658G>A	G1220R	1/96	0/192	Sporadic	Congenital	Severe	0	0.09
12	Motor	4322G>T	G1441V	1/96	0/192	Autosomal recessive	Congenital	Profound	0.785	0.01
30	MyTH4	6486delG	A2153fs	1/96	0/192	Sporadic	Congenital	Profound	-	-
57	MyTH4	9413T>A	L3138Q	1/96	0/192	Autosomal recessive	Congenital	Profound	0.791	0
65	-	10420A>G	S3474G	1/96	0/192	Sporadic	Congenital	Severe	0.427	-

Table 1: *MYO15A* variants found in this study.

Exon	Domain	Nucleotide change	Amino acid change	Mutation type	Age of onset	Hearing level	Origin of family	References
Exon 2	N-terminal extension	373delCG	R125VfsX101	Frameshift	-	-	Ashkenazi Jewish	12
Exon 2	N-terminal extension	867C>G	Y289X	Nonsense	Congenital or prelingual	Moderate to severe	Turkey	11
Exon 2	N-terminal extension	1185dupC	E396fsX431	Frameshift	10-14 yrs	Moderate to severe	Pakistan	13
Exon 2	N-terminal extension	1387A>G	M463V	Missense	-	Severe to profound	Iran	14
Exon 2	N-terminal extension	3313G>T	E1105X	Nonsense	-	Profound	Pakistan	7, 13
Exon 2	N-terminal extension	3334delG	G1112fsX1124	Frameshift	-	Severe to profound	Pakistan	7, 13
Exon 3	Motor	4023C>T	Q1229X	Nonsense	Congenital	Profound	Pakistan	6
Intron 4	Motor	IVS4+1G>T	D1232fsX1241	Splice donor site	Congenital	Profound	Pakistan	6
Exon 5	Motor	3758C>T	T1253I	Missense	-	Severe to profound	India	7
Intron 5	Motor	IVS5+1G>A	T1253fsX1277	Splice donor site	-	Severe to profound	Pakistan	7
Exon 10	Motor	4176C>A	Y1392X	Nonsense	-	Severe to profound	Pakistan	7
Exon 10	Motor	4198G>A	V1400M	Missense	Congenital or prelingual	Severe to profound	Turkey	11
Exon 11	Motor	4240G>A	E1414K	Missense	-	-	Palestinian Arab	12
Exon 11	Motor	4273C>T	Q1425X	Nonsense	-	-	Turkey	15
Exon 12	Motor	4351G>A	D1451N	Missense	-	Severe to profound	India	7
Exon 12	Motor	4441T>C	S1481P	Missense	Congenital or prelingual	Severe to profound	Turkey	11,15
Exon 14	Motor	4652C>A	A1551D	Missense	-	-	Turkey	15
Exon 15	Motor	4669A>G	K1557E	Missense	-	Severe to profound	Pakistan	7
Exon 17	Motor	4904-4907delGAG	E1637del	Frameshift	-	Severe to profound	Iran	14
Exon 17	Motor	4998C>A	C1666X	Nonsense	-	Severe to profound	Tunisia	10
Exon 18	Motor	5117_5118GC>TT	L1706V	Missense	-	Severe to profound	Pakistan	7
Exon 19	Motor	5189T>C	G1730P	Missense	-	Severe to profound	Pakistan	7
Exon 20	Motor	5305A>G	T1769A	Missense	-	Severe to profound	Iran	14
Exon 22	Motor	5419-21delT	F1807L fsX6	Frameshift	-	Severe to profound	Iran	14
Exon 22	Motor	5492G>T	G1831V	Missense	-	Profound	Turkey	8
Exon 24	Motor	5810G>A	R1937H	Missense	-	Severe to profound	Iran	14
Exon 24	Motor	5807_5813delCCCGTGG	R1937TfsX10	Frameshift	Congenital or prelingual	Severe to profound	Turkey	11
Exon 26	IQ Motif	5925G>A	W1975X	Nonsense	-	Severe to profound	Iran	14
Exon 28	-	6061C>T	Q2021X	Nonsense	-	Severe to profound	Pakistan	7
Exon 29	MyTH4	6217C>T	P2073S	Missense	Congenital	Profound	Iran	1
Exon 30	MyTH4	6331A>T	N2111Y	Missense	Congenital	Profound	India	5
Exon 30	MyTH4	6337A>T	I2113F	Missense	Congenital	Profound	Indonesia	5
Exon 30	MyTH4	6371G>A	R2124Q	Missense	Congenital	Profound	Iran	1
Exon 31	MyTH4	6952C>T	T2205I	Missense	Congenital	Moderate	North America*	6
Exon 32	-	6731G>A	G2244E	Missense	-	Severe to profound	Pakistan	7
Exon 33	-	6796G>A	V2266M	Missense	-	Severe to profound	Pakistan, Turkey	7

