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ORIGINAL ARTICLE

A Japanese family showing high-frequency hearing loss with *KCNQ4* and *TECTA* mutations

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Abstract

Conclusions: We describe a Japanese family with high-frequency sensorineural hearing loss (SNHL) harboring a c.211delC mutation in the *KCNQ4* gene. Families showing progressive high-frequency SNHL should be investigated for mutations in the *KCNQ4* gene. *Objective:* To determine the responsible deafness gene in a Japanese family with dominantly inherited high-frequency SNHL of unknown etiology. *Methods:* We performed hearing tests for five members of the family, and the three affected with hearing loss underwent further audiological and vestibular examinations. Genetic analysis was performed to identify any possible causative mutations, as well as analysis of detailed clinical findings to determine the phenotype. *Results:* The three affected subjects showed high-frequency SNHL. Extensive audiologic evaluation suggested cochlear involvement and progressive hearing loss. As for bilateral caloric testing, two of the three affected subjects showed hyporeflexia with recurrent vestibular symptoms. We identified the c.211delC mutation in the *KCNQ4* gene and the c.2967C>A (p.H989Q) mutation in the *TECTA* gene. Based on the genotype–phenotype correlation, the c.211delC mutation in the *KCNQ4* gene was associated with high-frequency SNHL in this family.

Keywords: *Progressive hearing loss, c.211delC mutation, hyporeflexia, deafness gene*

Introduction

There are over 100 loci associated with nonsyndromic sensorineural hearing loss (SNHL) in humans [1]. To date, more than 60 loci of DFNA, the gene locus responsible for autosomal dominant deafness, have been identified and 27 genes were defined as DFNA-causative (Van Camp G, Smith RJH. Hereditary Hearing Loss Homepage: http://hereditaryhearingloss.org). The *KCNQ4* and *TECTA* genes are frequently associated with autosomal dominant nonsyndromic SNHL [2]. *KCNQ4* is a member of the voltage-gated potassium channel family localized in inner and outer hair cells and plays a role in potassium recycling in the inner ear. *KCNQ4* is composed of 695 amino acids with 6 transmembrane domains and a hydrophobic P-loop region that forms a channel pore containing a potassium ion-selective filter located between the transmembrane domains S5 and S6 (residues 259–296) [3]. *KCNQ4*-associated hearing loss has been reported to be typically late-onset high-frequency-involved and progressive over time [4]. More than 20 pathologic mutations have been identified in *KCNQ4* and they are mostly missense mutations with a dominant-negative mechanism that causes progressive, predominantly high-frequency hearing impairment [3,5]. Recently, Naito et al. reported a novel recurrent deletion mutation, c.211delC, in 13 Japanese patients with high-frequency-involved hearing loss [5]. This

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deletion mutation located in the N-terminal site causes truncation of *KCNQ4* protein product, and might have insufficient function for inner ear potassium recycling [5]. In contrast, the *TECTA* gene encodes α-tectorin, the major component of noncollagenous glycoprotein of the tectorial membrane, and has a role in intracochlear sound transmission [6]. Mutations of the *TECTA* gene cause ultrastructural defects of the tectorial membrane, in turn causing hearing loss [7]. The α-tectorin is composed of three distinct modules: the entactin G1 domain, the zonadhesin (ZA) domain with von Willebrand factor type D repeats, and the zona pellucida (ZP) domain [7]. Missense mutations affecting the ZP domain are associated with mid-frequency hearing loss, whereas mutations in the ZA domain are associated with hearing impairment primarily affecting the high frequencies [8].

We investigated the genetic cause in a Japanese family carrying nonsyndromic high-frequency SNHL with an autosomal dominant inheritance pattern. In addition, we analyzed their detailed audiological and vestibular findings.

Material and methods

Medical history and otological examination

One proband, as well as two other affected and two unaffected family members, from one autosomal dominant inherited SNHL family participated in this study. A complete history concerning hearing loss and symptoms potentially related to syndromic hearing loss was taken from all subjects and they all underwent otoscopic examination. Pure-tone audiometry was conducted in an acoustically isolated room using an AA-78 audiometer (Rion, Tokyo, Japan). Air- and bone-conduction thresholds were measured as decibel hearing level.

Detailed audiological and vestibular examination

Two of the three affected subjects underwent self-recording audiometry and evoked and distortion-product otoacoustic emissions (EOAE and DPOAE) examinations. All three underwent speech discrimination testing and caloric testing. In caloric testing, electronystagmography was recorded by cold water irrigation (20°C, 5 ml, 20 s). The details of the methods used for these evaluations, including self-recording audiometry, EOAE and DPOAE, speech discrimination testing, and caloric testing have been described previously [9].

Sequencing analysis of the KCNQ4 gene and TECTA gene

All 14 exons and flanking intronic sequences of the *KCNQ4* gene and all 23 exons and flanking intronic sequences of the *TECTA* gene were amplified by polymerase chain reaction (PCR). Primers were designed to flank all of the exon–intron boundaries through use of the Primer3Plus web-based server (http://primer3-plus.com). Each genomic DNA sample (40 ng) was amplified using a Multiplex PCR Assay Kit (Takara, Shiga, Japan) for 5 min at 95°C, followed by 40 three-step cycles of 94°C for 30 s, 60–67.6°C for 90 s, and 72°C for 90 s, with a final extension at 72°C for 10 min, ending with a holding period at 4°C in a Perkin-Elmer thermal cycler. The PCR products varied in size at about 100–400 bp, and they were treated with ExoSAP-IT (GE Healthcare Bio, Santa Clara, CA) by incubation at 37°C for 30 min, and inactivation at 80°C for 15 min. After the products were purified, we performed standard cycle sequencing reaction with ABI Big Dye terminators in an ABI 3100 autosequencer (Applied Biosystems, Foster City, CA). Computer analysis to predict the effect of missense variants on the protein function was performed with wANNOVAR [10] (http://wannovar.usc.edu) including the following functional prediction software: PhyloP (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/phyloP44way/), Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/), Polymorphism Phenotyping (PolyPhen2; http://genetics.bwh.harvard.edu/pph2/), likelihood ratio test (LRT; http://www.genetics.wustl.edu/jflab/lrt_query.html), and MutationTaster (http:/www.mutationtaster.org/).

Ethics statement

All subjects gave prior written informed consent for participation in the project, and the Ethical Committee of Jichi Medical University approved the study.

Results

Mutation analysis

We identified the c.211delC mutation in the *KCNQ4* gene in four of the subjects (three with high-frequency SNHL and one without SNHL), and the c.2967C>A (p.H989Q) mutation in the *TECTA* gene in two subjects with high-frequency SNHL (Figure 1).

Medical history and clinical findings

Otoscopic examination demonstrated a normal tympanic membrane in both ears of all five subjects.

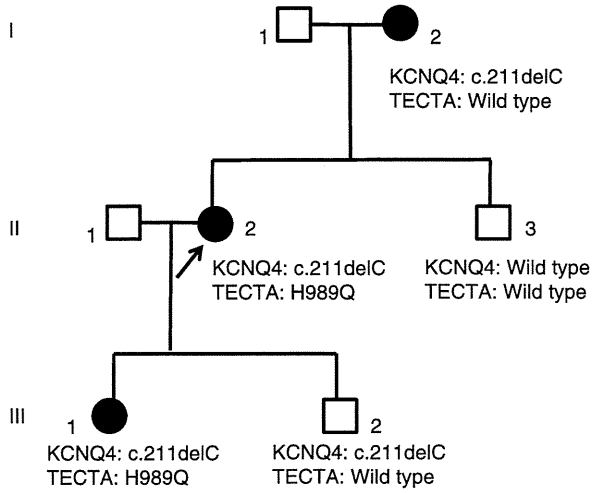

Figure 1. Pedigree of the family and the detected mutations in the *KCNQ4* and *TECTA* genes. The arrow indicates the proband.

