

# Analysis of mitochondrial A1555G mutation in infants with hearing impairment

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**Abstract.** Mutations in the mitochondrial 12S ribosomal RNA gene have been identified to be associated with deafness. Among these, the A to G transition at position 1555 is one of the most common pathogenic mutations associated with hearing loss. In order to evaluate the allele frequency of this mutation in infants with hearing loss, the A1555G mutation was screened in 300 deaf children and 100 age- and sex-matched healthy subjects. Consequently, 5 patients with this mutation were identified, whereas the mutation was absent in healthy controls. Among the patients with the mutation, only one had an obvious family history of hearing impairment. Notably, this pedigree manifested a high penetrance of deafness. In particular, the penetrance of deafness was 80 and 40%, when the aminoglycoside antibiotics (AmAn) was included or excluded, respectively. Clinical evaluation of this family exhibited a wide degree of hearing loss. Furthermore, screening for the complete mitochondrial genes revealed the occurrence of A1555G and transfer (t)RNA<sup>Thr</sup> T15943C mutations, together with other genetic variations belonging to East Asian haplogroup C. Notably, the T15943C mutation, located at the T arm of tRNA<sup>Thr</sup>, could disrupt the 63T-55A base-pairing and impair tRNA metabolism. Therefore, it was hypothesized that the combination of A1555G and T15943C mutations may result in mitochondrial dysfunction that is responsible for deafness. Screening for A1555G, as well as other potential pathogenic mutations in the mitochondrial genome, is critical for clinical diagnosis and prevention of hearing impairment.

## Introduction

Deafness is a serious health problem with unknown etiology, and it is estimated that 1 in 1,000 newborns suffer from the condition (1). It may be associated with numerous risk factors, including the administration of aminoglycoside antibiotics (AmAn) (2). Genetic alterations in human mitochondrial (mt)DNA are also thought to be associated with deafness (3). Human mtDNA is a double-stranded, circular molecule, encoding 13 protein subunits, 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs). In particular, the well-known 12S rRNA A1555G mutation has been demonstrated to be an important molecular basis of hearing loss (4,5). Furthermore, the A to G transition at position 1555 is located in the region of small ribosomal RNA that is highly conserved in bacteria and mammals (6). The corresponding region in *Escherichia coli* forms an essential part of the decoding site of the ribosome (7) and is crucial for subunit association either by RNA-protein or the RNA-RNA interaction (8). It is interesting to note that among individuals who carry this mutation, the use of AmAn for clinical treatment of fever is likely to result in irreversible hearing impairment (9). However, individuals who carry the A1555G mutation exhibit a wide range of clinical phenotypes, from profound hearing loss to normal hearing (4). Thus, if an individual carrying the A1555G mutation cannot use aminoglycosides, screening for the A1555G mutation is crucial for prevention and early diagnosis of maternally inherited deafness. In addition, lymphoblastoid cell lines derived from an Arab-Israeli family demonstrated more severe biochemical defects in those that were symptomatic to those that were not (10). Therefore, it is hypothesized that other unidentified risk factors, including polymorphisms in mtDNA, nuclear gene mutations, AmAn and epigenetic modification, may contribute to hearing impairment (10).

To explore the potential association between mtDNA polymorphisms and deafness, a mutational screening was conducted for the presence of A1555G in 300 infants with hearing impairment and 100 healthy controls. Consequently, 5 patients with the mutation were identified. Among these infants carrying the A1555G mutation, only one had a family history of exposure to AmAn.