Intron 37	-	IVS37 + 3G>C	-	Splice donor site	-	Severe to profound	Tunisia	10
Exon 41	-	7801A>T	K2601X	Nonsense	Congenital	Profound	India	5
Exon 44	FERM	8486G>T	Q2716H	Missense	Congenital	Profound	Pakistan	6
Exon 45	FERM	8158G>C	D2720H	Missense	-	Severe to profound	Pakistan	7
Exon 45	FERM	8183G>A	R2728H	Missense	-	-	Ashkenazi Jewish	12
Exon 48	FERM	8467G>A	D2823N	Missense	-	Severe to profound	Iran	14
Intron 50	-	IVS50-1G>C	-	Splice donor site	-	Profound	Turkey	8
Exon 51	SH3	8821_8822insTG	V2940fsX3034	Frameshift	-	Severe to profound	Pakistan	7
Intron 54	-	IVS54+1G>A	-	Splice donor site	-	Severe to profound	Tunisia	10
Exon 57	**MyTH4**	**9413T>A**	**L3138Q**	**Missense**	**Congenital or prelingual**	**Profound**	**Japan**	**This case**
Exon 57	MyTH4	9478C>T	L3160F	Missense	-	Severe to profound	Pakistan	7
Exon 62	FERM	9957_9960delTGAC	D3320fs	Frameshift	Frameshift	Severe to profound	Brazil**	9
Exon 62	FERM	9995_10002dupGCCG-GCCC	S3335AfsX121	Frameshift	Congenital or prelingual	Severe to profound	Turkey	11
Exon 65	-	10474C>T	Q3492X	Nonsense	-	Severe to profound	Pakistan	7
Exon 66	-	10573delA	S3525fs	Frameshift	Prelingual	Severe to profound	Brazil	9

*Mutation was found in a patient heterozygous at the DFNB3 locus with Smith-Magenis Syndrome.

**Mutation was found in a heterozygous individual.

Table 2: DFNB3-causing *MYO15A* mutations.

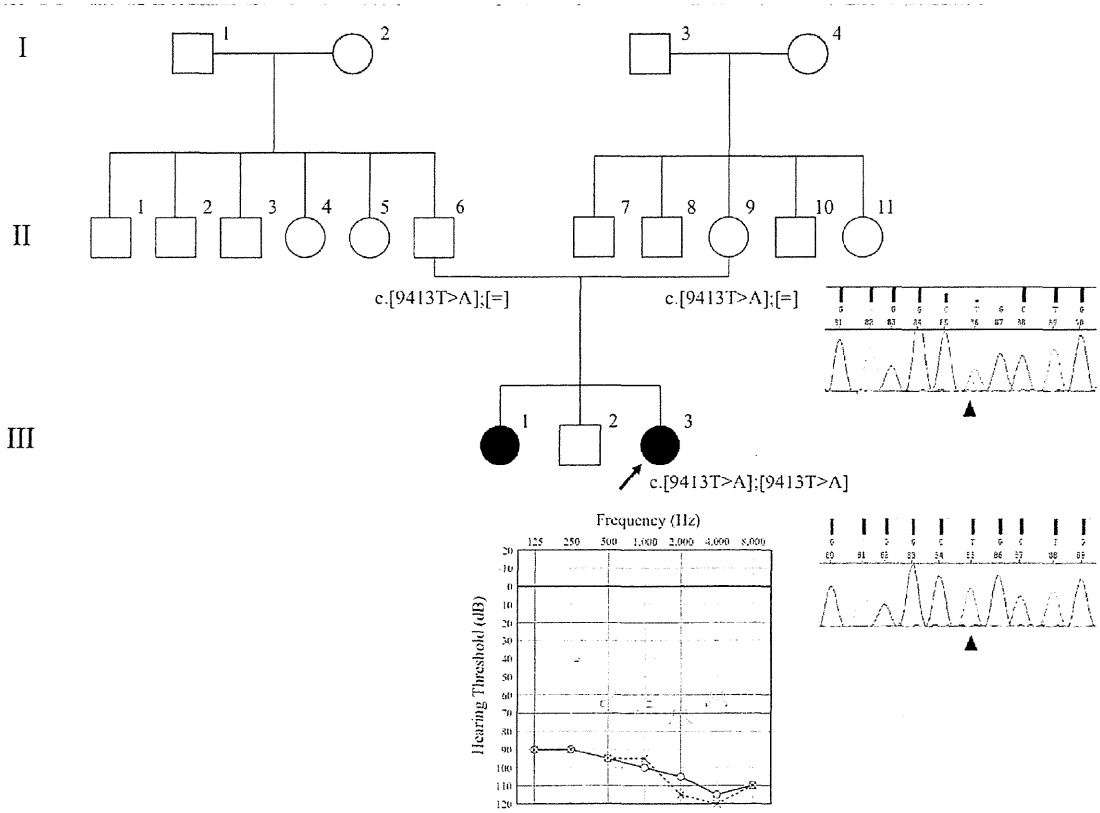

Figure 1: Family pedigree, audiogram of the patient, and lectropherogram analysis of MYO15A in the family. A novel homozygous mutation of *MYO15A* at exon 57 (c.9413T>A) is found in a patient with congenital severe to profound sensorineural hearing loss.

We also found other heterozygous variants: c.6824delG, p.G1441V, p.G1220R, and p.S3474G, each in a different independent patient, and none being found in the controls (Table 1).

Discussion

Myosin 15a protein is required for normal auditory function,

therefore *MYO15A* mutations cause ARSNHL. Mutations in this gene also cause the shaker 2(sh2) phenotype in mice. Sh2 mice are characterized by a vestibular defect and profound hearing loss [2,3] but such vestibular defects are not found in human carriers of *MYO15A* mutations. The stereocilia of hair cells of the sh2 mice are short and lack the characteristic staircase-like pattern [4].

In our patient, the novel *MYO15A* mutation located in the MyTH4 domain caused sensorineural hearing loss. In addition, this is the first *MYO15A* mutation found in an East Asian population. To date, 43 mutations in *MYO15A* were reported. Type of mutations, domains, and clinical features are summarized in Table 2 [1,5-15]. All *MYO15A* mutations previously reported were found in prelinguistic or congenital hearing loss patients, except for one Smith-Meganis syndrome patient [6]. Our patient had prelingual profound hearing loss, consistent with previous reports.