Audiometric examination confirmed high-frequency SNHL in three of the five subjects (I-2, II-2, and III-1 in Figure 2). Self-recording audiometry showed Jerger type I [11] hearing loss in both ears of subject III-1, indicating that they had normal hearing. On the other hand, subject I-2 showed Jerger type II [11] hearing loss in the high-frequency area in both ears, indicating that this subject's hearing loss was of cochlear origin (Figure 3). Maximum speech discrimination scores in the three subjects with hearing loss showed mild to moderate defects, with subject I-2 having the lowest scores (Table I). Subject I-2 had no detectable DPOAE, but in two subjects (II-2 and III-1), DPOAE were were detected only in the lower frequency area. Subject III-2 carried the c.211delC mutation but did not have SNHL and showed normal DPOAE (Figure 4). As for bilateral caloric testing, subjects II-2 and III-1 showed hyporeflexia in the right ear with recurrent vestibular symptoms, while subject I-2 showed normal response without vestibular symptoms (Figure 5).

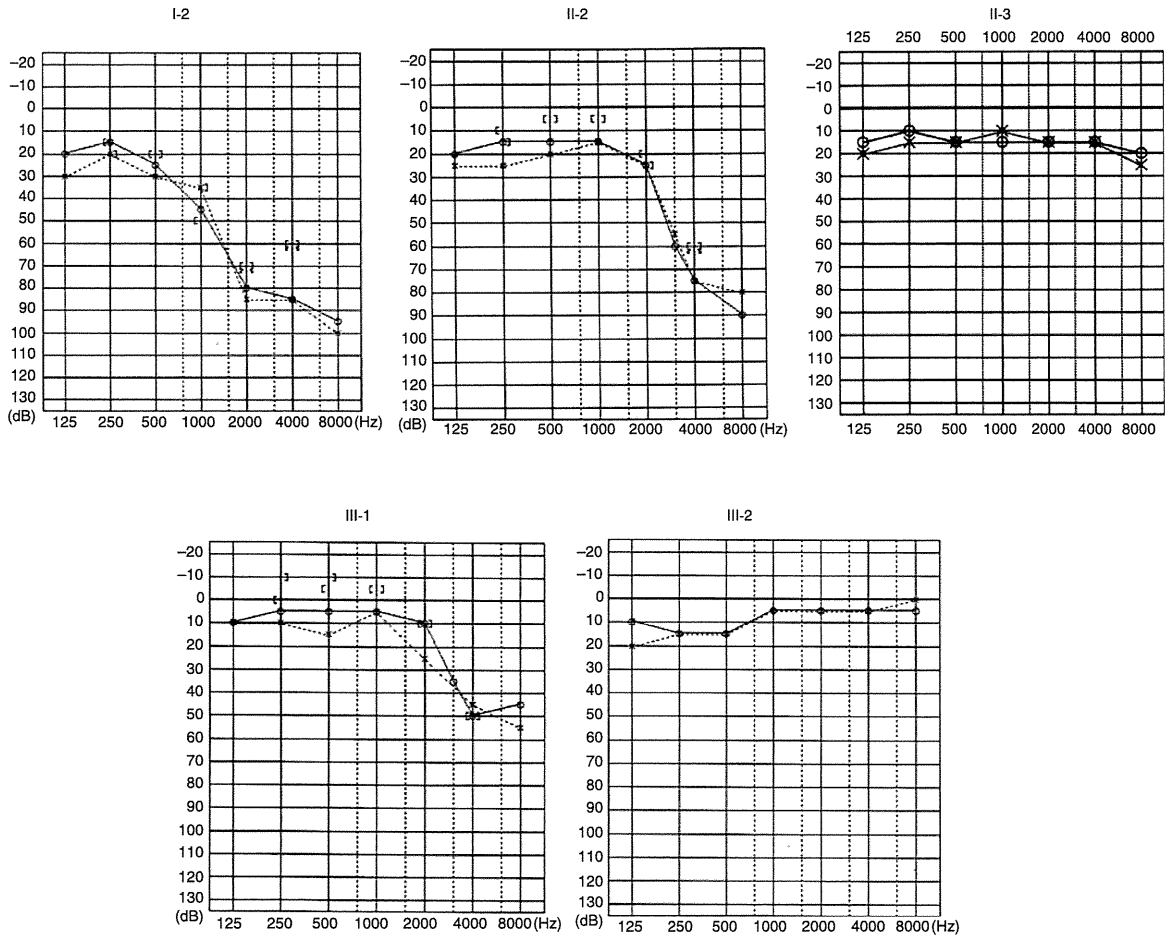

Figure 2. Pure-tone audiograms of the five family members shown in the Figure 1 pedigree.

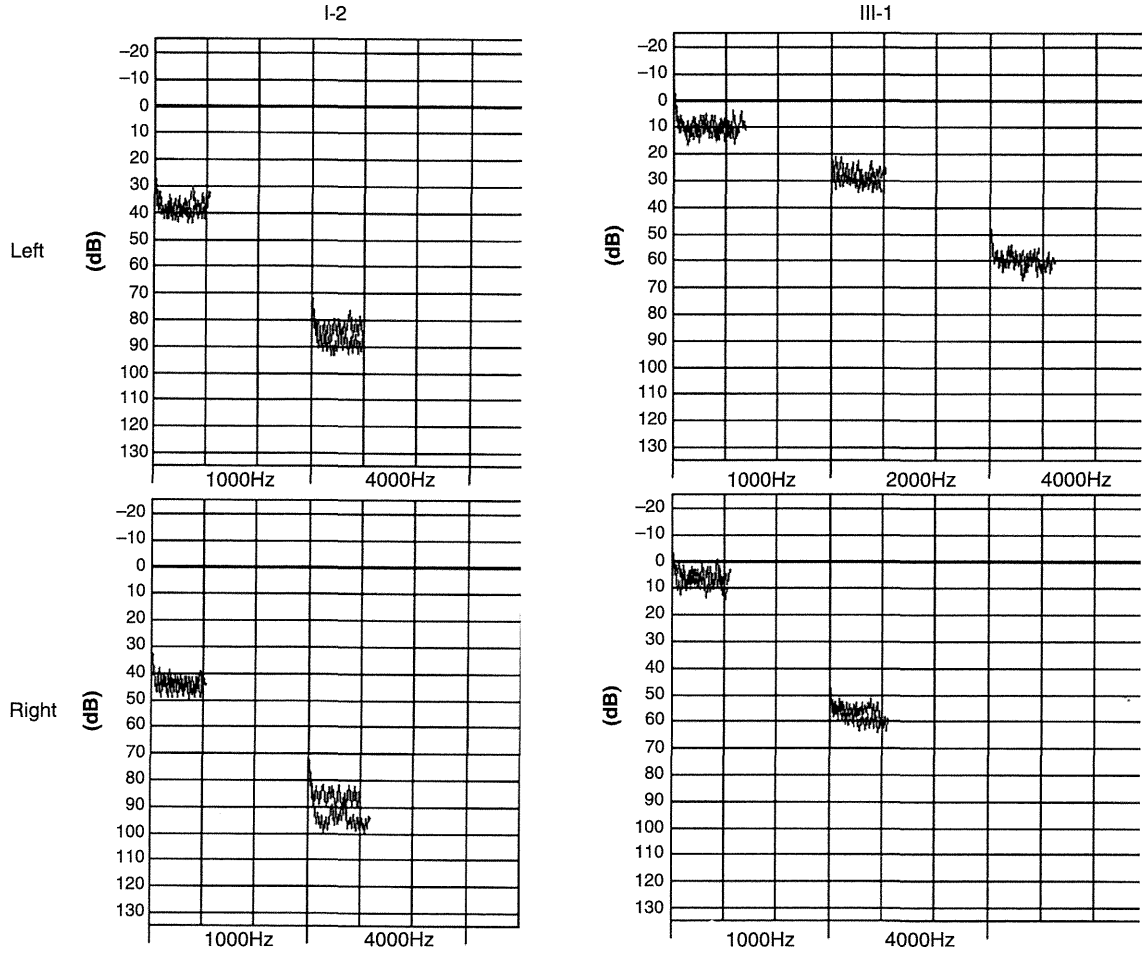

Figure 3. Self-recording audiometry results of two of the three subjects with high-frequency sensorineural hearing loss (SNHL).

Discussion

In the present study, we found a c.211delC mutation in the *KCNQ4* gene, as well as a c.2967C>A (p.H989Q) mutation in the *TECTA* gene, in an autosomal dominant inherited Japanese family with nonsyndromic high-frequency SNHL. The pathogenicity of the c.211delC mutation is strongly supported by the occurrence of the same mutation in several independent families with progressive nonsyndromic high-frequency SNHL [5,12]. Naito et al. reported that SNHL associated with the c.211delC mutation showed significant progression in only high frequencies by detailed progression analysis [5]. One subject (III-2), aged 6 years, carried the c.211delC mutation but did not have SNHL, suggesting that he may develop progressive high-frequency hearing loss in future. We explained this to the family, as it is the type of important information that we impart to patients during genetic counseling in our hospital.

