Mitochondrial $tRNA^{Ile}$ A4317G mutation may be associated with hearing impairment in a Han Chinese family

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Abstract. Mutations in the mitochondrial genome have been identified to be associated with hearing loss. The aim of the present study was to investigate the role of mitochondrial DNA (mtDNA) variants in a Chinese family with hearing loss. Polymerase chain reaction (PCR)-Sanger sequencing was used to screen the mtDNA variants and nuclear genes [gap junction protein β2 (*GJB2*) and transfer (t)RNA 5-methylaminomethyle-2-thiouridylate methyltransferase (*TRMU*)]; in addition, the mtDNA copy number was determined by quantitative PCR. The present study characterized the molecular features of a Chinese family with maternally-inherited hearing loss and identified mtDNA A1555G and $tRNA^{Ile}$ A4317G mutations. The A4317G mutation was localized at the TΨC arm of $tRNA^{Ile}$ (position 59) and created a novel base-pairing (G59-C54), which may alter the secondary structure of the tRNA. In addition, patients carrying the A4317G mutation exhibited a lower mtDNA copy number compared with the controls, suggesting that this mutation may cause mitochondrial dysfunction that is responsible for the deafness. However, no functional variants in the *GJB2* and *TRMU* genes were detected. mtDNA A1555G and A4317G mutations may contribute to the clinical manifestation of hearing loss in this family.

Introduction

Deafness is one of the most common human health problems, affecting one in 700-1,000 newborns (1). It has been estimated that ≥50% of cases of congenital deafness have genetic causes. At present, the etiology of hearing loss remains largely unknown, although gene alterations (2-15) and environmental factors, including aminoglycoside antibiotics (AmAn), are now generally considered to be involved in this condition (16). AmAn, including gentamicin and kanamycin, are of great clinical importance for the treatment of bacterial infections. However, the use of these drugs is frequently associated with toxicity, involving the renal, auditory and vestibular systems (17). In familial cases of ototoxicity, AmAn hypersensitivity is frequently maternally transmitted, suggesting that mutations in mitochondrial (mt)DNA may be the molecular basis for this susceptibility (18). Among the mtDNA genes, 12S ribosomal (r)RNA and transfer (t)RNA are hot-spots for pathogenic mutations associated with deafness (19,20). Indeed, it has been reported that deafness-associated mtDNA primary mutations impair mitochondrial translation, leading to deficient respiration (21). However, only mild mitochondrial dysfunctions are observed in cells carrying these mtDNA mutations, suggesting that these mutations are necessary but insufficient to produce a clinical phenotype (22). Therefore, there may be other modifying factors that modulate the phenotypic manifestation of these mtDNA mutations (23).

To investigate the modifying factors that contribute to the clinical expression of deafness-associated mtDNA mutations, a mutational screening on deaf patients from Tianjin, China was performed in the present study. The present study describes a Han Chinese family with maternally-transmitted hearing loss, in which analysis of the complete mtDNA sequence demonstrated the occurrence of 12S rRNA A1555G and $tRNA^{Ile}$ A4317G mutations. To further understand the possible role of A4317G mutation, the mtDNA copy number in the patients harboring this mutation was analyzed. In addition, to determine the contributions of the gap junction protein β2 (*GJB2*) and tRNA 5-methylaminomethyle-2-thiouridylate methyltransferase (*TRMU*) genes to the phenotypic manifestation of the A1555G mutation, a mutational analysis of these genes in individuals with hearing impairment in this family was conducted.

Patients and methods

Subjects. A screening program was performed for mtDNA mutations in deaf patients from Tianjin City, China. A Han Chinese family, as presented in Fig. 1, was enrolled in October, 2017 through the otology clinic of The PLA 254 Hospital (Tianjin, China). Informed consent was obtained from the participants prior to their participation in the study, in accordance with the requirements of the Ethics Committee of The PLA 254 Hospital. In addition, 300 healthy age- and

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sex-matched controls (including 100 females and 200 males; aged 20-50 years with an average of 30.5 years) were obtained from a panel of unaffected subjects with Han Chinese ancestry. The control subjects were healthy individuals, and did not have any family history of mitochondrial disorders. The present study was approved by the Ethics Committee of The PLA 254 Hospital.

