Low penetrance of hearing loss in two Chinese families carrying the mitochondrial tRNA$^{Ser(UCN)}$ mutations

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Abstract. Mutations in mitochondrial DNA (mtDNA), especially in mitochondrial 12S rRNA and transfer RNA(tRNA)$^{Ser(UCN)}$ genes, are important causes of non-syndromic hearing loss. However, the molecular mechanism underlying mt-tRNA mutations in clinical hearing impairment are not fully understood. The present study assessed the molecular characterization of two Chinese families with non-syndromic hearing loss, who both exhibited very low penetrance of deafness (9.1 and 12.5% for Family 1 and 2, respectively). Mutational analysis of the complete mtDNA genes identified the presence of cytochrome *c* oxidase 1/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations, together with polymorphisms belonging to human mitochondrial haplogroup D4 and G2b, respectively. Moreover, the G7444A and C7492T mutations occurred at highly conserved tRNA$^{Ser(UCN)}$ nucleotides and may cause tRNA metabolism failure, which is involved in mitochondrial translation defects. Therefore, the G7444A and C7492T mutations may lead to the mitochondrial dysfunction that responsible for deafness. However, the absence of any functional variants in Gap junction β-2, Solute Carrier Family 26 Member 4 and TRNA 5-methylaminomethyl-2-thiouridylate methyltransferase suggested that nuclear genes may not play active roles in the occurrence of deafness. In the present study, the observed incomplete penetrance of hearing loss and mild mitochondrial dysfunction indicated that mtDNA G7444A and C7492T mutations are insufficient to produce the deafness phenotype. Therefore, other risk factors such as environmental factors and epigenetic regulation may be involved in the pathogenesis of hearing loss in the families recruited in the present study.

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Key words: hearing loss, mitochondrial DNA-transfer RNA$^{Ser(UCN)}$, G7444A, C7492T, mutations, Chinese families

Introduction

Hearing loss is a common communication disorder, affecting 1-3 newborns out of every 1,000 live births globally (1). Survey data from the World Health Organization (http://www.who.int) indicates that 32 million individuals out of 360 million with hearing loss are pediatric patients (2,3). Hearing loss can be caused by gene alterations and environmental factors, including ototoxic drugs such as aminoglycoside antibiotics (AmAn) (4). Genetic alterations in human mitochondrial DNA (mtDNA) are also associated with deafness (5). Moreover, A1555G and C1494T mtDNA mutations have been implicated in both AmAn-induced and non-syndromic hearing loss in patients worldwide (6-8). mt-transfer RNA(tRNA)$^{Ser(UCN)}$ gene pathogenic mutations are associated with hearing loss (9) and include A7445G (10), 7472insC (11), T7510C (12) and T7511C (13). Furthermore, mt-tRNA mutations may have structural and functional implications, such as affecting the processing of RNA precursors, nucleotide modification and aminoacylation (14). However, the molecular mechanism underlying mt-tRNA$^{Ser(UCN)}$ mutations and deafness are not fully understood.

To investigate the association between mt-tRNA$^{Ser(UCN)}$ mutations and non-syndromic hearing loss, and to facilitate early diagnosis and prevention of mitochondrial-associated deafness, the present study performed genetic analyses for deafness-associated mtDNA mutations. The clinical and molecular features of two Chinese families with hearing impairment were assessed. PCR and Sanger sequencing analyses results suggested the presence of cytochrome *c* oxidase 1 (CO1)/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations in these families. Mutations in gap junction β-2 (GJB2), solute Carrier Family 26 Member 4 (SLC26A4) and TRNA 5-methylaminomethyl-2-thiouridylate methyltransferase (TRMU) genes are the most prevalent deafness causing genetic modifications worldwide (15-17). To examine the potential role of GJB2, SLC26A4 and TRMU in deafness, mutational analyses of these genes were performed in the matrilineal relatives in the two Chinese families.

Materials and methods

Subjects and clinical examinations. In the present genetic screening program for deafness-associated mt-tRNA$^{Ser(UCN)}$

