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ORIGINAL ARTICLE

THE MITOCHONDRIAL COI/tRNA$^{SER(UCN)}$ G7444A MUTATION MAY BE ASSOCIATED WITH HEARING IMPAIRMENT IN A HAN CHINESE FAMILY

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ABSTRACT

Variations in mitochondrial genome have been found to be associated with hearing loss. Of these, the mitochondrial 12S rRNA and tRNA$^{Ser(UCN)}$ are the hot-spots for pathogenic variants associated with deafness. To understand the putative role of mitochondrial DNA (mtDNA) variants in hearing loss, we recently screened the variants in mitochondrial genomes in patients with deafness from the Hangzhou area of Zhejiang Province, People's Republic of China (PRC). In this study, we describe a maternally-inherited Han Chinese family with high penetrance of hearing loss, notably, the penetrance of hearing loss in this family were 80.0 and 40.0%, when the aminoglycoside was included or excluded. Three matrilineal relatives in this pedigree exhibited different levels of hearing loss with different age at onset. In addition, sequence analysis of the complete mitochondrial genome showed the presence of the well-known C1494T pathogenic variant in the *12S rRNA* gene and the G7444A pathogenic variant in the COI/tRNA$^{Ser(UCN)}$. The C1494T anomaly had been reported to be a pathogenic mutation associated with aminoglycoside-induced and nonsyndromic hearing loss (AINHL), while the G7444A was considered as a secondary mutation associated with deafness. However, the lack of functional variants in *GJB2* and *TRMU* genes suggested that nuclear modified genes may not play important roles in deafness expression. Thus, the combination of G7444A and C1494T pathogenic variants in the mitochondrial genome may account for the high penetrance of hearing loss in this Chinese family.

Keywords: C1494T; G7444A; Chinese family; Deafness; Mitochondrial DNA (mtDNA); Pathogenic variants.

INTRODUCTION

Hearing loss is one of the most common human health problems, affecting one in 700-1000 newborns [1]. Deafness can be caused by gene alternations and environmental factors including the ototoxic drugs such as aminoglycoside antibiotics. Of the hereditary factors, variants in mitochondrial DNA (mtDNA), especially in *12S rRNA* and *tRNA$^{Ser(UCN)}$* genes, are the important causes of sensorineural hearing loss [2]; among these variants, the homo-plasmic A1555G and C1494T pathogenic variants in the highly conserved A-site of 12S rRNA has been associated with both aminoglycoside-induced and nonsyndromic hearing loss (AINHL) in many families worldwide [3-6]. Moreover, the A7445G, 7472insC, T7510C and T7511C pathogenic variants have been identified in the *tRNA$^{Ser(UCN)}$* gene [7]. However, matrilineal relatives within and among families carrying these mutations exhibited a wide range of penetrance, severity and age at onset in hearing loss [8-9], moreover, functional analysis of the cell lines derived from the matrilineal relatives carrying these primary mutations demonstrated that the A1555G or C1494T led to mild mitochondrial dysfunction and sensitivity to aminoglycosides [10-11]. These findings strongly indicated that the A1555G or C1494T pathogenic variants were insufficient to produce enough clinical phenotypes, thus, other factors, such as aminoglycosides, nuclear genes or mitochondrial haplotypes may contribute to the clinical expression of deafness-associated mtDNA variants.

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With the aim of elucidating the molecular basis of hearing loss, an extensive mutational screening for mitochondrial *12S rRNA* and *tRNA*$^{Ser(UCN)}$ genes were performed in the Hangzhou area of Zhejiang Province, People's Republic of China (PRC). In this report, we describe a Han Chinese family with maternally-inherited AINHL. Sequence analysis of the mitochondrial genome showed the presence of C1494T and G7444A pathogenic variants.

MATERIALS AND METHODS

Subjects. As a part of genetic screening program for hearing loss, a three-generation Han Chinese family (as shown in Figure 1) was found at the Department of Otolaryngology, Hangzhou First People's Hospital, Zhejiang Province, PRC. Informed consent was obtained from the participants. Blood samples were obtained from all participants prior to their participation in the study, in accordance with the Ethics Committee of Hangzhou First People's Hospital. In addition, a comprehensive history and physical examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, as well as the 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 auditory brainstem response (ABR), immittance testing and distortion product otoacoustic emissions. The PTA was calculated from the sum of the audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. The severity of hearing impairment was classified into five grades: normal <26 dB, mild 26-40 dB, moderate 41-70 dB, severe 71-90 dB and profound >90 dB. Moreover, DNA was obtained from 200 control subjects from a panel of unaffected Han Chinese subjects from the same region who were clinically tested.