Table I. Maximum speech discrimination scores of the three subjects with high-frequency sensorineural hearing loss (SNHL).

Subject	Age (years)	Side	Maximal speech discrimination (%)
I-2	55	Right	56
		Left	42
II-2	34	Right	74
		Left	78
III-1	14	Right	80
		Left	72

In the present family, subject I-2 (aged 55) showed the worst speech discrimination compared with II-2 (aged 34) and III-1 (aged 14), consistent with progressive hearing loss. Because subject I-2 also retained a nearly normal hearing level in low frequencies, it is highly likely that the c.211delC mutation does not cause profound deafness. This speculation is

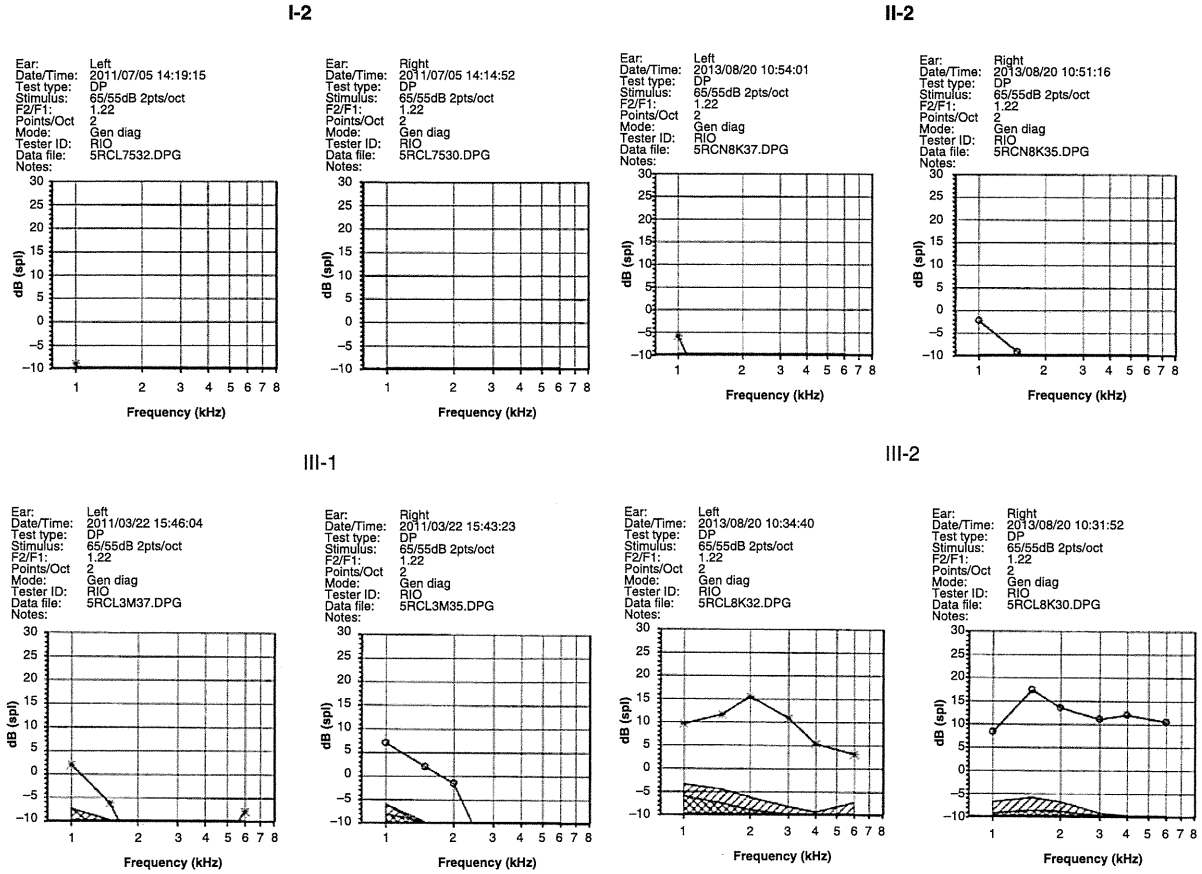

Figure 4. Distortion-product otoacoustic emissions (DPOAE) graphs of four subjects with the c.211delC mutation in the *KCNQ4* gene.

supported by the finding of Naito et al. that 20 patients with a maximum age of 73 who carried this mutation did not have progressive hearing loss. The detailed estimation of progression also does not suggest that development of profound hearing loss will occur.

Our findings from extensive audiological examination suggested that cochlear impairment induced by c.211delC mutation of the *KCNQ4* gene might start from the basal turn of the cochlea and progress to the middle turn. DPOAE in the middle frequency area was detectable in the youngest subject with high-frequency SNHL (III-1). Subject II-2 had detectable DPOAE only in the 1 kHz, at least in the right ear. The oldest subject (I-2) did not have detectable DPOAE in any frequency areas. These findings indicate that dysfunction of outer hair cells progressed from the basal turn to middle turn of the cochlea along with aging. In addition, this is supported by the results of self-recording audiometry, in which subject III-1 showed Jerger type I, indicating normal hearing, and subject I-2 showed Jerger type II (cochlear origin) hearing loss in the high-frequency area.

It is unclear whether vestibular symptoms are associated with the *KCNQ4* gene mutation. In the present study, two subjects carrying the c.211delC mutation showed recurrent vertigo and hyporeflexia in the right ear on caloric testing. However, one subject with this mutation showed normal caloric test responses without vestibular symptoms. In the two patients with vertigo, there was a unilateral decline of caloric response. Therefore, this vestibular dysfunction may not be due to the *KCNQ4* mutations, because if it were such a genetically determined vestibular response, it would usually be symmetric. Naito et al. also reported that in 20 patients carrying the c.211delC mutation, the majority did not have apparent vestibular symptoms, suggesting that this mutation is not associated with vestibular dysfunction [5].

We detected a novel missense mutation, p.H989Q, in the *TECTA* gene in two subjects with SNHL. This mutation is located in the TIL region of the zonadhesin-like domain D2 and is highly conserved in many species (from humans to fish). Alasti et al.

