

The mitochondrial transfer RNA^{Asp} A7551G mutation may contribute to the clinical expression of deafness associated with the A1555G mutation in a pedigree with hearing impairment

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Abstract. The role of mitochondrial (mt)DNA variations in hearing loss have been studied extensively; in particular, the well-known pathogenic A1555G mutation in the human mitochondrial 12S ribosomal RNA gene is associated with aminoglycoside-induced and non-syndromic hearing loss. The present paper described a Chinese pedigree with hearing impairments. We first performed polymerase chain reaction and direct sequence analysis for the mtDNA genes. Additionally, the *GJB2* gene mutations were also genotyped. Notably, this family had a very high penetrance of deafness (66.7 and 33.3%; including and excluding aminoglycoside use, respectively). Sequence analysis of the mtDNA genes from the matrilineal relatives identified the occurrence of A1555G mutation, as well as the tRNA^{Asp} A7551G mutation. The A7551G mutation occurred at position 37 in the anticodon stem of tRNA^{Asp}, which is extremely conserved among various species. The nucleotide at this position is often chemically modified and thus contributes to the maintenance of functional tRNA^{Asp}, therefore, this mutation may cause an imbalance in the level of tRNA^{Asp} and lead to mitochondrial dysfunction which is involved in the pathogenesis of hearing loss. Taken together, the findings of the present study demonstrated that the A7551G mutation may have contributed to the deafness phenotype caused by the A1555G mutation.

Introduction

Hearing loss is a common communication disorder that approximately 360 million people have worldwide. There are

two types of hearing loss: Non-syndromic and syndromic (1). The molecular mechanism behind deafness has not been well characterized; but it is generally thought to occur through a combination of genetic, environmental and lifestyle factors. In fact, the genetic aspects may account for >50% of cases of deafness (2). Specifically, variants in mitochondrial (mt)DNA have been reported to be associated with aminoglycoside-induced hearing loss (3,4).

Human mtDNA is a 16,569 bp molecule that encodes 13 oxidative phosphorylation (OXPHOS)-related protein subunits, 2 ribosomal (r)RNAs, as well as 22 transfer (t) RNAs (5). Of the mutations in human mtDNA, A1555G is one of the most common pathogenic mutations associated with aminoglycoside ototoxicity (6). However, increasing evidence has shown that the A1555G mutation cannot directly lead to hearing loss, as among pedigrees with maternally inherited deafness, a certain number of members (despite carrying the A1555G mutation) exhibit different levels of hearing, ranging from normal to profound hearing loss (7-11). Therefore, aminoglycosides, nuclear-encoded genes and the mtDNA genetic background may actively function in the occurrence of deafness (12,13).

In the present study, to determine the contribution of mtDNA mutations/polymorphisms to the occurrence of deafness, mtDNA gene mutations were screened from a cohort of deaf patients. The present study reported the molecular features of a deaf Chinese pedigree. Sequence analysis of the mtDNA genes identified the co-existence of tRNA^{Asp} A7551G and 12S rRNA A1555G mutations.

Materials and methods

Samples and clinical examinations. The current study was approved by the Ethics Committee of Zhoushan Hospital, and each participant provided written informed consent. A four-generation Han Chinese family (Fig. 1), comprising 30 individuals and 6 deaf patients, was enrolled from the Department of Otolaryngology, Zhoushan Hospital, the age, sex of each patient was summarized in Table I. First, a comprehensive physical examination of each patient was performed, including the determination of whether they were taking aminoglycosides. In addition, the levels of hearing loss were

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Table I. Summary of the clinical and molecular data of several deafness patients of a Chinese family.

