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ORIGINAL ARTICLE

Extensive and rapid screening for major mitochondrial DNA point mutations in patients with hereditary hearing loss

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Sensorineural hearing loss (HL) is one of the most frequent clinical features in patients with mitochondrial diseases caused by mitochondrial DNA (mtDNA) mutations, and hearing is impaired in over half of all cases with mitochondrial disorders. This study analyzed 373 patients with suspected hereditary HL using an extensive and rapid suspension-array screening system for 29 major mtDNA mutations, including the m.1555A>G homoplasmic mutation in the *MT-RNR1* gene, which causes non-syndromic sensorineural HL and aminoglycoside-induced HL, and the m.3243A>G heteroplasmic mutation in the *MT-TL1* gene. This method is rapid and suitable for large-scale screening because universal 96-well plates are available for use, and because an analysis of each plate can be completed within 1 h. This system detected five different mtDNA mutations in 24 of the 373 (6.4%) patients. The m.1555A>G and m.3243A>G mutations were detected in 11 (2.9%) and 9 (2.7%) patients, respectively. In addition, three mutations, that is, m.8348A>G in the *MT-TK* gene, m.11778G>A in the *MT-ND4* gene and 15498G>A in the *MT-CYB* gene were detected in one patient for each. This screening system is useful for the genetic diagnosis and epidemiological study of both syndromic and non-syndromic HL.
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INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common sensory disorder in humans. The prevalence of permanent SNHL continues to increase during childhood and it reaches a rate of about 2.7 per 1000 children before the age of 5 years and 3.5 per 1000 during adolescence.[1] Interestingly, hearing impairment is quite common in patients with mitochondrial disorders, affecting over half of all cases at some time during the course of the disease.[2] Therefore, many pathological mutations in mitochondrial DNA (mtDNA) have been reported to either cause or be associated with syndromic or non-syndromic HL.[3–6] A representative homoplasmic mutation at m.1555A>G in the *MT-RNR1* (12S ribosomal RNA) gene causes non-syndromic (isolated) hearing loss (HL) associated with a susceptibility to aminoglycoside antibiotics.[7–9] Moreover, it is not uncommon to find the m.1555A>G mutation in HL patients without defined past medication histories of aminoglycoside.[9–12] Other pathogenic mtDNA mutations have also been identified in syndromic HL with various clinical phenotypes, such as mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes, Kearns–Sayre syndrome, myoclonic epilepsy and ragged-red fibers[13] or maternally inherited diabetes and deafness.[14] Furthermore, the m.3243A>G heteroplasmic mutation in *MT-TL1* ($tRNA^{Leu(UUR)}$) gene is found in patients with non-syndromic HL.[15] Accordingly, certain mitochondrial diseases can arise from defects in the mtDNA transmitted from mothers, but there are also many cases without any apparent family history of the disease (sporadic).

It is therefore necessary to analyze the many presumable mutations in mtDNA because hearing impairment is a quite common clinical feature caused by mtDNA mutations. Over 200 point mutations in mtDNA have been listed in the mtDNA mutation database MITOMAP (http://www.mitomap.org/). However, mitochondrial single nucleotide polymorphisms (mtSNPs) are common in human mtDNA, according to the human mtSNP database (http://mtsnp.tmig.or.jp/mtsnp/index_e.shtml) and recent reports.[16–19] Consequently, it is also necessary to distinguish pathogenic mtDNA mutations from mtSNPs.

This study screened 373 unrelated Japanese patients with suspected hereditary HL by using an extensive and rapid suspension-array detection system for 29 major mtDNA mutations, including the m.1555A>G and m.3243A>G mutations, and verified the effectiveness of this system for identifying mtDNA mutations.

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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. The average age of the patients was 40 years, with an age range between 1 and 77 years. The patients included 144 males and 229 females, including four with the branchiootorenal syndrome, four with Pendred syndrome, one with Alport syndrome, one with Turner syndrome, one with spinocerebeller degeneration and one with renal tubular acidosis. Their mode of inheritance was autosomal dominant in 92, autosomal recessive in 52, maternal in 47, sporadic in 179 and unknown in 3. Their onset age of HL varied from newborn to 76 years of age (Table 1). The degree of HL was evaluated by pure-tone audiometry. The pure-tone average was calculated from the air-conduction audiometric thresholds at frequencies of 0.5, 1, 2 and 4 kHz.