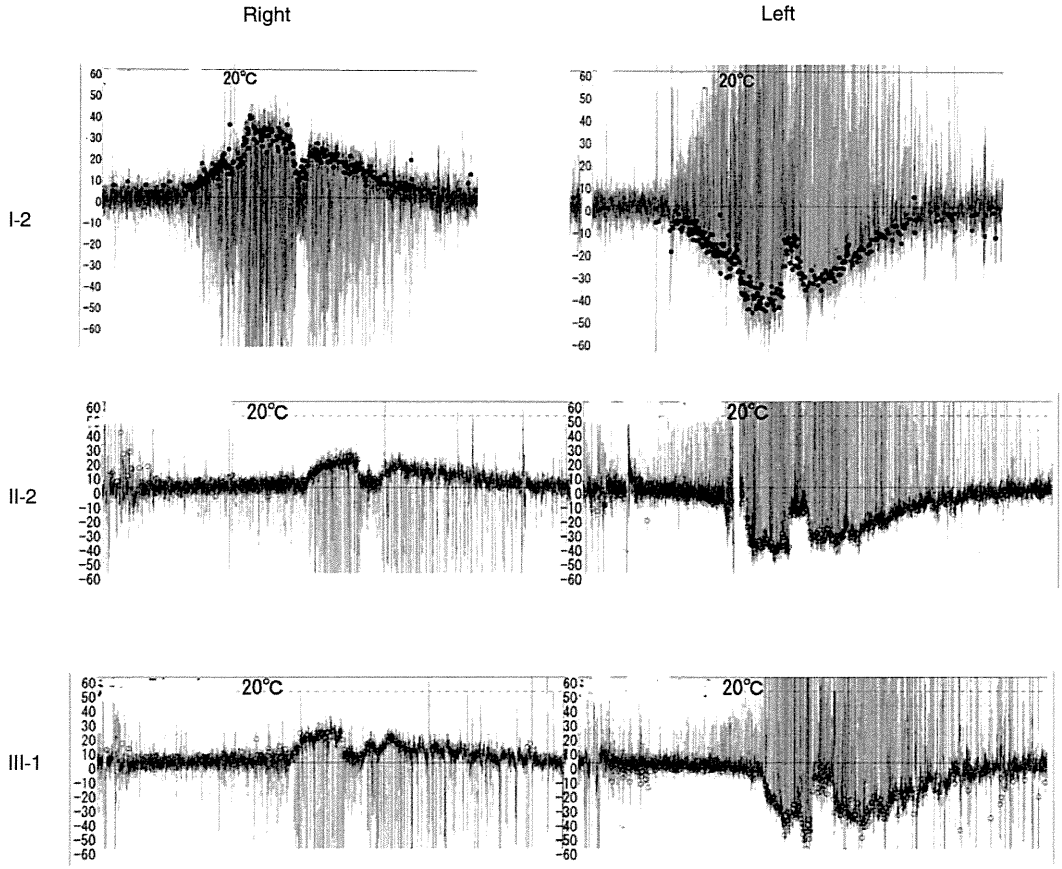

Figure 5. Caloric testing results of the three subjects with high-frequency sensorineural hearing loss (SNHL).

reported that ZA domain mutations cause progressive and high-frequency hearing loss [13]. However, our subject I-2 with high-frequency SNHL did not carry this mutation. Therefore we suspect that it was not associated with hearing loss in this family.

As technology develops and wide genome searches become more commonly performed, the detection of cases with two or more gene mutations is predicted to increase. It will be increasingly important to consider genotype–phenotype correlation of each mutation detected and to exercise due caution in determination of the causative mutation and selection of appropriate treatment.

Conclusion

In the present study, we found a c.211delC mutation in the *KCNQ4* gene in a Japanese family with autosomal dominant inherited progressive high-frequency SNHL, therefore the existence of this mutation should be considered in such families.

Acknowledgments

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

References

[1] Friedman TB, Griffith AJ. Human nonsyndromic sensorineural deafness. Annu Rev Genomics Hum Genet 2003;4: 341–402.

[2] Hilgert N, Smith RJ, Van Camp G. Forty-six genes causing nonsyndromic hearing impairment: which ones should be analyzed in DNA diagnostics? Mutat Res 2009;681:189–96.

[3] Kubisch C, Schroeder BC, Friedrich T, Lütjohann B, El-Amraoui A, Marlin S, et al. KCNQ4, a novel potassium channel expressed in sensory outer hair cells, is mutated in dominant deafness. Cell 1999;96:437–46.

[4] Dominguez LM, Dodson KM. Genetics of hearing loss: focus on DFNA2. Appl Clin Genet 2012;5:97–104.
[5] Naito T, Nishio SY, Iwasa Y, Yano T, Kumakawa K, Abe S, et al. Comprehensive genetic screening of KCNQ4 in a large autosomal dominant nonsyndromic hearing loss cohort: geneotype-phenotype correlations and a founder mutation. PLoS ONE 2013;8:e63231.
[6] Verhoeven K, Van Laer L, Kirschhofer K, Legan PK, Hughes DC, Schatteman I, et al. Mutations in the human α-tectorin gene cause autosomal dominant non-syndromic hearing impairment. Nat Genet 1998;19:60–2.
[7] Legan PK, Lukashkina VA, Goodyear RJ, Kössi M, Russell IJ, Richardson GP. A targeted deletion in alpha-tectorin reveals that the tectorial membrane is required for the gain and timing of cochlear feedback. Neuron 2000;28:273–85.
[8] Plantinga RF, Cremers CW, Huygen PL, Kunst HP, Bosman AJ. Audiological evaluation of affected members from a Dutch DFNA8/12 (TECTA) family. J Assoc Res Otolaryngol 2007;8:1–7.
[9] Tamagawa Y, Ishikawa K, Ishikawa K, Ishida T, Kitamura K, Makino S, et al. Phenotype of DFNA11: a nonsyndromic hearing loss caused by a myosin VIIA mutation. Laryngoscope 2002;112:292–7.
[10] Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.
[11] Jerger J. Békésy audiometry in analysis of auditory disorders. J Speech Hear Res 1960;3:275–87.
[12] Kamada F, Kure S, Kudo T, Suzuki Y, Oshima T, Ichinohe A, et al. A novel KCNQ4 one-base deletion in a large pedigree with hearing loss: implication for the genotype-phenotype correlation. J Hum Genet 2006;51:455–60.
[13] Alasti F, Sanati M, Behrouzifard AH, Sadeghi A, de Brouwer APM, Kremer H, et al. A novel TECTA mutation confirms the recognizable phenotype among autosomal recessive hearing impairment families. Int J Pediatr Otorhinolaryngol 2008;72:249–55.

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PLOS ONE

Deafness Gene Expression Patterns in the Mouse Cochlea Found by Microarray Analysis

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Abstract

Background: Tonotopy is one of the most fundamental principles of auditory function. While gradients in various morphological and physiological characteristics of the cochlea have been reported, little information is available on gradient patterns of gene expression. In addition, the audiograms in autosomal dominant non syndromic hearing loss can be distinctive, however, the mechanism that accounts for that has not been clarified. We thought that it is possible that tonotopic gradients of gene expression within the cochlea account for the distinct audiograms.

Methodology/Principal Findings: We compared expression profiles of genes in the cochlea between the apical, middle, and basal turns of the mouse cochlea by microarray technology and quantitative RT-PCR. Of 24,547 genes, 783 annotated genes expressed more than 2-fold. The most remarkable finding was a gradient of gene expression changes in four genes (*Pou4f3*, *Slc17a8*, *Tmc1*, and *Crym*) whose mutations cause autosomal dominant deafness. Expression of these genes was greater in the apex than in the base. Interestingly, expression of the *Emilin-2* and *Tectb* genes, which may have crucial roles in the cochlea, was also greater in the apex than in the base.

Conclusions/Significance: This study provides baseline data of gradient gene expression in the cochlea. Especially for genes whose mutations cause autosomal dominant non syndromic hearing loss (*Pou4f3*, *Slc17a8*, *Tmc1*, and *Crym*) as well as genes important for cochlear function (*Emilin-2* and *Tectb*), gradual expression changes may help to explain the various pathological conditions.

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The auditory systems of mammalians that perceive sounds are organized based on the separation of complex sounds into their component frequencies (tonotopy). Tonotopy begins at the level of the auditory sensory epithelium where specific frequencies are distributed along the tonotopic axis of the mammalian cochlea [1].

Gradients in morphological and physiological characteristics of the inner ear in different species have also been reported [1]. In addition, gene expression gradients along the tonotopic axis in chicken auditory epithelium have been also reported [2]. However, few reports are available on mammalian gene expression gradients.

Hearing loss that disturbs normal communication is a common sensory disorder worldwide. Most congenital or childhood onset hearing impairments are non syndromic. As of Apr. 2013, 27 dominant, 40 recessive and 3 X-linked genes whose mutations cause non syndromic hearing loss have been reported according to the Hereditary Hearing loss Homepage (http://hereditaryhearingloss.org/).

Interestingly, the audiograms of autosomal dominant non syndromic hearing loss (ADNSHL) can be distinctive, and thus useful to identify the gene responsible [3]. For example, mutations in *WFS1* are found in 75% of families with dominantly inherited hearing loss that initially affects the low frequencies while sparing the high frequencies [4,5]. On the contrary, many of the mutations in ADNSHL, like *KCNQ4*, *DFNA5*, *POU4F3*, and *SLC17A8*, affect the high frequencies [6]. However, the mechanism that accounts for the distinct frequency patterns has not been clarified. We hypothesized that certain gene expression patterns might show a gradient within the cochlea that could, at least in part, correspond with the distinct shapes of audiograms in ADNSHL.

Microarray analysis, which provides whole gene expression data, can be used to analyze differential gene expression among tissues [7]. In this study, to analyze the mechanism of the distinct audiograms in ADNSHL, we examined and compared gradient gene expression profiles, in particular ADNSHL genes, between the apical, middle, and basal turns of the cochlea by microarray technology.

