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ORIGINAL ARTICLE

Extended screening for major mitochondrial DNA point mutations in patients with hereditary hearing loss

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Hearing loss (HL) is the most common sensory disorder in humans. Many patients with mitochondrial diseases have sensorineural HL (SNHL). The HL of these patients manifests as a consequence of either syndromic or nonsyndromic mitochondrial diseases. Furthermore, the phenotypes vary among patients even if they are carrying the same mutation. Therefore, these features make it necessary to analyze every presumed mutation in patients with hereditary HL, but the extensive analysis of various mutations is laborious. We analyzed 373 patients with suspected hereditary HL by using an extended suspension-array screening system for major mitochondrial DNA (mtDNA) mutations, which can detect 32 other mtDNA mutations in addition to the previously analyzed 29 mutations. In the present study, we detected 2 different mtDNA mutations among these 373 patients; m.7444G>A in the *MT-CO1* gene and m.7472insC in the *MT-TS1* gene in 1 patient (0.3%) for each. As these two patients had no clinical features other than HL, they had not been suspected of having mtDNA mutations. This extended screening system together with the previous one is useful for the genetic diagnosis and epidemiological study of both syndromic and nonsyndromic HL.
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INTRODUCTION

We can find many patients with hearing loss (HL) among those with mitochondrial DNA (mtDNA) mutations. These patients are classified into two categories; those having only HL (nonsyndromic HL) and those with HL plus other symptoms of mitochondrial disease (syndromic HL). Furthermore, as the severities and phenotypes of mitochondrial diseases vary from patient to patient, we often find mtDNA mutations in unexpected cases.[1] The fact that there are many cases without any apparent family history makes it more difficult to diagnose mitochondrial diseases.[2] Sensorineural HL (SNHL) is the most common sensory disorder in humans, having a prevalence of 2.7 per 1000 in children under 5 years of age.[3] The frequency of patients with HL caused by mtDNA mutations increases with age, because mitochondrial diseases usually become aggravated with age. Therefore, it is necessary to analyze many different suspected mutations in mtDNA, but it is very exhaustive to examine these muations one by one.

Previously, we reported the results of extensive and rapid screening for major 29 major point mutations of mtDNA in patients with hereditary HL by using a suspension array technology.[4] Our previous survey of 373 patients with suspected HL by use of this screening system revealed the m.1555A>G mutation in 11 patients, the m.3243A>G mutation in 9 patients, and the m.8348A>G, m.11778G>A and m.15498G>A mutations in 1 patient each. In the present extended study, we increased the number of mutations that could be detected from 29 to 61. We examined the applicability of this extended screening system for genetic diagnosis of hereditary HL by analyzing these same 373 patients with suspected hereditary HL.

MATERIALS AND METHODS

Patients

The study population included 373 unrelated Japanese patients with suspected hereditary HL, who visited the outpatient clinic of the Department of Otolaryngology, University Hospital of Medicine, Tokyo Medical and Dental University. The subjects included patients with a family history of HL and those with no apparent cause of HL, even though they did not have any apparent family history of HL. Their detailed demographic and audiometric features are shown in Table 1. The average age of them was 40 years, with a range between 1 and 77 years.

The study protocol complied with the Declaration of Helsinki, and it was also approved by the Committee on the Ethics of Human Research of the Tokyo Metropolitan Institute of Gerontology and the Institutional Review Board (IRB no. 68) of Tokyo Medical and Dental University. This study was carried out only after obtaining the written informed consent of each individual and/or the parents in the case of children.

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Table 1 Demographic features of HL patients

Sex	
Male (%)	144 (38.6)
Female (%)	229 (61.4)
Onset age of HL (years)	
Newborn or 0 (%)	31 (8.3)
1∼3 (%)	23 (6.2)
4∼10 (%)	80 (21.4)
11∼20 (%)	43 (11.5)
21∼30 (%)	39 (10.5)
31∼40 (%)	50 (13.4)
41∼50 (%)	37 (9.9)
51∼60 (%)	31 (8.3)
61∼70 (%)	12 (3.2)
71∼80 (%)	5 (1.3)
Unknown (%)	22 (5.9)
Mode of inheritance	
Autosomal dominant (%)	92 (24.7)
Autosomal recessive (%)	52 (13.9)
Maternal (%)	47 (12.6)
X-linked (%)	0
Sporadic (%)	179 (48.0)
Unknown (%)	3 (0.8)
Type of audiogram	
High-frequency steeply sloping (%)	80 (21.4)
High-frequency gently sloping (%)	104 (27.9)
Flat (%)	39 (10.5)
U-shaped (Cookiebite) (%)	39 (10.5)
Reverse U-shaped (%)	4 (1.1)
Low frequency (%)	39 (10.5)
Deafness (%)	21 (5.6)
Others (%)	43 (11.5)
Unknown (%)	4 (1.1)
Total (%)	373 (100)

Abbreviation: HL, hearing loss.