Subject	Sex	Age at test (year)	Age at onset (year)	PTA (dB) right ear	PTA (dB) left ear	Level of hearing loss
II-4	Female	68	60	68	70	Severe
II-6	Female	59	55	87	65	Severe
II-8	Female	61	58	90	87	Severe
III-10	Female	33	28	80	73	Severe
III-12	Male	35	30	100	90	Profound
IV-6	Female	8	4	30	38	Mild

tested in each participant using pure-tone audiometry (PTA) as described (3). The average PTA level was measured using the total audiometric thresholds at 125, 250, 500, 1,000, 2,000, 4,000 and 8,000 Hz. The levels of hearing loss were divided into the following degrees: i) Normal hearing (PTA <26 dB); ii) mild hearing loss (PTA 26-40 dB); iii) moderate hearing loss (PTA 41-70 dB); iv) severe hearing loss (PTA 71-90 dB); and v) profound hearing loss (PTA >90 dB). In addition, 200 healthy subjects were enrolled from the Physical Examination Center of Zhoushan Hospital. The inclusion criteria for the healthy subjects were that they were age- and sex-matched, healthy, and had no family history of mitochondrial disorders.

Screening for mutations in mtDNA genes. To analyze the mtDNA mutations, PCR and direct sequencing analysis were performed. The genomic DNA was first isolated from blood samples using Puregene DNA Isolation kits (Gentra Systems; Thermo Fisher Scientific, Inc.). The complete mtDNA of the matrilineal relatives from this family were then PCR amplified using 24 oligonucleotides, as previously described (14). The PCR product was purified and analyzed by Sanger sequencing as described (3). The data were then compared with the updated mitochondrial genome sequence to detect mtDNA mutations (GenBank accession no. NC_001807) (15).

Genotyping analysis of gap junction protein β 2 (*GJB2*) variants. Variants in the *GJB2* gene were further investigated in matrilineal relatives. For amplification of the *GJB2* coding region, PCR was performed using the following primers: Forward, 5'-TTGGTGTTCAGGAAGA-3' and reverse, 5'-GGCCTACAGGGTTTCAAAT-3', as previously described (16). PCR fragments were sequenced and the data were analyzed with the wild-type *GJB2* sequence (accession no. NM_004004.5).

Statistical analysis. SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and Student's t-test were used to perform the statistical analysis; we compared the significance between deaf patients carrying mitochondrial A1555G and A7551G mutations and control subjects. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Pedigree information. The proband (III-12), aged 35, lived in Zhoushan (Zhejiang, China). He attended to the Otolary

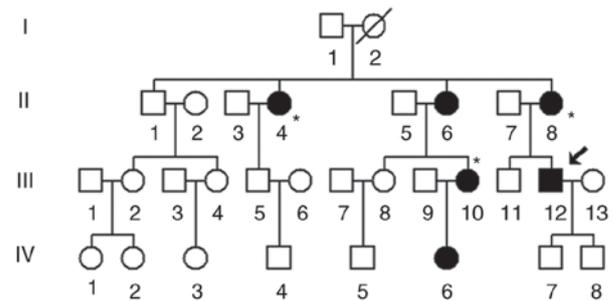


Figure 1. A four-generation pedigree with hearing loss. Filled symbols indicate the occurrence of deafness. The arrow indicates the proband. Asterisks denote individuals who had previously taken aminoglycosides.

Clinic of Zhoushan Hospital for deafness treatment. Clinical examination suggested that he had manifested bilateral hearing loss at 30 years old. As shown in Fig. 2, it was noted that the proband exhibited profound hearing loss (right ear, 100 dB; left ear, 90 dB). However, he did not have any associated medical history.

In addition, a physical examination was performed for each deaf patient from this pedigree, including the level of hearing loss, aminoglycosides usage, as well as other disorders. It was revealed that the family members did not have other common disorders, such as cardiovascular events, diabetes mellitus, visual impairment or neurological disorders. It was also noted that several individuals had a history of using aminoglycosides, such as individual II-8. She received kanamycin for fever when she was 58 years old, unfortunately, three weeks later, she developed bilateral deafness. As presented in Table I and Fig. 2, a variable degree of hearing loss was determined in this Chinese pedigree, ranging from mild (IV-6) to profound (III-12). Furthermore, the deafness exhibited a pattern of maternal transmission (Fig. 1).