Materials and Methods

Tissue dissection and RNA extraction

Four C57BL/6 mice aged 6 weeks were euthanized by decapitation under deep anesthesia induced by an intraperitoneal injection containing 75 mg/kg Ketamine (Daiichi Sankyo, Tokyo, Japan) and 32.4 mg/kg Pentobarbital Sodium (Kyoritsu, Tokyo, Japan). Inner ears were rapidly extracted from the temporal bone and transferred into RNA*later* solution (Ambion, Austin, TX, USA). After removing the otic capsule, the cochlea including the lateral wall comprising the stria vascularis, spiral ligament, and spiral prominence, the organ of Corti and the spiral ganglion neurons were dissected and separated into the apical, middle and basal turns (Fig. 1). All of these dissections were performed in RNA*later* solution to prevent RNA degradation. Total RNA was were extracted using the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The quality of the extracted total RNA was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and found to be adequate for microarray analysis (data not shown).

RNA labeling and purification

Total RNA (25 ng each) was reverse transcribed with the Low Input Quick Amp Whole Transcriptome Labeling Kit (Agilent Technologies). After reverse transcription process, labeled cRNA was synthesized from cDNA by using T7 RNA polymerase mix and cyanine 3-CTP according to the manufacturer's instructions. Labeled cRNA was purified using the Rneasy Mini kit (QIAGEN).

Microarray hybridization

To analyze gene expression of each cochlea turn, 12 SurePrint G3 Mouse Exon Microarrays (Agilent Technologies), which were spotted with 165,984 exon probes (24,547 genes), were hybridized to labeled cRNA (4 microarrays were used for each turn sample). Prior to the hybridization step, Cyanine 3-labeled cRNAs were fragmented using 25X fragmentation buffer at 60°C in a water bath for 30 min and then hybridized to a microarray slide for 17 hours at 65°C in a hybridization oven and washed using Gene Expression Wash Buffer (Agilent Technologies).

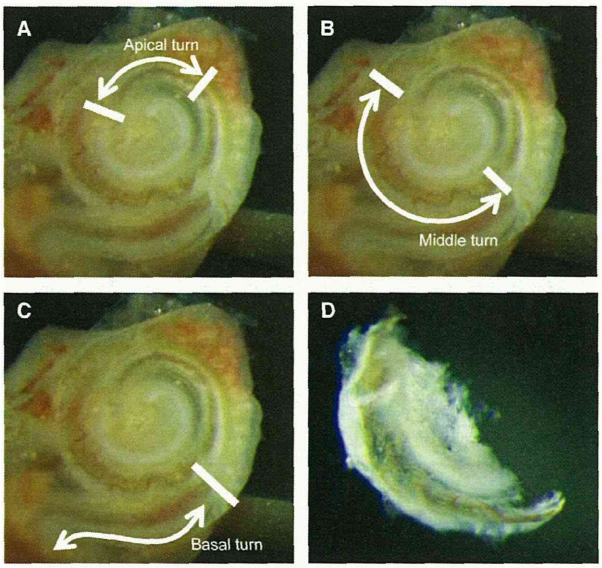

Figure 1. Microscopical image of the mouse cochlea (right ear). Bars indicate the incision points for each turn sample. A: apical turn, B: middle turn, C: basal turn, D: dissection example
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Microarray scanning and statistical analysis

Fluorescence intensities were measured with the Agilent Microarray Scanner (Agilent) using the scanning protocols specific for each microarray assay and raw microarray image files were created. The expression data were extracted from raw microarray image files using Agilent Feature Extraction Image Analysis Software (Version 10.7.3.1). The software also generated quality control reports using the protocol specific for the microarray assays as well as data files for analysis with GeneSpring GX (Version 11, Agilent Technologies). Signal intensities for each probe were normalized to the 75th percentile without baseline transformation.

Data for each microarray was analyzed using the manufacturer's workflow in GeneSpring GX. For gene-level analysis, the average expression levels of each exon probe were used. Then averages of four microarray data of each cochlea turn (base, middle, and apex) were used for comparison analysis (one-way analysis of variance (ANOVA)) by using GeneSpring GX. The microarray data have been lodged in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) as accession number: GSE53863.

Quantitative RT-PCR

To confirm the microarray analysis results, qPCR was performed on 9 deafness genes. Reverse transcription was performed with 4 total RNA samples of each cochlea turn by using High Capacity RNA-to-cDNA Kit (Life Technologies, Foster City, CA, USA) as described in the manufacturer's procedure. The TaqMan probe for each gene was selected from the TaqMan Gene Expression Assay system (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?;cmd = ABGEKeywordSearch,Life Technologies). *Gapdh*, *Actb*, *Rps17*, *Rpl30*, *Atp6*, and *Ipo8* were chosen as internal control genes. The estimated gene expression level (EL) was normalized to the internal control gene expression level and data are presented as the mean of $\log_2$EL.

Ethics Statement

All experimental procedures were performed in accordance with the regulations for animal experimentation of Shinshu University. These experiments were approved by Shinshu University institutional animal care and use committee.

Results

Scatter plot analysis

To confirm the technical stability of cochlear dissection and RNA extraction and to estimate global gene expression change, we performed scatter plot analysis of the gene expression profiles of each cochlear turn. In each comparison of basal, middle and apical cochlear turns, the gene expression patterns were quite similar and most gene expression changes were less than 2-fold (Fig. 2).

In detail, the gene expression profile of the apical turn of the cochlea was more similar to that of the middle turn than the basal turn. The scatter plot of the apical turn vs. basal turn showed lower correlation than the others. From these results, the gene expression of each cochlear turn clearly indicated gradual gene expression change according to the tonotopic axis.

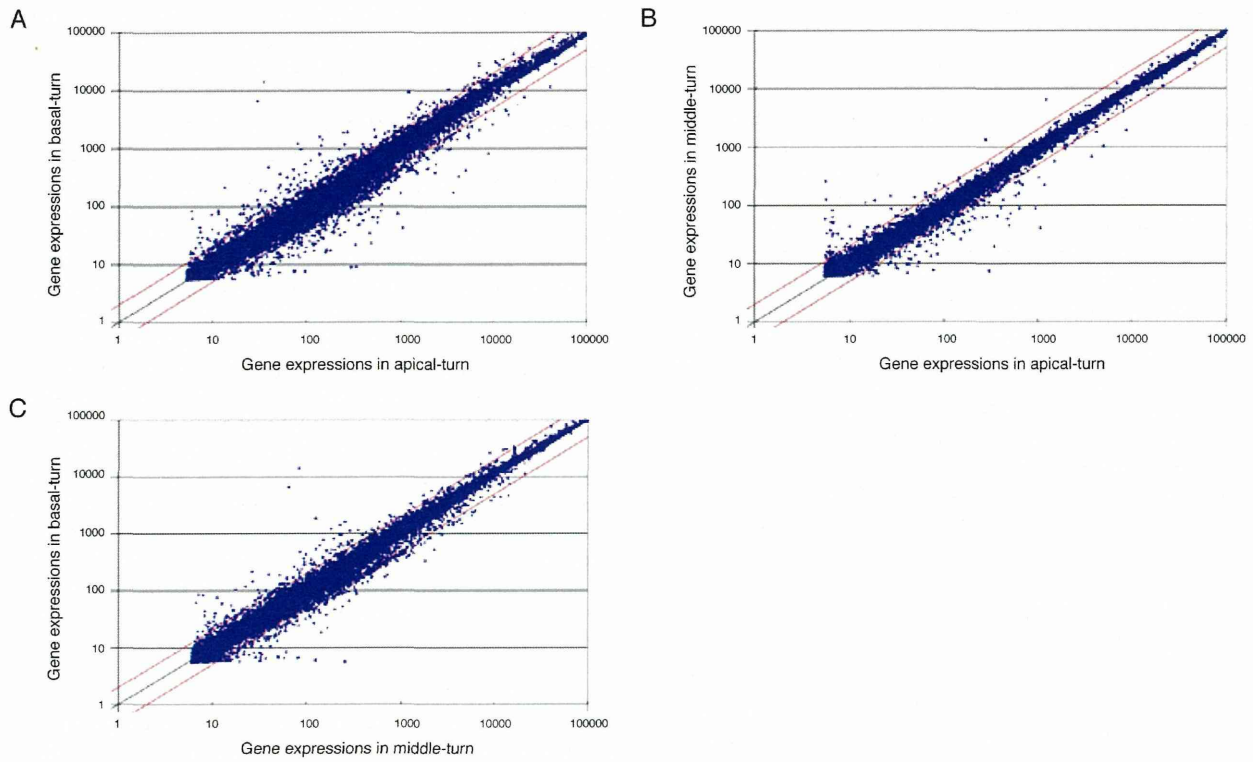

Figure 2. Scatter plot analysis of gene expression profile of each cochlear turn. Black lines indicate equal gene expression and red lines indicate 2-fold gene expression. A: apical turn vs. basal turn, B: apical turn vs. middle turn, C: middle turn vs. basal turn.
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Genes indicate differential expression in each cochlear turn

To analyze difference in gene expression, we focused on the genes, which were expressed 2-fold or more in one turn than in the other turn. Each of the gene expression levels was estimated from the average value of four microarray results for independent mouse samples and one-way ANOVA was employed before the comparative analysis as written in the material and methods. Of 24,547 genes, 941 differed more than 2-fold. Of these genes, 783 genes (3.2%: 783/24547) had been annotated and the others were predicted genes (Table S1).