Clinical examinations. A comprehensive history was taken and a physical examination was performed on all available members of this Chinese pedigree to identify any syndromic findings, history of exposure to AmAn and genetic factors associated with hearing impairment. An age-appropriate audiological examination was additionally performed, which included pure-tone audiometry (PTA), auditory brainstem response and distortion product otoacoustic emissions. The PTA was calculated from the sum of the audiometric thresholds at 500, 1,000, 2,000, 4,000 and 8,000 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).

Screening for variants in the mitochondrial genome. The maternally-transmitted pattern in the studied family suggested the involvement of the mitochondria and thus analysis of variants in the mitochondrial genome was performed. Genomic DNA was isolated from whole blood samples (3 ml) from the participants using PAXgene Blood DNA Isolation kits (Qiagen, Inc., Valencia, CA, USA). The entire mitochondrial genomes of the matrilineal relatives were amplified by polymerase chain reaction (PCR) in 24 overlapping fragments using sets of the light-strand (L) and heavy-strand (H) oligonucleotide primers, as described elsewhere (24). The sequences of the primers are the same as stated in a previous study (24). The PCR primers were supplied by BGI (Shenzhen, China) and the PCR mixture included 200 μM dNTP, 10X buffer, Taq DNA polymerase and 15 mmol/l Mg^{2+} (Takara Biotechnology Co., Ltd., Dalian, China). The PCR was performed as follows: 95˚C for 5 min, 94˚C for 10 sec, 60˚C for 30 sec and 72˚C for 1 min (for 30 cycles), followed by 72˚C extension for 5 min. Finally, 2 μl PCR product was analyzed using 1.5% agarose gel, which contained ethidium bromide, electrophoresis at 130 V for 30 min. Following electrophoresis, a BandPeeper (Maestrogen, Inc., Hsinchu, Taiwan) instrument was used to detect the results using the Invitrogen™ E-Gel™ Imager software (version 2.2.4; Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA). 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 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession no. NC_012920; www.ncbi.nlm.nih.gov/genbank) (25) and the Mitomap database (www.mitomap.org).

Phylogenetic analysis. A total of 17 vertebrate mtDNA sequences were used in the inter-species analysis. The conservation index (CI) was calculated by comparing the human nucleotide variants with 16 other vertebrates. The CI was defined as the percentage of species from the list of 16 different vertebrates that possessed the wild-type nucleotide at that position. CI ≥75% was regarded as being indicative of functional potential.

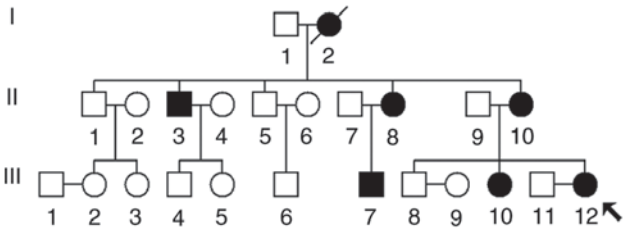

Figure 1. A Han Chinese family with aminoglycoside antibiotics-induced and non-syndromic hearing loss. Affected members are indicated by filled symbols, and the arrow denotes the proband.

Mutational analysis of the GJB2 gene. DNA fragments spanning the entire coding region of the *GJB2* gene were amplified by PCR using appropriate primers, in accordance with a previous investigation (26). The sequences of the primers are as stated in a previous study (26). PCR amplification and subsequent sequencing analysis were performed as described previously (26). The results were compared with the wild-type *GJB2* sequence to identify potential mutations (GenBank accession no. M86849).

Genotyping analysis of TRMU gene. A previous study demonstrated that the *TRMU* exon 1 A10S mutation may modulate the phenotypic manifestation of deafness-associated mitochondrial 12S rRNA mutations (27). To determine whether *TRMU* serves an active role in the expression of deafness, mutational screening on *TRMU* exon 1 was conducted in matrilineal relatives in this pedigree and in healthy controls. The primer information and PCR conditions have been described elsewhere (27). The PCR segments were analyzed and compared with the *TRMU* genomic sequence (GenBank accession no. AF_448221).

