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Screening for mitochondrial 12S rRNA C1494T mutation in 655 patients with non-syndromic hearing loss
An observational study

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Abstract
Mutations in mitochondrial DNA, especially in 12S rRNA gene, are the most important causes for hearing loss. In particular, the A1555G and C1494T mutations have been found to be associated with both aminoglycoside-induced and non-syndromic hearing loss in many families worldwide. To determine the frequency of C1494T mutation in deaf patients, in the current study, we screened this mutation in 655 patients with non-syndromic hearing loss and 300 control subjects. After PCR amplification of mitochondrial 12S rRNA gene and direct sequence analysis, we found that there were 2 patients carrying the C1494T mutation; however, this mutation was not detected in 300 healthy subjects. Further genetic counseling suggested that only 1 patient had an obvious family history of hearing impairment. Clinical evaluation showed that 3 of 10 matrilineal relatives suffered from hearing loss, with different age at onset of hearing loss. Molecular analysis revealed the presence of homoplasmic 12S rRNA C1494T and ND5 T12338C mutations, together with a set of polymorphisms belonging to human mitochondrial haplogroup F2. Interestingly, T12338C mutation resulted in the replacement of the first amino acid, a translation-initiating methionine with a threonine, shortening 2 amino acids of ND5 polypeptide. Moreover, this mutation is located in 2 nucleotides adjacent to the 3' end of the mt-tRNALeu(CUN) gene. Therefore, this mutation may alter ND5 mRNA metabolism and the processing of RNA precursors. Thus, the combination of T12338C and C1494T mutations may contribute to deafness expression in this family. Taken together, our data suggested that the C1494T mutation was the molecular basis for hearing loss, screening for the mitochondrial DNA pathogenic mutations was recommended for early detection, prevention, and diagnosis of mitochondrial deafness.

Abbreviations: AmAn = aminoglycosides antibiotics, CI = conservation index, mtDNA = mitochondrial DNA, PTA = pure-tone audiometry.

Keywords: C1494T mutation, frequency, hearing loss, T12338C mutation

1. Introduction

Mutations in human mitochondrial 12S rRNA gene were the important causes for hearing loss.[1] Of these, the homoplasmic

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A1555G and C1494T mutations in the highly conserved A-site of the mitochondrial 12S rRNA had been associated with both aminoglycoside-induced and non-syndromic hearing loss in many families worldwide.[2–7] In particular, the C1494T mutation was expected to form a novel U1494–1555A base pair, which was in the same position as the C1494–1555G pair created by the deafness-associated A1555G mutation.[8] Functional analysis of the cell lines carrying the C1494T mutation revealed a significant decrease in the rate of total oxygen consumption when compared with the controls, and exhibited ~38% to 43% decrease in the rate of mitochondrial protein labeling, which contributed to significant reductions in the rate of overall respiratory capacity.[8] However, the C1494T mutation was rarer than the A1555G mutation. Previous investigations suggested the highly variable penetrance and expressivity of hearing loss in several Han Chinese families carrying the C1494T mutation.[5–7] Matrilineal relatives within the families carrying this mutation showed a wide range of penetrances, severity, age-of-onset in hearing loss, and sensitivity to aminoglycosides antibiotics (AmAn), which indicated that the screening for the C1494T mutation was very important, especially for the individuals who had a family history of using AmAn.[9]

To determine the frequency of mitochondrial C1494T mutation in Shanghai City, China, in this study, we screened this mutation in 655 patients with non-syndromic hearing loss by using PCR and direct sequence analysis. In addition, we

performed the clinical, genetic, and molecular analysis of a 3-generation Han Chinese family carrying the C1494T mutation. To determine whether *GJB2* and *TRMU* genes played active roles in C1494T induced deafness, we screened the *GJB2* and *TRMU* mutations in matrilineal relatives in this family.