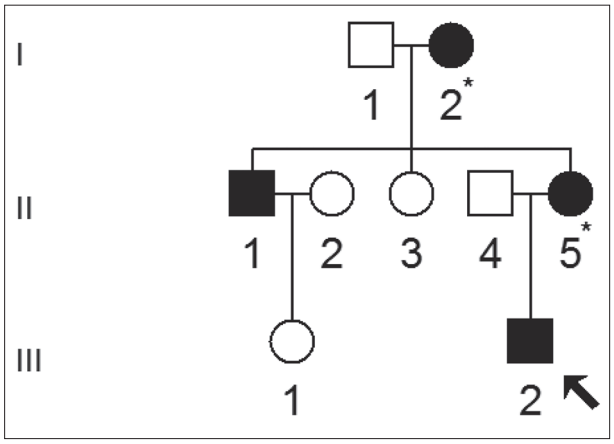

Figure 1. A three-generation Han Chinese family with AINHL. Hearing loss individuals are indicated by filled symbols. The arrow denotes the proband. Asterisks denote individuals who have a family history of exposure to amino glycosides.

Analysis of the Mutations in the Mitochondrial Genome. Genomic DNA was isolated from whole blood of participants using the Puregene DNA Isolation Kits (Gentra Systems, Minneapolis, MN, USA). First, three matrilineal relatives (I-2, II-5, III-2) and control subject's DNA fragments spanning the mitochondrial *12S rRNA* and *tRNA*$^{Ser(UCN)}$ genes were amplified by polymerase chain reaction (PCR) using oligodeoxynucleotides as previously described [12]. Subsequently, the entire mitochondrial genomes of the deaf patients (I-2, II-5, III-2) and controls were PCR-amplified in 24-overlapping fragments using the set of light-strand and the heavy-strand primers [12]. After PCR amplification, each fragment was purified and analyzed by direct sequencing in an ABI PRISM™ 3700 automated DNA sequencer using the BigDye Terminator Cycle sequencing reaction kit (Applied Biosystems Inc., Foster City, CA, USA). The sequence data were compared with the reversed Cambridge sequence to detect the mutations (GenBank accession no. NC_012920) [13].

Phylogenetic Analysis. A total of 17 vertebrates' mitochondrial genome sequences were used in the interspecific analysis. These include *Bos Taurus*, *Cebus albifrons*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Pongo pygmaeus*, *Pongo abelii*, *Papio hamadryas*, *Tarsius bancanus*, and *Xenopus laevis* (GenBank). The conservation index (CI) was calculated by comparing the human nucleotide variants with 16 other vertebrates. Notably, the CI ≥75.0% was regarded as having functional potential.

Mutational Screening for the *GJB2* Gene. The DNA fragments spanning the entire coding region of the *GJB2* gene were amplified by PCR using the following primers: forward (5'-TAT GA CAC TCC CCA GCA CAG-3') and reverse (5'-GGG CAA TGC TTA AAC TGG C-3'). Polymerase chain reaction amplification and sequencing analyses were performed as described elsewhere [14]. The results were compared with the wild-type *GJB2* sequence to identify the variants (GenBank Accession No. M86849).

Mutational Analysis of the *TRMU* Gene. A previous study showed that the *TRMU* exon 1 A10S variant may modulate the phenotypic manifestation of deafness-associated mitochondrial 12S rRNA mutations [15]. To see whether *TRMU* played an active role in deafness expression, we conducted a mutational screening for the *TRMU* exon 1 in matrilineal relatives in this pedigree and the healthy controls. The primers for detecting the A10S variant were as follows: forward (5'-ACA GCG CAG AAG AAG AGC

AGT-3') and reverse (5'-ACA ACG CCA CGA CGG ACG-3'). The PCR segments were analyzed and compared with the *TRMU* genomic sequence (Accession No. AF_448221).

RESULTS

Clinical Features of the Han Chinese Family with AINHL. All patients from the Han Chinese family lived in Hangzhou City of Zhejiang Province. The proband (III-2) was an infant born in Hangzhou First People's Hospital. As shown in Table 1 and Figure 2, the proband exhibited bilateral hearing impairment (90 dB right ear and 95 dB left ear). A comprehensive history and physical examination were performed to identify any syndromic findings, and the history of use of aminoglycosides. Moreover, we noticed that the proband's mother (II-5), a young woman at the age of 26 years; had been administered gentamicin (5 mg/kg/dose, 10 days) for fever when she was 18-years-old. She developed the profound hearing loss 2 months after the drug administration. It is interesting to note that two matrilineal relatives (I-2, II-5), who had a history of exposure to gentamicin and streptomycin, exhibited a severe hearing impairment in this maternal kindred, suggesting that the aminoglycosides may play an important role in this disorder.