Mutational analysis of mitochondrial genome. Owing to the apparent maternal transmission of deafness in this family, the proband's complete mtDNA was analyzed for mutations, together with those of other matrilineal relatives. A total of 24 overlapping DNA fragments spanning the 16,569 bp of mtDNA were PCR amplified and sequenced. It was shown that compared with the standard mitochondrial genome sequences, the proband and other matrilineal relatives exhibited 24 genetic polymorphisms (Table II), which were as follows: Seven variants in the D-loop gene, three in the 12S rRNA

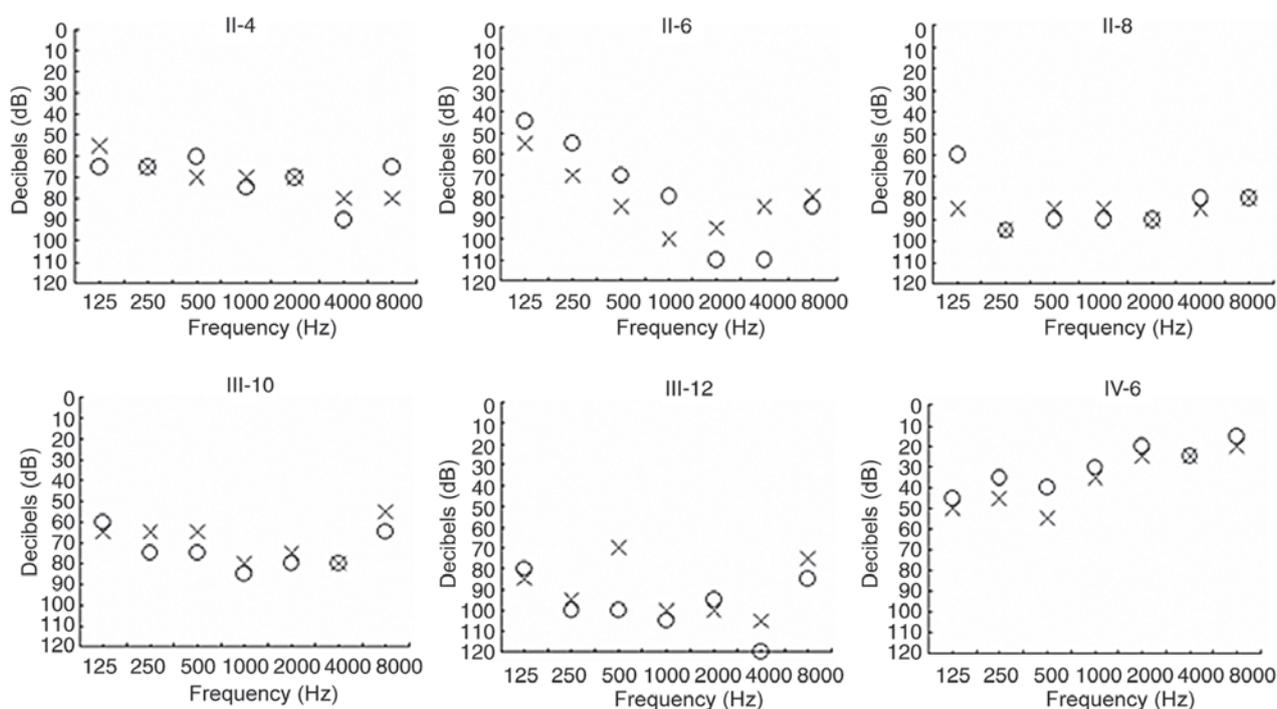


Figure 2. Air conduction audiogram of six affected subjects in the family. The x and y axes represent the sound level at decibel (dB) and frequency (Hz), respectively. X indicates the left ear, and O indicates the right ear.

