

# The Association Between Mitochondrial tRNA<sup>Glu</sup> Variants and Hearing Loss: A Case-Control Study

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**Purpose:** This study aimed to examine the frequencies of mt-tRNA<sup>Glu</sup> variants in 180 pediatric patients with non-syndromic hearing loss (NSHL) and 100 controls.

**Methods:** Sanger sequencing was performed to screen for mt-tRNA<sup>Glu</sup> variants. These mitochondrial DNA (mtDNA) pathogenic mutations were further assessed using phylogenetic conservation and haplogroup analyses. We also traced the origins of the family history of probands carrying potential pathogenic mtDNA mutations. Mitochondrial functions including mtDNA content, ATP and reactive oxygen species (ROS) were examined in cells derived from patients carrying the mt-tRNA<sup>Glu</sup> A14692G and CO1/tRNA<sup>Ser(UCN)</sup> G7444A variants and controls.

**Results:** We identified four possible pathogenic variants: m.T14709C, m.A14683G, m.A14692G and m.A14693G, which were found in NSHL patients but not in controls. Genetic counseling suggested that one child with the m.A14692G variant had a family history of NSHL. Sequence analysis of mtDNA suggested the presence of the CO1/tRNA<sup>Ser(UCN)</sup> G7444A and mt-tRNA<sup>Glu</sup> A14692G variants. Molecular analysis suggested that, compared with the controls, patients with these variants exhibited much lower mtDNA copy numbers, ATP production, whereas ROS levels increased ( $p < 0.05$  for all), suggesting that the m.A14692G and m.G7444A variants led to mitochondrial dysfunction.

**Conclusion:** mt-tRNA<sup>Glu</sup> variants are important risk factors for NSHL.

**Plain Language Summary:** The main aim of our study was to explore the association between the mt-tRNA<sup>Glu</sup> variants and hearing loss. We found that m.T14709C, m.A14683G, m.A14692G and m.A14693G variants were associated with hearing impairments, these variants localized at extremely conserved nucleotides of mt-tRNA<sup>Glu</sup> and may result a failure in tRNA metabolism, furthermore, patients with mt-tRNA<sup>Glu</sup> variants exhibited much lower levels of mtDNA copy number, ATP as compared with controls, whereas ROS increased. As a result, mt-tRNA<sup>Glu</sup> variants may serve as biomarkers for mitochondrial deafness, and screening for tRNA<sup>Glu</sup> variants is recommended for early detection and diagnosis of mitochondrial deafness.

**Keywords:** deafness, mitochondrial tRNA<sup>Glu</sup> variants, pediatrics, tRNA metabolism

## Introduction

Deafness is one of the most common human health problems, affecting one in 1000 newborns.<sup>1</sup> It is anticipated that the number of deaf people will be more than 28 million by the end of 2030 in the US.<sup>2</sup> To date, the etiology of hearing loss is not well understood, but increasing evidence has suggested that deafness can be caused by genetic and environmental factors.<sup>3</sup> In fact, the genetic impact has been found more than 50% patients with hearing loss. To date, around 124 genes, as well as 1000 variants have been identified to be related to hearing loss (<https://hereditaryhearingloss.org/>).<sup>4</sup> In addition to nuclear genes, mitochondrion is very important organelle whose primary role is to generate ATP via oxidative

phosphorylation (OXPHOS). Moreover, mitochondria have their own genetic codes, named mtDNA, which is 16,569 bp in length.<sup>5</sup> Variants in mtDNA are important causes of aminoglycoside-induced and non-syndromic hearing loss (AINSHL) in many families worldwide.<sup>6–8</sup> In addition to the well-known m.A1555G or m.C1494T mutation, mitochondrial tRNA (mt-tRNA) gene is another hot spot for pathogenic variants associated with deafness.<sup>9</sup> Variants in mt-tRNA genes may alter tRNA structure and functions, including the processing of RNA precursors, modification of specific nucleotides, and maintenance of secondary and tertiary structures. Failure of mt-tRNA metabolism and protein synthesis caused by these variants may lead to mitochondrial dysfunction, which is involved in deafness.<sup>10,11</sup> More recently, several mt-tRNA variants had been reported to be related to hearing loss, including mt-tRNA<sup>Ile</sup> A4317G,<sup>12</sup> mt-tRNA<sup>Cys</sup> C5783T,<sup>13</sup> mt-tRNA<sup>Ser(UCN)</sup> T7505C,<sup>14</sup> however, the pathophysiology of these variants remained poorly elucidated.

In the present study, we screened for the frequencies of mt-tRNA<sup>Glu</sup> variants in a cohort of 180 children with NSHL and 100 controls using PCR and direct sequencing analyses. Phylogenetic conservation and mtDNA haplogroup analyses were performed to assess the pathogenicity of mt-tRNA variants. We also performed clinical, genetic, molecular, and biochemical assessments of a Chinese pedigree carrying the putative pathogenic mt-tRNA<sup>Glu</sup> variant.

## Materials and Methods

### Patients

From January 2020 to January 2023, 180 deaf children including 100 males and 80 females, aged from 1 to 5 years, with an average age of 3.5 years were recruited from Hangzhou First People's Hospital and Quzhou People's Hospital. Furthermore, 100 healthy subjects including 60 males and 40 females, aged 3–8 years, with an average age of 6 years, were enrolled as controls. This study was reviewed by the ethics committees of Hangzhou First People's Hospital (Approval No: 2020–285-01) and Quzhou People's Hospital (Approval No: 2021–028). Informed consent was obtained by each individual (parents) enrolled in the study. All the procedures were performed with the principles of the Declaration of Helsinki.