Of the 43 reported *MYO15A* mutations, six were missense mutations in the MyTH4 domains. Five of those six were found in homozygous state: p.N2111Y in Indians [5]; p.I2113F in Indonesians [5]; p.R2124Q and p.P2073S in Iranians [1]; and p. L3160F in a Pakistani family [7]. The sixth missense mutation was a heterozygous mutation, p. T2205I, in a North American family affected by Smith-Magenis syndrome [6] (Table 2).

Furthermore, based on the prediction programs, two missense mutations, p. G1441V, p. L3138Q, are predicted to be pathologic variants (Table 1). However, except for p. L3138Q, all variants found in this study were identified as heterozygous and no associated mutation was found in the other allele.

The structure of the MyTH4 domain has not been fully characterized. In other myosins, it has been implicated in microtubule binding as well as actin binding to the plasma membrane. Some data suggest that the MyTH4/FERM domains are required for localization of Myosin15a to stereocilia tips. The co-localization of Myosin15a and whirlin proteins appears essential to form the complex at the stereocilia tips [16]. From our data combined with previous reports, the MyTH4 domain mutations interfere with the interaction between Myosin15a and whirlin, preventing the formation of the complex required for normal hearing [1]. *MYO15A* mutations have been found in each domain (Motor, MyTH4, N-terminal extension, FERM, and SH3) and caused similar clinical features including hearing level, implying the overall importance of *MYO15A* protein in cochlear function.

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ORIGINAL ARTICLE

Mutation spectrum and genotype–phenotype correlation of hearing loss patients caused by *SLC26A4* mutations in the Japanese: a large cohort study

Maiko Miyagawa[1], Shin-ya Nishio[1], Shin-ichi Usami[1] and The Deafness Gene Study Consortium[2]

Mutations in *SLC26A4* cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with enlarged vestibular aqueduct. Identification of these mutations is important for accurate diagnosis, proper medical management and appropriate genetic counseling and requires updated information regarding spectrum, clinical characteristics and genotype–phenotype correlations, based on a large cohort. In 100 patients with bilateral enlarged vestibular aqueduct among 1511 Japanese hearing loss probands registered in our gene bank, goiter data were available for 79, of whom 15 had Pendred syndrome and 64 had nonsyndromic hearing loss. We clarified the mutation spectrum for the *SLC26A4* mutations and also summarized hearing levels, progression, fluctuation and existence of genotype–phenotype correlation. *SLC26A4* mutations were identified in 82 of the 100 patients (82.0%). Of the Pendred syndrome patients, 93% (14/15) were carriers, as were 77% (49/64) of the nonsyndromic hearing loss patients. Clinical characteristics of patients with *SLC26A4* mutations were congenital, fluctuating and progressive hearing loss usually associated with vertigo and/or goiter. We found no genotype–phenotype correlations, indicating that, unlike in the case of *GJB2* mutations, the phenotype cannot be predicted from the genotype. Our mutation analysis confirmed the importance of mutations in the *SLC26A4* gene among hearing loss patients with enlarged vestibular aqueduct and revealed the mutation spectrum, essential information when performing genetic testing.
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Keywords: congenital hearing loss; DFNB4; enlarged vestibular aqueduct; goiter; Pendred syndrome; *SLC26A4*

INTRODUCTION

Based on our genetic screening, *SLC26A4* is the second most common responsible gene in Japanese deafness patients.[1] Mutations in the *SLC26A4* gene are known to be responsible for a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss with enlarged vestibular aqueduct (EVA). The prevalent association of *SLC26A4* mutations in these patients (90% in Pendred syndrome and 78.1% in nonsyndromic hearing loss associated with EVA) indicates the importance of this gene in the pathophysiology of this category of hearing impairment.[2] More than 160 mutations have been found in *SLC26A4* (Pendred/BOR Homepage, http://www.healthcare.uiowa.edu/labs/pendredandbor/), and different mutational spectrums among different ethnic groups have been reported.[2] The identification of *SLC26A4* mutations enables more appropriate genetic counseling and proper medical management for these patients. For such clinical application, updated information regarding mutation spectrum, clinical characteristics and genotype–phenotype correlations based on a large cohort is needed. In addition to our previous reports,[1–7] the present study was performed using a large cohort of patients to collect updated data and summarize these data to enable more precise decision making by ear, nose and throat clinicians.

MATERIALS AND METHODS

Subjects

Data on 1511 independent probands and 1545 family members were collected from 33 ear, nose and throat departments nationwide in Japan and registered in our gene bank. All subjects or next of kin, caretakers or guardians on behalf of the minors/children gave prior written informed consent for participation in the project, and the Shinshu University Ethical Committee as well as the respective Ethical Committees of the other participating institutions of the Deafness Gene Study Consortium (Hokkaido University, Hirosaki University, Iwate Medical University, Tohoku University, Yamagata University, Fukushima Medical University, Jichi Medical University, Gunma University, Nihon University, Nippon Medical School, Nippon Medical School Tama Nagayama

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Hospital, Jikei University, Toranomon Hospital, Kitasato University, Hamamatsu Medical University, Mie University, Shiga Medical Center for Children, Osaka Medical College, Hyogo College of Medicine, Kobe City Medical Center General Hospital, Wakayama Medical University, Okayama University, Yamaguchi University, Ehime University, Kyushu University, Fukuoka University, Nagasaki University, Kanda ENT Clinic, Miyazaki Medical College, Kagoshima University and Ryukyus University) approved the study.

Computerized tomography scan was used to diagnose EVA (according to the criteria of EVA: a diameter of >1.5 mm at the midpoint between the common crus and the external aperture), and they were clinically well characterized by repeated auditory examinations.

The 100 subjects (51 males and 49 females) from among the 1511 probands who met the criteria of bilateral EVA and who ranged in age from 0 to 59 years with a mean age of 13.9 years at the time of examination were enrolled in the current study. Fifteen subjects had Pendred syndrome and 64 had nonsyndromic hearing loss.