mtDNA copy number analysis. The mtDNA copy number was measured using quantitative (q)PCR and the $2^{-\Delta\Delta Cq}$ method (28). mtDNA content was normalized using a single copy nuclear β-globin gene. The following primers were used for qPCR analysis: β-Globin gene forward, 5'-CTATGGGAC GCTTGATGT-3' and reverse, 5'-GCAATCATTCGTCTG TTT-3'. For mtDNA forward, 5'-CACCAGCCTAACCAG ATTTC-3' and reverse, 5'-GGGTTGTATTGATGAGATTAG T-3'. Standard curves were generated for the two fragments and their respective amplification efficiencies calculated to test whether the $2^{-\Delta\Delta Cq}$ method was appropriate. The 20 μl PCR reaction solution contained 2X Taqman Universal PCR Master Mix (Takara Biotechnology Co., Ltd.), 500 nmol/l of each primer, 200 nmol/l Taqman Probe and 100 ng of total DNA. PCR conditions were 2 min at 50˚C and 10 min at 95˚C, followed by 40 cycles of denaturation for 15 sec at 95˚C and 60 sec annealing/extension at 60˚C. All experiments were repeated three times.

Statistical analysis. Statistical significance was evaluated by independent Student's t-test using SPSS software version 18.0

Table I. Clinical characterization of the deafness patients in the family.

Subject	Sex	Age at test, years	Age at onset, years	PTA (left ear)	PTA (right ear)	Level of hearing loss
II-3	Male	55	50	90	94	Profound
II-8	Female	63	53	96	93	Profound
II-10	Female	60	58	78	115	Profound
III-7	Male	35	30	78	91	Profound
III-10	Female	30	21	99	100	Profound
III-12	Female	28	1	108	108	Profound

PTA, pure tone audiometry.

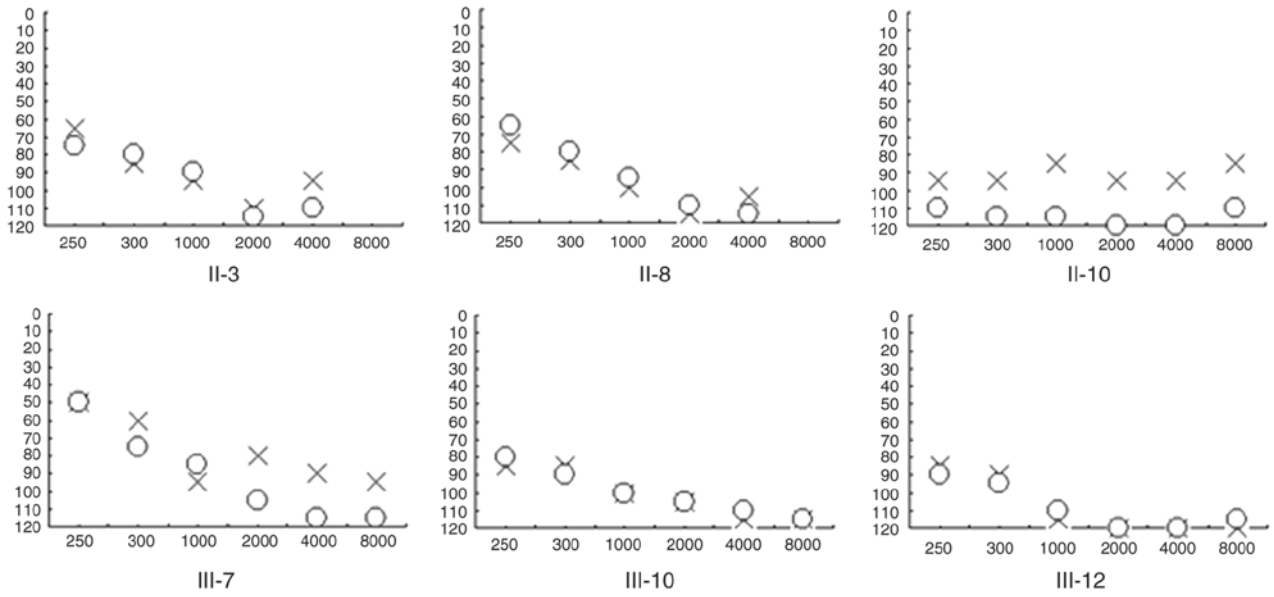

Figure 2. Air conduction audiograms of deaf individuals in the Chinese family. X, left ear; O, right ear. The y-axis represents hearing level (dB), and the x-axis represents frequency (Hz).

(SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical features of the Han Chinese family with hearing loss. The proband (III-12) was a 28-year-old woman from Tianjin, China. As presented in Fig. 1 and Table I, the proband began to suffer bilateral hearing loss at 1 year of age (108 dB right ear and 108 dB left ear). Subsequent to taking a comprehensive history and performing a physical examination, it was noted that the proband (III-12) had begun to use gentamycin (5 mg/kg/dose; 10 days) at the age of 15 years. Following 2 weeks of use, the proband exhibited irreversible hearing impairment. In addition, two relatives (II-3 and II-10) on the maternal side of the family had used AmAn in childhood and also developed profound hearing impairment. As presented in Fig. 2, audiological evaluation of the deaf patients in this family demonstrated that the majority of them had profound hearing loss with a slope-shaped pattern. In addition, this family exhibited a high penetrance of hearing loss, of 66.6% or 33.3% when AmAn was included or excluded, respectively.

Genotype analysis of mitochondrial genes. The fact that the deafness in this family was maternally inherited indicated that mtDNA dysfunction may be responsible for the clinical manifestation of hearing loss. The mtDNA sequence variants in matrilineal relatives in this pedigree were thus screened using PCR and direct sequencing. As presented in Table II, Sanger sequencing led to the identification of 27 genetic polymorphisms. These included 10 variants in the D-loop gene, three variants in the 12S rRNA gene, two variants in the 16S rRNA and one variant in the tRNA gene, in addition to a 9-bp common deletion in the junction between $tRNA^{Lys}$ and CO2, while other variants were primarily localized to protein-coding genes. In addition, six missense mutations were noted, including ND2 C5178A (Leu to Met), A8 C8414T (Leu to Phe), A6 A8701G (Thr to Ala) and A8860G (Thr to Ala), and CytB C14766T (Thr to Ile) and A15326G (Thr to Ala). These variants in rRNAs, tRNA and polypeptides were further evaluated by phylogenetic analysis against other organisms including mouse (29), bovine (30) and *Xenopus laevis* (31) sequences. However, none of these variants exhibited evolutionary conservation, with the exception of the A1555G and A4317G mutations (Figs. 3-5). In addition, A1555G and A4317G mutations were not detected in healthy

Table II. Mitochondrial DNA sequence variants in the family with hearing loss.

Gene	Position[b]	Alternations	Conservation (H/B/M/X)[a]	Previously reported
D-loop	73	A to G	-	Yes
	150	A to G	-	Yes
	199	T to C	-	Yes
	315	C to CC	-	Yes
	489	T to C	-	Yes
	16183	A to C	-	Yes
	16189	T to C	-	Yes
	16310	A to G	-	Yes
	16355	C to T	-	Yes
	16519	T to C	-	Yes
12S rRNA	750	A to G	A/A/A/-	Yes
	1438	A to G	A/A/A/G	Yes
	1555	A to G	A/A/A/A	Yes
16S rRNA	2706	A to G	A/G/A/A	Yes
	3010	G to A	G/G/A/A	Yes
ND1	3396	T to C	-	Yes
	3970	C to T	-	Yes
	4248	T to C	-	Yes
$tRNA^{Ile}$	4317	A to G	A/A/A/A	Yes
ND2	5178	C to A (Leu to Met)	T/T/T/T	Yes
CO1	7028	C to T	-	Yes
NC7	8281-8289	9-bp del	-	Yes
A8	8414	C to T (Leu to Phe)	F/M/L/W	Yes
A6	8701	A to G (Thr to Ala)	M/M/M/F	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	Yes
CytB	14766	C to T (Thr to Ile)	T/S/T/S	Yes
	15326	A to G (Thr to Ala)	T/M/I/I	Yes

[a]Conservation of amino acid for polypeptides or nucleotide for rRNAs in human (H), bovine (B), mouse (M) and *Xenopus laevis* (X). [b]See www.mitomap.org. rRNA, ribosomal RNA; tRNA, transfer RNA.