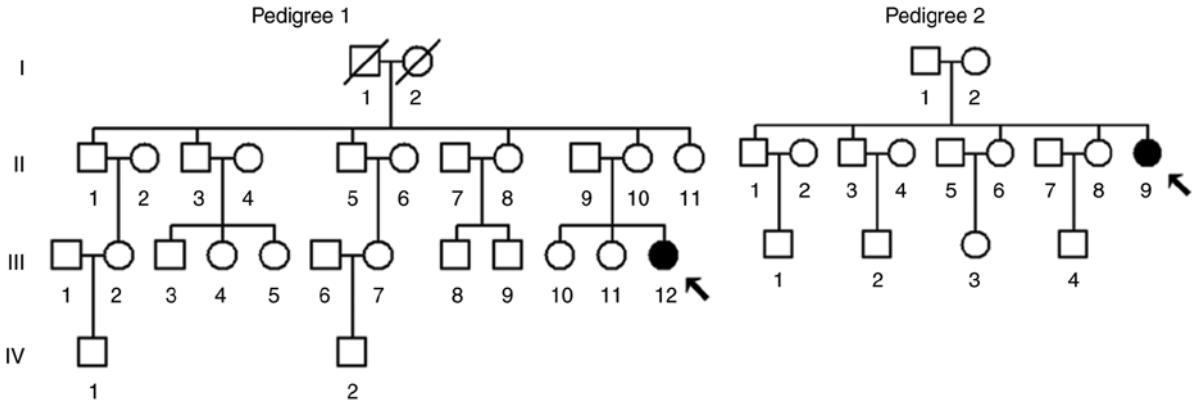

Figure 1. Pedigree chart of two Chinese families with hearing loss. The arrows indicate the probands and the open and filled symbols indicate unaffected and affected respectively.

mutations, two Chinese families with hearing loss were recruited from The Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between January 2018 and January 2019 (Fig. 1). There were 11 matrilineal relatives in Family 1 (5 males and 6 females), one of them (III-12) was the deaf patient. There were 8 matrilineal relatives in Family 2 (3 males and 5 females), one of them (II-9) was the deaf patient. Comprehensive medical histories and physical examination were assessed to identify any syndromic findings, history of AmAn use and genetic factors related to hearing impairment. An age-appropriate audiological examination was performed by an experienced audiologist, including pure-tone audiometry (PTA) and/or auditory brainstem response (ABR), immittance testing and distortion product otoacoustic emissions. PTA was calculated from the average 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: i) Normal, <26 decibel (dB); ii) mild =26-40 dB; iii) moderate =41-70 dB; iv) severe =71-90 dB; and v) profound >90 dB (18). The study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Signed written informed consent was obtained from the participants or their guardians. Control subjects (n=455; men, 200; women, 255; age, 20-45 years) were enrolled in the present study. The healthy subjects had normal hearing and did not have any mitochondrial disorders such as vision loss, neurological disorders, cancer or cardiovascular diseases. Subjects who had a family history of mitochondrial diseases or ongoing infectious disease, neoplastic disease, major surgery, severe liver dysfunction and inflammatory disease were excluded. Written informed consent was also provided by the control subjects.

Genetic screening for mtDNA mutations or variants. PCR and direct sequence analysis were performed to detect deafness related mtDNA mutations or variants. Blood samples (5 ml) from the family members (II-10, II-8 and II-5 in Family 1; I-2, II-6 and II-8 in Family 2; Fig. 1), as well as from 455 controls were collected. Genomic DNA was isolated from probands from the two families (III-12 and II-9) using the Puregene DNA Isolation kits (Gentra Systems, Inc.). DNA was preserved at 20˚C. Amplification of complete mtDNA genes was performed as previously described (19). A PCR mixture containing 200 μM dNTPs, 10X buffer, Taq DNA polymerase and 15 mmol/l Mg^{2+} (Takara Biotechnology Co., Ltd.) was used for PCR, the primers' sequences are listed in Table I. The following PCR thermocycling conditions were used: Initial denaturation at 95˚C for 5 min; 30 cycles of 94˚C for 10 sec, 60˚C for 30 sec and 72˚C for 1 min; and a final extension at 72˚C for 5 min. Subsequently, the 24 PCR products were purified and analyzed using Sanger sequence technology as previously described (20). The complete mtDNA genes of the matrilineal relatives from the two families (II-10, II-8 and II-5 in Family 1; I-2, II-6 and II-8 in Family 2) were also amplified by PCR and sequenced as above. Data were then compared with the revised Cambridge Reference sequences (rCRS) from GenBank database to detect mtDNA mutations (GenBank accession no. NC_001807) (21).

Conservation assessments. The sequence alignment was performed by using the ClustalW program version 2.0 (http;//www.ebi.ac.uk/Tools/msa/clustalw2/) (22). The conservation index (CI) of each mtDNA variants or mutations identified in these two families were analyzed as previously described (23). The CI was determined as the percentage of the sequence, from a list of 16 different vertebrates that possessed the wild-type nucleotide at the same position (24), these species included *Bos Taurus*, *Cebus albifrons*, *Gorilla gorilla*, *Homo sapiens*, *Hylobates lar*, *Lemur catta*, *Macaca mulatta*, *Macaca sylvanus*, *Mus musculus*, *Nycticebus coucang*, *Pan paniscus*, *Pan troglodytes*, *Pongo pygmaeus*, *Pongo abelii*, *Papio hamadryas* and *Tarsius bancanus*.