### Audiological Examinations

Age-appropriate audiological and neurotological examinations of hearing loss were performed as detailed previously, including pure-tone audiometry (PTA), auditory brainstem response, acoustic immittance measurement and distortion product otoacoustic emission.<sup>15</sup> The PTA was performed in a sound-controlled room at frequencies ranging from 250 to 8000 Hz, as suggested in a recent study.<sup>16</sup> The levels of hearing loss were divided into five grades according to a previous study: normal: <20 decibels (dB); mild: 20–40 dB; moderate: 41–70 dB; severe: 71–95 dB; and profound >95 dB.<sup>17</sup>

### Mutational Analysis of Mt-tRNA<sup>Glu</sup> Gene

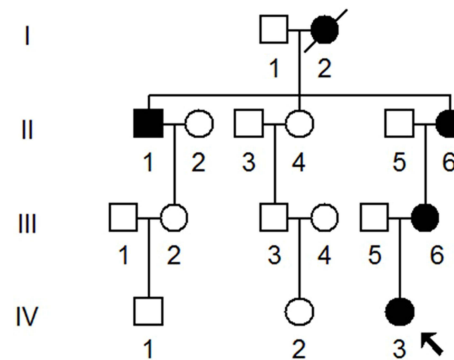
To screen for mt-tRNA<sup>Glu</sup> variants, we first performed PCR amplification of mt-tRNA<sup>Glu</sup> in all participants enrolled in this study. The primer sequence for amplification of mt-tRNA<sup>Glu</sup> was: forward-5'-GCA TAA TTA AAC TTT ACT TC-3', reversed-5'-AGA ATA TTG AGG CGC CAT TG-3'. After amplification, the PCR products were purified and analyzed by direct Sanger sequencing. The sequence data were then compared with the revised Cambridge reference sequences (rCRS) to detect the mt-tRNA<sup>Glu</sup> variants (GenBank accession number: NC\_012920.1).<sup>18</sup>

### Data Analysis

A total of 14 species were selected for conservation analysis, and the conservation index (CI) was calculated by comparing the human mtDNA with that of the other 13 vertebrates. A CI of  $\geq 75\%$  was considered functional significance.<sup>19</sup>

### Molecular Characterization of One Chinese Family with Mt-tRNA<sup>Glu</sup> A14692G Variant

We ascertained a Chinese pedigree (Figure 1) via Hangzhou First People's Hospital. The entire mitochondrial genomes of matrilineal relatives (II-1, II-6, III-6, and IV-3) was PCR-amplified using 24 primers and sequenced. The mtDNA variants were detected by comparison with the rCRS (GenBank accession number: NC\_012920.1).<sup>18</sup> Furthermore, Phylotree (<http://www.phylotree.org/>) and East Asian phylogeny were used to determine the mtDNA haplogroup of this pedigree.<sup>20,21</sup>



**Figure 1** One Han Chinese family with NSHL, arrow indicated the proband.

## Mutational Analysis of Common Deafness-Related Genes

To further explore the roles of nuclear genes variants to deafness expression, we conducted mutational screening of common deafness associated genes (*GJB2*, *GJB3*, *GJB6*, *TRMU* and *SLC26A4*) in matrilineal relatives (II-1, II-6, III-6 and IV-3), together with other healthy subjects from this family. The primers for *GJB2* amplification were: forward-5'-TAT GAC ACT CCC CAG CAC AG-3', and reverse-5'-GGG CAA TGC TTA AAC TGG C-3'.<sup>22</sup> The primers for *GJB3* were: forward-5'-GTC ACC TAT TCA TTC ATA CGA TGG-3' and reverse-5'-TCA CTC AGC CCC TGT AGG AC-3'.<sup>23</sup> The primer sequences for amplification of the *GJB6* were: forward-5'-CCT TAA AAT AAA GTT GGC TTC AG-3', reverse-5'-GGA ACT TTC AGG TTG GTA TTG-3'.<sup>24</sup> The primer sequences for *TRMU* were: forward-5'-ACA GCG CAG AAG AAG AGC AGT-3', reverse-5'-ACA ACG CCA CGA CGG ACG-3'.<sup>25</sup> The five primer sequences for *SLC26A4* were as follows: forward-5'-CGT GTA GCA GCA GGA AGT AT-3', and reverse-5'-TTA AAT AAA AAA GAC TGA CT-3'; forward-5'-TGG GGA AAA AGG ATG GTG GT-3', and reverse-5'-CCA ACC CCT TCT TTA GCT GA-3'; forward-5'-GCA GGA TAG CTC AAG GAA TT-3', and reverse-5'-TCA TCA GGG AAA GGA AAT AA-3'; forward-5'-TCT CCT TGA TGT CTT GCT TA-3', and reverse-5'-CCC ATG TAT TTG CCC TGT TG-3'; and forward-5'-CTG GGC AAT AGA ATG AGA CT-3', and reverse-5'-ATC TGT AGA AAG GTT GAA TA-3'.<sup>26</sup> After PCR amplification and direct Sanger sequencing, the data were compared with the wild-type sequences of *GJB2*, *GJB3*, *GJB6*, *TRMU* and *SLC26A4* (GenBank accessible numbers: M86849, AF052692, NG\_008323, AF448221 and NM\_000441.1, respectively) to detect variants.