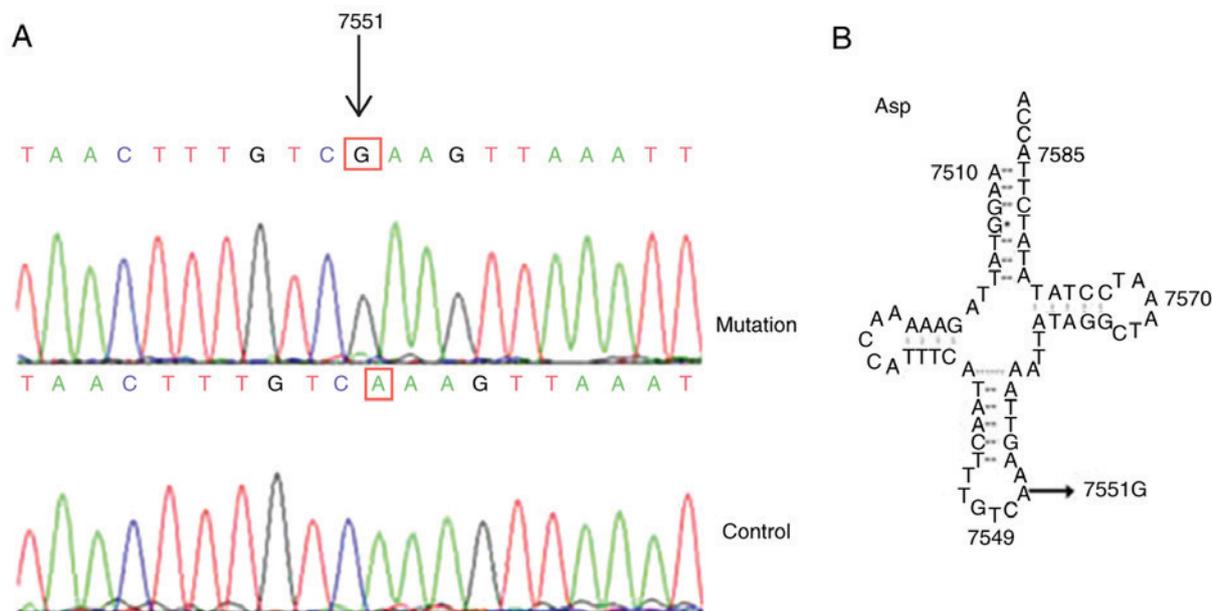


Figure 3. Characterization of mitochondrial A7551G mutation. (A) Identification of the A7551G mutation in the mitochondrial tRNA^{Asp} gene using polymerase chain reaction and Sanger sequencing. (B) The location of A7551G mutation in tRNA^{Asp} gene.

gene, two in the 16S rRNA gene and one in a tRNA gene (Fig. 3A and B), as well as a nine bp deletion in the non-coding region between tRNA^{Lys} and cytochrome C oxidase II (*CO2*). These polymorphisms have all been previously reported (www.mitomap.org). The other variants were located in oxidative phosphorylation (OXPHOS) encoding genes. In addition, six missense mutations were identified, namely, the NADH:Ubiquinone (*ND2*) C5178A (Leu→Met) and T5442C (Phe→Leu) mutations, the *ATPase 6* A8701G (Thr→Ala)

and A8860G (Thr→Ala) mutations, the NADH:Ubiquinone oxidoreductase core subunit 5 (*ND5*) G13928C (Ser→Thr) mutation, and the cytochrome B (*Cyt b*) A15326G (Thr→Ala) mutation. Phylogenetic analysis of these mutations in 11 vertebrates was performed, including mouse (17), bovine (18), and *Xenopus laevis* (19) (Table III). The results revealed that only the A1555G and A7551G mutations exhibited a high level of conservation. Further, the A1555G and A7551G mutations were not detected in the 200 control subjects, suggesting that

Table II. Sequence analysis of the family's complete mitochondrial genome.