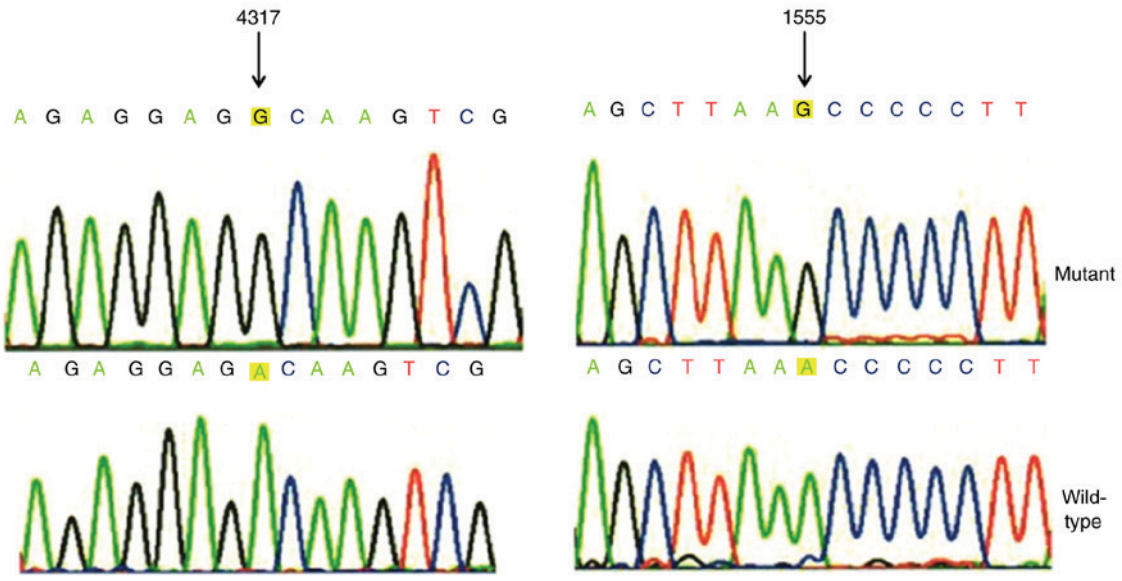

Figure 3. Identification of mitochondrial A4317G and A1555G mutations using polymerase chain reaction-Sanger sequencing.

Organism	Acc-stem		D-stem	D-loop	D-stem		Ac-stem	Anticd-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem	
	1	8	10		22	26	27	32	39	44	49	59	61	66	73
Cavia porcellus	AGAAATA	TG	TCTG	ATAA	AAGA	G	TTACT	TTGATAG	AGTAA	ACAAT	AGGGG	TTTAAAC	CCCCT	TATTTCT	A
Mus musculus	AGAAATA	TG	TCTG	ATAA	AAGA	A	TTACT	TTGATAG	AGTAA	ATTAT	AGAGG	TTCAAGC	CCTCT	TATTTCT	A
Myoxus glis	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATCAT	AGAGG	TTTAAAG	CCTCT	TATTTCT	A
Gorilla gorilla	AGAAATA	TG	TCTG	ATAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATAAT	AGAGG	TTTAAAC	CCCCT	TATTTCT	A
Rattus norvegicus	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATAAT	AGAGG	TTTAAAT	CCTCT	TATTTCT	A
Homo sapiens	AGAAATA	TG	TCTG	ATAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATAAT	AGGAG	CTTAAAC	CCCCT	TATTTCT	A
Pan troglodytes	AGAAATA	TG	TCTG	ATAA	AAGA	A	TTACT	TTGATAG	AGTAA	ATAAT	AGGAG	TTCAAAT	CCCCT	TATTTCT	A
Papio hamadryas	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	ACAAT	AGAGG	CCCCAAT	CCTCT	TATTTCT	A
Tarsius bancanus	AGAAATA	TG	TCTG	ACAA	AAGA	A	TTACT	TTGATAG	AGTAA	ATAAT	AGAGG	TTCAAGC	CCTCT	TATTTCT	A
Lemur catta	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATAAT	AGAGG	TTTAAAT	CCCCT	TATTTCT	A
Macaca sylvanus	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	ATAAT	AGAGG	CCCCAAC	CCTCT	TATTTCT	A
Pongo pygmaeus	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACT	TTGATAG	AGTAA	AAAAT	AGAGG	TCTAAAT	CCCCT	TATTTCT	A
Vombatus ursinus	AGAAATA	TG	TCTG	ACAA	AAGA	A	TTATC	TTGATAG	GATAA	ACTAT	AGGGG	TGCAAGC	CCCCT	TATTTCT	A
Zaglossus bruijni	AGAAATA	TG	TCTG	ATAA	AAGA	G	TTACA	TTGATAG	CGTAA	ATAAT	AGAGG	TTAAAAT	CCTCT	TATTTCT	A
Ornithorhynchus anatinus	AGAAATA	TG	TCTG	ACAA	AAGA	G	TTACA	TTGATAG	CGTAA	ATTAT	AGAGG	TTAAAAT	CCTCT	TATTTCT	A
Phascogale tapoatafa	AGAAATA	TG	TCTG	AGAA	TAGA	G	TTATC	TTGATAG	GATAA	AAAAT	AGGGG	TGCAAAC	CCCCT	TATTTCT	A
Thylamys elegans	AGAAATA	TG	TCTG	ATAA	AAGA	A	TTATC	TTGATAG	GATAA	ATTAT	AGGGG	TTTAAAC	CCCCT	TATTTCT	A