Out of the 783 annotated genes, 747 were differentially expressed between apex and base; 51 were differentially expressed between apex and middle, and 458 genes were differentially expressed between middle and base (some genes were in more than one group) (Table 1). This is consistent with the notion that those genes whose expression changes showed an apical-to-basal gradient along the tonotopic axis. The complete list of differentially expressed genes for each of the three comparisons (i.e., apex vs. base, apex vs. middle, middle vs. base) is indicated as a supporting information file (Tables S2, S3, and S4).

Overall tonotopic expression pattern

Most (96.2%) genes expression changes did not differ and only a limited number of genes showed tonotopic expression pattern.

Tonotopic expression of the genes responsible for hearing loss

The results of gene expression analysis of each cochlear turn showed that 4 ADNSHL genes (*Pou4f3*, *Slc17a8*, *Tmc1*, and *Crym*) and 9 autosomal recessive non syndromic hearing loss genes (*Otof*, *Strc*, *Ush1c*, *Pcdh15*, *Grxcr1*, *Dfnb59*, *Slc26a5*, *Lhfpl5*, and *Ptprq*) were changed 2-fold or more (Table 2). Interestingly, expression of

Table 1. The numbers of differentially expressed genes for apex vs. base, apex vs. middle, middle vs. base.

apex vs. base		apex vs. middle		middle vs. base	
up in apex	up in base	up in apex	up in middle	up in middle	up in base
571/783	176/783	38/783	13/783	389/783	69/783
747/783		51/783		458/783	

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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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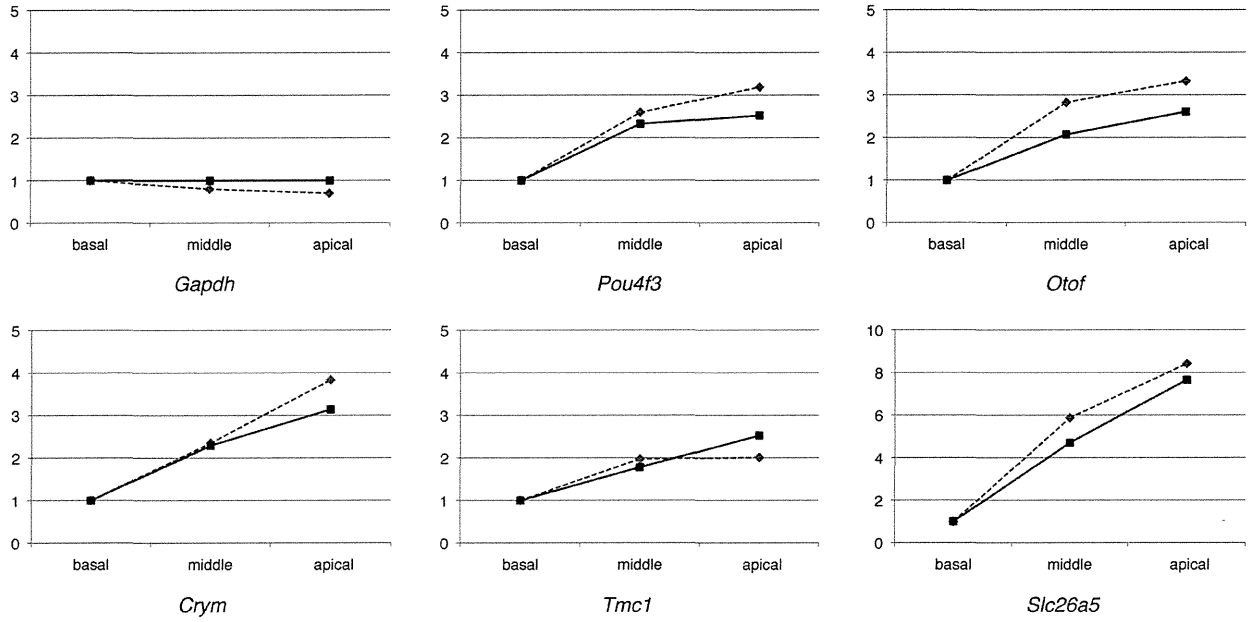

Figure 3. Gene expression patterns found by microarray analysis and quantitative RT-PCR. Values of each gene expression are indicated as a relative value to the basal turn. The expression level of each gene measured by microarray analysis (solid lines) was comparable with the level measured by quantitative 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.

References

1. Mann ZF, Kelley MW (2011) Development of tonotopy in the auditory periphery. Hear Res 276: 2–15.
2. Frucht CS, Uduman M, Kleinstein SH, Santos-Sacchi J, Navaratnam DS (2011) Gene expression gradients along the tonotopic axis of the chicken auditory epithelium. J Assoc Res Otolaryngol 412: 23–35.
3. Hildebrand MS, Tack D, McMordie SJ, DeLuca A, Hur IA, et al. (2008) Audioprofile-directed screening identifies novel mutations in KCNQ4 causing hearing loss at the DFNA2 locus. Genet Med 10: 797–804.
4. Fukuoka H, Kanda Y, Ohta S, Usami S (2007) Mutations in the WFS1 gene are a frequent cause of autosomal dominant nonsyndromic low-frequency hearing loss in Japanese. J Hum Genet 52: 510–515.
5. Cryns K, Sivakumaran TA, Van den Ouewland JM, Pennings RJ, Cremers CW, et al. (2003) Mutational spectrum of the WFS1 gene in Wolfram syndrome, nonsyndromic hearing impairment, diabetes mellitus and psychiatric disease. Hum Mut 22: 275–287.
6. Smith RJH, Hildebrand MS, Van Camp G (1999) Deafness and Hereditary Hearing Loss Overview. GeneReviews. Available: http://www.ncbi.nlm.nih.gov/books/NBK 1434/#deafness-overview.REF.cryns.2003.275. Accessed 10 Apr 2013.
7. Morris KA, Snir E, Pompeia C, Koroleva IV, Kachar B, et al. (2005) Differential expression of genes within the cochlea as defined by a custom mouse inner ear microarray. J Assoc Res Otolaryngol 6: 75–89.
8. Sato T, Doi K, Hibino H, Kubo T (2009) Analysis of gene expression profiles along the tonotopic map of mouse cochlea by cDNA microarrays. Acta Otolaryngol Suppl 129(Supple 562): 12–17.
9. Bosl WJ, Li R (2010) The role of noise and positive feedback in the onset of autosomal dominant diseases. BMC Syst Biol 4: e93.
10. Cook DL, Gerber AN, Tapscott SJ (1998) Modeling stochastic gene expression: implications for haploinsufficiency. Proc Natl Acad Sci U S A 95: 15641–15646.
11. Lee HK, Park HJ, Lee KY, Park R, Kim UK. (2010) A novel frameshift mutation of POU4F3 gene associated with autosomal dominant non-syndromic hearing loss. Biochem Biophys Res Commun 396: 626–630.
12. de Heer AM, Huygen PL, Collin RW, Kremer H, Cremers CW. (2009) Mild and variable audiometric and vestibular features in a third DFNA15 family with a novel mutation in POU4F3. Ann Otol Rhinol Laryngol 118: 313–320.
13. Ruel J, Emery S, Nouvian R, Bersot T, Amilhon B, et al. (2008) Impairment of SLC17A8 encoding vesicular glutamate transporter-3, VGLUT3, underlies nonsyndromic deafness DFNA25 and inner hair cell dysfunction in null mice. Am J Hum Genet 83: 278–292.
14. Kurima K, Peters LM, Yang Y, Riazuddin S, Ahmed ZM, et al. (2002) Dominant and recessive deafness caused by mutations of a novel gene, TMC1, required for cochlear hair-cell function. Nat Genet 30: 277–284.
15. Hilgert N, Alasti F, Dieltjens N, Pawlik B, Wollnik B, et al. (2008) Mutation analysis of TMC1 identifies four new mutations and suggests an additional deafness gene at loci DFNA36 and DFNB7/11. Clin Genet 74: 223–232.
16. Abe S, Katagiri T, Saito-Hisaminato A, Usami S, Inoue Y, et al. (2003) Identification of CRYM as a candidate responsible for nonsyndromic deafness, through cDNA microarray analysis of human cochlear and vestibular tissues. Am J Hum Genet 72: 73–82.
17. Amma LL, Goodyear R, Faris JS, Jones I, Ng L, et al. (2003) An emilin family extracellular matrix protein identified in the cochlear basilar membrane. Mol Cell Neurosci 23: 460–472.
18. Ghaffari R, Aranyosi AJ, Richardson GP, Freeman DM (2010) Tectorial membrane travelling waves underlie abnormal hearing in Tectb mutant mice. Nat Commun 1: e96
19. Russell IJ, Legan PK, Lukashkina VA, Lukashkin AN, Goodyear RJ, et al. (2007) Sharpened cochlear tuning in a mouse with a genetically modified tectorial membrane. Nat Neurosci 10: 215–223.