2. Materials and methods

2.1. Subjects and clinical examinations

From January 2016 to January 2019, a total of 655 patients were studied with non-syndromic hearing loss including 315 males and 340 females, aged from 22 to 77 years, with the average of 43 years. In addition, 300 control subjects (180 males and 120 females, aged from 20 to 52 years, with the average of 38 years) were recruited from Eye & ENT Hospital, Fudan University. The 655 patients expressed the non-syndromic hearing loss as sole clinical phenotype. Exclusion criteria were ongoing maintenance dialysis, a grave acute infectious disease, neoplastic disease, severe liver dysfunction, major surgery, a chronic inflammatory disease, and systemic lupus erythematosus. Furthermore, 300 control subjects were healthy individuals, who did not have any family history of mitochondrial diseases such as vision loss, diabetes mellitus, cardiovascular diseases, renal disorders, cancers, or neurological diseases.

The study protocol was approved by the Ethics Committee of Eye & ENT Hospital, Fudan University. Written informed consent was obtained from all participants prior to their participation in the study, in accordance with the Ethics Committee of Eye & ENT Hospital, Fudan University. An age-appropriate audiological examination was performed, and this examination included pure-tone audiometry (PTA) and/or auditory brainstem response, immittance testing, and distortion product otoacoustic emissions. The PTA was calculated from the sum of the audiometric thresholds at 250, 500, 1000, 2000, 4000, and 8000 Hz. The severity of hearing impairment was classified into 5 grades: normal <26 dB; mild = 26–40 dB; moderate = 41–70 dB; severe = 71–90 dB; and profound >90 dB.

2.2. Screening for the mitochondrial C1494T mutation

To detect the deafness-associated mitochondrial 12S rRNA C1494T mutation, we first extracted the genomic DNA of each participant using Puregene DNA Isolation Kits (Gentra Systems, Minneapolis, MN). Subject's DNA fragments spanning the entire mitochondrial 12S rRNA gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 618 to 635 and 1988 to 2007, as described in a previous study.[10] The amplified PCR fragment was purified and subsequently analyzed by ABI 3730 automated DNA sequencer. The resultant sequence data were compared with the default revised Cambridge Reference sequence (GenBank accession No. NC_012920.1) to screen the C1494T mutation.[11]

2.3. Molecular characterization of 1 Chinese family with C1494T mutation

As part of genetic screening program for the deafness-related mitochondrial C1494T mutation, 1 Han Chinese family, as shown in Figure 3, was ascertained in Eye & ENT Hospital, Fudan University. A comprehensive history and physical examination were performed to identify any syndromic findings, the history of the use of AmAn, and genetic factors related to the hearing impairment in members of this pedigree. In addition, we measured PTA of each deaf patient and the controls, using the methods as previously described.[7]

To assess the role of mitochondrial DNA (mtDNA) variants in the phenotypic expression of the C1494T mutation, the entire mitochondrial genomes of the proband and the matrilineal relatives (II-10, II-12, and III-9) carrying the C1494T mutation were PCR amplified in 24 overlapping fragments by use of sets of the light strand and the heavy strand oligonucleotide primers, as described elsewhere.[12] 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. The resultant sequence data were compared with the updated revised Cambridge Reference sequence (GenBank accession No. NC_012920.1).[11]

2.4. Phylogenetic conservation analysis

A total of 17 vertebrates mtDNA sequences were used in the interspecific analysis. The conservation index (CI) was then calculated by comparing the human mtDNA variants with 16 other vertebrates; we regarded the CI > 75% as having functional potential.[13]

2.5. Analysis of mutations in GJB2 gene

The DNA fragments spanning the entire coding region of *GJB2* gene were amplified by PCR using the following primers: forward: 5'-TATGACACTCCCCAGCACAG-3', reverse: 5'-GGGCAATGCTTAAACTGGC-3'. PCR amplification and subsequent sequencing analysis were performed as detailed elsewhere.[14] The results were compared with the wild-type *GJB2* sequence (GenBank accession No. M86849) to identify the mutations or variants.