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## Materials and methods

**Subjects and clinical evaluations.** From January 2015 to January 2017, a total of 300 infants with hearing loss (180 females and 120 males), aged from 1-3 years (with an average age of 2 years), as well as 100 age- and sex-matched control subjects (60 females and 40 males) were recruited from Fujian Provincial Hospital (Fuzhou, China). A physical examination was performed for each participant that was enrolled. In addition, the level of hearing impairment was tested using pure-tone audiometry. Furthermore, the level of hearing loss was classified as follows: Normal,  $\leq 25$  dBHL; mild, 26-40 dBHL; moderate, 41-70 dBHL; severe, 71-90 dBHL; profound,  $>90$  dBHL. Written informed consent was obtained from the patients' parents or guardians, and the study was approved by the Ethics Committee of Fujian Provincial Hospital. Patients were included if the infants possessed the sole clinical phenotype. Those patients who carried other mitochondrial disorders, including Leigh syndrome, exercise intolerance and developmental delay were excluded. In addition, healthy patients exhibited normal hearing, and did not carry any diseases.

**Screening for the mitochondrial A1555G mutation.** Genomic DNA was extracted from blood samples obtained from each participant using Puregene DNA Isolation kits (Gentra; Qiagen, Inc., Valencia, CA, USA). The mitochondrial 12S rRNA gene was amplified using polymerase chain reaction (PCR; Takara, Bio, Inc., Otsu, Japan), the primer sequence for amplification the 12S rRNA gene was as follows: (5): Forward, 5'-CGATCAACCTCACCACCTCT-3' and reverse, 5'-TGGACAACCAGC TATCACCA-3'. The thermocycling conditions for PCR were as follows: 95°C for 5 min, 94°C for 10 sec, 60°C for 30 sec, 30 cycles at 72°C for 60 sec and 72°C for 10 min for extension. Following amplification, the PCR product was digested with the restriction enzyme *BsmAI* to screen for the occurrence of the A1555G mutation. Equal amounts (5  $\mu$ l) of various digested samples were then analyzed using electrophoresis with 1.5% agarose gel. Following electrophoresis, a BandPeeper (MaestroGen Inc., Hsinchu, Taiwan) instrument was utilized to detect the results using the Invitrogen™ E-Gel™ Imager software (version 2.2.4; Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA).

**Molecular features of the pedigree harboring mitochondrial A1555G mutation.** A Chinese pedigree was collected in Fujian Provincial Hospital. The complete mitochondrial genomes of the matrilineal individuals (I-2, II-1, II-5, II-8, II-10, III-9, III-10 and III-12) were amplified using 24 primers, as previously described (6). The PCR reagents (all obtained from Takara Bio, Inc.) were as follows: 200  $\mu$ M dNTPs, 2  $\mu$ l 10X PCR buffer (10X0.2  $\mu$ l Taq DNA polymerase and 15 mmol/l  $Mg^{2+}$ ). Subsequently, the PCR products were purified and sequenced using the ABI PRISM™ 3700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) (11). A total of 24 PCR product sequences were compared with the reverse Cambridge sequence, using DNA Star software version 5.01 (DNASTAR Inc., Madison, WI, USA) to detect variations in mitochondrial genes (GenBank accession no. NC\_012920) (12).

**Phylogenetic conservation analysis.** To discern the potential pathogenic mtDNA mutations, the phylogenetic approach was utilized to assess the conservation index (CI) of each mtDNA mutation. A total of 17 species were used to analysis the CIs. Notably, CI $>75\%$  was regarded as exhibiting functional potential (13).

**Detecting GJB2 mutations.** In order to identify whether *GJB2* mutations were involved in hearing impairment, variants of *GJB2* were screened using PCR and direct sequencing. The primers for amplification of the *GJB2* coding region were as follows: Forward, 5'-TATGACACTCCCCAGCACAG-3' and reverse, 5'-GGGCAATGCTTAAACTGGC-3' (14). The PCR reagents (all obtained from Takara Bio, Inc.) were as follows: 200  $\mu$ M dNTPs, 2  $\mu$ l 10X PCR buffer (10x0.2  $\mu$ l Taq DNA polymerase) and 15 mmol/l  $Mg^{2+}$ . PCR was performed under the following thermocycling conditions: 95°C for 5 min, 94°C for 10 sec, 60°C for 30 sec, 30 cycles at 72°C for 60 sec, and 72°C for 10 min for extension. Then, the PCR products were sequenced using the ABI PRISM™ 3700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) and compared with the wild-type *GJB2* sequence using DNA Star software version 5.01 (GenBank accession no. M86849) to detect potential pathogenic mutations.