Extended screening of mtDNA pathological mutation by use of suspension-array technology

DNA samples were purified from the blood by using a standard procedure. The mtDNA from each patient was analyzed with the previously described extended suspension array-based screening system.[5] The targets of the present analysis were 32 mtDNA mutations in 15 genes: 1 in each of *MT-TQ* (*tRNA*^{Gln}), *MT-TW* (*tRNA*^{Trp}), *MT-TC* (*tRNA*^{Cys}), *MT-TH* (*tRNA*^{His}), *MT-TL2* (*tRNA*$^{Leu(CUN)}$), *MT-ND6* and *MT-CYB* genes; 2 in each of *MT-TN* (*tRNA*^{Asn}), *MT-ATP6*, *MT-TG* (*tRNA*^{Gly}) and *MT-TE* (*tRNA*^{Glu}) genes; 3 in the *MT-ND3* gene; 4 in the *MT-CO1* gene; and 5 in each of the *MT-TI*(*tRNA*^{Ile}) and *MT-TS1* (*tRNA*$^{Ser(UCN)}$) genes as shown in Table 2. The mtDNA mutations reported in our previous study were within the DNA fragments amplified by multiplex PCR for mtDNA haplotyping, which was mainly designed for anthropological purposes. For the present study, we newly designed a second multiplex PCR system to analyze the 32 additional mtDNA mutations (including m.7445, which is known to cause of HL).

Comparison of results between suspension array and direct DNA sequencing

DNA sequencing was carried out by using an Applied Biosystems 3130 × l Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and Sequencher version 4.2.2 (Gene Codes, Ann Arbor, MI, USA) to compare the sequences with the revised Cambridge reference sequence,[6,7] while following the standard procedure.[8,9]

Table 2 List of 32 mutations examined by use of the extended suspension array-based system for the detection of mtDNA mutation detection system

Nucleotide position (m)	*Nucleotide change*	*Amino acid change*	*Locus*	*Clinical phenotype*
4269	A>G		MT-TI	Encephalopathy/FICP
4295	A>G		MT-TI	MHCM
4298	G>A		MT-TI	CPEO/MS
4300[a]	A>G		MT-TI	MICM
4320	C>T		MT-TI	MHCM
4332	G>A		MT-TQ	MELAS/encephalopathy
5537	A>insT		MT-TW	MILS
5698	G>A		MT-TN	CPEO/MM
5703	G>A		MT-TN	CPEO/MM
5814	T>C		MT-TC	Encephalopathy
7443[b]	A>G	Ter-G	MT-CO1	DEAF
7444[b]	G>A	Ter-K	MT-CO1	LHON/SNHL/DEAF
7445[b]	A>C	Ter-S	MT-CO1	DEAF
7445[a]	A>G	Ter-Ter	MT-CO1	SNHL
7472[b]	C>insC		MT-TS1	PEM/AMDF
7497[a]	G>A		MT-TS1	MM/exercise intolerance
7510	T>C		MT-TS1	SNHL
7511[a]	T>C		MT-TS1	SNHL
7512[a]	T>C		MT-TS1	PEM/MERRF + MELAS
8993	T>C	L>P	MT-ATP6	NARP/MILS
8993	T>G	L>R	MT-ATP6	NARP/MILS
9997	T>C		MT-TG	MHCM
10010	T>C		MT-TG	PEM
10158[a]	T>C	S>P	MT-ND3	MILS
10191	T>C	S-P	MT-ND3	ESOC/Leigh-like disease/MILS
10197[a]	G>A	A-T	MT-ND3	MILS/dystonia/stroke
12147	G>A		MT-TH	MERRF + MELAS/cerebral edema
12297	T>C		MT-TL2	Dilated cardiomyopathy
14568[b]	C>T	G-S	MT-ND6	LHON
14709[a]	T>C		MT-TE	MM + DMDF/encephalomyopathy
14710	G>A		MT-TE	Encephalomyopathy + retinopathy
15243	G>A	G>E	MT-CYB	MHCM