2.6. Mutational analysis of TRMU gene

Previous studies showed that the *TRMU* A10S mutation may modulate the phenotypic manifestation of deafness-associated 12S rRNA mutations.[15] To determine whether *TRMU* played an active role in C1494T-induced deafness, we used the PCR-Sanger sequence to detect *TRMU* exon 1 A10S mutation. The PCR primer was: forward: 5'-ACAGCGCAGAAGAAGAGCAGT-3'; reverse: 5'-ACAACGCCACGACGGACG-3'. The PCR product was purified and analyzed by direct sequencing as described above. The result was compared with the wild-type *TRMU* sequence (GenBank accession No. AF448221).

2.7. Statistical analysis

SPSS 17.0 software (SPSS Inc.) was used for statistical analysis. Fisher exact test was used to assess the differences between groups. $P < .05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Genetic screening of mitochondrial C1494T mutations

To screen the deafness-associated mitochondrial C1494T mutation, a total of 655 deaf patients, as well as 300 controls, were recruited from Eye & ENT Hospital, Fudan University. We first performed PCR amplification of 12S rRNA gene and
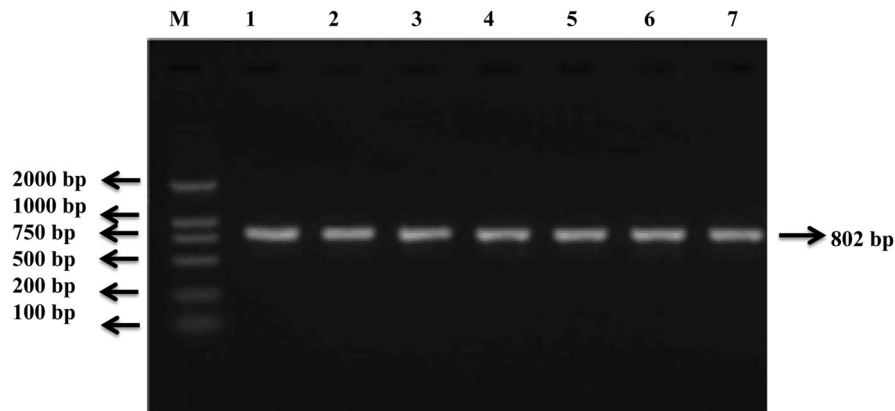
Figure 1. PCR amplification of mitochondrial 12S rRNA gene in deaf patients, arrow indicates the PCR product, which was 802-bp. M: DL2000 DNA marker.

subsequently sequenced the PCR products. As shown in Figure 1, after electrophoresis, the size of the PCR product spanning the entire human mitochondrial 12S rRNA gene was 802-bp. In addition, we identified 2 deaf patients carrying the C1494T mutation by direct sequence analysis (Fig. 2), with the frequency of 0.31% (2/655); however, we did not detect this mutation in 300 controls.