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 from each individual and/or the parents in the case of children.

Extensive mtDNA pathological mutation screening by suspension array

The DNA samples were purified from the blood using a standard procedure. The mtDNA from the patients was analyzed using an extensive and rapid suspension array-based screening system that has been originally designed to identify various mtDNA point mutations that cause mitochondrial diseases.[20] In brief, multiplex PCR and a flow-metric analysis using a suspension array system (Luminex, Austin, TX, USA) was used to detect any mtDNA mutations. A total of 11 pairs of primers were used for PCR amplification and 65 sequence-specific oligonucleotide probes, which were carefully designed to detect either mutant or wild-type mtDNA even when a certain mtSNP was present in the vicinity of the mutation site. Two pairs of sequence-specific oligonucleotide probes that matched the mutant and wild-type with or without the polymorphic sites were used when mtSNPs were present in the vicinity of target mutations. Four probes were designed for the analysis of the m.1606G>A mutation in the *MT-RNR1* (12S ribosomal RNA) gene causing ataxia, myoclonus and deafness to avoid interference by the mtSNP m.1598G>A, which is found in haplogroups B5b, N9b1 and N1b in the Japanese population (frequency: 4.2%).[21] Similarly, for the detection of the m.15498G>A mutation in the *MT-CYB* (cytochrome *b*) gene,[22] two pairs of probes were designed to avoid interference by the mtSNP m.15497G>A, which is characteristic of haplogroup G1a in the Japanese population (frequency: 3.7%). The detection of the m.12706T>C mutation in the *MT-ND5* gene can be inhibited by the presence of mtSNP m.12705C>T, which is found in haplogroups A and N9a, as well as in macrohaplogroup M among the Japanese population (frequency, 81%). Nevertheless, the m.12706T>C mutation is expected to be detected even in the presence of mtSNP m.12705C>T, because four probes were designed to detect the mutant and wild-type mtDNA with or without the polymorphic sites. Therefore, this system can be used for the screening of various mtDNA point mutations, even in the presence of

Table 1 Demographic features of 373 patients with HL

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 (0.0%)
Sporadic	179 (48.0%)
Unknown	3 (0.8%)

Abbreviation: HL, hearing loss.

Table 2 List of 29 mtDNA mutations analyzed by the array-based extensive detection system

Nucleotide change (m.)	*Locus*	*Amino- acid change*	*Clinical phenotype*
1555A>G	*MT-RNR1*		DEAF
1606G>A	*MT-TV*		AMDF
3243A>G	*MT-TL1*		MELAS, DM, DMDF, CPEO, MM
3254C>T	*MT-TL1*		CPEO
3255G>A	*MT-TL1*		MERRF+KSS overlap
3256C>T	*MT-TL1*		MELAS
3260A>G	*MT-TL1*		MMC
3271T>C	*MT-TL1*		MELAS, DM
3280A>G	*MT-TL1*		Myopathy
3291T>C	*MT-TL1*		MELAS
3302A>G	*MT-TL1*		MM
3303C>T	*MT-TL1*		MMC
3460G>A	*MT-ND1*	Ala>Thr	LHON
8344A>G	*MT-TK*		MERRF
8348A>G	*MT-TK*		Cardiomyopathy
8356T>C	*MT-TK*		MERRF
8363G>A	*MT-TK*		MERRF, MICM+DEAF, Autism
9176T>C	*MT-ATP6*	Leu>Pro	FBSN, MILS
11777C>A	*MT-ND4*	Arg>Ser	MILS
11778G>A	*MT-ND4*	Arg>His	LHON
12315G>A	*MT-TL2*		CPEO
12706T>C	*MT-ND5*	Phe>Leu	MILS
13513G>A	*MT-ND5*	Asp>Asn	MELAS, MILS
13514A>G	*MT-ND5*	Asp>Gly	MELAS
14459G>A	*MT-ND6*	Ala>Leu	LDYT, MILS
14482C>G	*MT-ND6*	Met>Ile	LHON
14484T>C	*MT-ND6*	Met>Val	LHON
14487T>C	*MT-ND6*	Met>Val	Dystonia, MILS
15498G>A	*MT-CYB*	Gly>Asp	Histiocytoid cardiomyopathy