Abbreviations: AMDF, ataxia, myoclonus and deafness; ATP6, ATP synthase F_0 subunit 6; CO1, cytochrome *c* oxidase subunit I; CPEO, chronic progressive external ophthalmoplegia; CYB, cytochrome *b*; DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DMDF, diabetes mellitus + deafness; ESOC, epilepsy, strokes, optic atrophy and cognitive decline; FICP, fatal infantile cardiomyopathy, plus a MELAS-associated cardiomyopathy; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibers; MHCM, maternally inherited hypertrophic cardiomyopathy; MICM maternally inherited cardiomyopathy; MILS, maternally inherited Leigh syndrome; MR, mental retardation; MS, multiple sclerosis; mtDNA, mitochondrial DNA; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; ND, NADH dehydrogenase subunit; SNHL, sensorineural hearing loss; PEM, progressive encephalomyopathy.
Abbreviations and information about mutations are annotated in the MITOMAP database.
[a]Mutation reported as both homoplasmic and heteroplasmic.
[b]Mutation reported as homoplasmic.

RESULTS AND DISCUSSION

In the present extended study, 2 of the 32 mtDNA mutations, m.7444G>A and m.7472insC, were detected by the screening system, each in 1 patient out of the 373 patients with SNHL. The median fluorescent intensities for the m.7444G>A mutation and the 7472insC mutation are displayed in scatter diagrams (Figure 1). When the median fluorescent intensity values for the wild-type signals were below the cut-off values, we regarded the mutations as homoplasmic. The m.7444G>A mutation was homoplasmic and the 7472insC mutation was heteroplasmic. On the basis of the

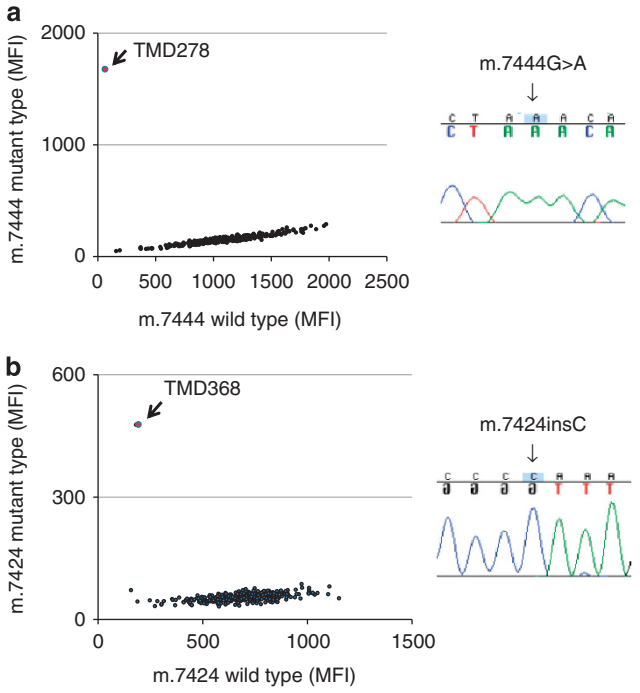

Figure 1 Scatter diagrams with mutant median fluorescent intensity values on the y axis and wild-type ones on the x axis and electropherograms of DNA sequences for the m.7444G>A homoplasmic mutation (**a**) and the m.7472insC heteroplasmic mutation (**b**). All 373 DNA samples were analyzed by the m.7444G>A and m.7472insC mutation detection systems, using universal 96-well plates. Later on, each result was merged into the two separate scatter diagrams. Red circles indicate median fluorescent intensity values for mutation-positive DNAs.