Figure 4. Sequence alignment of transfer RNA^{Ile} from 17 species. The arrow indicates position 59, corresponding to the A4317G mutation.

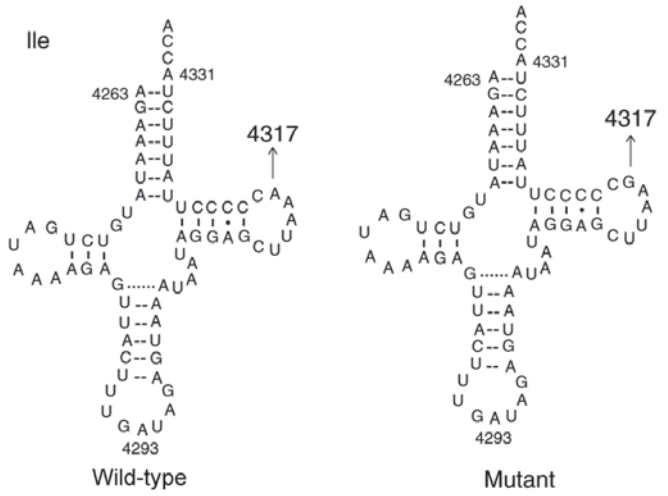

Figure 5. Location of the A4317G mutation in the mitochondrial tRNA^{Ile} gene. The cloverleaf structure of human mitochondrial tRNA^{Ile} was derived from Mitomap database (www.mitomap.org). Arrow indicates the position of the A4317G mutation. tRNA, transfer RNA.

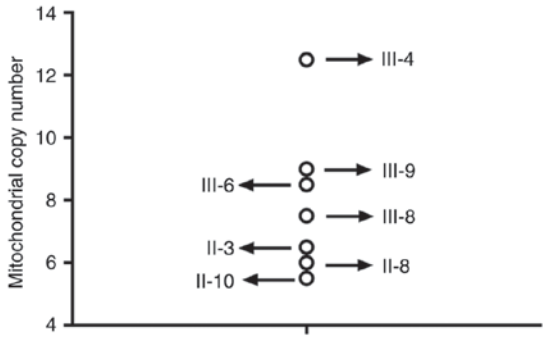

Figure 6. Qualification of mitochondrial DNA copy number in deaf patients from the family.

subjects, which indicated that these mutations may be involved in the pathogenesis of deafness in this family (P<0.05; data is not shown).

Screening for mutations in the GJB2 gene. Mutations in the *GJB2* gene have been reported to be important causes of non-syndromic hearing loss (32). Therefore, to determine the contribution of *GJB2* to the expression of deafness mutational analysis of the *GJB2* coding region in the matrilineal relatives and controls in this family was conducted. For this, PCR was performed, the obtained products purified, and subsequently analyzed by Sanger sequencing. However, no functional variants in the *GJB2* gene were detected (data not shown).

Mutational analysis of the TRMU gene. A previous study demonstrated that *TRMU* acts as a nuclear modified gene and is responsible for the 2-thiolation modification of mt-tRNAs, in addition to modulating the phenotypic manifestation of deafness-associated 12S rRNA mutations (27). Therefore, the *TRMU* A10S mutation was screened in matrilineal relatives and controls in the family in the present study. However, no variants in *TRMU* exon 1 were identified.