Screening for the GJB2, SLC26A4 and TRMU mutations. Mutations in GJB2, SLC26A4 and TRMU are associated with hearing impairment (15-17). To assess whether these nuclear genes play important roles in the clinical manifestation of hearing loss, a mutational screening for GJB2, SLC26A4 and TRMU was performed in the probands from these families (III-12 and II-9; Fig. 1). The primers used were as described previously (17,19,20). The primers for PCR amplification of GJB2 were: Forward, 5'-TATGACACTCCCCAGCACAG-3' and reverse, 5'-GGGGCAATGCTTAAACTGGC-3'. The five primer sequences for SLC26A4 were: Forward, 5'-CGTGTA GCAGCAGGAAGTAT-3' and reverse, 5'-TTAAATAAAAAA GACTGACT-3'; forward, 5'-TGGGGAAAAGGATGGTG

Table I. Primer sequence information for amplification of complete mitochondrial genome.

Primer name	Direction	Primer Sequence (5'-3')	Target region (bp)	Tm (°C)
Mit-1	F	CTCCTCAAAGCAATACACTG	592-1430	59.7
	R	TGCTAAATCCACCTTCGACC		
Mit-2	F	CGATCAACCTCACCACCTCT	1226-2026	59.7
	R	TGGACAACCAGCTATCACCA		
Mit-3	F	GGACTAACCCCTATACCTTCTGC	1930-2688	58
	R	GGCAGGTCAATTTCACTGGT		
Mit-4	F	AAATCTTACCCCGCCTGTTT	2480-3365	58
	R	AGGAATGCCATTGCGATTAG		
Mit-5	F	TACTTCACAAAGCGCCTTCC	3150-3980	58
	R	ATGAAGAATAGGGCGAAGGG		
Mit-6	F	TGGCTCCTTTAACCTCTCCA	3777-4679	59
	R	AAGGATTATGGATGCGGTTG		
Mit-7	F	ACTAATTAATCCCCTGGCCC	4466-5443	59
	R	CCTGGGGTGGGTTTTGTATG		
Mit-8	F	CTAACCGGCTTTTTGCCC	5238-6050	56
	R	ACCTAGAAGGTTGCCTGGCT		
Mit-9	F	GAGGCCTAACCCCTGTCTTT	5835-6661	59
	R	ATTCCGAAGCCTGGTAGGAT		
Mit-10	F	CTCTTCGTCTGATCCGTCCT	6450-7334	59
	R	AGCGAAGGCTTCTCAAATCA		
Mit-11	F	ACGCCAAAATCCATTTCACT	7129-8114	59.7
	R	CGGGAATTGCATCTGTTTTT		
Mit-12	F	ACGAGTACACCGACTACGGC	7908-8816	59.7
	R	TGGGTGGTTGGTGTAAATGA		
Mit-13	F	TTTCCCCCTCTATTGATCCC	8602-9416	59
	R	GTGGCCTTGGTATGTGCTTT		
Mit-14	F	CCCACCAATCACATGCCTAT	9211-10149	58
	R	TGTAGCCGTTGAGTTGTGGT		
Mit-15	F	TCTCCATCTATTGATGAGGGTCT	9967-10858	58
	R	AATTAGGCTGTGGGTGGTTG		
Mit-16	F	GCCATACTAGTCTTTGCCGC	10653-11511	59
	R	TTGAGAATGAGTGTGAGGCG		
Mit-17	F	TCACTCTCACTGCCCAAGAA	11295-12095	59
	R	GGAGAATGGGGGATAGGTGT		
Mit-18	F	TATCACTCTCCTACTTACAG	11929-12793	54
	R	AGAAGGTTATAATTCCTACG		
Mit-19	F	AAACAACCCAGCTCTCCCTAA	12551-13526	59
	R	TCGATGATGTGGTCTTTGGA		
Mit-20	F	ACATCTGTACCCACGCCTTC	13319-14287	55
	R	AGAGGGGTCAGGGTTCATTC		
Mit-21	F	GCATAATTAAACTTTACTTC	14081-15017	55
	R	AGAATATTGAGGCGCCATTG		
Mit-22	F	TGAAACTTCGGCTCACTCCT	14837-15997	58
	R	AGCTTTGGGTGCTAATGGTG		
Mit-23	F	TCATTGGACAAGTAGCATCC	15792-31	59.7
	R	GAGTGGTTAATAGGGTGATAG		
Mit-24	F	CACCATTCTCCGTGAAATCA	16401-794	59.7
	R	AGGCTAAGCGTTTTGAGCTG		

F, forward; R, reverse; Tm, Annealing Temperature.