**Screening for the TRMU A10S variant.** Nuclear modified gene *TRMU* was previously identified to be associated with hearing loss (15). To understand the role of *TRMU* in hearing impairment, the *TRMU* A10S variant was screened in the matrilineal relatives using PCR amplification of exon 1 of this gene. The PCR reagents (all obtained from Takara Bio, Inc.) were as follows: 200  $\mu$ M dNTPs, 2  $\mu$ l 10X PCR buffer (10x0.2  $\mu$ l Taq DNA polymerase) and 15 mmol/l  $Mg^{2+}$ . The primer sequence for exon 1 was as described previously (15). PCR was performed under the following thermocycling conditions: 95°C for 5 min, 94°C for 10 sec, 58°C for 30 sec, 30 cycles at 72°C for 60 sec, and 72°C for 10 min for extension. Then, the PCR product was sequenced using the ABI PRISM™ 3700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) and compared with the wild-type version of *TRMU* and DNA Star software version 5.01 (GenBank accession no. AF448221) (16).

**Bioinformatics analysis.** To investigate whether T15943C mutation altered the structure of tRNA<sup>Thr</sup>, the RNA Fold web server was employed to analyze the secondary structure of tRNA<sup>Thr</sup> with and without the T15943C mutation (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (17). The wild-type version of tRNA<sup>Thr</sup> gene was: 5'-GTCCTTGTAGTATAAACTAATACA CCAGTCTTGTAACCGGAGATGAAAACCTTTTCCAAGG ACA-3'. The sequence of tRNA<sup>Thr</sup> gene carrying the T15943C mutation was: 5'-GTCCTTGTAGTATAAACTAATACACCA GTCTTGTAACCGGAGATGAAAACCTTTCTCCAAGG ACA-3'.

## Results

**Frequency of mitochondrial A1555G mutation in infants with deafness.** To detect the allele frequency of the A1555G mutation, 300 infants were enrolled who were confirmed to have non-syndromic hearing loss by a clinical physician in

Table I. Summary of clinical and molecular data for members of a Han Chinese family with AmAn-induced hearing loss.

Subject	Sex	Age at onset (years)	Age at test (years)	Use of AmAn	PTA (dB), left ear	PTA (dB), right ear	Level of hearing loss
I-2	Female	55	62	Yes	90	90	Profound
II-1	Male	18	30	No	75	80	Severe
II-5	Male	25	32	Yes	35	40	Moderate
II-8	Female	23	28	Yes	88	85	Severe
II-10	Female	21	26	Yes	75	88	Severe
III-9	Male	3	5	No	70	80	Severe
III-10	Female	1	2	No	95	90	Profound
III-12	Female	1	1	No	55	65	Severe
III-1	Male	N/A	5	No	18	20	Normal

PTA, pure tone audiometry.

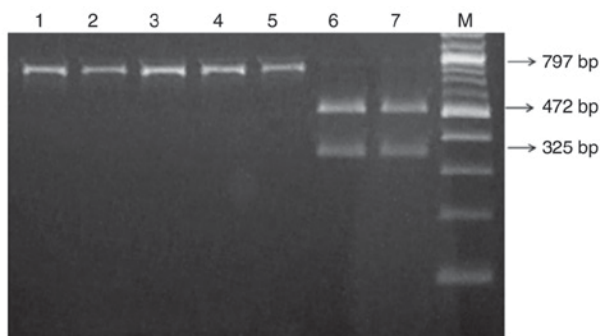
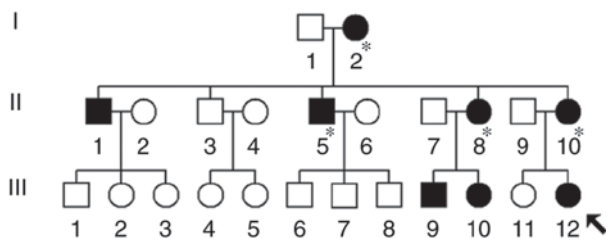
Figure 1. Detection of the A1555G mutation using *Bsm*AI. Lanes 1-5 indicate samples with the A1555G mutation. Lanes 6 and 7 indicate samples without the A1555G mutation. M, DNA marker.