Gene	Position	Alternation	Conservation (H/B/M/X)
D-loop	73	A to G	
	152	T to C	
	310	T to CTC	
	489	T to C	
	16189	T to C	
	16261	C to T	
	16519	T to C	
12S rRNA	750	A to G	A/A/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
	3010	G to A	G/G/A/A
<i>ND1</i>	3970	C to T	
<i>ND2</i>	5178	C to A (Leu to Met)	L/T/T/T
	5442	T to C (Phe to Leu)	F/F/M/L
CO1	7028	C to T	
tRNA ^{Asp}	7551	A to G	A/A/A/A
NC_7	8271-8279	9-bp del	
<i>A6</i>	8701	A to G (Thr to Ala)	T/S/L/Q
	8860	A to G (Thr to Ala)	T/A/A/T
<i>ND4</i>	11719	G to A	
<i>ND5</i>	13928	G to C (Ser to Thr)	S/T/S/T
<i>Cyt b</i>	15301	G to A	
	15326	A to G (Thr to Ala)	T/M/I/I

H, human; B, bovine; M, mouse; X, *Xenopus laevis*.

these mutations may have a functional impact associated with deafness (P<0.05; Table IV). Notably, the homoplasmic A7551G mutation occurred at position 37 in the anticodon stem of tRNA^{Asp}, which may have caused tRNA metabolism failure.

Screening for *GJB2* mutations. To examine whether the *GJB2* gene had critical function in deafness expression, the coding regions of *GJB2* were analyzed. However, no functional variants in this gene were identified (data not shown).

Discussion

The present paper reported the molecular features of a Chinese pedigree with maternally inherited hearing loss. Overall, a total of six matrilineal relatives of this family exhibited hearing loss of various degrees. Besides the deafness phenotype, no other clinical disorders were detected in this family. As the deafness was shown to be maternally transmitted in this family, the mtDNA variants of the proband and other matrilineal relatives were analyzed in this pedigree. There were six deaf patients in total, while the number of matrilineal relatives was nine,

Table III. Alignment of mt-tRNA^{Asp} gene from different species, arrow indicated the position 37, corresponding to the A7551G mutation.

Organism	Acc-stem	D-stem	D-loo	D-stem	Ac-Stem	Anti CD-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem
<i>Homo sapiens</i>	AAGGATTA	GAAA	AACCA	TTTCA	TAACT	TTGTCAA	AGTTA	AATT	ATAGG	CTAAAT	CCTAT	ATATCTTA
<i>Gorilla gorilla</i>	AAGATATTA	GAAA	AACCA	TTTCA	TAACT	TTGTCAA	GGTTA	AATT	ACGGG	TTAAAC	CCCCT	ATATCTTA
<i>Lemur catta</i>	AAGATATTA	GAAA	AATCA	TTTCA	TAACT	TTGTCAA	AGTTA	AATT	ATAGG	TTAAACC	CCTAT	GTATCTTT
<i>Pan troglodytes</i>	AAGATATTA	GAAA	AACCA	TTTCA	TAACT	TTGTCAA	AGTTA	AATT	ACAGG	TTAACC	CCCCT	ATATCTTA
<i>Hylobates lar</i>	AAGATATTA	GAAA	AACCA	TTTCA	TAACT	TTGTCAA	AGTTA	AGTT	ACAGG	TTCAAAC	CCCCT	ATATCTTA
<i>Trachypithecus obscurus</i>	GAGATATTA	GAAA	AATTA	TTTCA	TAGCT	TTGTCAA	AGCTA	AATT	ATAGG	ACATAT	CCTAT	ATATCTTA
<i>Muntiacus reevesi</i>	GAGGATTA	GAAA	AATA	TTTCA	TAACT	TTGTCAA	AGTTA	AGTT	ACAGG	TGAAAAT	CCCCT	ATATCTCA
<i>Mus musculus</i>	AAGATATTA	GAAA	AATCAA	TTTCA	TAACT	TTGTCAA	AGTTA	AATT	ATAGA	TCAATAA	TCTAT	ATATCTTA
<i>Balaenoptera bonaerensis</i>	GAGATATTA	GAAA	AATC	TTTCA	TAACT	TTGTCAA	AGTTA	AGTT	ACAAG	TGAGAAT	CCTGT	ATATCTCC
<i>Cynocephalus variegatus</i>	GAGATATTA	GAAA	AAACA	TTTCA	TGACT	TCGTGAG	AGTCA	ACTT	ATAGG	TCTAAT	CCITT	ATATCTTT
<i>Pongo pygmaeus abelii</i>	GAGATACTA	GAAA	AACCA	TTTCA	TGACT	TTGTCAA	AGTTA	AGTT	ACAGG	CCAAAAC	CCTGT	GTATCTTA

Table IV. Mitochondrial A1555G and A7551G mutations in affected individuals and controls.