## Qualification of mtDNA Copy Number

mtDNA copy number was assessed using real-time PCR in four patients with NSHL (II-1, II-6, III-6 and IV-3) carrying the m. A14692G and m.G7444A variants, as well as in four control subjects (II-2, II-5, III-1 and III-5). The primers used for amplification of mt-*ND1* were as follows: forward: 5'-AAC ATA CCC ATG GCC AAC CT-3', revised: 5'-AGC GAA GGG TTG TAG TAG CCC-3'. The primers for the amplification of  $\beta$ -globin were as follows: forward: 5'-GAA GAG CCA AGG ACA GGT AC-3'; reversed: 5'-CAA CTT CAT CCA CGT TCA CC-3'. Real-time PCR was performed using a LightCycler<sup>®</sup> 480II system (Roche Diagnostics GmbH, Mannheim Germany) and each measurement was repeated in triplicate.

## Isolation of Polymorphonuclear Leukocytes (PMNs)

The PMNs from four subjects with NSHL (II-1, II-6, III-6 and IV-3) carrying the m. A14692G and m.G7444A variants, as well as in four control subjects (II-2, II-5, III-1 and III-5) without these variants were isolated according to our previous study.<sup>27</sup> PMNs were counted in a Neubauer chamber and cultured in Hanks' buffered salt solution.

## ATP Analysis

The cellular ATP levels in eight cells were measured by using the Cell Titer-Glo<sup>®</sup> Luminescent Cell Viability Assay kit (Promega), according to the protocols provided by the manufacturer.<sup>28</sup>

## Analysis of ROS Production in Cells

The fluorometry was used to determine the ROS level. Approximately  $2 \times 10^6$  cells were first incubated with the fluorescent probe 2,7-dichlorodihydrofluorescein (DCFH) for about 30 min, subsequently, the cells were analyzed by fluorescence plate reader, as mentioned in our previous study.<sup>29</sup>

## Statistical Analysis

Statistical significance was evaluated by an independent Student's *t*-test using SPSS software (version 22.0; SPSS, Inc., Chicago, IL, USA).  $p < 0.05$  was considered statistically significant.

## Results

### Screening for Deafness-Associated Mt-tRNA<sup>Glu</sup> Variants

To determine the frequencies of the deafness-related mt-tRNA<sup>Glu</sup> variants, we first performed PCR amplification of mt-tRNA<sup>Glu</sup> gene in all participants (Figure 2), which indicated that the targeted PCR product was 1050-bp. Sanger-sequencing suggested that four variants were identified in the current study: m.T14709C, m.A14683G, m.A14692G, and m.A14693G (Table 1). Among these, the m.T14709C variant was found in two subjects with hearing loss (1.11%), the m.A14692G and m.A14683G variants were identified in one individual with deafness (0.56%), and the m.A14693G variant occurred in four patients with hearing loss (2.22%). These variants were not detected in the control groups.

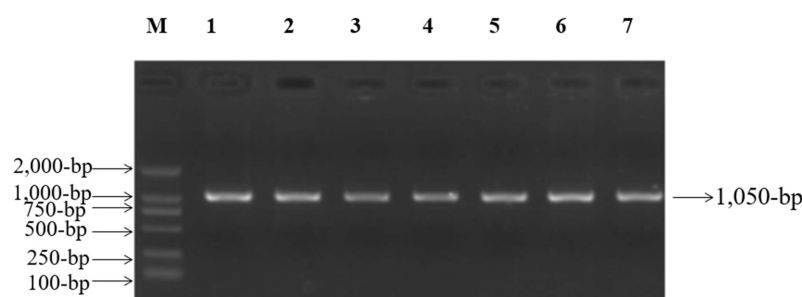
### Molecular Features of Deafness-Associated Mt-tRNA<sup>Glu</sup> Variants

Next, we performed phylogenetic conservation analysis of the variants identified in this study. For this purpose, a total of 14 species of mtDNA sequences were selected. As shown in Table 1 and Figure 3, we found that all variants were highly conserved (CI=100% for all).

Structurally, as shown in Figure 4, the m.T14709C variant was located at the anticodon stem of mt-tRNA<sup>Glu</sup> gene (position 37), whereas the m.A14683G variant was localized at the T $\psi$ C loop of mt-tRNA<sup>Glu</sup> gene (position 64), which created a novel base-pairing (64A-50G) and may change the secondary structure of this mt-tRNA. Moreover, both m.A14692G and m.A14693G variants occurred in the T $\psi$ C loop (positions 55 and 54). Nucleotide at positions 55 and 54 are often chemically modified and thus contribute to the structural and functional importance of mt-tRNA.<sup>30,31</sup> Therefore, it can be anticipated that the alteration of mt-tRNA structure by these variants may lead to the failure of mt-tRNA<sup>Glu</sup> metabolism.

### Clinical and Molecular Analysis of One Pedigree with NSHL

We ascertained a Chinese pedigree with maternally inherited NSHL at Hangzhou First People's Hospital. Of the seven matrilineal relatives, four had suffered varying degrees of hearing loss. The proband (IV-3), aged 2 years, suffered from profound bilateral hearing loss (105 dB for the right ear and 116 dB for the left ear). As shown in Figure 5 and Table 2, subjects (II-1, II-6 and III-6) were also deaf. Intriguingly, none of the patients had a history of aminoglycoside use.



**Figure 2** PCR amplification of mt-tRNA<sup>Glu</sup> gene in subjects with NSHL, arrow indicated the PCR product, which was 1050-bp.