Screening for the Mutations in the Mitochondrial Genome. The maternal transmission of hearing loss in this family suggested a mitochondrial involvement and led us to analyze the mitochondrial genome of matrilineal relatives (I-2, II-5, III-2) and the healthy subjects. We

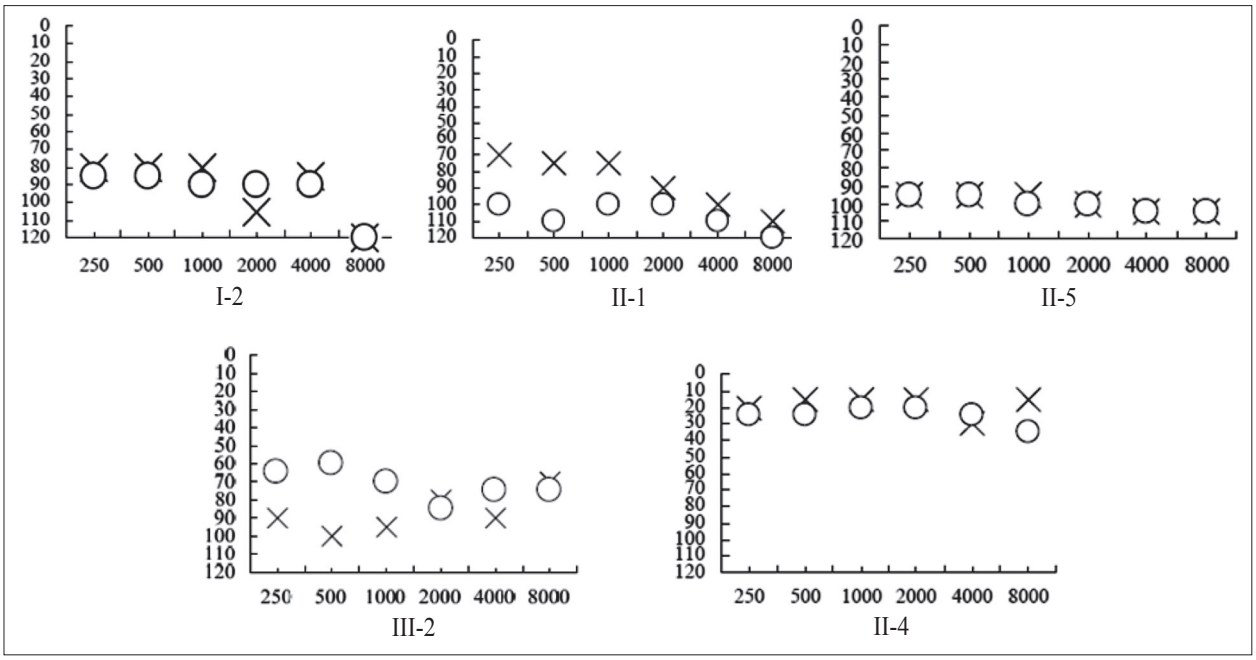

Figure 2. Air conduction audiogram of family members with the mitochondrial C1494T and G7444A pathogenic variants, subject II-4 was used as a control. Symbols: X: left ear, O: right ear.

Table 1. Summary of clinical data for several members of this family.

Subjects	I-2	II-1	II-5	III-2	II-4
Gender	female	male	female	male	male
Age when tested	50	26	30	1	39
Age at onset	46	18	25	1	–
Use of aminoglycoside	yes	no	yes	no	no
PTA (dB) right ear	90	90	100	85	25
PTA (dB) left ear	92	85	100	75	25
Level of hearing loss	profound	profound	severe	severe	normal

PTA: pure tone audiometry; dB: decibel.

first examined the known mtDNA pathogenic variants associated with deafness by PCR amplification (A1555G, C1494T, A7445G, T7510C and T7511C). As shown in Figure 3, the PCR-Sanger sequencing identified two known pathogenic variants: the C1494T in the *12S rRNA* gene and the G7444A in the *COI/tRNA*$^{Ser(UCN)}$ gene. However, we did not detect the presence of the A1555G pathogenic variant in the *12S rRNA* gene or the A7445G, T7510C, T7511C pathogenic variants in the *tRNA*$^{Ser(UCN)}$ gene in these matrilineal relatives.