3.2. Clinical features of a Chinese family carrying the mtDNA C1494T mutation

In this case-control study for genetic screening of deafness-related mtDNA C1494T mutation, a Chinese pedigree with maternally transmitted hearing loss, as shown in Figure 3, was ascertained in the Department of Otology and Skull Base Surgery, Eye & ENT Hospital, Fudan University. The proband (III-9) was a 41-year-old man who lived in Shanghai City of P.R. China and went to the Otology Clinic of Eye & ENT Hospital, Fudan University, for treatment of hearing loss. In fact, he began to suffer from bilateral hearing loss at the age of 33, as shown in Figure 4 with a slope-shaped pattern. He had a profound hearing loss (100 dB in left ear and 103 dB in right ear), and his mother (II-12) exhibited the profound hearing loss after an administration with streptomycin (0.75 g/dose for 15 days) for high fever at the age of 49, while another family member (II-10) exhibited profound hearing impairment (96 dB in left ear and 95 dB in right ear); moreover, the proband's grandma (I-2), who was also a deaf patient, died 3 years ago.
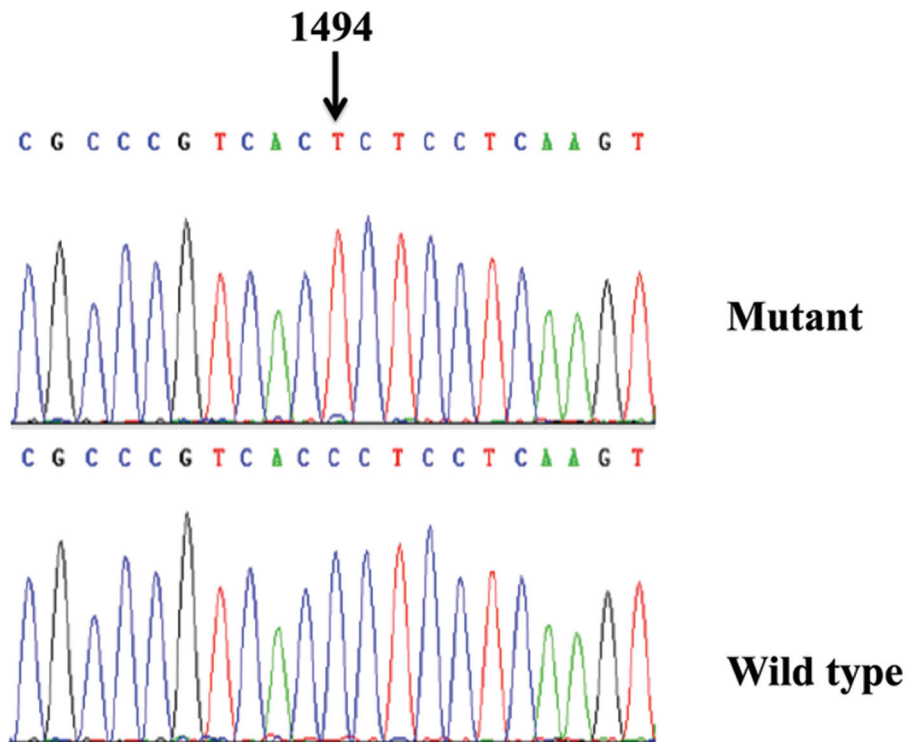

Figure 2. Identification of 12S rRNA C1494T mutation using PCR and sequence analysis.
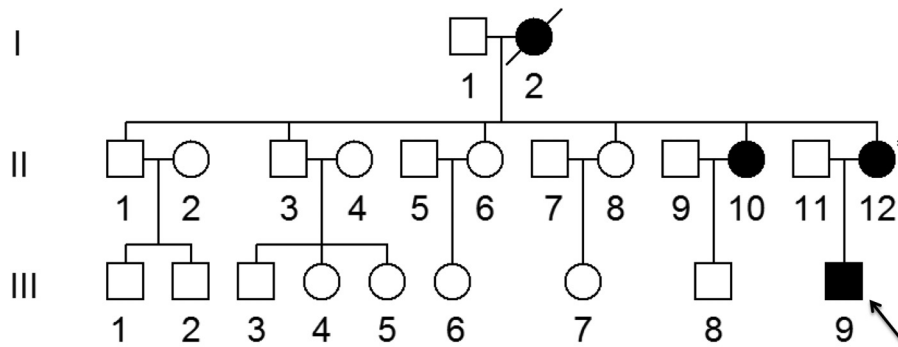
Figure 3. A Han Chinese pedigree with hearing loss. Hearing impaired individuals are indicated by filled symbols. An arrow denotes proband. Asterisks denote individuals who had a history of exposure to AmAn. AmAn = aminoglycoside antibiotics.