The controls were 192 unrelated Japanese healthy individuals with normal hearing evaluated by auditory testing.

Mutation analysis

To identify *SLC26A4* mutations, a DNA fragment containing all the exons of *SLC26A4*, including flanking intronic sequences, was sequenced as described elsewhere.[4] New variants were tested in 192 unrelated normal hearing controls.

Possible pathologic mutations were defined as (1) mutations found to be homozygotes or compound heterozygotes (and determined by segregation study); (2) variants that were not found, or were very few, in the 192 control subjects; and (3) amino acids that were well conserved among various species.

Clinical evaluations

Hearing levels were determined by pure-tone audiometry in adults. For the young patients, conditioned orientation response audiometry or auditory steady-state response was used. Clinical data, including hearing loss progression, fluctuation, episodes of tinnitus and vestibular dysfunction (vertigo, dizziness) and goiter, were collected by anamnestic evaluation. For genotype–phenotype correlation analysis, one-way analysis of variance (Tukey's honest significant difference (HSD) test), Kruskal–Wallis test and multivariate statistics (multiple regression analysis and logistic regression analysis) were used.

RESULTS

SLC26A4 mutation spectrum

There were a total of 39 *SLC26A4* mutations found in the probands with bilateral EVA (Table 1). These mutations were either homozygous, compound heterozygous or heterozygous with no other mutations being detectable. There were two nonsense mutations (p.S610X, p.L727X), three deletion frameshift mutations (c.322delC, c.917delT, c.1219delCT) and three insertion frameshift mutations (c.139insC, c.1652insT, c.2111ins GCTGG). Seven splice site mutations were found (c.416-1G>A, c.600+1G>T, c.601-1G>A, c.919-2A>G, c.1001+1G>A, c.1002-9A>G and c.1707+5G>A).

There were 24 missense mutations (p.P76S, p.T94I, p.P123S, p.M147V, p.P297Q, p.K369E, p.A372V, p.N392Y, p.G396E, p.T410M, p.A434T, p.G439R, p.S448L, p.T527P, p.I529S, p.S532I, p.C565Y, p.R581S, p.S657N, p.V659L, p.S666F, p.T721M, p.H723R and p.H723Y). To evaluate the evolutionary conservation of the amino acids affected by these missense mutations, we made an alignment of the *SLC26A4* amino acid sequence of four mammalian species: human, rat, cow and mouse. On the basis of this alignment, all missense mutations had changed evolutionary conserved amino acids. Of these mutations, nine variants had not been reported. We checked the 192 control subjects with normal hearing, but with the exception of p.H723R in 1 case, no mutations were detected.

Sequencing identified mutations in 82 of the 100 patients (82.0%). Mutations were detected in 93% of those with Pendred syndrome (14/15) and 77% (49/64) of those with nonsyndromic hearing loss. Of these, 15/100 (15.0%) were homozygous, 51/100 (51.0%) were compound heterozygous and 16/100 (16.0%) were heterozygous (Table 2).

The most frequent mutation was p.H723R that accounted for 36.0%, and the second was c.919-2A>G found in 7.0%, followed by c.1707+5G>A (4.0%). Frequency of the other 36 mutations was very low (0.5–2.0%).

Clinical findings

Table 2 shows the clinical details for the 100 subjects.

The subjects had an average hearing level of 80.9 dB (7.5–112.5 dB), with hearing loss that was mild in 5, moderate in 22, severe in 37, profound in 19 and unknown in 12. Regarding onset age of hearing loss, 45 patients were congenital, 18 were prelingual (1–3 years old), 20 were from 4 to 14 years and 17 were unknown. These results clearly indicated that early onset is dominant in patients with EVA. Also, 70 patients (70%) showed progressive hearing loss and 56 patients (56.0%) felt fluctuation of hearing. With regard to the 79 patients for whom data on vertigo were available, 41 patients complained of vertigo and 38 did not. Of the 79 patients for whom data on goiter were available, 15 had goiter and 64 did not, with an onset age from 12 to 33 years. As to family history, all families were recessive inheritance or sporadic cases.

Genotype–phenotype correlations for diagnostic age, fluctuation, vertigo, tinnitus and goiter are summarized in Figure 1.

We defined nonsense or frameshift mutations as truncating (T) and missense mutations as nontruncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/nontruncating (T/NT) or nontruncating/nontruncating (NT/NT). Significant differences were not found between the groups in any of the clinical features (Tukey's HSD test was used for diagnostic age and Kruskal–Wallis test was used for fluctuation, vertigo, tinnitus and goiter, all tests indicated $P>0.05$; Figure 1). Figure 2 shows the relationship between hearing loss severity and the mutation (T or NT) that also showed no significant differences (Tukey's HSD test, $P>0.05$). We also performed multivariate statistics (multiple regression analysis and logistic regression analysis) and we found that only the age of the patients correlated with the hearing loss severity while the genotype of *SLC26A4* mutations did not significantly affect the hearing loss severity ($P>0.05$).

DISCUSSION

The present large cohort study revealed a high prevalence (82%; 82/100) of *SLC26A4* mutations in sensorineural hearing loss patients with EVA in Japanese. The frequency (8.7%) is the second most common next to *GJB2* that is found in 16.2% of overall and 25.6% of congenital hearing loss patients.[1]

Our mutation analysis results confirmed the previous reports that indicated the importance of this gene among hearing loss patients with EVA. This study also added novel mutations and summarized updated data for the precise molecular diagnosis.

