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The ND4 G11696A mutation may influence the phenotypic manifestation of the deafness-associated 12S rRNA A1555G mutation in a four-generation Chinese family

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Abstract

We report here the clinical, genetic and molecular characterization of a large Han Chinese family with aminoglycoside induced and nonsyndromic hearing loss. The penetrance of hearing loss (affected matrilineal relatives/total matrilineal relatives) in this pedigree was 53%, when aminoglycoside-induced deafness was included. When the effect of aminoglycosides was excluded, the penetrance of hearing loss in this pedigree was 42%. These matrilineal relatives exhibited a wide range of severity of hearing loss, varying from profound to normal hearing. Furthermore, these affected matrilineal relatives shared some common features: bilateral hearing loss of high frequencies and symmetries. Sequence analysis of mitochondrial DNA (mtDNA) in the pedigree identified the homoplasmic 12S rRNA A1555G mutation and other 35 variants belonging to Eastern Asian haplogroup D4. Of these, the V313I (G11696A) mutation in ND4 was associated with vision loss. However, the extremely low penetrance of visual loss, and the mild biochemical defect and the presence of one/167 Chinese controls indicated that the G11696A mutation is itself not sufficient to produce a clinical phenotype. Thus, the G11696A mutation may act in synergy with the primary deafness-associated 12S rRNA A1555G mutation in this Chinese family, thereby increasing the penetrance and expressivity of hearing loss in this Chinese pedigree.

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INTRODUCTION

Mutations in mitochondrial 12S rRNA gene are the hot spot for mutations hearing loss [1–2]. Of these, the 12S rRNA C1494T mutation has been associated with both aminoglycoside-induced and non-syndromic hearing loss in only eight Chinese families and three Spanish pedigrees [3–8]. The 12S rRNA A1555G mutation has been associated with both aminoglycoside-induced and nonsyndromic hearing loss in many families worldwide [9–23]. Matrilineal relatives within and among families carrying the A1555G or C1494T mutation exhibited a wide range of penetrance and expressivity, including severity and age at onset of hearing loss [3,9,12,14,15,20]. Functional characterization of cell lines derived from matrilineal relatives of a large Arab-Israeli family or one Chinese family demonstrated that the A1555G or C1494T mutation led to mild mitochondrial dysfunction and sensitive to aminoglycosides [24–27]. These findings strongly indicated that the A1555G or C1494T mutation itself is insufficient to produce the deafness phenotype. Therefore, other modifier factors including aminoglycosides, nuclear and mitochondrial modifiers contribute to phenotypic variability of hearing loss associated with the A1555G or C1494T mutation [20, 24–28].

To further understand the role of mitochondrial modifiers in the phenotypic manifestation of these deafness-associated mtDNA mutations, we have initiated a systematic and extended mutational screening of mtDNA in several cohorts of hearing-impaired subjects [15–23]. In the previous investigation, we showed the variable penetrance and expressivity of hearing loss in 40 Chinese families carrying the A1555G mutation [17–23]. Five mitochondrial variants: A14693G, T15908C, T10454C, G7444A and G5821A, were implicated to influence the phenotypic manifestation of the A1555G mutation [18–22], while the T5628C and G7444A variants may modulate the phenotypic manifestation of the C1494T mutation in a large Chinese family [5,7]. In the present study, a mutational screening of mitochondrial DNA in a large cohort of hearing-impaired Chinese subjects led to the identification of 12S rRNA A1555G and ND4 G11696A mutations in a Han Chinese family with aminoglycoside-induced and nonsyndromic hearing loss. We further performed the clinical, molecular and genetic characterization of this Chinese pedigree. To assess the contribution that mtDNA variants make toward the phenotypic expression of the A1555G mutation, we performed a PCR-amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in the matrilineal relatives of this family.

SUBJECTS AND METHODS

Subjects and audiological examinations

As a part of genetic screening program for the hearing impairment, a four-generation Han Chinese family, as shown in Figure 1, was ascertained through the Otology Clinic at Wenzhou Medical College. A comprehensive history and physical examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment in members of this pedigree. An age-appropriate audiological examination was performed and this examination included pure-tone audiometry (PTA) and/or auditory brainstem response (ABR), immittance testing and Distortion product otoacoustic emissions (DPOAE). The PTA was calculated from the sum of the audiometric thresholds at 500, 1000 and 2000, 4000 and 8000 Hz. The severity of hearing impairment was classified into five grades: normal <26dB; mild =26–40 dB; moderate=41–70 dB; severe=71–90 dB; and profound >90 dB. Informed consent was obtained from participants prior to their participation in the study, in accordance with the Cincinnati Children's Hospital Medical Center Institutional Review Board and Ethnic Committee of Wenzhou Medical College.