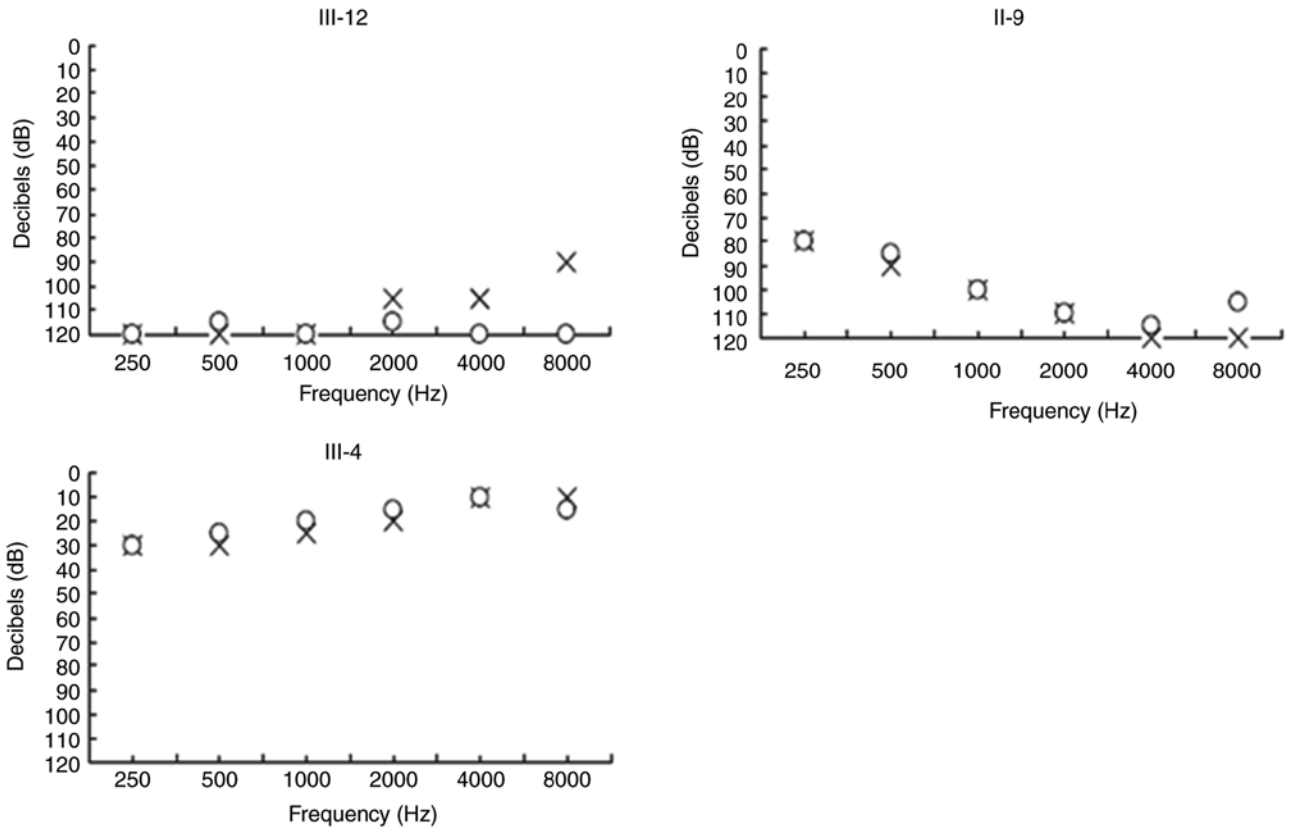

Figure 2. Air conduction audiogram of probands in the two Chinese families. X, left ear; O, Right ear.

GT-3' and reverse, 5'-CCAACCCCTTCTTTAGCTGA-3'; forward, 5'-GCAGGATAGCTCAAGGAATT-3' and reverse, 5'-TCATCAGGGAAAGGAAATAA-3'; forward, 5'-TCTCCT TGATGTCTTGCTTA-3' and reverse, 5'-CCCATGTATTTG CCCTGTTG-3'; and forward, 5'-CTGGGCAATAGAATG AGACT-3' and reverse, 5'-ATCTGTAGAAAGGTTGAA TA-3'. The primers for TRMU amplification were: Forward, 5'-ACAGCGCAGAAGAAGAGCAGT-3' and reverse, 5'-ACA ACGCCACGACGGACG-3'. The PCR products were analyzed by direct Sanger sequencing as mentioned in a previous study (19). The data were compared with published National Center for Biotechnology Information database sequences (https://www.ncbi.nlm.nih.gov/pubmed; GJB2, NM_004004; SLC26A4, NM_000441.1; TRMU, AF448221).

Assessment of pathogenicity. The updated pathogenicity scoring system (25) was used to assess the pathogenic status of CO1/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations. Using this approach, a mutation was classified as a 'neutral polymorphism' with a score ≤6, as 'possibly pathogenic' with a score from 7-10 and 'definitely pathogenic' with a score ≥11. The status of G7444A and C7492T mutations were also searched on the MITOMAP database (www.mitomap.org) to confirm their pathogenicity (26).