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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					
				Total	Cohort 1	Cohort 2	Control	Hearing	Progression of hearing			Associated	
Allele	*Locus*	*Status*[a]	*Disease*	*(/394)*	*(/254)*	*(/140)*	*(/96)*	*characteristics*	*loss*	*Tinnitus*	*Vertigo*	*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

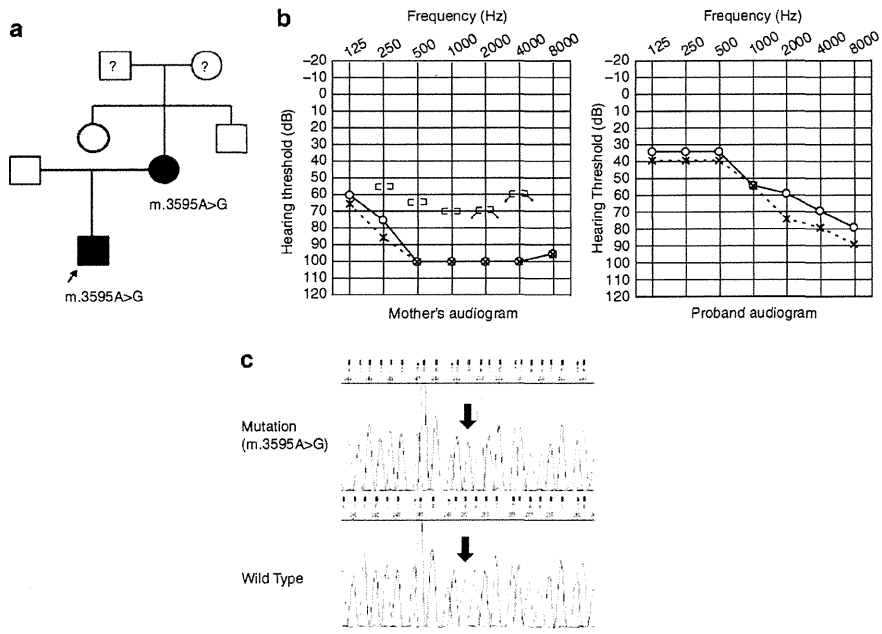

Figure 1 Clinical features of the proband carrying the homoplasmic 3595A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiograms of the proband and mother. (**c**) Electropherogram depicting the 3595A>G sequence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