To elucidate the molecular basis for maternally transmitted deafness, 24-overlapping DNA fragments spanning the entire mitochondrial genome were PCR-amplified and sequenced. The comparison of the resultant sequence with the Cambridge consensus sequence identified a set of polymorphisms, as shown in Table 2. Among these, there were five variants in the D-loop, two known variants in the *12S rRNA* and two variants in the *16S rRNA* genes, while other variants were mainly localized at protein-coding genes. Moreover, we noticed that there were four amino acid substitutions caused by corresponding mtDNA variants occurring in different polypeptides. These missense variants included the *ND1* C3497T (A64V), *A6* A8860G (T112A), *ND3* A10398G (T114A) and *Cytb* A15326G (T194A). These variants in rRNAs and polypeptides were further evaluated by phylogenetic analysis from other organisms including mouse [16], bovine [17] and *Xenopus laevis* [18]. However, none of the variants in the polypeptides were highly evolutionarily conserved and implicated to have functional consequences.

Mutational Analysis of the *GJB2* and *TRMU* Genes. To examine the role of the *GJB2* and *TRMU* genes in phe-notypic expression of the C1494T pathogenic variant, we performed the mutational screening of *GJB2* and *TRMU* exon 1 in matrilineal relatives who carried the C1494T pathogenic variant. However, none of the variants were found in the *GJB2* and *TRMU* genes, suggesting that the *GJB2* and *TRMU* genes may not play an important role in this Chinese family.

DISCUSSION

In this study, we have performed clinical, genetic and molecular characterization of a three-generation Han Chinese family with AINHL. Hearing impairment as a sole clinical phenotype was mostly present in the maternal lineage of this pedigree, suggesting that the mtDNA variant was the molecular basis for this disorder. As shown in Figure 1, this family exhibited a high penetrance of hearing loss, in particular, the penetrance of hearing loss in this family was 80.0 and 40.0%, when aminoglycoside was included and excluded.

Table 2. mtDNA sequence variants in this family with hearing impairment.

Gene	Position	Replacement	Conservation[a]	Previously Reported[b]
D-Loop	73	A>G	–	yes
	152	T>C	–	yes
	263	A>G	–	yes
	16223	C>T	–	yes
	16519	T>C	–	yes
12S rRNA	827	A>G	–	yes
	1438	A>G	–	yes
	1494	C>T	C/C/C/C	yes
16S rRNA	2706	A>G	A/G/A/A	yes
	3010	G>A	G/G/A/A	yes
ND1	3497	C>T (Ala→Val)	–	yes
	3970	C>T	–	yes
ND2	4883	C>T	–	yes
CO1	7444	G>A (Term→Lys)	–	yes
A6	8860	A>G (Thr→Ala)	–	yes
ND3	10398	A>G (Thr→Ala)	–	yes
	10400	C>T	–	yes
ND4	11719	G>A	–	yes
ND5	12705	C>T	–	yes
Cyt b	15301	G>A	–	yes
	15426	A>G (Thr→Ala)	T/M/I/I	yes

[a] Conservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).

[b] See the online mitochondrial genome database (http://www.mitomap.org).

Sequence analysis of the mitochondrial genome showed the presence of C1494T pathogenic variant in the *12S rRNA* gene, in fact, this pathogenic variant was first identified in a large Chinese family with AINHL [6]. Functional characterizations of cell lines derived from the C1494T pathogenic variant led to only mild mitochondrial dysfunction and sensitivity to aminoglycosides [11]. In addition, three affected matrilineal relatives exhibited the various severities, age at onset of hearing loss, suggesting that the C1494T pathogenic variant itself was insufficient to produce the clinical phenotypes; other modifying factors such as environmental factors, aminoglycosides, mitochondrial haplotype and nuclear genes were involved in deafness expression.