Abbreviations: AMDF, ataxia, myoclonus, and deafness; ATP6, ATP synthase F_0 subunit 6; CPEO, chronic progressive external ophthalmoplegia; CYB, cytochrome *b*; DEAF, maternally inherited deafness or aminoglycoside-induced deafness; DM, diabetes mellitus; DMDF, diabetes mellitus+deafness; FBSN, familial bilateral striatal necrosis; KSS, Kearns–Sayre's syndrome; LDYT, Leber hereditary optic neuropathy and dystonia; LHON, Leber hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, myopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibers; MICM, maternally inherited cardiomyopathy; MILS, maternally inherited Leigh syndrome; MM, mitochondrial myopathy; MMC, maternal myopathy and cardiomyopathy; mtDNA, mitochondrial DNA; ND, NADH dehydrogenase subunit.
Abbreviations and information about mutations are annotated in the MITOMAP database.

polymorphisms at least in an East Asian population. As shown in Table 2, this detection system simultaneously identified 29 different heteroplasmic or homoplasmic mutations in 11 genes: one in each of *MT-ND1*, *MT-ATP6*, *MT-CYB*, *MT-RNR1*, *MT-TV* ($tRNA^{Val}$) and *MT-TL2* ($tRNA^{Leu(CUN)}$) genes; two in the *MT-ND4* gene; three in the *MT-ND5* gene; four in each of *MT-TK* ($tRNA^{Lys}$) and *MT-ND6* genes; and 10 in the *MT-TL1* ($tRNA^{Leu(UUR)}$) gene. All of the 29 mtDNA mutations are listed in the MITOMAP. The median fluorescence intensity (MFI) values were calculated from an analysis of 50 microspheres of each set, which represented 50 replicate measurements, using a flowmeter equipped with a Luminex XY Platform plate reader and the Luminex 1.7 proprietary software package.[23,24] Both the MFI values for the two corresponding alleles were displayed on scatter diagrams. The mutations were considered to be heteroplasmic when both the mutant and wild-type signal intensities were detected above the cutoff values. In contrast, the mutations were homoplasmic when the mutant signal intensities were above the cutoff values, whereas the wild-type signals were below them.

Comparison of results between suspension array and direct DNA sequencing

The DNA sequences were analyzed by using an Applied Biosystems 3130*xl* 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,[25,26] while following the standard procedure.[27,28]

Quantification of mutation load of m.3243A>G. by restriction fragment length polymorphism analysis

The last cycle PCR-restriction fragment length polymorphism analysis on mtDNA was conducted with the m.3243A>G mutation in *MT-TL1* gene to compare MFI values, obtained by the m.3243G detection system, with the mutation loads. The 3243A>G mutant mtDNA introduces an *Apa*I restriction site detectable by restriction fragment length polymorphism analysis. The *Apa*I-digested 6-carboxyfluorescein-labeled PCR products were analyzed with the Applied Biosystems 3130*xl* Genetic Analyzer using GeneMapper software program Version 4.0 (Applied Biosystems) to quantify the proportion of the mutation as a percentage, as described previously.[20] The mutation load was also compared with the pure-tone average value.

RESULTS

Five of the 29 mutations were detected in 23 of 373 patients with SNHL by the extensive and rapid screening system (Table 3). All 373 DNA samples were analyzed by the m.1555A>G mutation detection system and the results were displayed in the scatter diagram, as shown in Figure 1a. Only the MFI value of the m.1555A>G homoplasmic mutation was detected in 11 samples (2.9%). Only 2 of the 11 patients with the m.1555A>G mutation had an apparent history of aminoglycoside exposure.