**Table I** Molecular Characterization of Deafness-Associated Mt-tRNA<sup>Glu</sup> Mutations

tRNA Species	Position	Alternation	Homoplasmy/Heteroplasmy	Location	Numbering in tRNA <sup>Glu</sup>	CI (%)	Numbers of 180 Deaf Patients (%)	Numbers of 100 Controls (%)
tRNA <sup>Glu</sup>	14,709	T to C	Heteroplasmy	Anticodon stem	37	100	2 (1.11)	0
tRNA <sup>Glu</sup>	14,692	A to G	Homoplasmy	TψC loop	54	100	1 (0.56)	0
tRNA <sup>Glu</sup>	14,693	A to G	Homoplasmy	TψC loop	55	100	4 (2.22)	0
tRNA <sup>Glu</sup>	14,683	A to G	Homoplasmy	TψC loop	64	100	1 (0.56)	0

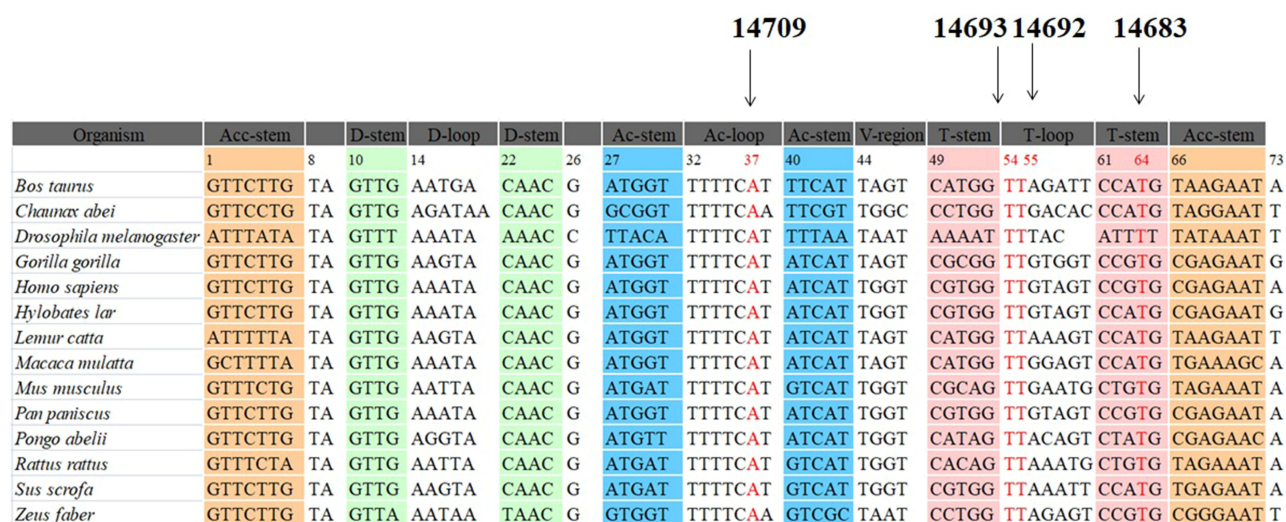
**Abbreviation:** CI, conservation index.

## Screening for mtDNA Variants

Because of maternal transmission, which suggested that mtDNA dysfunction may be involved in the pathogenesis of NSHL. We conducted mutational screening of the entire mitochondrial genome of four matrilineal relatives (II-1, II-6, III-6 and IV-3). As shown in Table 3, PCR-Sanger sequencing revealed a set of genetic polymorphisms belonging to mitochondrial haplogroup B4c1.<sup>21</sup> Of these, there were eight variants in the D-loop, two variants in 12S rRNA, three variants in 16S rRNA, and two variants in mt-tRNA genes (m.G7444A and m.A14692G), while the rest were mainly located in protein-coding genes. In addition, seven missense variants were identified: *ND2* C5178A (Leu to Met), *COI*/tRNA<sup>Ser(UCN)</sup> G7444A (Ter to Lys), *A6* A8701G (Thr to Ala) and A8860G (Thr to Ala), *ND3* A10398G (Thr to Ala), *Cytb* C14766T (Thr to Ile) and A15326G (Thr to Ala). These missense variants and tRNA variants were further assessed by phylogenetic analysis, especially in bovine,<sup>32</sup> mouse<sup>33</sup> and *Xenopus laevis*.<sup>34</sup> We found that except for the m.G7444A and m.A14692G variants (Figures 6 and 7), others were not well conserved, suggesting that these variants may be involved in deafness expression.

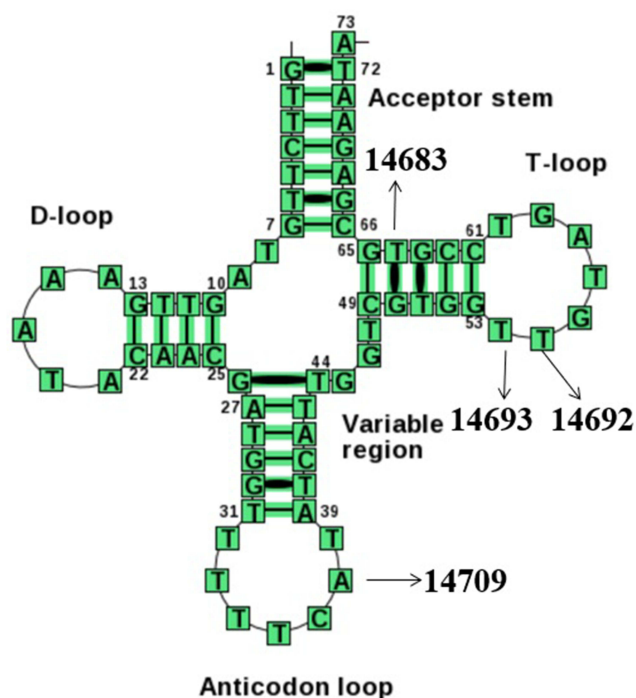
## Mutational Analysis of Nuclear Genes

Variants in *GJB2*, *GJB3*, *GJB6*, *TRMU* and *SLC26A4* played active roles in NSHL.<sup>35,36</sup> To examine the contributions of these gene variants to NSHL, we analyzed the exons of these genes in matrilineal relatives of this pedigree (II-1, II-6, III-6, and IV-3), as well as in controls. However, we did not identify any variants of these genes, suggesting that nuclear modified genes may not play a role in hearing impairment.