First, the high prevalence (82%) of *SLC26A4* mutations in EVA patients is compatible with the high prevalence of *SLC26A4* mutations reported in eastern Asians; that is, 97.9% in Chinese,[8] and 92% in Koreans.[9] These frequencies are higher than those reported in Caucasoid populations (20% in Americans,[10] 40.0% in French[11] and 28.4% in Spanish[12]). It is still an open question whether other genes are involved in the EVA patients without *SLC26A4* mutations.

Table 1 Possible pathogenic variants found in enlarged vestibular aqueduct (EVA) subjects ($n = 100$)

			Frequency (n = 100)				
Nucleotide change	*Amino acid change*	*Exon*	*Homozygote*	*Compound heterozygote*	*Heterozygote*	*Allele frequency (in 200 alleles)*	*References*
c. 139insC		1		1		0.50	This study
c. 266C>T	p. P76S	2		1		0.50	Suzuki *et al.*[5,6]
c. 281C>T	p. T94I	3		1		0.50	Wang *et al.*[7,8]
c. 322delC		4		1		0.50	Tsukamoto *et al.*[2,4]
c. 367C>T	p. P123S	4		1		0.50	Tsukamoto *et al.*[2,4]
c. 416-1G>A		Intron 4		2		1.00	Tsukamoto *et al.*[2,4]
c. 439A>G	p. M147V	5		2		1.00	Tsukamoto *et al.*[2,4]
c. 600+1G>T		Intron 5		1		0.50	This study
c. 601-1G>A		Intron 5		1		0.50	Tsukamoto *et al.*[2,4]
c. 890C>A	p. P297Q	7		1		0.50	This study
c. 917delT		7			1	0.50	Tsukamoto *et al.*[2,4]
c. 919-2A>G		Intron 7	1	11	1	7.00	Coucke *et al.*[21]
c. 1001+1G>A		Intron 8		2		1.00	Coyle *et al.*[22]
c. 1002-9A>G[a]		Intron 8		1		0.50	This study
c. 1105A>G	p. K369E	9		1		0.50	Usami *et al.*[2,3]
c. 1115C>T	p. A372V	9		1		0.50	Usami *et al.*[2,3]
c. 1174A>T	p. N392Y	10		3		1.50	Park *et al.*[14,16]
c. 1187G>A	p. G396E	10		1		0.50	This study
c. 1219delCT		10		1		0.50	This study
c. 1229C>T	p. T410M	10	1	1		1.50	Coyle *et al.*[22]
c. 1300G>A	p. A434T	11			1	0.50	This study
c. 1315G>A	p. G439R	11		1		0.50	Suzuki *et al.*[5,6]
c. 1343C>T	p. S448L	11		1		0.50	Wang *et al.*[7,8]
c. 1579A>G	p. T527P	14		2		1.00	Suzuki *et al.*[5,6]
c. 1586T>G	p. I529S	14		1		0.50	Wang *et al.*[7,8]
c. 1595G>T	p. S532I	14		2		1.00	Usami *et al.*[3,17]
c. 1652insT		15		3	1	2.00	Tsukamoto *et al.*[2,4]
c. 1694G>A	p. C565Y	15		1		0.50	Tsukamoto *et al.*[2,4]
c. 1707+5G>A		Intron 15	1	6		4.00	Park *et al.*[8,9]
c. 1743G>C	p. R581S	16		2		1.00	Iwasaki *et al.*[5,18]
c. 1829C>A	p. S610X	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1970G>A	p. S657N	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 1975G>C	p. V659L	17		3		1.50	Wang *et al.*[7,8]
c. 1997C>T	p. S666F	17		1		0.50	Tsukamoto *et al.*[2,4]
c. 2111ins GCTGG		19		1	1	1.00	Usami *et al.*[2,3]
c. 2162C>T	p. T721M	19		1	1	1.00	Usami *et al.*[2,3]
c. 2168A>G	p. H723R	19	11	40	10	36.00	Usami *et al.*[2,3]
c. 2168C>T	p. H723Y	19	1			1.00	This study
c. 2180T>A	p. L727X	19		1		0.50	This study

[a]c. 1002-9A>G, uncertain pathogenicity.

Mutations in *FOXI1*,[13] a modulatory gene of *SLC26A4*, were not found in our series of patients (data not shown). As seen in previous mutation screening reports, we encountered a significant number of heterozygous cases without a second mutation even after direct sequencing of the coding region of the gene. It is highly likely that there is one more occult mutation somewhere because patients with heterozygous mutation are associated with EVA.

Second, it is evident that the mutation spectrum found in the Japanese population is quite different from that in Caucasoid populations, but similar to the mutation spectrum reported in the Asian populations, especially Koreans.[8–12,14] There are two frequent mutations in east Asians, namely p.H723R and c.919-2A>G. p.H723R is most prevalent in the Japanese and Korean populations,[8] whereas c.919-2A>G is most common in the Chinese.[7]

The existence of a genotype–phenotype correlation is still controversial.[6,12,15] Mutations in *SLC26A4* can cause a broad phenotypic spectrum, from typical Pendred syndrome to nonsyndromic hearing loss associated with EVA. In the present study, various features of the phenotype were compared with the genotypes. We defined nonsense or frame shift mutations as truncating (T) and missense mutations as non-truncating (NT) and classified the genotypes as truncating/truncating (T/T), truncating/non-truncating (T/NT), or non-truncating/non-truncating (NT/NT). However, statistical differences were not found between the groups in any of the clinical features (χ^2 tests, $P > 0.05$; Figure 1).