The relationship between the mutation loads and signal intensity values (MFI: 9190 ± 1590, mean ± s.d.) in the nine test DNA samples with the m.3243A>G mutation (mutational load: 23.1% ± 16.5, mean ± s.d.; range: 4–59%) was evaluated to verify the detection level of the 3243A>G detection system. The signal intensity values correlated significantly with the mutation loads (R=0.80 and P<0.001). The minimum detection limit of the m.3243A>G mutation was estimated to be approximately 2% (Supplementary Figure 1). As shown in Figure 1b, results obtained by the m.3243A>G detection system were displayed in a scatter diagram. Both mutant- (m.3243G) and wild-type-positive (m.3243A) signals were detected in nine (2.4%) samples, thus this mutation was considered heteroplasmic. The mutation loads of the m.3243A>G detected in these nine patients were between 16 and 46%, as determined by the last cycle PCR-restriction fragment length polymorphism analysis (Table 4). The clinical findings in these nine patients were evaluated with the m.3243A>G mutation, as shown in Table 4. There was no correlation between the mutation loads in the DNA extracted from the blood and the clinical severities of those patients. The onset age of HL ranged from 10 to 42 years. Although four patients suffered from diabetes and two patients showed renal dysfunction, other three patients presented isolated HL without a family history of HL.

Interestingly, one sample each harbored the m.8348A>G mutation in the *MT-TK* ($tRNA^{Lys}$) gene (Figure 2a), the m.11778G>A mutation in the *MT-ND4* gene (Figure 2b) and the m.15498G>A mutation in the *MT-CYB* gene (Figure 2c). None of these three mutations had been suspected until the samples were analyzed by this screening system. The sample with m.8348A>G and that with m.15498G>A were revealed as either homoplasmy or heteroplasmy with a high mutational load. The mutation load of the m.11778G>A

Table 3 The 373 DNA samples with hereditary hearing loss screened by the extensive detection system for 29 mtDNA mutations and direct DNA sequencing

mtDNA mutation	*Number*	*Frequency (%)*
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
Undetected	350	93.8

Abbreviation: mtDNA, mitochondrial DNA.

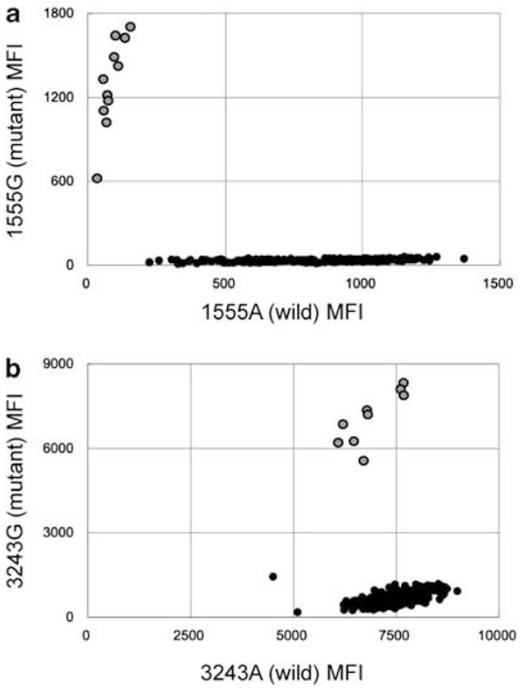

Figure 1 Scatter diagrams with the mutant median fluorescence intensity (MFI) values on the *y*-axis and the wild-type ones on the *x*-axis for the 1555A>G homoplasmic mutation (**a**) and the 3243A>G heteroplasmic mutation (**b**). All 373 DNA samples were analyzed by the m.1555A>G and m.3243A>G mutation detection systems using universal 96-well plates. Each result was merged into the two separate scatter diagrams. Red circles indicate MFI values of mutation-positive DNAs. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 4 Clinical and genetic features of the nine HL patients with m.3243A>G heteroplasmic mutation detected by the extensive mtDNA pathological mutation screening system

TMD no.	*Sex*	*PTA right (dB)*	*PTA left (dB)*	*Onset of HL (y)*	*DM*	*Renal dysfunction*	*Family history*	*Mutation load (%)*
18	M	56.3	51.3	42	+	–	–	16
22	F	30	30	25	–	–	–	26
27	M	28.8	36.3	Unknown	–	+	+	24
49	F	46.3	56.3	Childhood	+	–	+	8.6
169	F	57.5	60	10	+	–	+	25
219	F	62.5	87.5	40	–	–	–	17
268	F	31.3	38.8	27	+	–	–	23
325	M	55.0	53.8	25	–	–	–	43
583	F	43.8	45	31	–	+	+	46

Abbreviations: DM, diabetes mellitus; F, female; HL, hearing loss; M, male; mtDNA, mitochondrial DNA; PTA, pure-tone average; RFLP, restriction fragment length polymorphism.
The 3243A>G mutation loads were evaluated by RFLP analysis. Two patients, TMD 219 and 325, were not examined on pure-tone audiometry in our hospital.