Figure 2. A Han Chinese family with AmAn-induced hearing loss. Males are indicated with squares and females are indicated with circles. Subjects with hearing loss are indicated by filled symbols. The arrow denotes the proband. Asterisks denote subjects with a history of AmAn exposure. AmAn, aminoglycoside antibiotic.

Fujian Provincial Hospital. The restriction enzyme *Bsm*AI was used to screen the mitochondrial A1555G mutation following PCR amplification of the 12S rRNA gene. As indicated in Fig. 1, following electrophoresis, it was observed that samples carrying the A1555G mutation exhibited only one 797 bp band, while the samples without this mutation resulted in two specific bands: 472 and 325 bp. Consequently, 5 patients with this mutation were identified (5/300; 1.67%). The mutation was not detected in the 100 healthy controls. Further clinical evaluation indicated that 1 patient with the mutation had an obvious family history of using AmAn.

**Molecular characterization of the pedigree carrying mitochondrial A1555G mutation.** The proband's mother (II-10) was 26 years old and came from the Fuzhou area of Fujian Province, China. She received AmAn (gentamycin, 5 mg/kg/dose) for clinical treatment of a high fever when she was 21 years old. Unfortunately, 2 weeks following the antibiotic treatment, she suffered from bilateral hearing loss and clinical examination indicated that she had severe deafness (75 dB in left ear, 88 dB in right ear; see Table I). Further physical and audiological examinations were performed for the matrilineal relatives in this family. It was observed that I-2, II-5 and II-8 had a history of exposure to AmAn.

As indicated in Fig. 2, this pedigree had an obvious maternal transmission pattern. It was noted that the pedigree manifested a high penetrance and expressivity of deafness: The penetrance of deafness was 80 and 40% when patients who were exposed to AmAn were included and excluded, respectively. Furthermore, it was identified that the age at onset of deafness varied from 1 to 55 years (Table I). Other members of this family did not exhibit any other mitochondrial disorders, including type 2 diabetes, cardiovascular events, vision loss or neurological diseases.

**Screening for mutations in mitochondrial genes.** The maternally inherited pattern of this family indicated that mitochondrial dysfunction may be involved in the pathogenesis of hearing loss. In order to investigate this, the variants in mtDNA were screened using PCR and direct sequencing. The results indicated that matrilineal relatives (I-2, II-1, II-5, II-8, II-10, III-9, III-10 and III-12) in this family harbored 22 mtDNA polymorphisms, as well as the well-known A1555G mutation (Table II). Of these, six variants were identified in the D-loop, three variants in 12S rRNA, one variant in 16S rRNA and one mutation in tRNA<sup>Thr</sup>. The other sequence alterations were primarily in oxidative phosphorylation-associated genes. Furthermore, there were six missense mutations, including *ND2* C5178A (L237M) and A5301G mutations (I278V), *ATP6* A8860G mutation (T112A), *ND3* A10398G mutation (T114A), and *CytB* C14766T (T71) and A15326G (T194A) mutations. In addition, evolutionary conservation analysis was performed for these variants between 17 vertebrates, including

Table II. Mitochondrial DNA sequence variants in a Han Chinese family with hearing loss.