As can be seen in Figure 3, the family history was consistent with a maternal inheritance. Notice that the penetrance of hearing loss of this pedigree was 30% (including the AmAn) and 20% (excluding the AmAn). Moreover, comprehensive family history of the proband (III-9) and other members (II-10, II-12) of this family showed no other clinical abnormalities, including diabetes mellitus, cardiovascular diseases, muscular diseases, visual loss, cancer, and neurological disorders. In addition, there was a wide range in the age at onset of deafness in this family, varying from 33 to 52 years. The clinical and molecular features of the deaf patients and control are listed at Table 1.

3.3. Screening for the mtDNA variants

To assess the role of mtDNA variants in the phenotypic manifestation of mitochondrial C1494T mutation, we performed PCR amplification of the fragments spanning the complete mtDNA genome and subsequently sequenced the PCR products in matrilineal relatives (II-10, II-12, and III-9). Besides the identical C1494T mutation, as shown in Table 2, these matrilineal relatives exhibited a set of mtDNA polymorphisms belonging to human mitochondrial haplogroup F2.[16] Among these, 7 variants were localized at D-loop region, 2 variants at 12S rRNA, 2 in 16S rRNA, 1 deletion in non-coding region, whereas others were localized at oxidative phosphorylation-related genes. Notably, there were
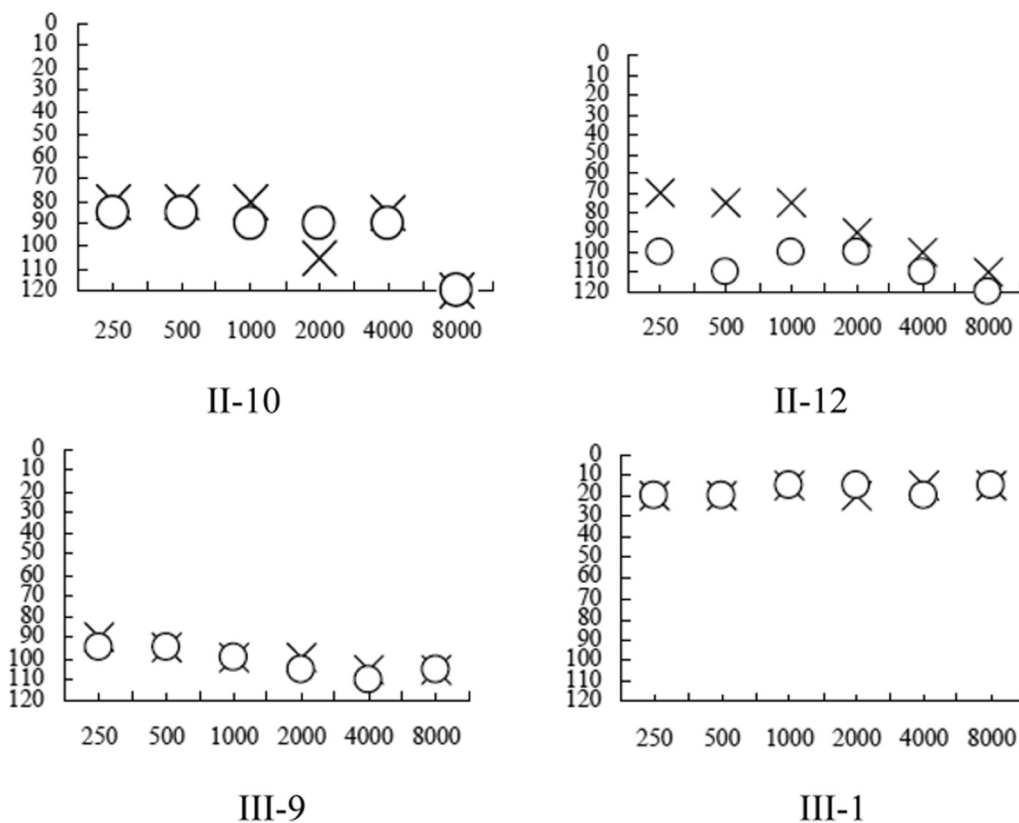

Figure 4. Air conduction audiogram of three affected subjects and control from this pedigree. Symbols: X, left ear; O, right ear.