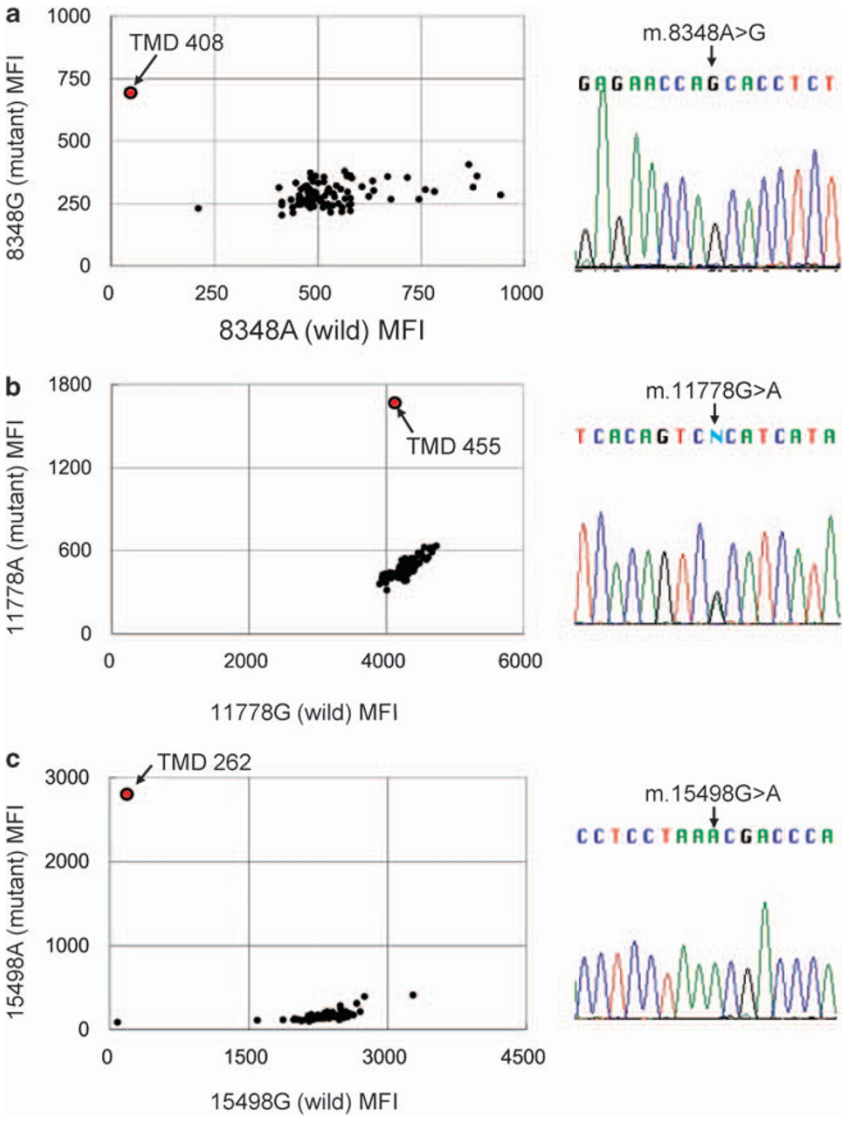

Figure 2 Scatter diagrams with the mutant median fluorescence intensity (MFI) values on the *y*-axis and wild-type ones on the *x*-axis and the electropherograms of DNA sequences for the 8348A>G homoplasmic mutation (**a**), the 11778G>A heteroplasmic mutation (**b**) and the 15498G>A homoplasmic mutation (**c**). Red circles indicate MFI values of mutation-positive DNAs. The samples were analyzed with an Applied Biosystems 3130*xl* Genetic Analyzer (Applied Biosystems) and Sequencher version 4.2.2 (Gene Codes).

heteroplasmy was approximately 45%, as determined on the electropherogram of the DNA sequence. None of the 29 major mtDNA mutations was detected in the other 350 (93.8%) patients among the patients with suspected hereditary HL.