Gene	Position	Nucleotide change	Conservation (H/B/M/X) <sup>a</sup>
D-loop	73	A to G	
	263	A to G	
	310	T to CTC	
	16189	T to C	
	16355	C to T	
	16519	T to C	
12S rRNA	750	A to G	A/G/G/-
	1438	A to G	A/A/A/G
	1555	A to G	A/A/A/A
16S rRNA	2706	A to G	A/G/A/A
	4769	A to G	
	5178	C to A (Leu to Met)	L/T/T/T
ND2	5301	A to G (Ile to Val)	I/I/M/L
	7028	C to T	
	8860	A to G (Thr to Ala)	T/A/A/T
ND3	10398	A to G (Thr to Ala)	T/T/T/A
ND4	11719	G to A	
	12705	C to T	
	13344	A to G	
CytB	14766	C to T (Thr to Ile)	T/S/T/S
	15301	G to A	
	15326	A to G (Thr to Ala)	T/M/I/I
tRNA <sup>Thr</sup>	15943	T to C	T/T/T/T

<sup>a</sup>Conservation of amino acid (for polypeptides) or nucleotide (for rRNAs) in H/B/M/X. H, human; B, bovine; M, mouse; X, *Xenopus laevis*; rRNA, ribosomal RNA; tRNA, transfer RNA; ND, NADH dehydrogenase; CO1, cytochrome c oxidase 1; ATP6, adenosine triphosphate 6; CytB, cytochrome B.

the mouse (18), bovine (19) and *Xenopus laevis* (20). However, it was identified that none of these variants were conserved, with the exception of A1555G and T15943C mutations (Fig. 3 and Table III). In addition, the A1555G and T15943C mutations were absent in the control subjects. This suggested that the A1555G and T15943C mutations were involved in the pathogenesis of deafness in this family.

**Genotyping analysis of *GJB2* gene.** To evaluate the contribution of *GJB2* gene variants to deafness expression, *GJB2* mutations were analyzed in the matrilineal relatives (I-2, II-1, II-5, II-8, II-10, III-9, III-10 and III-12). However, no functional variants were observed in the *GJB2* gene.

**Mutational analysis of *TRMU* gene.** A previous study indicated that the *TRMU* A10S variant may contribute to the clinical expression of hearing loss associated with mitochondrial A1555G mutation (15). Therefore, the *TRMU* A10S variant was screened in matrilineal members from the current pedigree (I-2, II-1, II-5, II-8, II-10, III-9, III-10 and III-12). However, the variant was not detected in these subjects.

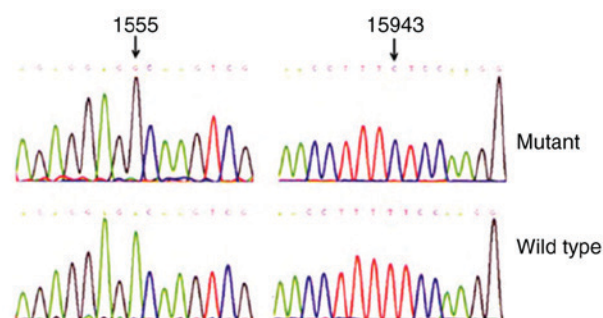


Figure 3. Identification of the mitochondrial A1555G and T15943C mutations using polymerase chain reaction and Sanger sequencing.

**T15943C mutation affects the secondary structure of tRNA<sup>Thr</sup>.** To evaluate the effect of T15943C mutation on tRNA<sup>Thr</sup>, a bioinformatics tool was used to predict the tRNA<sup>Thr</sup> secondary structure with and without this mutation (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (17). As indicated in Fig. 4, the T15943C mutation notably altered the tRNA<sup>Thr</sup> secondary structure, strongly indicating that this mutation was pathogenic.

## Discussion

The current study screened the occurrence of mitochondrial A1555G mutation in pediatric subjects with hearing loss. As early as 1993, the A1555G mutation was reported in an Arab-Israeli family with non-syndromic hearing impairment (4). In fact, this mutation occurred in the A-site of 12S rRNA gene, which was strongly conserved between multiple species and was critical for the binding of AmAn (21,22). Therefore, patients carrying A1555G mutation were more vulnerable to developing hearing loss when using AmAn.