Results

Clinical presentation of two families with hearing loss. The present study investigated deafness related mt-tRNA$^{Ser(UCN)}$ polymorphisms in two Chinese families with hearing impairment (Fig. 1). In Family 1, the proband (III-12) was a 15-year-old female patient who began to suffer bilateral hearing impairment at the age of 10 years old. No family members in Family 1 had a history of using AmAn. Audiological evaluation indicated that the 15-year-old female had profound hearing loss (118 and 110 dB for right and left ears, respectively; Fig. 2; Table II). Furthermore, clinical examination identified that no other matrilineal relatives in Family 1, including the mother of the patient (II-10), had hearing loss.

In Family 2, the proband (II-9) was a 38-year-old female who was treated at the Otology Clinic of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology for deafness. A comprehensive history and physical examination indicated that the proband (II-9) suffered bilateral hearing impairment at the age of 20 years old. Furthermore, the proband had profound hearing loss (103 and 99 dB for right and left ears, respectively; Table II). However, all matrilineal relatives in Family 2 had normal hearing and none had a history of AmAn use (Fig. 1).

Mitochondrial genome analysis. To investigate the molecular basis of hearing loss, mutational screening of mtDNA genes was performed in these families. First, the present study amplified the complete mitochondrial genomes of the probands in the families (III-12 and II-9) and the corresponding matrilineal relatives (II-10, II-8 and II-5 in Family 1; I-2, II-6 and II-8 in Family 2; Fig. 1). The resultant 24 PCR products were sequenced as previously described (20). By comparing the present results with previous data from the rCRS (21), a number of genetic variants were identified, in

Table II. Summary of the clinical data for probands in two Chinese families.

Proband	Sex	Age at audiological test, year	Age at onset, year	Use of AmAn	PTA, dB Right ear	PTA, dB Left ear	Level of hearing loss
III-12	Female	15	10	No	118	110	Profound
II-9	Female	38	20	No	103	99	Profound
III-4	Female	10	-	No	21	19	Normal

AmAn, aminoglycoside antibiotics.

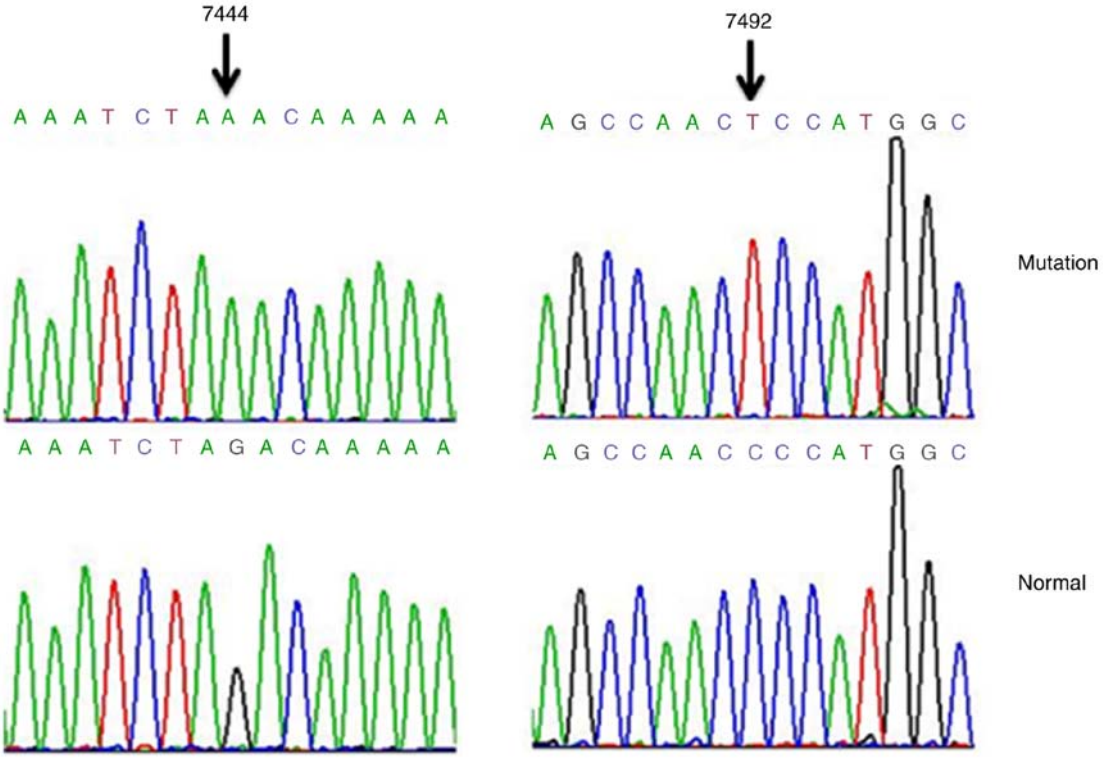

Figure 3. Sequence analysis of mitochondrial G7444A and C7492T mutations.