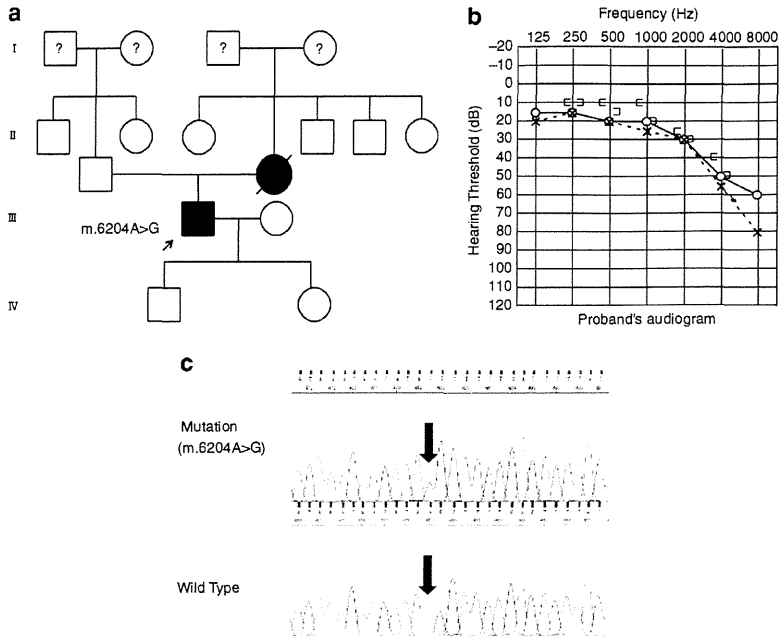

Figure 2 Clinical features of the proband carrying the homoplasmic 6204A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiogram of the proband. (**c**) Electropherogram depicting the 6204A>G sequence and its 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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1 Jacobs, H. T., Hutchin, T. P., Kappi, T., Gillies, G., Minkkinen, K., Walker, J. *et al.* Mitochondrial DNA mutations in patients with postlingual, nonsyndromic hearing impairment. *Eur. J. Hum. Genet.* **13,** 26–33 (2005).
2 Prezant, T. R., Agapian, J. V., Bohlman, M. C., Bu, X., Oztas, S., Qiu, W. Q. *et al.* Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness. *Nat. Genet.* **4,** 289–294 (1993).
3 Fischel-Ghodsian, N., Prezant, T. R., Bu, X. & Oztas, S. Mitochondrial ribosomal RNA gene mutation in a patient with sporadic aminoglycoside ototoxicity. *Am. J. Otolaryngol.* **14,** 399–403 (1993).
4 Usami, S., Abe, S., Akita, J., Namba, A., Shinkawa, H., Ishii, M. *et al.* Prevalence of mitochondrial gene mutations among hearing impaired patients. *J. Med. Genet.* **37,** 38–40 (2000).
5 Zhao, H., Li, R., Wang, Q., Yan, Q., Deng, J. H., Han, D. *et al.* Maternally inherited aminoglycoside-induced and nonsyndromic deafness is associated with the novel C1494T mutation in the mitochondrial 12S rRNA gene in a large Chinese family. *Am. J. Hum. Genet.* **74,** 139–152 (2004).
6 van den Ouweland, J. M., Lemkes, H. H. P., Ruitenbeek, W., Sandkjujl, L. A., deVijlder, M. F., Struyvenberg, P. A. *et al.* Mutation in mitochondrial tRNALeu(UUR) gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness. *Nat. Genet.* **1,** 368–371 (1992).
7 Reid, F. M., Vernham, G. A. & Jacobs, H. T. A novel mitochondrial point mutation in a maternal pedigree with sensorineural deafness. *Hum. Mutat.* **3,** 243–247 (1994).
8 Fischel-Ghodsian, N., Prezant, T. R., Fournier, P., Stewart, I. A. & Maw, M. Mitochondrial mutation associated with nonsyndromic deafness. *Am. J. Otolaryngol.* **16,** 403–408 (1995).
9 Hutchin, T. P., Parker, M. J., Young, I. D., Davis, A. C., Pulleyn, L. J. & Deeble, J. A novel mutation in the mitochondrial tRNA(Ser(UCN)) gene in a family with non-syndromic sensorineural hearing impairment. *J. Med. Genet.* **37,** 692–694 (2000).
10 Li, R., Greinwald, J. H. Jr., Yang, L., Choo, D. I., Wenstrup, R. J., Guan, M. X. *et al.* Molecular analysis of the mitochondrial 12S rRNA and tRNASer(UCN) genes in paediatric subjects with non-syndromic hearing loss. *J. Med. Genet.* **41,** 615–620 (2004).
11 Thyagarajan, D., Bressman, S., Bruno, C., Przedborski, S. & Shanske, S. A novel mitochondrial 12SrRNA point mutation in parkinsonism, deafness, and neuropathy. *Ann. Neurol.* **48,** 730–736 (2000).
12 Mutai, H., Kouike, H., Teruya, E., Takahashi-Kodomari, I., Kakishima, H., Taiji, H. *et al.* Systematic analysis of mitochondrial genes associated with hearing loss in the Japanese population: dHPLC reveals a new candidate mutation. *BMC. Med. Genet.* **12,** 135 (2011).
13 Bonneux, S., Fransen, E., Van Eyken, E., Van Laer, L., Huyghe, J., Van de Heyning, P. *et al.* Inherited mitochondrial variants are not a major cause of age-related hearing impairment in the European population. *Mitochondrion* **11,** 729–734 (2011).
14 Zaragoza, M. V., Brandon, M. C., Diegoli, M., Arbustini, E. & Wallace, D. C. Mitochondrial cardiomyopathies: how to identify candidate pathogenic mutations by mitochondrial DNA sequencing, MITOMASTER and phylogeny. *Eur. J. Hum. Genet.* **19,** 200–207 (2011).
15 Leveque, M., Marlin, S., Jonard, L., Procaccio, V., Reynier, P., Amati-Bonneau, P. *et al.* Whole mitochondrial genome screening in maternally inherited non-syndromic hearing impairment using a microarray resequencing mitochondrial DNA chip. *Eur. J. Hum. Genet.* **15,** 1145–1155 (2007).
16 Li, X., Fischel-Ghodsian, N., Schwartz, F., Yan, Q., Friedman, R. A. & Guan, M. X. Biochemical characterization of the mitochondrial tRNASer(UCN) T7511C mutation associated with nonsyndromic deafness. *Nucleic Acids. Res.* **32,** 867–877 (2004).
17 Chamkha, I., Mkaouar-Rebai, E., Aloulou, H., Chabchoub, I., Kifagi, C., Fendri-Kriaa, N. *et al.* A novel m.3395A>G missense mutation in the mitochondrial ND1 gene associated with the new tRNA(Ile) m.4316A>G mutation in a patient with hypertrophic cardiomyopathy and profound hearing loss. *Biochem. Biophys. Res. Commun.* **404,** 504–510 (2011).
18 Mkaouar-Rebai, E., Tlili, A., Masmoudi, S., Belguith, N., Charfeddine, I., Mnif, M. *et al.* Mutational analysis of the mitochondrial tRNALeu(UUR) gene in Tunisian patients with mitochondrial diseases. *Biochem. Biophys. Res. Commun.* **355,** 1031–1037 (2007).
19 Chen, F. L., Liu, Y., Song, X. Y., Hu, H. Y., Xu, H. B., Zhang, X. M. *et al.* A novel mitochondrial DNA missense mutation at G3421A in a family with maternally inherited diabetes and deafness. *Mutat. Res.* **602,** 26–33 (2006).
20 Pandya, A., Xia, X. J., Erdenetungalag, R., Amendola, M., Landa, B., Radnaabazar, J. *et al.* Heterogenous point mutations in the mitochondrial tRNASer(UCN) precursor coexisting with the A1555G mutation in deaf students from Mongolia. *Am. J. Hum. Genet.* **65,** 1803–1806 (1999).
21 Brown, M. D., Voljavec, A. S., Lott, M. T., Torroni, A., Yang, C. C. & Wallace, D. C. Mitochondrial DNA complex I and III mutations associated with Leber's hereditary optic neuropathy. *Genetics* **130,** 163–173 (1992).
22 Torroni, A., Campos, Y., Rengo, C., Sellitto, D., Achilli, A., Magri, C. *et al.* Mitochondrial DNA haplogroups do not play a role in the variable phenotypic presentation of the A3243G mutation. *Am. J. Hum. Genet.* **72,** 1005–1012 (2003).
23 Sue, C. M., Tanji, K., Hadjigeorgiou, G., Andreu, A. L., Nishino, I., Krishna, S. *et al.* Maternally inherited hearing loss in a large kindred with a novel T7511C mutation in the mitochondrial DNA tRNA(Ser(UCN)) gene. *Neurology* **52,** 1905–1908 (1999).
24 Lu, J., Li, Z., Zhu, Y., Yang, A., Li, R., Zheng, J. *et al.* Mitochondrial 12S rRNA variants in 1642 Han Chinese pediatric subjects with aminoglycoside-induced and nonsyndromic hearing loss. *Mitochondrion.* **10,** 380–390 (2010).
25 Sawano, T., Tanaka, M., Ohno, K., Yoneda, M., Ota, Y., Terasaki, H. *et al.* Mitochondrial DNA mutations associated with the 11778 mutation in Leber's disease. *Biochem. Mol. Bio. Int.* **38,** 693–700 (1996).
26 Li, Z., Li, R., Chen, J., Liao, Z., Zhu, Y., Qian, Y. *et al.* Mutational analysis of the mitochondrial 12S rRNA gene in Chinese pediatric subjects with aminoglycoside-induced and non-syndromic hearing loss. *Hum. Genet.* **117,** 9–15 (2005).
27 Jaksch, M., Hofmann, S., Kaufhold, P., Obermaier-Kusser, B., Zierz, S. & Gerbitz, K. D. A novel combination of mitochondrial tRNA and ND1 gene mutations in a syndrome with MELAS, cardiomyopathy, and diabetes mellitus. *Hum. Mutat.* **7,** 358–360 (1996).
28 Chen, F. L., Liu, Y., Song, X. Y., Hu, H. Y., Xu, H. B., Zhang, X. M. *et al.* A novel mitochondrial DNA missense mutation at G3421A in a family with maternally inherited diabetes and deafness. *Mutat. Res.* **602,** 26–33 (2006).

29 Spagnolo, M., Tomelleri, G., Vattemi, G., Filosto, M., Rizzuto, N. & Tonin, P. A new mutation in the mitochondrial tRNA(Ala) gene in a patient with ophthalmoplegia and dysphagia. *Neuromuscul. Disord.* **11**, 481–484 (2001).
30 Wang, Q., Li, R., Zhao, H., Peters, J. L., Liu, Q., Yang, L. *et al.* Clinical and molecular characterization of a Chinese patient with auditory neuropathy associated with mitochondrial 12S rRNA T1095C mutation. *Am. J. Med. Genet. A* **133A**, 27–30 (2005).
31 Terasaki, F., Tanaka, M., Kawamura, K., Kanzaki, Y., Okabe, M., Hayashi, T. *et al.* A case of cardiomyopathy showing progression from the hypertrophic to the dilated form: association of Mt8348A–>G mutation in the mitochondrial tRNA(Lys) gene with severe ultrastructural alterations of mitochondria in cardiomyocytes. *Jpn. Circ. J.* **65**, 691–694 (2001).
32 De Vries, D. D., Went, L. N., Bruyn, G. W., Scholte, H. R., Hofstra, R. M., Bolhuis, P. A. *et al.* Genetic and biochemical impairment of mitochondrial complex I activity in a family with Leber hereditary optic neuropathy and hereditary spastic dystonia. *Am. J. Hum. Genet.* **58**, 703–711 (1996).
33 Tzen, C. Y., Thajeb, P., Wu, T. Y. & Chen, S. C. Melas with point mutations involving tRNALeu (A3243G) and tRNAGlu(A14693g). *Muscle Nerve.* **28**, 575–581 (2003).
34 Chen, B., Sun, D., Yang, L., Zhang, C., Yang, A., Zhu, Y. *et al.* Mitochondrial ND5 T12338C, tRNA (Cys) T5802C, and tRNA (Thr) G15927A variants may have a modifying role in the phenotypic manifestation of deafness-associated 12S rRNA A1555G mutation in three Han Chinese pedigrees. *Am. J. Med. Genet. A* **146A**, 1248–1258 (2008).