Using PCR and direct sequencing, 5 infants with hearing loss were identified to be carrying the A1555G mutation in the current study. Its frequency was 1.67% in patients, but the mutation was not detected in the control subjects. The frequency of the A1555G mutation varies worldwide. For patients with hearing loss who used AmAn, the frequency of the A1555G mutation was reported to be 33% in a Japanese population (23), whereas the incidence was reported to be 13, 10.4 and 5% in three Han Chinese populations (24-26). These data are important, since children who carry this mutation should not be exposed to AmAn. Thus, screening for the occurrence of the A1555G mutation is advisable, particularly for those who have a family history of using AmAn.

In the current study, 5 infants with hearing loss were identified to be carrying the homoplasmic A1555G mutation. However, only 1 of these patients had an obvious family history of using AmAn. Following comprehensive genetic counseling, it was identified that the family members only exhibited the deafness phenotype, without other common diseases, including type 2 diabetes mellitus, cardiovascular events and neurological disorders. In addition, the pedigree manifested a high expressivity of deafness. PCR-Sanger sequencing was performed to identify the occurrence of the homoplasmic A1555G mutation, together with a further 22 genetic variations belonging to the East Asian haplogroup C (20). Among these,



Table III. Sequence alignment of tRNA<sup>Thr</sup> gene from different species.

Organism	Acc-stem	D-stem	D-loop	D-stem	Ac-Stem	Anticd-loop	Ac-stem	V-region	T-stem	T-loop	T-stem <sup>a</sup>	Acc-stem
<i>Homo sapiens</i>	GTCCTTGTA	GTAT	AAACTA	ATACA	CCAGT	CTTGTA	ACCGG	AGAT	GAAAA	CCT	TTTTC	CAAGGACA
<i>Mus musculus</i>	GTCCTTGATA	GTAT	AAACA	TTACT	CTGGT	CTTGTA	ACCTG	AAAT	GAAGA	TCTTC	TCTTC	TCAAAGACA
<i>Myoxus glis</i>	GTCCTGGTA	GTAT	AAAGTTA	TTACT	CTGGT	CTTGTA	ACCG	AAAT	GGAAA	TCTCAAT	TTTTCC	CCAGGACA
<i>Colobus guereza</i>	GCCCCTCGTA	GTAC	AAACTA	GTATA	CCGGT	CTTGTA	ACCGA	AGAT	GGAGA	CT	TCTCC	CTAGGACA
<i>Pan paniscus</i>	GCCCCTTGTA	GTAT	AAGCTA	ATACA	CCGGT	CTTGTA	ACCGG	AAAC	GAAAA	CTT	TATTC	CAAGGACA
<i>Ceratotherium sinum</i>	GTCCTTGTA	GTAT	ATGAA	TTACT	CTGGT	CTTGTA	ACCG	AAAA	GGAAA	GCACCAC	TTTTCC	CCAAAGACT
<i>Arctocephalus forsteri</i>	GTCCTTCGTA	GTAT	AACAA	TTACC	TTGGT	CTTGTA	ACCAA	AAAT	GGAGA	ACACCA	TCTCC	CTAAGACT
<i>Elephas maximus</i>	ACCCCTATA	GTAT	AAGACA	TTACA	ATGGT	CTTGTA	GCCAT	AAAT	GAAAA	CCA	CTTTC	TAAAGGTA
<i>Bradypus tridactylus</i>	GCCCCTCGTA	GTAT	AACAAA	TTACA	CTAGC	CTTGTA	ACCG	AAAT	GAAAT	ACGC	ATTTC	CAAGGGCA
<i>Gorilla gorilla</i>	GCCCCTTGTA	GTAC	AGACCA	ATACA	CCAGT	CTTGTA	ACCGG	AAAC	GAAGA	CCT	CCTTC	CAAGGGCA

<sup>a</sup>Third nucleotide in this sequence represents position 63, corresponding to the T15943C mutation.

