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G. 知的所有権の取得状況

1.特許取得

なし

2.実用新案登録

なし

3.その他

なし

Ⅲ.　研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻	ページ		出版年
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	Spectrum of Patients in Japan.					
Miyagawa M, Nishio S, Ichinose A, Iwasaki S, Murata T, Kitajiri S, Usami S.	Mutational Spectrum and Clinical Features of patients with *ACTG1* Mutations identified by Massively Parallel DNA Sequencing.	Ann Otol Rhinol Laryngol	124	84	93	2015
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Miyagawa M, Nishio S, Hattori M, Moteki H, Kobayashi Y, Saton H, Watanabe T, Naito Y, Oshikawa C, Usami S.	Mutations in the *MYO15A* Gene are a Significant Cause of Nonsyndromic Hearing Loss: Massively Parallel DNA Sequencing-Based Analysis.	Ann Otol Rhinol Laryngol	124	158	168	2015
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Ⅳ.　研究成果の刊行物

OPEN

Journal of Human Genetics (2014), 1–7

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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.
Journal of Human Genetics advance online publication, 9 January 2014; doi:10.1038/jhg.2013.128

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 characteristics	Progression of hearing loss	Associated			
Allele	Locus	Status[a]	Disease	(/394)	(/254)	(/140)	(/96)			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

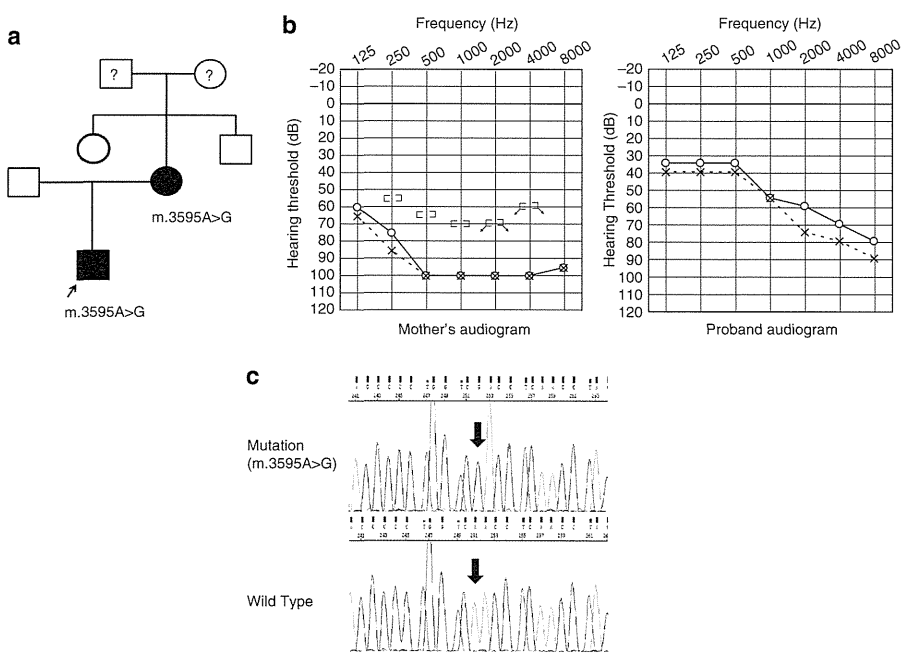

Figure 1 Clinical features of the proband carrying the homoplasmic 3595A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiograms of the proband and mother. (**c**) Electropherogram depicting the 3595A>G sequence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

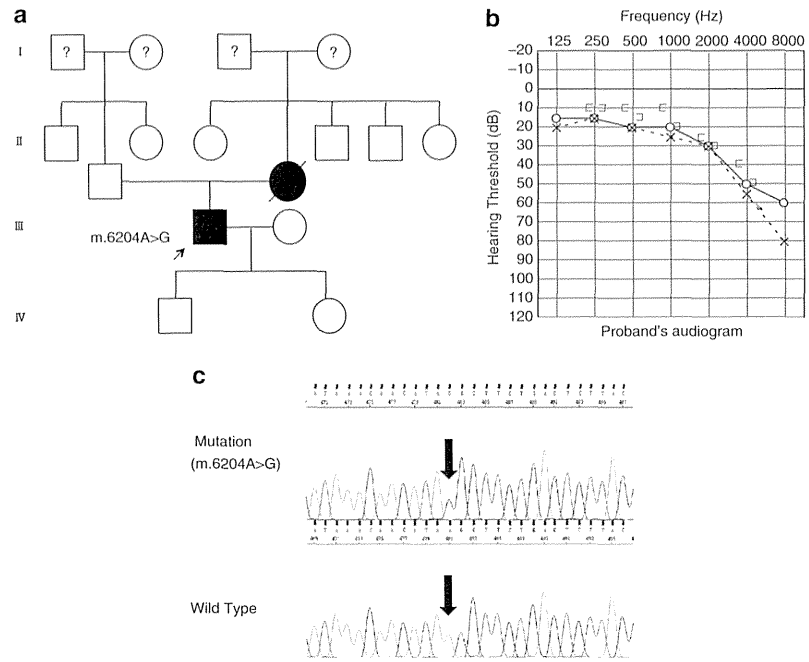

Figure 2 Clinical features of the proband carrying the homoplasmic 6204A>G variant. (**a**) Family pedigree. Individuals with hearing loss are indicated by filled symbols. The arrow indicates the proband. (**b**) Audiogram of the proband. (**c**) Electropherogram depicting the 6204A>G sequence and its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

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

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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.