Mutational screening of the mitochondrial genome

Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kits (Gentra Systems). First, affected and control subject's DNA fragments spanning the entire mitochondrial 12S rRNA gene were amplified by PCR using oligodeoxynucleotides corresponding to the mtDNA at positions 618–635 and 1988–2007 [16], respectively. For the detection of the A1555G mutation, the amplified segments were digested with a restriction enzyme *BsmAI* [16]. Equal amounts of various digested samples were then analyzed by electrophoresis through 1.5% agarose gel. The proportions of digested and undigested PCR product were determined by using the IMAGE-QUANT program after ethidium bromide staining to determine if these mtDNA mutations are in the homoplasmy in these subjects. Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. mtDNA sequence alignments were carried out using seqweb program GAP (GCG).

The entire mitochondrial genome of one affected matrilineal relative IV-1 was PCR amplified in 24 overlapping fragments by use of sets of the light-strand and the heavy strand oligonucleotide primers, as described elsewhere [29]. Each fragment was purified and subsequently submitted for sequence analysis as described above. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_001807) [30].

Mutational analysis of *GJB2* gene—The DNA fragments spanning the entire coding region of *GJB2* gene were amplified by PCR using the following oligodeoxynucleotides: forward-5'TATGACACTCCCCAGCACAG3' and reverse-5'GGGCAATGCTTAACTGGC3'. PCR amplification and subsequent sequencing analysis were performed as detailed elsewhere [16]. The results were compared with the wild type *GJB2* sequence (GenBank accession number: M86849) to identify the mutations.

RESULTS

Clinical presentation

The proband (IV-1) came to the otology clinic at the Wenzhou Medical College at the age of 17 years. She was administrated with gentamicin (3–5 mg/kg/dose every 8 h) for fever at the age of 1 year old. She began suffering hearing loss within one month after drug administration. As illustrated in Figure 2, audiological evaluation showed that she exhibited moderate bilateral hearing impairment (58 and 75 dB at right ear and left ear, respectively; with a sloped-shaped pattern). However, she had no other significant medical history. A comprehensive history and physical examination as well as audiological examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment in all available members of this family.

Clinical evaluation showed that ten of 19 matrilineal relatives in this family suffer from bilateral and sensorineural hearing impairment as the sole clinical symptom, while other members of this family exhibited normal hearing. Of these, subject IV-2, who received the gentamicin treatment at the age of one year old, suffered from severity hearing impairment. In the absence of aminoglycosides, other affected matrilineal relatives exhibited a variable degree of hearing impairment in this maternal kindred, ranging from severe hearing impairment (II-6, II-9, III-14, and III-15), to moderate hearing impairment (III-6, III-10, III-17), to mild hearing impairment (III-11). These subjects showed variable patterns of audiometric configuration, including the the sloping-shaped pattern (subject III-6 and IV-2). All affected individuals displayed the loss of the high frequencies and their hearing impairments were symmetric.

Mitochondrial genome analysis

The maternal transmission of hearing impairment in this family suggested the mitochondrial involvement and led us to analyze mtDNA of matrilineal relatives. First, DNA fragments spanning 12S rRNA gene were PCR amplified from derived from four matrilineal relatives (proband IV-1, her sister IV-2, affected female III-10, unaffected male III-7) and two unrelated Chinese controls. Each fragment was purified and subsequently analyzed by DNA sequencing. As shown in Figure 3, the A1555G mutation in the 12S rRNA was found in these subjects. The restriction enzyme digestion and subsequent electrophoresis analysis indicated that the A1555G mutation was indeed present in homoplasmy in those subjects and other matrilineal relatives of this family but not other members including III-5 of this family (data not shown).