Table 1

Summary of clinical and molecular data for several members in this family with hearing loss.

Subjects	Gender	Age at test	Age at onset	Use of AmAn	PTA (dB) Left ear	PTA (dB) Right ear	Level of hearing loss
II-10	Female	66	52	No	96	95	Profound
II-12	Female	60	49	Yes	84	106	Profound
III-9	Male	41	33	No	100	103	Profound
III-1	Male	35	/	No	18	16	Normal

AmAn = aminoglycoside antibiotics; PTA = pure-tone audiometry.

8 missense mutations, including the *ATP8* C8414T (Leu to Phe), *ATP6* A8701G (Thr to Ala) and A8860G (Thr to Ala), *ND5* T12338C (Met to Thr) and G13708A (Ala to Thr), *CytB* C14766T (Thr to Ile) and A15326G (Thr to Ala). To assess the pathogenicity of these mtDNA variants, a phylogenetic approach was used to analyze the CIs of each mtDNA variant, including the sequences from mouse,[17] bovine,[18] and *Xenopus laevis*.[19] We found that only the C1494T and *ND5* T12338C mutations were highly conserved, whereas other mtDNA variants showed no evolutionary conservation. Further genetic analysis revealed that the C1494T and T12338C mutations were not detected in 300 control subjects ($P < .05$ for all), suggesting that these mutations may have functional potential.

As shown in Figure 5, the T12338C mutation resulted in the replacement of the first amino acid, translation-initiating methionine with a threonine. Thus, the truncated protein was expected to be shortened by 2 amino acids.[20]

3.4. Genotyping analysis of GJB2 gene

Mutations in *GJB2* gene were the important causes for non-syndromic hearing loss.[21] To examine the contributions of *GJB2* to deafness expression, we conducted the mutational screening of exons of *GJB2* in matrilineal relatives (II-10, II-12, and III-9) from this pedigree. However, no functional variants were found in these subjects with hearing loss.

3.5. Analysis of mutations in TRMU gene

Previous studies indicated that the *TRMU*, a nuclear modified gene that was involved in mt-tRNA modification, contributed to the phenotypic manifestation of deafness expression in Israeli/European pedigrees.[15] To determine whether the A10S mutation also played an active role in C1494T expression, we performed the mutational screening of *TRMU* A10S mutation in matrilineal relatives (II-10, II-12, and III-9) of this family. But

Table 2

MtDNA sequence variants in this Chinese pedigree with non-syndromic hearing loss.

Gene	Position	Replacement	Conservation (H/B/M/X)[a]	rCRS[b]	GenBank frequency[c]
D-loop	73	A to G		E	0.76
	263	A to G		A	0.948
	310	T to TC		T	0.00
	489	T to C		T	0.257
	16140	T to C		T	0.019
	16189	T to C		T	0.259
	16519	T to C		T	0.631
12S rRNA	750	A to G	A/G/G/-	A	0.983
	1494	C to T	C/C/C/C	C	0.00
16S rRNA	2706	A to G	A/G/A/A	A	0.79
	3010	G to A	G/G/A/A	G	0.144
ND1	3970	C to T		C	0.037
ND2	4769	A to G		A	0.977
	5201	T to C		T	0.001
CO1	7028	C to T		C	0.809
CO2	7822	A to G		A	0.00
NC7	8271-8279	9-dp del			0.041
ATP8	8414	C to T (Leu to Phe)	L/F/M/W	C	0.039
ATP6	8701	A to G (Thr to Ala)	T/S/L/Q	A	0.336
	8860	A to G (Thr to Ala)	T/A/A/T	A	0.987
CO3	9540	T to C		T	0.336
ND3	10398	A to G (Thr to Ala)	T/T/T/A	A	0.443
	10400	C to T		C	0.213
ND5	12338	T to C (Met to Thr)	M/M/M/M	T	0.003
	12705	C to T		C	0.418
	13708	G to A (Ala to Thr)	A/L/A/A	G	0.071
CytB	14766	C to T (Thr to Ile)	T/S/I/S	C	0.77
	15301	G to A		G	0.287
	15326	A to G (Thr to Ala)	T/M/I/I	A	0.987
	15749	C to T		C	0.00