A total of 10,817 mutant sites in 373 DNA samples were screened using the system for detecting the 29 major mtDNA mutations. Importantly, 99.8% of the targeted mutant sites were detected by the system and only 25 (0.2%) of the mutant sites in 15 DNA samples could not be determined, because the signals of both wild-type and mutant were negative. In each instance, the DNA fragment flanking the putative mutation sites was sequenced and rare polymorphisms were found within the binding sites for both the mutant-specific and the wild-type-specific probes. Conventional DNA sequencing confirmed all the mtDNA mutations detected by this system. The evaluation of the system in this study was initially limited because DNA samples with the other mtDNA mutations were not available. The oligonucleotides complementary to the mutant-specific probes were synthesized to overcome that problem. This confirmed the specificity of all the probes for these mutations (data not shown).

DISCUSSION

This study screened 373 DNA samples from patients with suspected hereditary HL using the extensive and rapid detection system for 29 major mtDNA mutations. Accordingly, m.1555A>G and m.3243A>G mutations were detected in 11 (2.9%) and 9 (2.4%) patients, respectively. In addition, three other mutations (m.8348A>G, m.11778G>A and m.15498G>A) were detected by this screening system.

Sensorineural hearing loss is a quite common clinical feature in patients with mitochondrial diseases caused not only by m.1555A>G and m.3243A>G mutations, but also by the other mtDNA mutations. According to the most recent study on this topic, the prevalence of the m.1555A>G mutation is 0.21% in adults of European descent in Australia.[29] In Japan, the mutation was detected in 3% of all patients with SNHL.[30] As patients with the m.1555A>G mutation are susceptible to aminoglycoside ototoxicity,[7–9] screening for this mutation is therefore beneficial for people who are going to be administered aminoglycoside. Furthermore, this mutation has also been demonstrated in patients with non-syndromic HL without defined past medication histories of aminoglycoside, and even in patients without any apparent maternal inheritance.[9–12]

In contrast, the m.3243A>G mutation causes several major clinical phenotypes of mitochondrial disease such as myopathy, encephalopathy, lactic acidosis, stroke-like episodes, maternally inherited diabetes and deafness, chronic progressive external ophthalmoplegia and mitochondrial diabetes.[31–33] However, epidemiological evidence of the prevalence of the patients with the m.3243A>G mutation in the population still has limitations. The patients with m.3243A>G including asymptomatic cases in the general population are thought to be as common as 6.57–16.3 per 100 000.[34–36] According to the findings of recent epidemiological studies, however, Manwaring *et al.*[36] reported that the population prevalence was much higher, at 236/100 000. A population-based study of the m.3243A>G mutation revealed the mutation in 7.4% of patients with maternally inherited HL in northern Finland.[38] In this study, this mutation was demonstrated to have a prevalence of 2.4% in the cases of Japanese hereditary HL, and this frequency is very close to that previously reported in Japan.[39]

This screening system detected the m.8348A>G mutation in the *MT-TK* gene in a 6-year-old boy (patient TMD 408) with SNHL (Figure 2a). The m.8348A>G heteroplasmic mutation was reported in a patient with severe cardiomyopathy in adulthood.[40] In this study, the proband presented with SNHL at age of 3 years; but he did not display any other clinical features including cardiac symptoms. Both the 32-year-old mother and younger sister (II-2 and III-2, respectively, in Figure 3a) of the proband (TMD 408, III-1) also suffered from HL. The results of audiometry showed that the proband demonstrated a high-frequency SNHL, whereas his mother (II-2) showed moderate SNHL at the frequencies of 2 and 4 kHz. He had no apparent family history of cardiomyopathy. The grandfather (I-1) of the proband probably suffered from HL of an unknown origin, although his hearing level was not examined by audiological testing. The m.8348A>G homoplasmic transition is one of the characteristic mtSNPs of mitochondrial haplogroup H1b, and the pathogenicity of this mutation still remains controversial.[41,42] The clinical course of this patient must therefore be followed closely and further detailed investigation is required.