To assess the role of mtDNA variants in the phenotypic expression of the A1555G mutation, we performed a PCR-amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in this proband. In addition to the identical A1555G mutation, as shown in Table 1, these subjects exhibited a distinct set of mtDNA polymorphism. Of other nucleotide changes in these mitochondrial genomes, there are 10 variants in the D-loop, two known variants in 12S rRNA gene, three known variants in the 16S rRNA gene, 14 known silent variants in the protein encoding genes as well as 7 missense mutations in the protein encoding genes [31]. These missense mutations are the C5178A (L237M) in the ND2 gene, the C8414T (L17F) in the A8 gene, the A8701G (T59A), and A8860G (T112A) in A6 gene, the A10398G (T114A) in the ND3 gene, the G11696A (V312I) in the ND4 gene, the A15326G (T194A) in the cyto b gene. These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis of these variants and sequences from other organisms including mouse [32], bovine [33], and *Xenopus laevis* [34]. None of other variants showed evolutionary conservation. However, the G-to-A transition at position 11696 (G11696A) in ND4, caused the substitution of an isoleucine for valine at amino acid position 313, as shown in Figure 3, has been found in those subjects. Indeed, this mutation has been associated with LHON and hereditary spastic dystonia in a large Dutch family [35] and LHON in Chinese families [36–37].

Mutational analysis of GJB2

To examine the role of *GJB2* gene in phenotypic expression of the A1555G mutation, we performed the mutational screening of *GJB2* gene in six affected matrilineal relatives of the Chinese pedigree. None of variants in *GJB2* gene was found in these affected matrilineal relatives of the Chinese pedigree. Indeed, the absence of variant in the *GJB2* gene in those subjects with hearing impairment indicates that the *GJB2* gene may not be a modifier of the phenotypic effects of the A1555G mutation in those subjects.

DISCUSSION

In the present study, we have performed the clinical, genetic and molecular characterization of a large Han Chinese pedigree with aminoglycoside-induced and nonsyndromic hearing impairment. Hearing impairment as a sole clinical phenotype was mostly present in the maternal lineage of those pedigrees, suggesting that the mtDNA mutation is the molecular basis for this disorder. In fact, the A1555G mutation was identified to be homoplasmy in matrilineal relatives of this Chinese family. The Chinese family exhibit much higher penetrance and expressivities of hearing loss than in other 27 Chinese pedigrees carrying the A1555G mutation [18,20,23]. In particular, as shown in Table 2, the penetrance of hearing loss (affected matrilineal relatives/total matrilineal relatives) in this pedigree was 53%, when aminoglycoside-induced deafness was included. When the effect of aminoglycosides was excluded, the penetrance of hearing loss in this pedigree was 42%. These are comparable with the penetrance of hearing loss in previously identified five Chinese pedigrees, with the average

of 54% and 31.7%, respectively, when aminoglycoside-induced deafness was included or excluded [19,20,22]. However, the average penetrances of hearing loss in other 27 pedigrees carrying the A1555G mutation were 9.3% and 1.7%, respectively, when aminoglycoside-induced deafness was included or excluded [18,21,23].

The wide range of penetrance and severity of hearing loss among these pedigrees carrying the identical A1555G mutation strongly indicated the involvement of modifier factors including aminoglycosides and nuclear and mitochondrial modifiers in the phenotypic manifestation of the A1555G mutation. The nuclear modifier genes apparently contributed to the phenotypic variability of matrilineal relatives in this Chinese family including the variable severity and audiometric configuration of hearing impairment as described other pedigrees [24,28]. However, the lack of mutation in the *GJB2* gene ruled out its involvement in the phenotypic expression of the A1555G mutation in those affected subjects, as in the cases of other Chinese pedigrees carrying the A1555G mutation [18,21]. The observation that two of 10 affected matrilineal relatives of these pedigrees suffered from aminoglycoside-induced hearing loss indicated that aminoglycosides indeed led to the deafness expression of the A1555G mutation. In addition, the mtDNA variants/haplotypes have been shown to modulate the phenotypic manifestation of the deafness-associated mtDNA mutations. In particular, mitochondrial tRNA variants A14693G, T15908C, T10454C, G7444A and G5821A may contribute to higher penetrance of hearing loss in five Chinese pedigrees carrying the A1555G mutation [19,20,22].

The mtDNAs of this Chinese pedigree, as shown in Table 2, belong to Easter Asian haplogroups D4 [38], while other 17 Chinese mitochondrial genomes carrying the A1555G mutation belong to haplogroups B, D, F, M, and N [18–22], respectively. Of these mtDNA variants, the V313I (G11696A) mutation in ND4 was first identified in a large Dutch family [35] and subsequently in five Chinese pedigrees [36] and coexisted with ND4 G11778A mutation in a Chinese family with higher penetrance of vision loss [37]. However, the extremely low penetrance of visual loss, and the mild biochemical defect [35] and the presence of one/167 Chinese controls indicated that the G11696A mutation is itself not sufficient to produce a clinical phenotype [36]. Thus, the G11696A mutation may act in synergy with the primary deafness-associated 12S rRNA A1555G mutation in this Chinese family, thereby increasing the penetrance and expressivity of hearing loss in this Chinese pedigree.