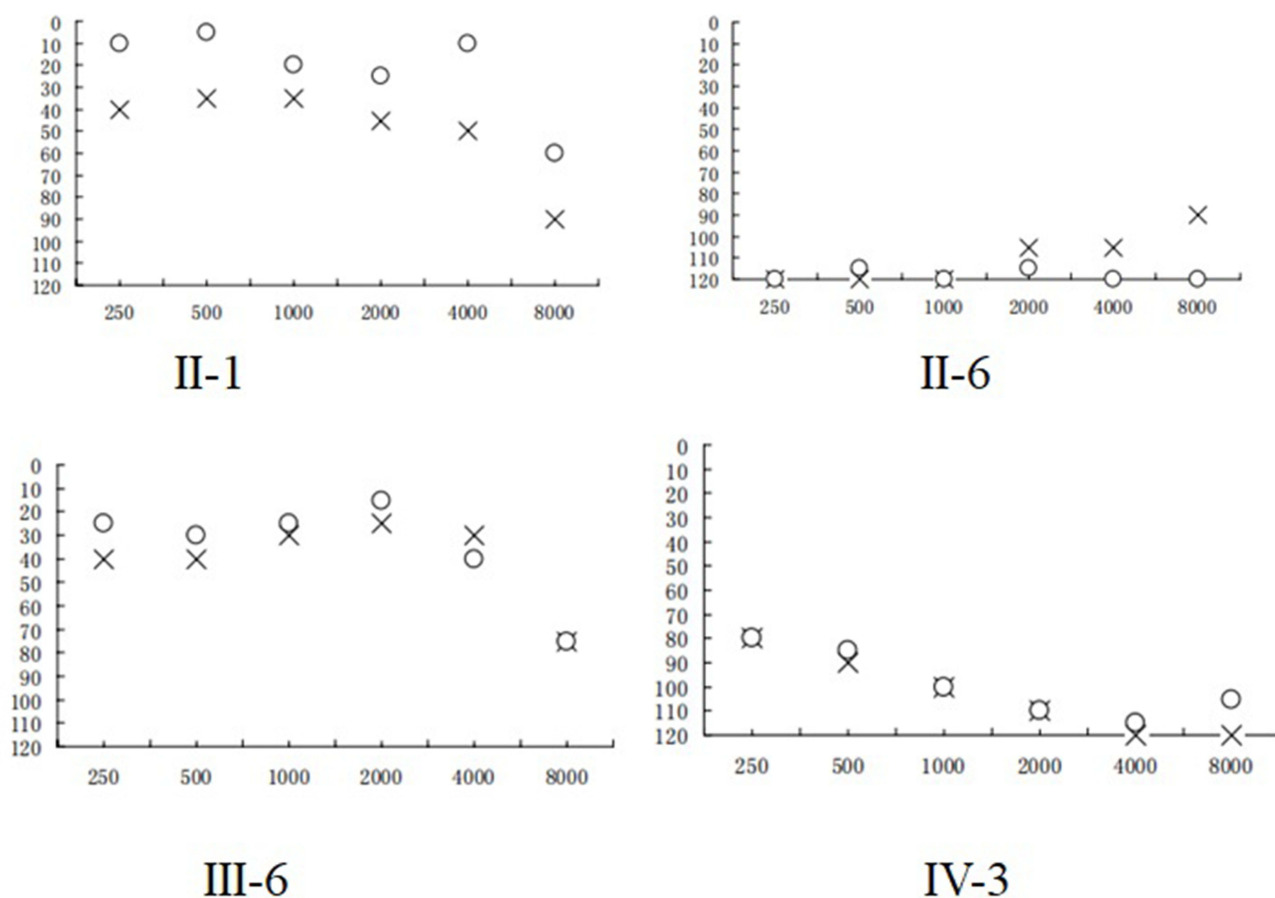


**Figure 3** Sequence alignment of mt-tRNA<sup>Glu</sup> gene from different species, arrows indicated the positions of 37, 54, 55 and 64, corresponding to the m.T14709C, m.A14693G, m.A14692G and m.A14683G variants.





**Figure 4** Cloverleaf structure of mt-tRNA<sup>Glu</sup> gene, arrows indicated the positions of m.T14709C, m.A14693G, m.A14692G and m.A14683G variants.



**Figure 5** Audiological examination of matrilineal relatives of one pedigree with NSHL, (X) left ear; (O) right ear.

**Table 2** Summary of Clinical and Molecular Data for Several Members of the Chinese Family

Subject	Gender	Audiometric Configuration	Age at Test (Year)	Age at Onset (Year)	Use of Aminoglycoside	PTA (dB) Right Ear	PTA (dB) Left Ear	Level of Hearing Loss
II-1	Male	Slope	78	69	No	103	99	Profound
II-6	Female	Rising	60	55	No	110	118	Profound
III-6	Female	Valley	30	28	No	40	35	Mild
IV-3	Female	Slope	2	1	No	105	116	Profound