addition to the CO1/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations (Fig. 3; Table III). Moreover, ten variants were identified in the D-loop region, four known variants in 12S rRNA, four known variants in 16S rRNA and one 9-bp deletion in the non-coding region located between the CO2 and tRNA^{Lys} genes (21). In addition, 26 variants were found in protein coding genes. These included five missense mutations: A8701G (Thr→Ala), A8860G (Thr→Ala) in ATP synthase membrane subunit 6, A10398G (Thr→Ala) in NADH-ubiquinone oxidoreductase chain 3 (ND3), C14766T (Thr→Ile) and A15326G (Thr→Ala) in Cytochrome B. Only the CO1/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations are highly conserved in various species, including humans (21), bovines (27), mice (28) and *Xenopus laevis* (29). The present phylogenetic analysis results indicated that none of the other identified variants were conserved, suggesting that the G7444A and C7492T mutations may have functional significance in deafness phenotype expression (Figs. 3 and 4). In addition, G7444A and C7492T mutations were not detected in 455 control subjects. Moreover, these mt-tRNA$^{Ser(UCN)}$ mutations were not found in the matrilineal relatives (II-10, II-8 and II-5 in Family 1; I-2, II-6 and II-8 in Family 2).

In fact, the C7492T mutation occurred at position 26 in the anticodon stem of tRNA$^{Ser(UCN)}$, nucleotide at that position was very conserved from different species (21), while the G7444A mutation was localized at the conjunction between CO1 and tRNA$^{Ser(UCN)}$ (21).

Genotyping analysis of GJB2, SLC26A4 and TRMU genes. Previous studies showed that GJB2 (15), SCL26A4 (17) and TRMU (16) gene mutations are important causes of hereditary hearing loss. To investigate whether these nuclear genes played active roles in the phenotypic expression of deafness, mutational screening was performed. The present results suggested that no functional variants were identified in these nuclear genes.

G7444A and C7492T are 'possibly pathogenic' mutations associated with deafness. A pathogenicity scoring system (25)

Table III. MtDNA mutations in the two Chinese families with hearing loss.

Gene	Position	Mutation	Conservation (H/B/M/X)	rCRS	Family 1	Family 2	Previously reported[a]
D-Loop	73	A to G		A	G		Yes
	150	C to T		C			Yes
	263	A to G		A		G	Yes
	310	T to CTC		T	CTC	CTC	Yes
	489	T to C		T		C	Yes
	16093	T to C		T		C	Yes
	16111	C to T		C	T		Yes
	16189	T to C		T	C		Yes
	16362	T to C		T		C	Yes
	16519	T to C		T	C	C	Yes
12S rRNA	750	A to G	A/A/G/-	A	G	G	Yes
	827	A to G		A		G	Yes
	1048	C to T		C	T		Yes
	1438	A to G	A/A/A/G	A	G	G	Yes
16S rRNA	2706	A to G	A/G/A/A	A	G	G	Yes
	3010	G to A	G/G/A/A	G	A		Yes
	3107	delN		N		delN	Yes
	3206	C to T		C	T		Yes
ND1	3759	A to G		A		G	Yes
	3771	A to G		A	G		Yes
	3970	C to T		C	T	T	Yes
ND2	4685	A to G		A		G	Yes
	4769	A to G		A	G		Yes
	4883	C to T		C		T	Yes
CO1	6284	A to G		A	G		Yes
	7028	C to T		C		T	Yes
	7066	C to T		C	T		Yes
CO1/tRNA$^{Ser(UCN)}$	7444	G to A (Ser to Lys)	G/G/G/G	G	A		Yes
tRNA$^{Ser(UCN)}$	7492	C to T	C/C/C/C	C		T	Yes
CO_2	7976	G to A		G		A	Yes
	8080	C to G		C		G	Yes
NC7	8271-8279	9-bp del	T/S/L/Q	9-bp		9-bp del	Yes
ATP6	8701	A to G (Thr to Ala)	M/M/M/F	A	G	G	Yes
	8725	A to G		A	G		Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G	Yes
	9128	T to C		T		C	Yes
CO3	9540	T to C		T	C		Yes
ND3	10398	A to G (Thr to Ala)	T/T/T/A	A	G		Yes
ND4L	10493	T to C		T		C	Yes
ND4	10873	T to C		T	C		Yes
	11440	G to A		G		A	Yes
ND5	12705	C to T		C	T		Yes
ND6	14455	C to T		C	T		Yes
CytB	14766	C to T (Thr to Ile)	T/S/T/S	C		T	Yes
	15301	G to A		G	A	A	Yes
	15326	A to G (Thr to Ala)	T/M/I/I	A	G	G	Yes
	15784	T to C		T	C	C	Yes

[a]Human MITOMAP database (www.mitomap.org). H, human; B, bovine; M, mouse; X, *Xenopus laevis*; rCRS, revised Cambridge Reference Sequences; ND, NADH-ubiquinone oxidoreductase chain; CO, cytochrome *c* oxidase; CytB, Cytochrome B; ATP6, ATP synthase membrane subunit 6; tRNA, transfer RNA; Lys, lysine; Thr, threonine; Ala, alanine; Ile, isoleucine; del, deletion.