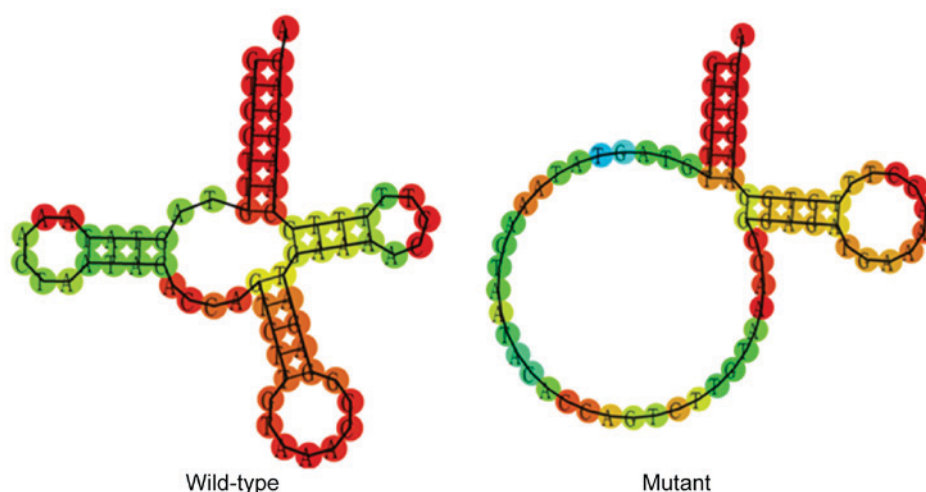


Figure 4. Prediction of the secondary structure of transfer RNA<sup>Thr</sup> with and without the T15943C mutation.

the T15943C mutation in tRNA<sup>Thr</sup> was of particular interest. This mutation was identified in this Chinese family with hearing loss (Patients: I-2, II-1, II-5, II-8, II-10, III-9, III-10 and III-12) but absent in 100 healthy subjects. In addition, this mutation occurred at an evolutionary conserved position of tRNA<sup>Thr</sup> (conventional position 63), which could disrupt 63T-55A base pairing and may result in failures in tRNA metabolism. In fact, RNA Fold results also indicated that this mutation caused a structural change of tRNA<sup>Thr</sup>. Thus, it was speculated that the T15943C mutation affects tRNA<sup>Thr</sup> function via the alteration of its secondary structure.

The phenotypic variability of matrilineal relatives within the pedigree suggested that nuclear genes or mtDNA genetic modifiers may serve key functions in deafness expression. For example, the *TRMU* A10S variant has previously been reported to increase the penetrance and expressivity of deafness-associated mitochondrial A1555G mutation (15). Mutations in the *GJB2* gene have been regarded as important causes for non-syndromic hearing loss (14). However, an absence of functional sequence variants in *TRMU* and *GJB2* genes in the current study indicated that these genes may not play active roles in deafness expression. Hence, other unidentified nuclear genes may be involved in the clinical manifestation of deafness in this pedigree.

In conclusion, the tRNA<sup>Thr</sup> T15943C may be a secondary mutation for the phenotypic expression of mitochondrial deafness, and should be regarded as a risk factor for hearing impairment. Furthermore, screening for the presence of mitochondrial A1555G mutation is advisable, particularly for those who have a family history of using AmAn. The primary limitation of this study was the small sample size; further studies including larger cohorts of patients with hearing impairment should be performed in the future.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

LW, RL and JC designed the project, YC, MY and QW performed the experiments and LW wrote the manuscript.

### Ethics approval and consent to participate

The present study was approved by the Ethics of Fujian Provincial Hospital (Fuzhou, China) and all parents/guardians of participants provided written informed consent to participate.

### Consent for publication

The patients' parents provided written informed consent for the publication of any associated data and accompanying images.

### Competing interests

The authors declare that they have no competing interests.

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