In addition, the mitochondrial haplotype has been shown to influence the penetrance of hearing loss associated with mtDNA primary mutations. In particular, mtDNA variants at positions 4216 and 13708, acting as second Lebers' hereditary optic neuropathy (LHON) variants, were implicated to increase the penetrance of the deafness-associated A7445G pathogenic variant [19]. Moreover, the T5628C variant in $tRNA^{Ala}$ was thought to have a modifying role in the phenotypic manifestation of the C1494T pathogenic variant in a Han Chinese family [20]. In this study, the sequence analysis of the entire mitochondrial genome identified a set of polymorphisms, apart from C1494T and G7444A pathogenic variants, other variants in the mitochondrial genome showed no evolutionary conservation. As shown in Figures 3 and 4, the G7444A pathogenic variant resulted in a read-through of the stop condon AGA of the COI message, thereby adding three amino acids (Lys-Gln-Lys) to the C-terminal of the polypeptide. Thus, the mutated polypeptide may retain a partial function. Alternatively, the G7444A pathogenic variant was adjacent to the site of 3' end endonucleolytic processing of the L-strand RNA precursor, spanning $tRNA^{Ser(UCN)}$ and *ND6* mRNA [19]. The previous study showed that the A7445G pathogenic variant in the precursor of $tRNA^{Ser(UCN)}$ led to a failure in the processing of the L-strand RNA precursor, thereby causing a marked decrease of the steady-state levels of $tRNA^{Ser(UCN)}$ and *ND6* mRNA [19]. Thus, the G7444A pathogenic variant, similar to the A7445G pathogenic variant, may also cause a defect in the processing of the L-strand RNA precursor, thus causing mitochondrial dysfunction. Although aminoglycoside was the predominant factor for hearing impairment, the G7444A pathogenic variant may also play an important role in the phenotypic expression of the C1494T pathogenic variant in this Chinese family. Moreover, due to the lack of any functional variants in *GJB2* and *TRMU* genes, those nuclear genes may not play active roles in deafness expression. Taken together, our data showed that the combination of the C1494T and G7444A pathogenic variants in the mitochondrial genome, as well as the aminoglycosides may account for the high penetrance and expression of hearing loss in this family.

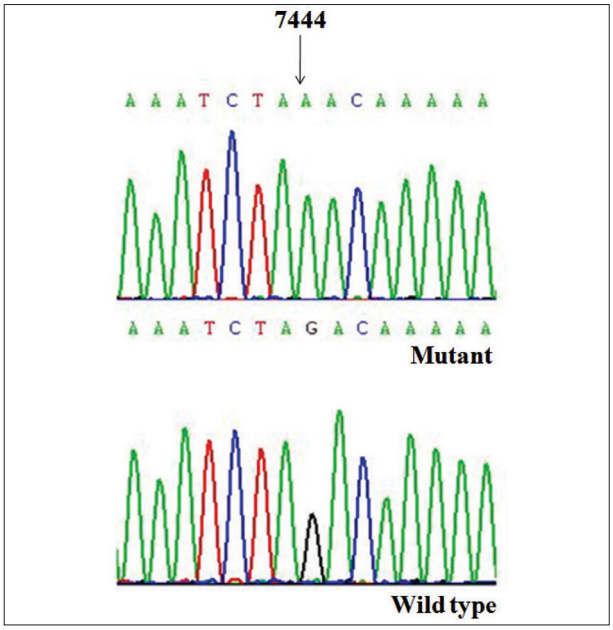

Figure 3. Identification G7444A pathogenic variant in the *CO1/tRNA*$^{Ser(UCN)}$ gene. Partial sequence chromatograms of COI/$tRNA^{Ser(UCN)}$ from affected individuals and the healthy control.

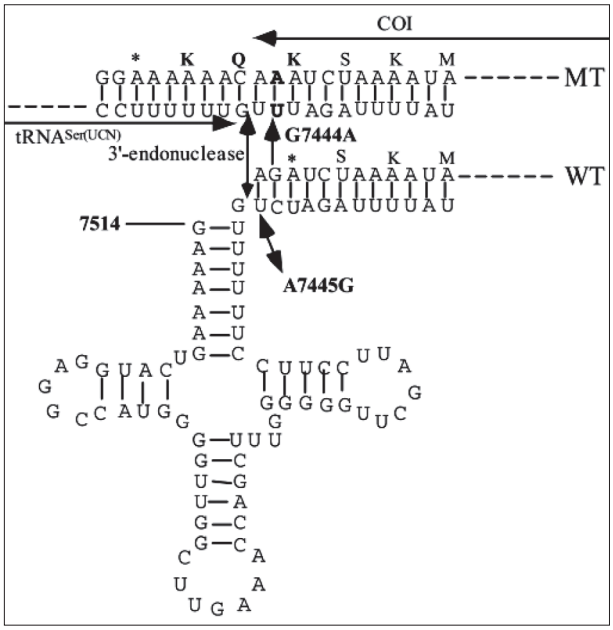

Figure 4. Location of deafness-associted mutations in $tRNA^{Ser(UCN)}$ and adjacent COI. The arrow indicates the A7445G and G7444A pathogenic variants in the precursor of this tRNA and adjacent sequence of COI from wild-type (WT) and mutant (MT).

In summary, our study indicated that the combination of the C1494T and G7444A pathogenic variants in the mitochondrial genome, combined with the aminoglycosides, may account for the high penetrance and expression of AINHL in this family. Moreover, the incomplete penetrance, variable degree of hearing loss in matrilineal relatives suggested that other modified factors, such as epigenetic modification and environmental factors may contribute to the clinical expression of hearing loss in this family.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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