**Abbreviations:** PTA, pure-tone audiometry; dB, decibels.

**Table 3** mtDNA Variants in One Chinese Family with Hearing Impairment

Gene	Position	Nucleotide Change	Amino Acid Change	rCRS <sup>a</sup>	Conservation (H/B/M/X) <sup>b</sup>	Previously Reported <sup>c</sup>
D-loop	73	A to G		A		Yes
	263	A to G		A		Yes
	310	T to TCC		T		Yes
	524	delC		C		Yes
	16,093	T to C		T		Yes
	16,189	T to C		T		Yes
	16,223	C to T		C		Yes
	16,519	T to C		T		Yes
12S rRNA	750	A to G		A	A/A/A/-	Yes
	1438	A to G		A	A/A/A/G	Yes
16S rRNA	2706	A to G		A	A/G/A/A	Yes
	3010	G to A		G	G/G/A/A	Yes
	3107	delN		N		Yes
ND1	3970	C to T		C		Yes
ND2	4769	A to G		A		Yes
	4833	C to T		C		Yes
	5178	C to A	Leu to Met	C	L/T/T/T	Yes
CO1	7028	C to T		C		Yes
CO1/tRNA <sup>Ser(UCN)</sup>	7444	G to A	Ter to Lys	G	G/G/G/G	Yes
A6	8701	A to G	Thr to Ala	A	T/S/L/Q	Yes
	8860	A to G	Thr to Ala	A	T/A/A/T	Yes
CO3	9540	T to C		T		Yes
ND3	10,398	A to G	Thr to Ala	A	T/T/T/A	Yes
	10,400	C to T		C		Yes

(Continued)

**Table 3** (Continued).

Gene	Position	Nucleotide Change	Amino Acid Change	rCRS <sup>a</sup>	Conservation (H/B/M/X) <sup>b</sup>	Previously Reported <sup>c</sup>
ND4	11,719	G to A		G	G/G/G/G	Yes
	11,914	G to A		G		Yes
ND5	12,361	A to G		A		Yes
	12,705	C to T		C		Yes
ND6	14,668	C to T		C		Yes
tRNA <sup>Glu</sup>	14,692	A to G		A	A/A/A/A	Yes
Cytb	14,766	C to T	Thr to Ile	C	T/S/T/S	Yes
	15,301	G to A		G		Yes
	15,326	A to G	Thr to Ala	A	T/M/I/I	Yes
	15,784	T to C		T		Yes

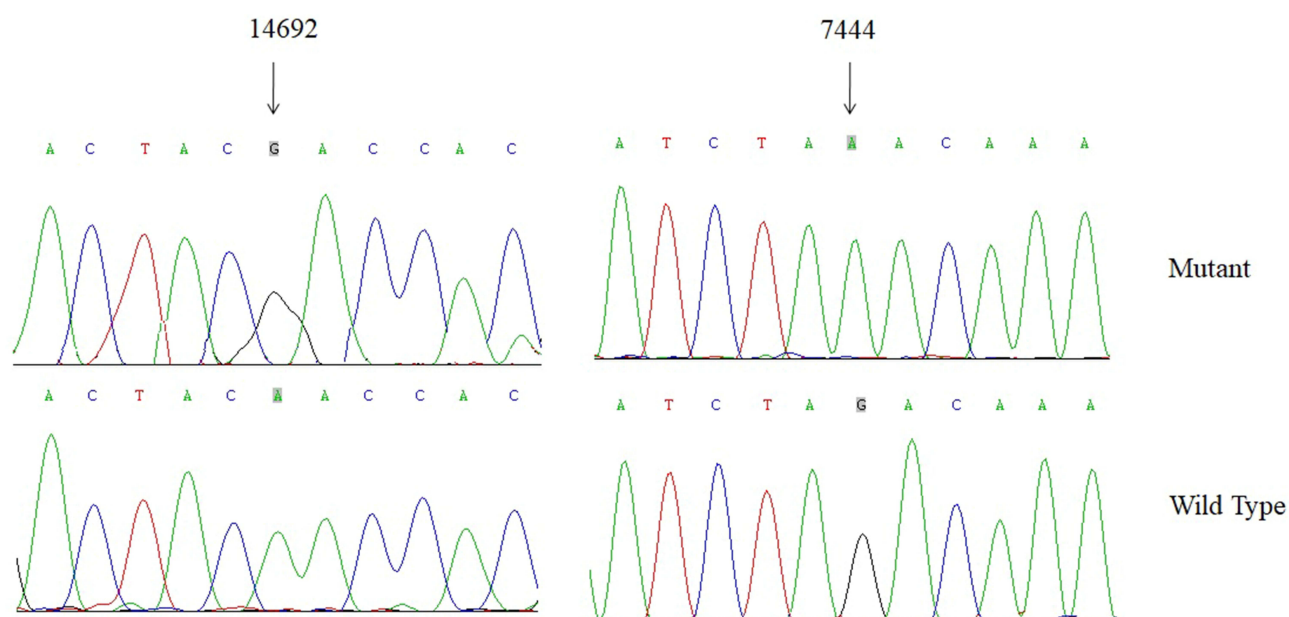
**Notes:** <sup>a</sup>rCRS: revised Cambridge Reference Sequences. <sup>b</sup>Conservation assessment in human, bovine, mouse and *Xenopus laevis*. <sup>c</sup>Please see online databases: mitomap ([www.mitomap.org](http://www.mitomap.org)) or mtDB (<http://www.genpat.uu.se/mtDB>).