The m.11778G>A mutation in the *MT-ND4* gene was detected in a 28-year-old female (TMD 455, Figure 2b). At age 10, the proband developed a hearing impairment and was diagnosed to have renal dysfunction at 12 years of age. The mother (I-2 in Figure 3b), older sisters (II-2 and -4) and nieces (III-1 and -2) of the proband also suffered from HL. Her audiogram showed profound bilateral SNHL (Figure 3b). The m.11778G>A mutation in *MT-ND4* gene is the most common mutation in patients with Leber hereditary optic neuropathy.[43,44] Although impaired hearing does not generally accompany Leber hereditary optic neuropathy,[45,46] appreciable progressive auditory neuropathy in two patients with Leber hereditary optic neuropathy has been reported.[47] However, the proband (TMD 455) in this study had never complained of any visual problems as is normally observed in cases with Leber hereditary optic neuropathy, nor did any of her family members. The clinical courses of the proband and her family must therefore be closely followed because the profound HL of the proband differed from the moderate HL in the reported cases.

The screening system detected the m.15498G>A mutation in the *MT-CYB* gene in a 54-year-old female (TMD 262) with HL. The m.15498G>A (p.MT-CYB: Gly251Asp) mutation can cause histiocytoid cardiomyopathy that usually occurs in infancy or childhood.[48,49] In this study, the proband developed HL at the age of 35 years. The results of the pure tone audiogram showed high-frequency SNHL (Figure 3c). Her electrocardiogram indicated Wolff–Parkinson–White syndrome but no apparent evidence of cardiomyopathy. The proband's sister (II-4 in Figure 3c) was reported to have HL, whereas the cause of HL in their 77-year-old father (I-1) may not be the same as the proband and her sister. Assuming that the HL in the proband and her sister (II-4) is caused by the m.15498G>A mutation, the HL of the proband's father probably has a different cause. In addition, none of the children of proband was found to have HL. It is therefore necessary to follow the clinical courses of both the proband and her family.

Over the past 5 years, new technologies have been developed to screen for mtDNA mutations, including denaturing high-performance liquid chromatography;[50,51] a detection system using a mismatch-specific DNA endonuclease;[52] the Biplex Invader assay by hybridization of two overlapping oligonucleotides to the target sequence;[53] the matrix-associated laser desorption/ionization time of flight mass spectrometry assay;[54] the pyrosequncing technology to detect and estimate heteroplasmic mtDNA point mutations;[55] and an entire mtDNA resequencing chip: Mitochip.[56,57] The Mitochip uses hybridization for detecting mutations or polymorphisms, so that the probes