Please see human Mitomap (https://mitomap.org/MITOMAP) database.
B = bovine, H = human, M = mouse, mtDNA = mitochondrial DNA, rCRS = revised Cambridge Reference Sequence, X = *Xenopus laevis*, were used for the evolutionary conservation analysis.
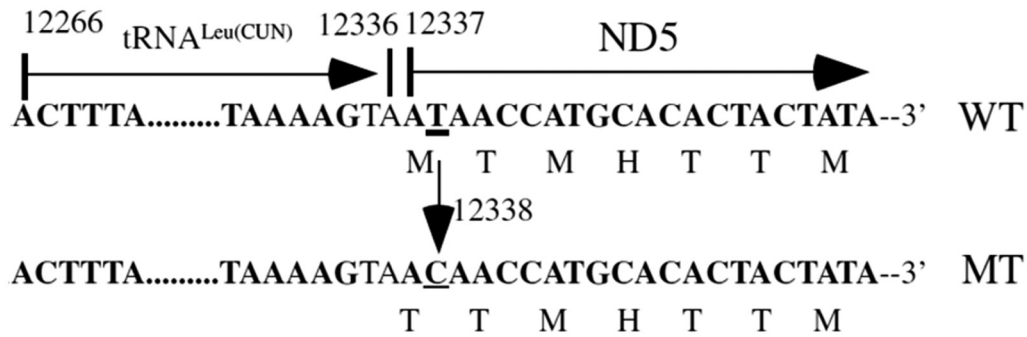
Figure 5. A schema of mtDNA sequence at position 12338 and adjacent sequence of *ND5* and tRNA[Leu(CUN)] from wild-type (WT) and mutant (MT). Arrow indicates the position of the T12338C mutation.

we did not detect any variants in *TRMU* exon 1, suggesting that *TRMU* may not play an important role in C1494T-induced deafness.

4. Discussion

In this study, we screened the frequency of deafness-associated mtDNA C1494T mutation in 655 patients with non-syndromic hearing loss and 300 control subjects. In fact, the C1494T mutation was expected to create a new U1494–1555A base pair at the highly conserved A-site of the12S rRNA [22] and was crucial for subunit association either by RNA-protein or by RNA-RNA interactions.[23] In addition, subjects with this mutation were sensitive to AmAn;[24] therefore, screening for the C1494T mutation was recommended, especially for those who had a family history of using AmAn.[25] In our study, we identified 2 deaf patients with the C1494T mutation; nevertheless, this mutation was not detected in 300 control subjects.

Among these deaf cases harboring the 12S rRNA mutation, only 1 patient with C1494T mutation manifested the maternally inherited pattern of hearing impairment. As shown in Figure 3, hearing loss as a sole clinical phenotype occurred in matrilineal relatives but not in other members in this family. The penetrances of hearing loss in this family were 30% and 20%, respectively, when the AmAn was included and excluded. In fact, the C1494T mutation was first reported in a large Chinese family with AmAn-induced and non-syndromic hearing loss.[5] Later, this mutation was found in several genetically unrelated Chinese pedigrees with hearing impairment [6,7,9,24,25] and Spanish families with hearing loss.[26] Matrilineal relatives within and among families carrying the C1494T mutation exhibited a wide range of penetrance, severity, and age-of-onset in hearing loss. These results strongly indicated that the C1494T mutation itself was insufficient to produce the clinical phenotype. Therefore, other modifiers such as AmAn, nuclear genes, and mitochondrial haplogroups may modulate the phenotypic manifestations of the C1494T mutation.[8,9,27] However, the absence of any functional variants in nuclear genes (*GJB2* and *TRMU*) suggested that nuclear modified genes may not have potential roles in the development of hearing impairment associated with the C1494T mutation.