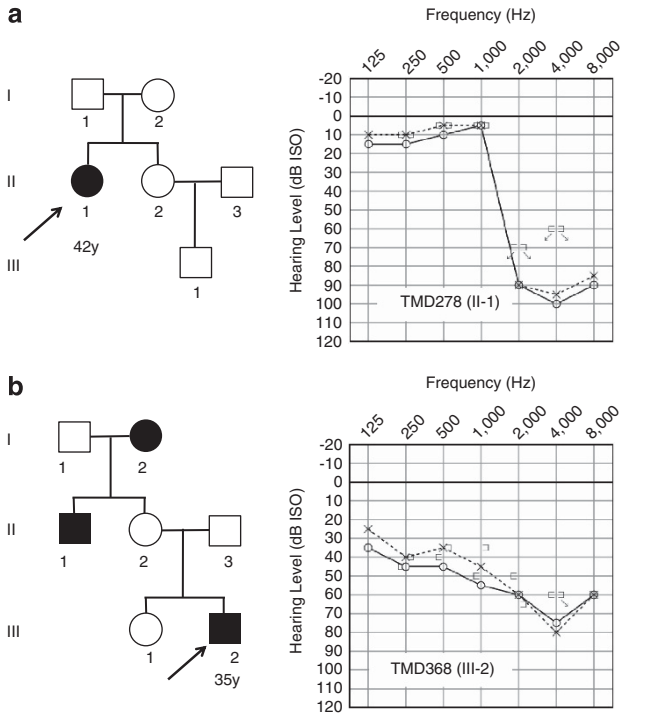

Figure 2 Pedigrees of the families and audiograms of patient TMD278 (**a**) and patient TMD368 (**b**). Clinical features are depicted: black-filled circles or squares as individuals with deafness. Arrows indicate probands. Symbols on pure tone audiograms: dB, decibels; ISO, international standards organization.], left-ear bone conduction; [, right-ear bone conduction; O, right-ear air conduction; X, left-ear air conduction.

Table 3 Mitochondrial DNA mutations detected in 373 patients with hereditary HL screened by the previous and present detection system

mtDNA mutation	*Number*	*Frequency (%)*
Previous study (Kato, 2010)		
m.1555A>G	11	2.9
m.3243A>G	9	2.4
m.8348A>G	1	0.3
m.11778G>A	1	0.3
m.15498G>A	1	0.3
Present study		
m.7444G>A	1	0.3
m.7472insC	1	0.3
Undetected	348	93.3
Total	373	100

Abbreviation: HL, hearing loss.

chromatogram, the mutation load was estimated as 59%. None of the other 30 mutations were detected in these patients with SNHL. We summarized the mtDNA mutations detected by the previous and the present analytical systems in Table 3.

SNHL is one of the most common disorder in patients with mitochondrial diseases,[10] which is represented by the mutations of the homoplasmic m.1555A>G and the heteroplasmic m.3243A>G.[11–16] We previously reported that not only these mutations but also other mutations could be detected in the patients with either nonsyndromic or syndromic hereditary HL. The m.7444G>A mutation was earlier reported as a cause of aminoglycoside-induced and nonsyndromic HL.[17] However, patient TMD278, carrying this mutation, had no history of aminoglycoside injection in her detailed clinical history. On the other hand, we should mention that this mutation characterizes haplogroup V7 and H40b, and there is no direct evidence that these haplogroups tend to have HL.[18] Furthermore, it was also reported that the m.7444G>A mutation is a secondary mutation found in patients with Leber's hereditary optic neuropathy (LHON) and that this mutation has an additional role in the pathogenesis of LHON.[19,20] Primary mutations, m.11778G>A, m.3460G>A and m.14484T>C can cause LHON. However, these mutations had already been examined in our previous study and the patient TMD278 was negative for them. With regard to her clinical data, she had high-tone SNHL as shown in Figure 2a, although she was 42 years of age. The onset of her HL occurred during her childhood, after which the HL became progressive. She had started wearing hearing aids 3 years before visiting our clinic. Her clinical feature seemed sporadic because she had neither other clinical disorders such as LHON nor a family history of HL.

The m.7472insC was reported as a pathogenic heteroplasmic mutation within the coding region of $tRNA^{Ser(UCN)}$. This mutation was previously reported to be associated with progressive myoclonus epilepsy or syndromic disorders including HL, ataxia and myoclonus in previous reports.[21] The phenotypes of this mutation, however, vary even among individuals within the same family.[22] The audiogram of TMD368 showed moderate SNHL (Figure 2b), although this patient

was still just 35 years old. He had no other clinical disorders such as myoclonus or family history of HL or neurological disorders. It should also be noted that recently the m.7472insC mutation was identified in gastric cancer tissues.[23] This report suggested that somatic mtDNA mutations may have an important role in the progression of gastric cancer.

In conclusion, the present extended screening system by use of a suspension array for major mtDNA mutations was demonstrated to be powerful, because we could detect both major causative and unexpected mtDNA mutations. The present system is helpful for both the diagnosis and epidemiological studies. Detecting mtDNA mutations in the early stage of HL could be meaningful both to select the optimal therapeutic strategies for the patients and to provide appropriate genetic counseling.

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