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Figure 1. A four-generation Han Chinese pedigree with aminoglycoside-induced and nonsyndromic hearing impairment

Hearing-impaired individuals are indicated by filled symbols. Arrowhead denotes proband. Asterisks denote individuals who had a history of exposure to aminoglycosides.

Figure 2. Air conduction audiogram of three members in the Chinese family
 Symbols: X-left, O-right ear.

Figure 3. Identification and qualification of A1555G and G11696A mutations
Partial sequences chromatograms of 12S rRNA and ND4 genes from two matrilineal relatives and one married-in Chinese control.

Table 1
mtDNA variants in one Chinese family with hearing loss

Gene	position	Replacement	Conservation (H/B/M/X) ^a	Previously reported ^b
D-loop	73	A to G		Yes
	152	T to C		Yes
	263	A to G		Yes
	310	C or TC		Yes
	373	A to G		Yes
	489	T to C		Yes
	16184	T to C		Yes
	16223	C to T		Yes
	16311	T to C		Yes
	16362	T to C		Yes
12S rRNA	1438	A to G	A/A/A/G	Yes
	1555	A to G	A/A/A/A	Yes
16S rRNA	2706	A to G	C/A/A/A	Yes
	3010	G to A	G/G/A/A	Yes
	3107	C del	C/T/T/T	Yes
ND2	4769	A to G		Yes
	4883	C to T		Yes
	4985	G to A		Yes
	5178	C to A (Leu to Met)	L/T/T/T	Yes
CO1	7028	C to T		Yes
A8	8414	C to T (Leu to Phe)	L/F/M/W	Yes
A6	8701	A to G (Thr to Ala)	T/S/L/Q	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	Yes
CO3	9540	T to C		Yes
ND3	10398	A to G (Thr to Ala)	T/T/T/A	Yes
	10400	C to T		Yes
ND4	10873	T to C		Yes
	11335	T to C		Yes
	11696	G to A (Val to Ile)	V/T/T/M	Yes
	11719	G to A		Yes
ND5	12705	C to T		Yes
ND6	14668	C to T		Yes
Cyt b	14783	T to C		Yes
	15043	G to A		Yes
	15301	G to A		Yes
	15326	A to G (Thr to Ala)	T/M/I/I	Yes

^aConservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X);

^bSee the online mitochondrial genome database <http://www.mitomap.org>.

Table 2
Summary of clinical and molecular data for 17 Chinese families carrying the A1555G mutation

Pedigree	Number of matrilineal relatives	^a Penetrance (including the use of drugs) (%)	Penetrance (excluding the use of drugs) (%)	Second mtDNA mutations	mtDNA haplogroup
WZD11	19	53	42	ND4 G11696A	D4
WZD1 ^b	17	5.9	0	none	B4C1C1
WZD2	21	9.5	4.7	none	D4a
WZD3	16	12.5	0	none	D5a2
WZD4	24	29.2	16.7	none	F1a1
WZD5	31	3.2	0	none	D52b
WZD6	8	25	12.5	none	D4b2b
WZD7	30	10	3.3	none	D5a2
^c BJ101	14	5	0	none	F3
BJ102	13	13	8	none	N9a1
BJ103	20	5	0	none	D4a
BJ104	11	4	0	none	D4b2b
^d BJ105	15	67	45	tRNA ^{Cys} G5821A	F3
^e BJ106	18	33	33	none	M7b
^e BJ107	34	35	24	tRNA ^{Thr} T15908C	N
BJ108	16	63	38	tRNA ^{Glu} A14693G	F
BJ109	9	67	44	tRNA ^{Arg} T10454C	M
^f BJ110	17	59	5.9	CO1/tRNA ^{Ser(UCN)} G7444A	D4a

^a affected matrilineal relatives/total affected matrilineal relatives

^b Tang et al. 2007 [18];

^c Young et al. 2005 [21];

^d Zhao et al. 2005 [22];

^e Young et al. 2006 [20];

^f Yuan et al. 2005 [19]