## Analysis of mtDNA Content

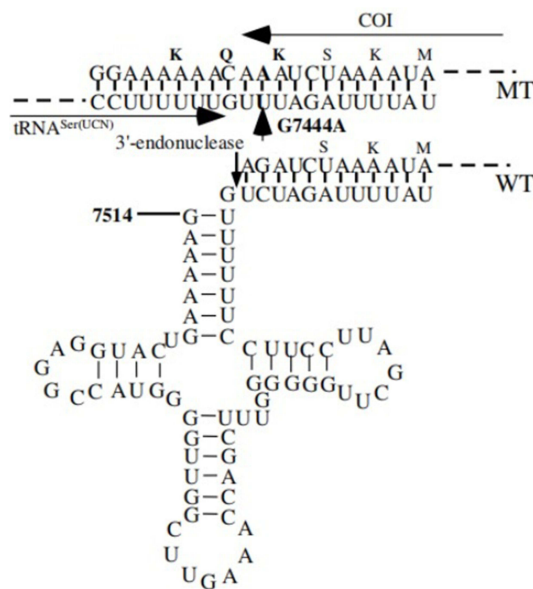
To test whether the m.A14692G and m.G7444A variants affected mitochondrial function, we examined the mtDNA content in four individuals with hearing loss and four control subjects using Real-time PCR. As can be seen in Figure 8A, deaf patients exhibited much lower mtDNA copy numbers than controls ( $p=0.0010$ ).

## ATP Decreased in Mutant Cells

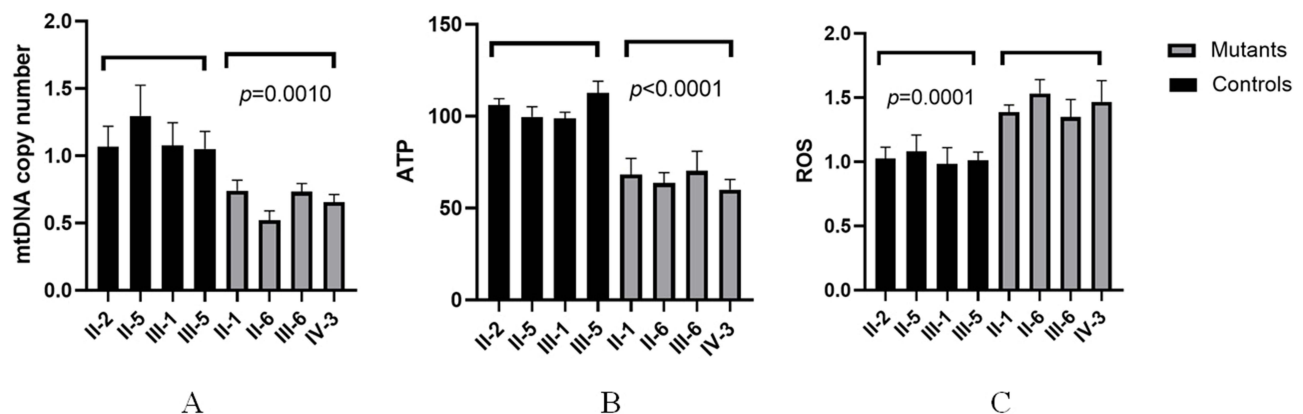
We next measured the ATP levels in mutant and control cell lines, as shown in Figure 8B, cells with m.A14692G and m.G7444A variants showed markedly decreased in ATP levels as compared with controls ( $p<0.0001$ ).

**Figure 6** Identification of m.A14692G and m.G7444A variants by direct sequencing analysis.





**Figure 7** Location of deafness-associated variants in tRNA<sup>Ser(UCN)</sup> and adjacent COI. Arrow indicated the m.G7444A variant in the precursor of this tRNA and adjacent sequence of COI from wild-type (WT) and mutant (MT).



**Figure 8** Analysis of mtDNA copy number in matrilineal relatives of the pedigree with NSHL. (A) mtDNA copy number; (B) ATP content; (C) ROS qualifications.

## ROS Levels Increased in Cells with m.A14692G and m.G7444A Variants

We also examined the levels of ROS production in cell lines with and without m.A14692G and m.G7444A variants (Figure 8C), as compared with controls, we noticed that mutant cells exhibited much higher levels of ROS production ( $p=0.0001$ ).

## Discussion

In the present study, we examined the frequency of mt-tRNA<sup>Glu</sup> variants in a cohort of 180 children with NSHL. Four potential pathogenic variants: m.T14709C, m.A14692G, m.A14693G and m.A14683G were identified using Sanger-Sequencing. These variants were further evaluated using the following criteria: (1) present in <1% of controls; (2) evolutionary conservation; and (3) potential structural and functional alterations. Of these, the m.T14709C variant was first reported in a patient with maternally inherited diabetes and deafness (MIDD).<sup>37</sup> Biochemical analysis revealed that the m.T14709C variant affected the Complexes I and IV activities,<sup>38</sup> furthermore, this variant decreased the steady-state level of tRNA<sup>Glu</sup> and subsequently influenced the protein synthesis.<sup>39</sup> Thus, the m.T14709C was definitely pathogenic for NSHL.

Moreover, the m.A14693G variant occurred at the extremely conserved position of mt-tRNA<sup>Glu</sup> (position 55), a position that was critical for tRNA chemical modification.<sup>3</sup> Previously study indicated that the m.A14693G variant may modulate the clinical expression of deafness-associated m.A1555G mutation.<sup>40</sup> Besides, the m.A14683G variant created a new Watson-Crick base-pairing (64A-50G).<sup>41</sup> Intriguingly, m.T15965C variant which occurred at the same position as the T $\Psi$ C stem of mt-tRNA<sup>Pro</sup> gene has been associated with Parkinson diseases.<sup>42</sup> Therefore, the m.A14683G variant, which was similar to the m.T15965C variant, may also cause mitochondrial dysfunction that was responsible for NSHL.