Concerning the relationship between the severity of hearing loss and individual *SLC26A4* mutations, several functional studies have demonstrated the property of transporter function.[16–18] Furthermore,

Table 2 Phenotypes and genotypes of affected EVA subjects

ID	*Age*	*Mutation allele 1/allele 2*	*Age of awareness*	*Progression*	*Fluctuation*	*Tinnitus*	*Vertigo*	*Goiter*	*Threshold (Rt) (dB)*[a]	*Threshold (Lt) (dB)*[a]	*Hearing level in the low frequencies*[b]
77	12	p.[917delT];[=]	12	+	+	+	+	−	58.75	45	49.375
237	7	p.[T721M];[H723R]	0	+	−	−	+	−	112.5	68.75	83.75
334	23	p.[A372V];[H723R]	0	NA	NA	+	NA	NA	96.25	83.75	81.9
695	4	p.[K369E];[H723R]	0	+	−	NA	NA	−	100	90	89.4
752	18	p.[1652insT];[=]	1	−	−	+	+	+	98.75	102.5	96.3
1045	25	p.[H723R];[H723R]	0	+	NA	−	+	+	78.75	90	85.6
1306	3	p.[919-2A>G];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
1365	20	p.[T721M];[=]	2	NA	NA	NA	NA	NA	96.25	105	96.9
1379	10	p.[1001+1G>A];[H723R]	0	+	+	−	−	NA	66.25	46.25	57.5
1432	6	p.[H723R];[=]	0	+	−	−	−	NA	102.5	105	100.0
1625	16	p.[919-2A>G];[H723R]	0	+	+	NA	+	NA	100	95	88.1
1795	NA	p.[H723R];[=]	NA	NA	N/A	NA	NA	NA	NA	NA	NA
1820	12	p.[H723R];[H723R]	5	+	+	−	−	NA	72.5	73.75	61.3
1957	7	p.[S666F];[H723R]	3	+	+	NA	NA	−	95	101.25	93.8
1961	12	p.[C565Y];[H723R]	0	+	N/A	NA	NA	NA	108.75	110	103.8
2010	12	p.[416-1G>A];[H723R]	9	+	+	−	−	+	80	91.25	81.3
2202	4	p.[P297Q];[T527P]	3	+	−	−	−	−	77.5	76.25	73.8
2331	31	p.[H723R];[H723R]	0	+	+	+	+	+	90	100	87.5
2449	1	p.[139insC];[322delC]	0	NA	NA	−	+	−	100	85	92.5
2462	52	p.[M147V];[H723R]	2	+	+	−	−	−	98.75	95	88.1
2498	0	p.[919-2A>G];[1001+1G>A]	0	+	+	NA	−	−	86.25	86.25	83.8
2538	10	p.[H723R];[H723R]	3	+	+	−	−	+	81.25	55	66.9
2621	3	p.[R581S];[H723R]	0	+	+	−	−	−	91.25	91.25	90.0
2695	13	p.[T527P];[H723R]	2	+	+	+	+	−	62.5	61.25	63.1
2728	3	p.[919-2A>G];[H723R]	1	+	+	−	−	−	97.5	97.5	93.8
2798	15	p.[H723R];[H723R]	4	+	+	NA	+	+	52.5	96.25	66.3
2804	2	p.[1707+5G>A];[H723R]	0	+	+	−	−	−	78.75	78.75	82.5
3072	44	p.[G439R];[H723R]	6	+	+	+	+	−	110	108.75	105.0
3074	21	p.[H723R];[=]	2	+	+	+	+	+	105	106.25	99.4
3298	6	p.[919-2A>G];[H723R]	0	+	+	+	+	−	73.75	110	86.9
3301	4	p.[416-1G>A];[H723R]	0	+	+	+	+	−	65	72.5	68.1
3442	6	p.[919-2A>G];[H723R]	NA	+	NA	+	+	−	81.25	50	60.0
3450	14	p.[H723R];[H723R]	0	+	+	+	+	−	110	73.75	87.5
3561	6	p.[H723Y];[H723Y]	4	NA	NA	NA	NA	NA	83.75	65	71.3
3994	59	p.[601-1G>A];[H723R]	10	+	+	+	+	+	96.0	94	91.3
3996	8	p.[H723R];[1652insT]	0	+	−	+	−	−	100	110	98.1
3999	8	p.[H723R];[1652insT]	0	+	+	−	+	−	30	50	40.0
4050	5	p.[M147V];[H723R]	1	+	+	+	+	−	107.5	85	93.8
4097	3	p.[N392Y];[1002-9A>G]	0	−	−	−	−	−	106.25	85	93.1
4098	26	p.[N392Y];[919-2A>G]	2	−	+	+	+	−	110	37.5	71.3
4102	5	p.[N392Y];[H723R]	0	+	+	+	+	−	95	78.75	83.1
4131	10	p.[H723R];[=]	8	+	+	−	−	−	81.25	60	70.6
4144	21	p.[H723R];[H723R]	4	+	NA	+	+	−	93.75	105	95.6
4232	15	p.[V659L];[H723R]	NA	−	+	+	+	−	60	92.5	69.4
4299	4	p.[S532I];[2111ins GCTGG]	3	−	+	−	+	−	17.5	70	42.5
4305	14	p.[A434T];[=]	0	+	−	+	−	−	110	110	105.0
4320	10	p.[G396E];[S532I]	NA	+	+	+	−	−	72.5	80	72.5
4338	6	p.[R581S];[H723R]	0	+	+	+	+	−	78.75	52.5	64.4
4380	10	p.[1707+5G>A];[H723R]	2	+	+	−	−	−	96.25	81.25	84.4
4386	21	p.[H723R];[H723R]	NA	+	+	+	+	+	77.5	93.75	85.0
4398	4	p.[1652insT];[H723R]	2	+	+	+	+	−	70	97.5	86.9
4434	8	p.[T410M];[1707+5G>A]	1	+	+	−	+	−	92.5	100	91.3
4469	11	p.[H723R];[=]	0	+	NA	−	−	−	20	21.25	16.9
4485	40	p.[H723R];[=]	10	+	+	+	+	−	56.25	65	58.8