Gene	Position	Replacement	Number of patients (%)	Number of 200 controls (%)	P-value
12S rRNA	1555	A to G	6 (60)	0 (0)	<0.05
tRNA ^{Asp}	7551	A to G	6 (60)	0 (0)	<0.05

therefore, the penetrance of hearing loss was 6/9=66.7% when the aminoglycoside was included, however, in the absence of aminoglycoside, there were three deaf patients, thus, the penetrance of hearing loss was 3/9=33.3%.

The maternal inheritance of deafness suggested that mitochondrial dysfunction may be the molecular basis of this hearing loss. Therefore, the sequence variants of mtDNA from the matrilineal relatives and the proband were detected. Screening of the mtDNA genes from the members of this family identified 24 genetic polymorphisms, belonging to human mitochondrial haplogroup B5a (20). Among these variants, the homoplasmic tRNA^{Asp} A7551G mutation was of particular interest. At the molecular level, this mutation was localized at the 3' end to the anticodon stem (position 37). The nucleotide at position 37 in tRNA^{Asp} is highly conserved among different vertebrates (21). In fact, A37 in tRNAs is often chemically modified (by thiolation or methylation) (22), therefore, the A7551G mutation may contribute to maintenance of the level of tRNA^{Asp} (23). Notably, the well-known A4435G mutation occurring at the same position in the tRNA^{Met} gene was previously found to be a risk factor for hypertension (24) and Leber's hereditary optic neuropathy (LHON) (25). Thus, the A7551G mutation, which is similar to the A4435G mutation, may have also resulted in failure of tRNA metabolism. A previous study also reported that the A7551G mutation, combined with *GJB2* 235delC, was involved in the progression of deafness in a Chinese family (26). Consequently, a decrease in the tRNA^{Asp} steady-state level may affect mitochondrial protein translation. Thus, the A7551G mutation would lead to defects in the synthesis of components of the mitochondrial respiration chain and subsequently, a decline in ATP production and a decrease in reactive oxygen species (ROS), which was similar to the tRNA^{Leu} A4317G mutation (27). These mitochondrial dysfunctions, caused by the A7551G mutation, may be involved in the pathogenesis of hearing loss (28,29). However, the penetrance of hearing loss and haplogroup in the present study differed from those reported by Wu *et al* (26). The current study identified the co-existence of 12S rRNA A1555G and tRNA^{Asp} A7551G mutations in a Chinese family, indicating that the A7551G mutation may increase the penetrance and expression of the deafness-associated A1555G mutation.

However, the incomplete penetrance of hearing loss, the homoplasmic form of the mtDNA mutations, the variable degrees of hearing impairment, as well as the absence of any functional variants in the *GJB2* gene suggested that the A7551G mutation itself was insufficient to produce the clinical phenotypes. Therefore, other risk-modifying factors, such as nuclear DNA, the use of aminoglycosides and environmental factors may have contributed to the clinical expression of the deafness-associated A1555G mutation in this family. Taking together, the findings of the present study indicated that the tRNA^{Asp} A7551G mutation

may have contributed to the clinical manifestation of hearing loss-associated mitochondrial A1555G mutation in this family. The main limitations of the current study was the relatively small sample size. Further assays including more deafness patients are required to verify the results. In addition, functional experiments, such as the measurement of ROS production, ATP and oxygen consumption levels, should be performed.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JZ, BL, W-WX and BF made substantial contributions to the design of the present study. G-WH collected the samples and performed the clinical analysis. X-XD performed the molecular and statistical analysis. G-WH wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Zhoushan Hospital, and each participant provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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