Table IV. Pathogenicity scoring system for G7444A and C7492T mutations.

Scoring criteria	G7444A mutation	Score/20	C7492T mutation	Score/20
>1 independent report	Yes	2	Yes	2
Evolutionary conservation of the base pair	No changes	2	No changes	2
Variant heteroplasmy	No	0	No	0
Segregation of the mutation with disease	Yes	2	Yes	2
Histochemical evidence of mitochondrial disease	No evidence	0	No evidence	0
Biochemical defect in complex I, III or IV	No	0	No	0
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Weak evidence	3	Weak evidence	3
Maximum score	Possibly pathogenic	9	Possibly pathogenic	9

Classification: ≤6 points: 'neutral polymorphisms'; 7-10 points: 'possibly pathogenic'; 11-13 points (not including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): 'probably pathogenic'; ≥11 points (including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): 'definitely pathogenic'. mt-tRNA, mitochondrial DNA-transfer RNA.

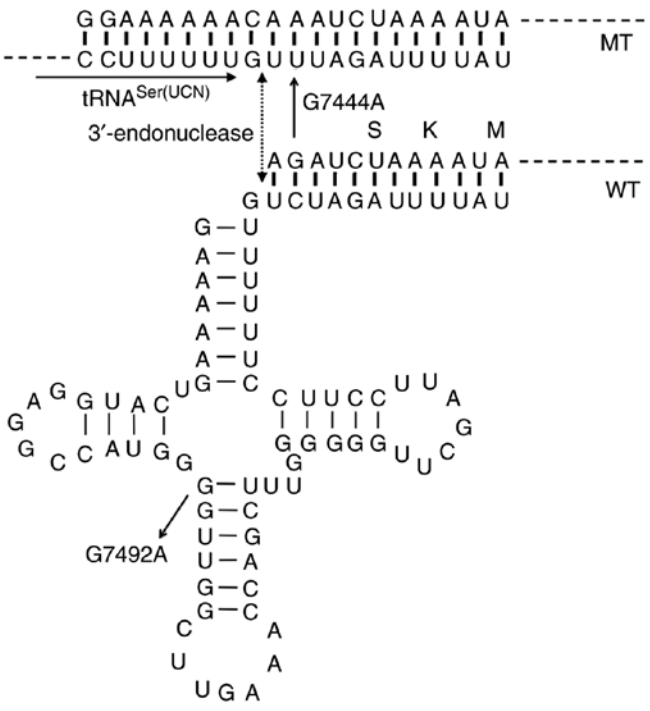

Figure 4. Locations of mitochondrial G7444A and C7492T mutations. tRNA, transfer RNA; WT, wild-type; MT, mutant.

was used to evaluate the status of CO1/tRNA$^{Ser(UCN)}$ G7444A and tRNA$^{Ser(UCN)}$ C7492T mutations (Table IV). It was found that the total scores of G7444A and C7492T mutations were each nine points, and that these variants were 'possibly pathogenic' changes associated with hearing impairment.

Discussion

The present study investigated the molecular and genetic features of two Chinese family with deafness related mt-tRNA$^{Ser(UCN)}$ gene mutations. The mt-tRNA$^{Ser(UCN)}$ gene has been implicated as a key site for mutations and variants associated with hearing impairment (30-32). In a previous genetic screening program for mt-tRNA$^{Ser(UCN)}$ variants in 2,651 patients with deafness, the incidence rate of T7511C, T7505C and A7445C mutations were all 0.04% (9). It has been suggested that the primary defect in mt-tRNA$^{Ser(UCN)}$ mutations is tRNA metabolism failure, which affects mitochondrial translation and respiratory chain function (33).

Using PCR and direct sequencing analysis, the present results identified two potential pathogenic mutations in the two families with hearing loss; G7444A and C7492T. The CO1/tRNA$^{Ser(UCN)}$ G7444A mutation is on the mtDNA heavy strand and causes a read-through of the AGA stop codon in the CO1 gene. The CO1/tRNA$^{Ser(UCN)}$ G7444A mutation adds three amino acids, Lys-Gln-Lys, to the C-terminus of the polypeptide (34). In addition, the G7444A mutation is adjacent to the 3' terminal endonucleolytic processing site of the L-strand RNA precursor, spanning tRNA$^{Ser(UCN)}$ and ND6 mRNA (35). Moreover, the deafness-associated A7445G mutation causes a significant decrease in the steady-state level of tRNA$^{Ser(UCN)}$ and ND6 mRNA (35,36). Thus, the G7444A mutation, which is similar to the A7445G mutation, may also cause impaired tRNA$^{Ser(UCN)}$ metabolism, which plays an important role in deafness (37). Kokotas *et al* (38) investigated a Greek family with hearing loss and identified the co-existence of the G7444A mutation and the GJB2 c.35delG mutation.