Table 2 (Continued)

ID	Age	Mutation allele 1/allele 2	Age of awareness	Progression	Fluctuation	Tinnitus	Vertigo	Goiter	Threshold (Rt) (dB)[a]	Threshold (Lt) (dB)[a]	Hearing level in the low frequencies[b]
4486	20	p. [1707 + 5G > A]; [1707 + 5G > A]	4	+	+	+	+	+	72.5	95	78.1
4490	25	p. [T410M];[T410M]	0	−	−	+	+	+	87.5	92.5	90.0
4508	29	p. [H723R];[H723R]	5	+	+	−	−	−	85	110	91.9
4518	26	p. [H723R];[919-2A > G]	0	+	+	+	+	−	105	97.5	98.1
4530	5	p. [H723R];[919-2A > G]	0	+	+	−	+	−	67.5	86.25	71.9
4545	12	p. [1707 + 5G > A];[H723R]	4	+	+	+	+	+	86.25	28.75	53.1
4549	13	p. [V659L];[1219delCT]	NA	+	+	+	+	−	38.75	50	38.1
4663	0	p. [1707 + 5G > A];[H723R]	0	−	+	NA	NA	−	68.75	68.75	99.2
4696	0	p. [V659L];[H723R]	0	+	−	NA	NA	−	NA	NA	97.5
4362	26	p. [H723R]; [=]	6	+	−	−	−	−	70	68.75	63.8
4513	34	p. [H723R]; [=]	NA	+	+	+	NA	−	71.25	53.75	61.3
4645	23	p. [919-2A > G]; [=]	14	+	−	+	−	−	96.25	105	93.8
723	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
724	NA	p. [2111ins5bp]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
742	NA	p. [H723R]; [=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
1975	3	p. [H723R];[H723R]	0	NA	NA	NA	NA	NA	80	70	62.5
2082	2	p. [H723R];[H723R]	0	−	−	−	−	−	NA	NA	NA
4735	9	p. [H723R];[919-2A > G]	0	+	+	+	+	−	107.5	110	103.8
195	20	p. [=];[=]	2	+	+	+	+	−	83.75	83.75	81.9
670	8	p. [=];[=]	3	+	−	+	−	−	26.25	107.5	62.5
1755	16	p. [=];[=]	NA	NA	NA	NA	NA	NA	NA	NA	NA
2607	5	p. [=];[=]	0	−	+	−	−	−	97.5	105	98.8
3851	33	p. [=];[=]	0	+	+	+	−	+	103.75	103.75	100.6
4194	11	p. [=];[=]	NA	+	+	−	−	−	67.5	80	76.3
4215	5	p. [=];[=]	0	+	+	−	−	−	98.75	93.75	93.8
4216	55	p. [=];[=]	NA	+	+	+	+	NA	51.25	78.75	68.8
4258	30	p. [=];[=]	28	NA	−	+	−	−	17.5	7.5	13.8
4281	6	p. [=];[=]	2	−	−	−	−	−	57.5	61.25	63.1
4324	37	p. [=];[=]	6	−	−	−	−	−	10	27.5	22.5
4352	3	p. [=];[=]	0	+	+	−	−	−	86.25	88.75	88.1
4357	6	p. [=];[=]	4	+	+	+	−	−	71.25	72.5	67.5
4397	5	p. [=];[=]	0	−	−	−	−	−	102.5	105	100.6
4402	8	p. [=];[=]	0	+	−	−	−	−	100	90	88.8
4450	12	p. [=];[=]	NA	+	+	+	−	−	NA	NA	NA
4462	8	p. [=];[=]	7	+	−	+	−	−	63.75	20	41.3
4488	1	p. [=];[=]	0	−	−	NA	−	−	97.5	97.5	95.0
4671	2	p. [H723R];[600 + 1G > T]	0	+	−	−	+	−	NA	NA	NA
3253	NA	p. [I529S];[H723R]	NA	NA	NA	NA	NA	NA	NA	NA	NA
4949	0	p. [L727X];[H723R]	0	+	−	−	−	−	NA	NA	51.7
J27	NA	p. [H723R];[S448L]	NA	NA	NA	NA	NA	NA	NA	NA	90.6
3309	5	p. [919-2A > G];[P76S]	0	+	+	+	+	−	106.25	106.25	101.3
J15	0	p. [P123S];[H723R]	0	NA	NA	NA	NA	NA	NA	NA	NA
FUK2004	1	p. [H723R];[T94I]	0	NA	NA	N/A	NA	NA	NA	NA	85.0
1299	NA	p. [S610X];[S657N]	0	NA	NA	NA	NA	NA	NA	NA	NA
SNS5500	42	p. [919-2A > G];[919-2A > G]	4	+	+	+	+	+	70	81.3	64
SNS5503	37	p. [H723R];[1707 + 5G > A]	5	+	+	+	+	+	67.5	70	NA

Abbreviation: EVA, enlarged vestibular aqueduct; Lt, left; NA, not available; Rt, right.
[a]Average of 500, 1000, 2000 and 4000 Hz.
[b]Average of 125, 250 and 500 Hz.

retention of improperly folded Pendrin mutants in the endoplasmic reticulum has been suggested as the major pathological mechanism for Pendred syndrome.[19,20] In this study, we compared not only the difference between the T and NT mutations, but also compared the individual mutations and severity of hearing. However, there were no correlations (data not shown). Indeed, there was great variation regarding hearing loss severity even with the same mutations. For example, in the patients homozygous for the most prevalent mutation, p.H723R, hearing level at low frequency varied from 61 to 99 dB (Table 2). In addition, many reports have described intrafamilial