MtDNA content analysis. As presented in Fig. 6, it was observed that the patients carrying the mitochondrial A4317G and A1555G mutations had a lower mtDNA copy number than the healthy controls, suggesting that these mutations may decrease mtDNA content and cause mitochondrial dysfunction.

Discussion

The present study reported the molecular and genetic characterizations of a Han Chinese family with maternally-transmitted and AmAn-induced hearing loss. Hearing impairment as the sole clinical phenotype was presented only in the maternal lineage of this pedigree. This family exhibited high penetrance and expressivity of deafness; in particular, the penetrance of hearing loss was 66.6% when the effect of AmAn was included and 33.3% when it was excluded. In addition, the age at onset of deafness ranged between 1 and 58 years, with an average of 35 years and the matrilineal relatives in this family had an earlier age at onset of hearing loss, suggesting that mitochondrial sequence variants

may be risk factors that may be used for early molecular diagnosis and prevention of deafness.

Mutational analysis of the complete mitochondrial genome demonstrated the presence of the A1555G mutation, together with a set of genetic polymorphisms belonging to the human mitochondrial haplogroup B4c1b2 (33). The well-known A1555G mutation, which was first described in a large Arab-Israel pedigree with hearing loss (34), has been associated with AmAn-induced and non-syndromic hearing loss in numerous families worldwide (35-37). Although biochemical data indicated that the A1555G mutation led to sensitivity to AmAn (21,22), the incomplete penetrance and mild mitochondrial dysfunction suggested that this mutation alone was insufficient to produce the clinical phenotype. Therefore, other modifying factors, including environmental factors, the use of AmAn, mitochondrial variants/haplogroups and nuclear genes, may contribute to the expression of deafness. Notably, the absence of any functional variants in nuclear genes (*GJB2* and *TRMU*) suggested that these genes may not serve important roles in the phenotypic expression of the A1555G mutation in this family; therefore, other nuclear genes may contribute to the phenotypic variability.

The mitochondrial haplogroup has been demonstrated to modulate the phenotypic expression of the deafness-associated A1555G mutation (23). For example, the haplogroup Y2 specific $tRNA^{Glu}$ A14693G variant may increase the penetrance and expressivity of the deafness-associated A1555G mutation (23). In the present study, sequence analysis of the complete mitochondrial genomes of the affected individuals in this family identified the occurrence of the A4317G mutation. This mutation was localized at the TΨC arm of the $tRNA^{Ile}$ gene (position 59) using the Mitomap database, which is highly conserved from bacteria to human mitochondria (38,39). Notably, the A4317G mutation creates a novel base-pairing and may exert a structural effect. In fact, the presence of G59 facilitates the formation of an additional base pair with C54. This may in turn lead to complete rearrangement of the T stem and shortening of the T loop (40,41). Furthermore, this mutation has been reported to be associated with essential hypertension in the Han Chinese population (42). Biochemical analysis has also demonstrated that the A4317G mutation decreases the aminoacylation ability of $tRNA^{Ile}$ (43). In addition, it was identified that this mutation significantly decreased the mtDNA copy number when compared with that in controls. Indeed, mtDNA content has been shown to be a measure indicative of the cell number or mass of mitochondria (44). A previous experimental study suggested that alterations in mtDNA serve a fundamental role in the increase in reactive oxygen species; maintenance of mtDNA copy number was demonstrated to be essential for the preservation of mitochondrial function and cell growth (45). Therefore, it may be speculated that A4317G mutation may lead to the failure of tRNA metabolism, and subsequently impair mitochondrial protein synthesis, thereby worsening mitochondrial dysfunction (represented by a malfunction in biochemical processes, characterized by mitochondrial membrane potential collapse and decreased ATP production, which also has an essential role in the mediation of apoptosis) induced by A1555G mutation (46). Therefore, the $tRNA^{Ile}$ A4317G mutation may contribute to the high penetrance and expressivity of the hearing loss associated with the mtDNA A1555G mutation in this Chinese pedigree. The principal limitation of the present study study was the small sample size; further studies are required, with a greater number of deaf patients and controls.

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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

YC and D-JH designed the study. YC performed the experiments and analyzed the data. D-JH wrote the paper. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Informed consent was obtained from the participants prior to their participation in the present study, in accordance with the Ethics Committee of PLA 254 Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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