Among the cases harboring the mt-tRNA<sup>Glu</sup> variants, only one child with the m.A14692G variant had an obvious family history of NSHL. Interestingly, none of the members in this pedigree had a history of aminoglycoside use, notably, the age at onset of NSHL varied from 1 to 69 years, with an average of 38 years. Sequence analysis of the entire mitochondrial genome from matrilineal relatives suggested the presence of two interesting variants: mt-tRNA<sup>Glu</sup> A14692G and CO1/tRNA<sup>Ser(UCN)</sup> G7444A. At the molecular level, the m.A14692G variant affected a highly conserved uridine at position 55 in the T $\Psi$ C loop of mt-tRNA<sup>Glu</sup>. The uridine was modified to pseudouridine ( $\Psi$ 55), which plays an important role in the structure and function of this mt-tRNA.<sup>43,44</sup> The destabilization of base-pairing (18A- $\Psi$ 55) caused by the m.A14692G variant perturbed the conformation and stability of mt-tRNA<sup>Glu</sup>. An approximately ~65% decrease in the steady-state level of mt-tRNA<sup>Glu</sup> was observed in the cells carrying this variant. Failure in mt-tRNA<sup>Glu</sup> metabolism impaired mitochondrial translation, especially for polypeptides with a high proportion of glutamic acid codons such as MT-ND1, MT-ND6 and MT-CO2 in mutant cells.<sup>45</sup> Thus, the m.A14692G variant was definitely pathogenic as it caused mitochondrial dysfunction.

In addition, the m.G7444A variant resulted in a read-through of the stop codon AGA of the *COI* message, thereby adding three amino acids (Lys-Gln-Lys) to the C-terminal of the polypeptide.<sup>46</sup> Thus, the variant polypeptide may retain a partial function (Figure 7). Alternatively, the m.G7444A variant was adjacent to the site of 3' end endonucleolytic processing of the L-strand RNA precursor, spanning mt-tRNA<sup>Ser(UCN)</sup> and *ND6* mRNA.<sup>47</sup> Furthermore, the homoplasmic m.A7445G variant was reported to reduce tRNA<sup>Ser(UCN)</sup> levels by ~70% and to cause a 45% reduction in mitochondrial protein synthesis in cybrid cells containing this variant.<sup>47</sup> Therefore, the m.G7444A variant, which was similar to the m.A7445G variant, may have an impact on mitochondrial function. Our results indicated that patients with both the m.A14692G and m.G7444A variants had lower mtDNA copy numbers, ATP production than controls, whereas the levels of ROS increased significantly. In fact, mtDNA copy number was a relative measure of the cellular number or mass of mitochondria.<sup>48</sup> Recent experimental study suggested that alterations in mtDNA copy number played a fundamental role in the increase in ROS, maintenance of mtDNA copy number was essential for the preservation of mitochondrial function and cell growth.<sup>49</sup> Moreover, the increased ROS would affect the quality of gametes, lead to the cochlear cell death and apoptosis,<sup>50,51</sup> and in turn, impaired OXPHOS and decreased the ATP production. Therefore, the m.A14692G and m.G7444A variants led to mitochondrial dysfunctions that were involved in NSHL. However, the absent of any functional variants in *GJB2*, *GJB3*, *GJB6*, *TRMU* and *SLC26A4* suggested that nuclear genes may not play important roles in deafness expression. Hence, the combination of m.A14692G and m.G7444A variants may account for high penetrance and expressivity of NSHL in this pedigree.

In conclusion, our study indicated that mt-tRNA<sup>Glu</sup> variants were important risk factors for NSHL, m.T14709C, m.A14692G, m.A14693G and m.A14683G were associated with NSHL. Screening for mt-tRNA<sup>Glu</sup> variants was recommended for early diagnosis and prevention of NSHL. However, the homoplasmic forms of mtDNA variants suggested that the mutation itself was not sufficient to produce the clinical phenotypes, hence, other modified factors such as environmental factors, epigenetic modifications and personal lifestyles contributed to deafness expression.

## Limitations

The main limitations of the current study were the relatively small sample size, and further studies including more deaf patients and controls are needed to verify these conclusions.

## Conclusions

In summary, our data indicated that variants in the mt-tRNA<sup>Glu</sup> gene were important contributors to NSHL, and screening for variants in this gene is recommended for the early diagnosis and detection of NSHL.

## Abbreviations

mtDNA, mitochondrial DNA; NSHL, non-syndromic hearing loss; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; AINSHL, aminoglycoside-induced and non-syndromic hearing loss; mt-tRNA, mitochondrial tRNA; PTA, pure-tone audiometry; dB, decibels; rCRS, revised Cambridge reference sequences; CI, conservation index; DCFH, 2,7-dichlorodihydrofluorescein; MIDD, maternally inherited diabetes and deafness.

## Data Sharing Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request (Yu Ding: dingyu\_zj@126.com).

## Ethics Approval and Consent to Participate

This study was approved by the Ethics Committees of Hangzhou First People's Hospital (Approval No: 2020-285-01) and Quzhou People's Hospital (Approval No: 2021-028). Prior to the commencement of the research, our team obtained written informed consent from each patient.

## Consent for Publication

Each participant provided their consent for publication.

## Acknowledgment

We thank the members of the Department of Clinical Laboratory, Quzhou People's Hospital for their discussion.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Funding

This work was supported by the grants from Ministry of Public Health of Zhejiang Province (No: 2021RC022) and Quzhou Bureau of Science and Technology (No: 2022K51).

## Disclosure

The authors declare that they have no competing interests in this work.

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