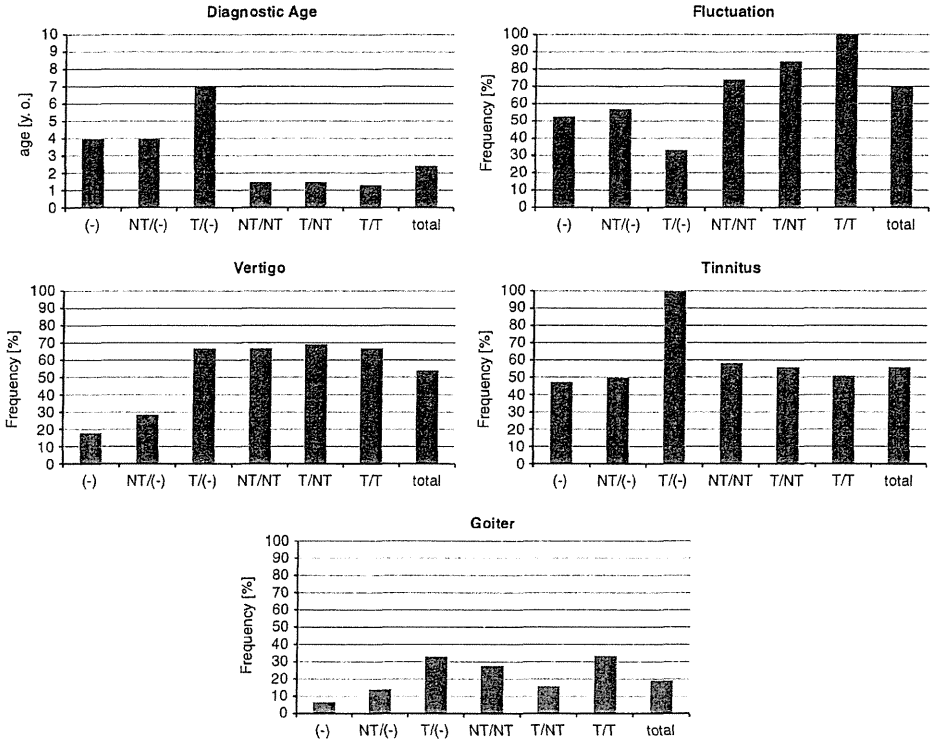

Figure 1 Genotypes and phenotypes (diagnostic age, fluctuation, vertigo, tinnitus and goiter) in the current study. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

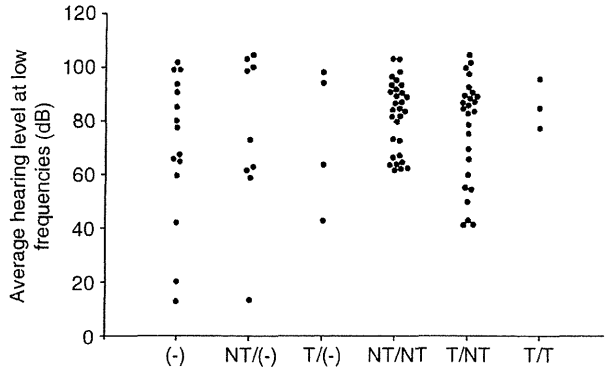

Figure 2 The relationship between hearing level at the lower frequencies and genotype. Hearing level was the average of 125, 250 and 500 Hz. NT/(−), heterozygote of nontruncating mutation; NT/NT, nontruncating/nontruncating; NT/T, nontruncating/truncating; T/(−), heterozygote of truncating mutation; T/T, truncating/truncating; (−), wild type.

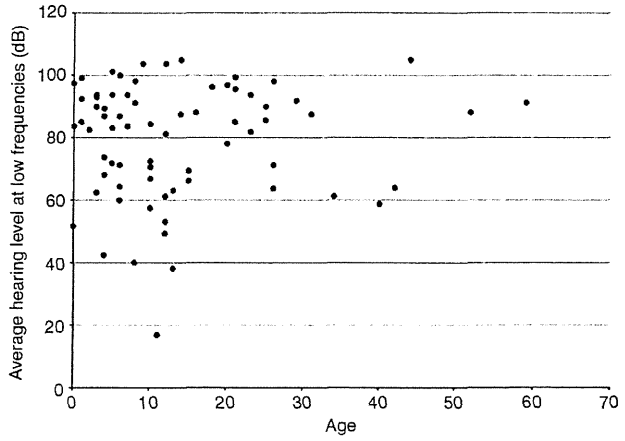

Figure 3 The relationship between hearing level and age in subjects with biallelic *SLC26A4* mutations. Hearing level was calculated as the average of 250, 500, 1000 and 2000 Hz in both sides.

phenotypic variation.[8–12] Therefore, phenotype may be determined not only by *SLC26A4* mutations but also other factors (genetic as well as environmental), contributing to such variability (Figure 2).

Unlike in the case of *GJB2*, phenotype cannot be predicted from the genotype;[6] however, the clarification of clinical features will enable more appropriate genetic counseling and proper medical management for these patients.

The present study confirmed clinical characteristics of 66 patients with EVA caused by biallelic *SLC26A4* mutations. These included congenital (5/63, 7.9%), fluctuated (42/52, 80.8%) and progressive (49/56, 87.5%) hearing loss usually associated with vertigo (35/52, 67.3%) and/or goiter (12/53, 22.6%) during long-term follow-up, in accordance with our previous study.[6] It is known that goiter sometimes becomes apparent between 10 and 20 years of age. The present cohort included young children, and therefore the frequency of goiter may be underestimated. As seen in Figure 3, in 66 patients with biallelic mutations for whom data were available, onset of hearing loss was likely to be early onset, and progressive with age.