Moreover, we noticed that many disorders associated with mitochondrial dysfunction were caused by inherited heteroplasmic point mutations.[28] For example, Yan et al reported a novel heteroplasmic mt-tRNAHis T12201C mutation that was associated with maternally inherited hearing impairment.[29] In

addition, the heteroplasmic A1555G mutation had been reported in a family with maternally inherited cardiomyopathy.[30] The A1555G mutation was found to be heteroplasmy in several tissues including the heart muscle. Moreover, the heteroplasmic A1555G mutation was identified in Chinese families with hearing loss.[31] However, in our study, the C1494T mutation was in homoplasmic form; to date, by literature searching, there are no reports regarding the deafness-associated heteroplasmic C1494T mutation.

In addition, it has been suggested that mitochondrial haplogroup F1 specific variant tRNAAla T5628C and haplogroup C4a1 specific variant CO1/ tRNASer(UCN) G7444A may increase the penetrance and expressivities of the C1494T-induced deafness.[9] Sequencing analysis of the whole mitochondrial genomes from the matrilineal relatives of this family identified a set of polymorphisms, which belonged to East Asian haplogroup F2.[16] Of these variants, the *ND5* T12338C was of special interest; as shown in Figure 5, this mutation resulted in the replacement of the first amino acid, translation-initiating methionine with a threonine in the *ND5* polypeptide. In fact, the first methionine in *ND5* polypeptide was an extraordinarily conserved residue in every sequenced *ND5* from bacteria to human mitochondria.[20] As a result, the truncated *ND5* protein was expected to be shortened by 2 amino acids. Moreover, the T12338C mutation was located in 2 nucleotides adjacent to the 3' end of the tRNALeu(CUN).[32] Therefore, it can be speculated that the T12338C mutation, similar to the *ND1* T3308C mutation, [33] may cause a decrease in *ND5* mRNA level as well as alter the processing of RNA precursors, thereby leading to a reduction in the tRNALeu(CUN) level. Moreover, previous studies showed that cell lines derived from patients carrying the T12338C mutation significantly reduced the *ND5* polypeptide, perturbed assemble and activity of complex I. [34] Furthermore, the T12338C mutation promoted apoptosis, increased the ROS production, and diminished ATP level and mitochondrial membrane potential.[34] As a result, this mutation altered the respiratory function, leading to the mitochondrial dysfunction which was responsible for hearing loss. The functional significance of the T12338C mutation in terms of mitochondrial physiology was further supported by recent observations that this mutation was associated with polycystic ovary syndrome,[35] Leber's hereditary optic neuropathy,[36] and hypertrophic cardiomyopathy.[37] Thus, the *ND5* T12338C mutation may modulate the clinical manifestation of deafness-associated 12S rRNA C1494T mutation in this pedigree.

In conclusion, our study indicated that the mtDNA C1494T mutation was the important risk factor for AmAn-induced and non-syndromic hearing loss, and screening for the C1494T mutation was recommended, especially for the individuals who had a history of using AmAn. The main limitation of the current study was the relatively small sample size and the lack of analyzing the tissue-specific mtDNA mutations; further studies including more deaf patients and the analysis of muscle biopsy are needed to verify the conclusion.

Author contributions

Conceptualization: Zhen Gao.
Data curation: Zhen Gao and Ya-Sheng Yuan.
Formal analysis: Ya-Sheng Yuan.
Supervision: Zhen Gao.
Writing – original draft: Zhen Gao and Ya-Sheng Yuan.

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