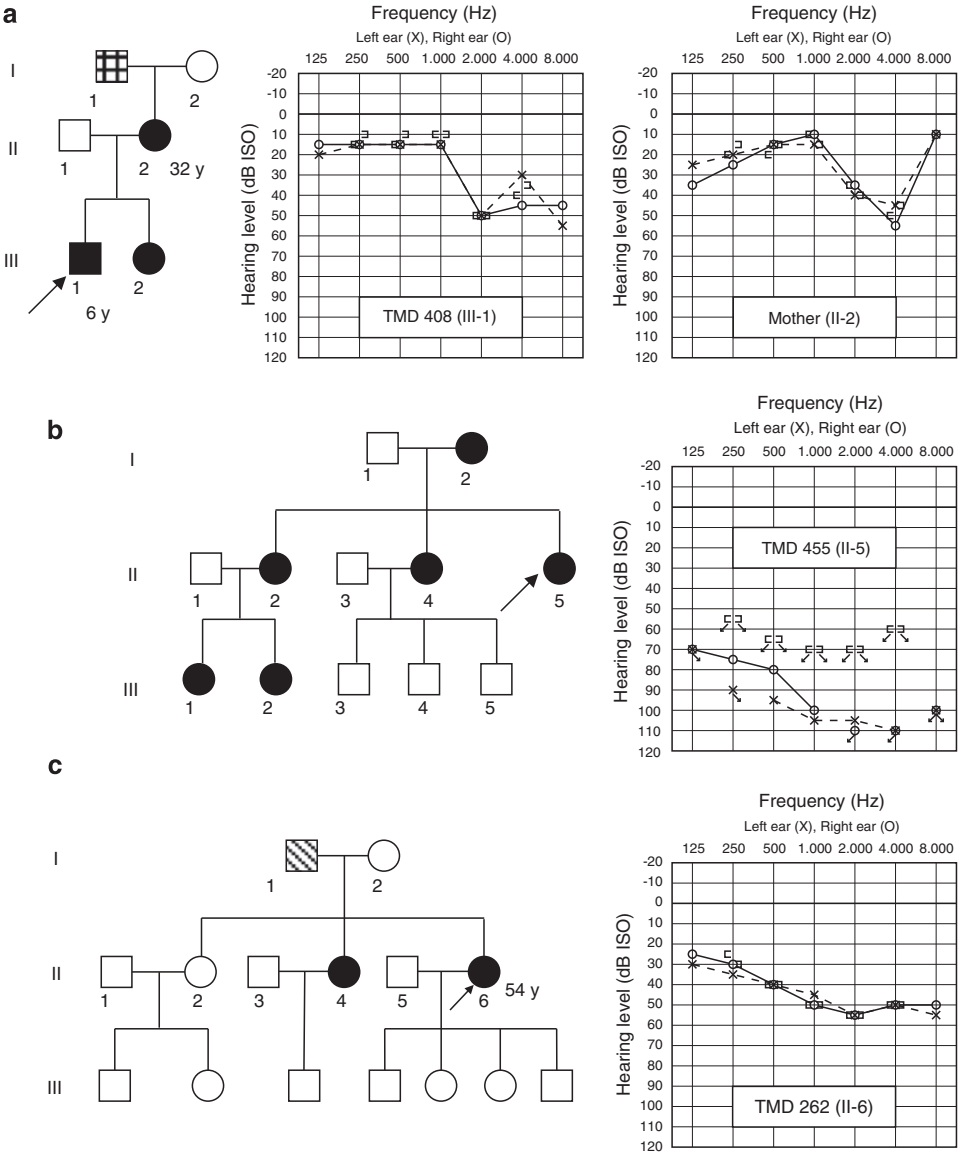

Figure 3 Pedigrees of the families and audiograms of patient TMD408 and his mother (**a**), patient TMD455 (**b**) and patient TMD262 (**c**). Clinical features are depicted: black-filled circles or squares as individuals with deafness, and pattern-filled squares as individuals with deafness of unknown cause. Arrows indicate probands. KEY on pure tone audiograms: dB, decibel hearing loss; ISO, international standards organization;], left ear bone conduction; [, right ear bone conduction; O, right ear air conduction; X, left ear air conduction.

should be designed.[56] Although version 2.0 of Mitochip has been improved for detecting mutations, the present version has not been designed for analysis of Asian mtDNA, as it is based on the revised Cambridge reference sequence.[25,26] As it is necessary to distinguish pathological mtDNA mutations from non-pathogenic mtSNPs, this study used 11 pairs of primers for multiple PCR amplifications and multiple sequence-specific oligonucleotide probes customized for the Japanese population, which were designed to carefully detect either mutant or wild-type mtDNA, even when mtSNPs were present in the vicinity of the mutation sites.

In terms of epidemiological studies, the extensive and rapid mtDNA mutation detection system using the suspension array reported in this study is suitable for large-scale screening owing to the following three advantages: first, the universal 96-well plates are available for the analysis. Second, the analysis of each plate can be completed within 1 h. Third, this system is adequate to detect heteroplasmic mutations that are a common type of mtDNA pathological mutation. The nine samples with the m.3243A>G mutation revealed positive signals with both mutant- and wild type-specific probes in this detection system. The detection limit of m.3243A>G was estimated to be approximately only 2%. The mtDNA mutation load in the impaired tissue of the patients is thought to closely correlate with the severity. In this study, however, no correlation was observed between the percentage of the m.3243A>G mutation in the DNA extracted from the blood and the clinical severities of the patients.

In conclusion, an extensive and rapid screening system using a suspension array for 29 major mtDNA mutations in patients with HL was found to be useful for the diagnosis and epidemiological study of both syndromic and non-syndromic HL. The diagnosis of mitochondrial disease is complex, but detecting mtDNA mutations in the early

stage of HL using this screening system is considered to be extremely important to select the optimal therapeutic strategies and also provide the appropriate genetic counseling for the patients.

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