Furthermore, in the present study, the homoplasmic C7492T mutation was identified in Family 2. Structurally, this mutation is located at position 26 of the tRNA$^{Ser(UCN)}$ gene anticodon stem (39). The nucleotide at this position is highly conserved between species, indicating that it plays a critical role in tRNA stability and normal function. Moreover, the heteroplasmic T4295C mutation, located at the same position in the tRNA^{Ile} gene, is a pathogenic mutation causing chronic progressive external ophthalmoplegia (40). In addition, the C7492T mutation disrupts conserved base-pairing (A26-U44) and may cause the tRNA metabolism failure. The C7492T mutation is also associated with polycystic ovary

syndrome (PCOS) (41) and hypertension (42). Thus, the present study hypothesized that the C7492T mutation may lead to mitochondrial dysfunction and may be involved in the pathogenesis of hearing loss. However, sequence analysis results for the complete mtDNA genes from the matrilineal relatives (II-10, II-8 and II-5 in Family 1; I-2, II-6 and II-8 in Family 2) indicated that the G7444A and C7492T mutations were only presented in the probands (III-12 in Family 1; II-9 in Family 2), but were absent in matrilineal relatives. Therefore, the present results suggested that G7444A and C7492T may be *de novo* mutations.

In total >130 genes have been associated with hearing loss (43). GJB2 encodes a gap junction protein that is expressed in the cochlea and is thought to be important for recycling potassium ions that flow into sensory hair cells as part of the transduction current (44). Mutations in the GJB2 gene are a major cause of non-syndromic hearing loss (45). Among these mutations, c.235delC and c.167delT are the most frequent variants among Eastern Asian populations (46). Furthermore, SLC26A4 mutations contribute to non-syndromic enlarged vestibular aqueduct (MIM 600791) and Pendred syndrome (MIM 274600) (47). Moreover, c.919>2A>G is the most frequent SLC26A4 gene mutation associated with non-syndromic hearing loss (48). The TRMU gene, which encodes the mitochondrial tRNA-specific 2-thiouridylase, is regarded as a nuclear modified gene for the phenotypic manifestation of deafness-associated 12S rRNA mutations (16). In a previous study, the A10S mutation in TRMU exon 1 was found to modulate the clinical expression of deafness related A1555G mutation in an Arab-Israel family (16). However, in the present study, the absence of GJB2, SLC26A4 and TRMU variants in the two families suggested that these genes may not play a role in the clinical expression of hearing impairments.

Mitochondrial haplogroups may influence the phenotypic expression of hearing loss associated with mtDNA pathogenic mutations (49). Mitochondrial haplogroup B may increase the risk for hearing impairment among patients with the A1555G mutation (50). In addition, mitochondrial haplogroup specific variants including G15927A of haplogroup B5b, T12338C of haplogroup F2, T5802C, T10454C, C12224T and G11696A of haplogroup D4, G5821A of haplogroup C, A14693G of haplogroups Y2 and F, and T15908C of haplogroup Y2 may enhance the penetrance of hearing loss carrying the 12S rRNA A1555G mutation (51). Sequence characterization of mitochondrial genomes in the present study identified sets of genetic polymorphisms belonging to the D4 and G2b Eastern Asian haplogroups (52). However, phylogenetic conservation analysis showed that, except for the G7444A and C7492T mutations, the variants were not conserved. Collectively, the present results suggested that the mitochondrial genetic background may not play a significant role in the expression of deafness-associated pathogenic mtDNA mutations. Moreover, the pathogenicity scoring system indicated that the total scores of G7444A and C7492T mutations were both nine points, and belonged to the 'possibly pathogenic' classification associated with deafness; however, scoring needs to be examined using cybrid cells carrying the G7444A or C7492T mutation in future studies. Furthermore, data from the MITOMAP suggested that the C7492T mutation was likely benign. However, MITOMAP reports the published data on human mtDNA mutations or variants and does not analyze functions (53). The C7492T mutation had been reported to be associated with PCOS (41) and hypertension (42), thus C7492T is a disease-associated mutation. However, in the present study, the incomplete penetrance of hearing loss and mild mitochondrial dysfunction indicated that G7444A and C7492T mutations are insufficient to produce the observed clinical phenotypes. Therefore, environmental factors and epigenetic modification may contribute to the expression of the deafness phenotype.

There are several limitations in the present study. Functional analysis was not performed for the C7492T and G7444A mutations, and the small sample size is major limitation. Further studies, including more patients with deafness and using trans-mitochondrial cybrid cells are required to investigate the present results in more depth.

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Availability of data and materials

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

Authors' contributions

WP and YZ designed the studies, XZ collected the samples and performed the clinical analysis of two pedigrees, JY performed the molecular analysis of mitochondrial genomes. WP and YZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Signed written informed consent was obtained from the participants or their guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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