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## Mitochondrial tRNA<sup>Thr</sup> G15927A mutation may modulate the phenotypic manifestation of ototoxic 12S rRNA A1555G mutation in four Chinese families

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### Abstract

**Objective**—To investigate the role of mitochondrial modifiers in the development of deafness associated with 12S rRNA A1555G mutation.

**Methods**—Four Chinese families with nonsyndromic and aminoglycoside-induced deafness were studied by clinical and genetic evaluation, molecular and biochemical analyses of mitochondrial DNA (mtDNA).

**Results**—These families exhibited high penetrance and expressivity of hearing impairment. Penetrances of hearing loss in WZD31, WZD32, WZD33, and WZD34 pedigrees ranged from 50 to 67% and from 39 to 50%, respectively, when aminoglycoside-induced hearing loss was included or excluded. Matrilateral relatives in these families developed hearing loss at the average of 14, 13, 16, and 15 years of age, respectively, when aminoglycoside-induced deafness was excluded. Mutational analysis of entire mtDNA in these families showed the homoplasmic A1555G mutation and distinct sets of variants belonging to haplogroup B5b1. Of these, the tRNA<sup>Thr</sup> G15927A mutation locates at the fourth base in the anticodon stem (conventional position 42) of tRNA<sup>Thr</sup>. A guanine (G42) at this position of tRNA<sup>Thr</sup> is highly conserved from bacteria to human mitochondria. The lower levels and altered electrophoretic mobility of tRNA<sup>Thr</sup> were observed in cells carrying A1555G and G15927A mutations or only G15927A mutation but not cells carrying only A1555G mutation. The abolished base pairing (28C-42G) of this tRNA<sup>Thr</sup> by the G15927A mutation caused a failure in tRNA metabolism, worsening the mitochondrial dysfunctions altered by the A1555G mutation.

**Conclusion**—The G15927A mutation has a potential modifier role in increasing the penetrance and expressivity of the deafness-associated 12S rRNA A1555G mutation in those Chinese pedigrees.

### Keywords

aminoglycoside; Chinese; haplogroup; hearing loss; mitochondrial; modifier; mutation; ototoxicity; penetrance; ribosomal RNA; tRNA; variant

### Introduction

Hearing loss is a very common congenital disorder affecting one in 1000 newborns. Hearing loss can be caused by hereditary and environmental factors including ototoxic drugs such as aminoglycoside antibiotics. Mutations in mitochondrial DNA (mtDNA) have been found to be one of the most important causes of sensorineural hearing loss in some countries [1,2]. Of these, the C1494T mutation in the 12S rRNA has been associated with both aminoglycoside induced and nonsyndromic hearing loss in some Chinese and Spanish pedigrees [3–5]. The 12S rRNA A1555G mutation, however, has been associated with both aminoglycoside-induced and nonsyndromic hearing loss in many families worldwide [6–16]. The administration of aminoglycosides induces or worsens hearing loss in these participants carrying the A1555G or C1494T mutation. In the absence of aminoglycosides, matrilineal relatives within and among families carrying the A1555G or C1494T mutation exhibited a wide range of penetrance, severity and age-of-onset in hearing loss [3,9,10,12,14]. Functional characterization of cell lines derived from matrilineal relatives of a large Arab–Israeli family or one large Chinese family demonstrated that the A1555G or C1494T mutation conferred mild mitochondrial dysfunction and sensitivity to aminoglycosides [3,17–20]. These findings strongly indicated that the A1555G and C1494T mutations by themselves are insufficient to produce the deafness phenotype. Therefore, other modifier factors including aminoglycosides, nuclear and mitochondrial genetic modifiers modulate the expressivity and penetrance of hearing loss associated with the A1555G or C1494T mutation [19–22].

These modifier factors, however, remain poorly defined. To investigate the role of mitochondrial genetic factors in the development of hearing loss, a systematic and extended mutational screening of mtDNA has been initiated in several cohorts of hearing-impaired participants. In the earlier studies, we showed the highly variable penetrance and expressivity of hearing loss in 40 Han Chinese families carrying the A1555G mutation [12–15,22–25]. Our data suggested that the tRNA<sup>Glu</sup> A14693G, tRNA<sup>Thr</sup> T15908C, tRNA<sup>Arg</sup> T10454C, tRNA<sup>Ser(UCN)</sup> G7444A, and tRNA<sup>Cys</sup> G5821A variants may influence the phenotypic manifestation of the A1555G mutation [15,22,24]. In this study, we characterized another four Han Chinese pedigrees carrying the A1555G mutation with high penetrance and expressivity of hearing loss. A wide range of penetrance and severity of hearing loss, however, was observed in the matrilineal relatives within and among these families. To assess the contribution that mtDNA variants make toward the phenotypic expression of the A1555G mutation, we performed PCR amplification of fragments spanning entire mtDNA and subsequent DNA sequence analysis in the matrilineal relatives of those families. Interestingly, the G15927A mutation, which locates at the fourth base in the anticodon stem, corresponding to conventional position 42 of the tRNA<sup>Thr</sup>, is implicated to influence the penetrance and expressivity of hearing loss associated with the A1555G mutation. Functional significance of the G15927A mutation was evaluated by examining for the steady-state levels and aminoacylation capacities of mitochondrial tRNA<sup>Thr</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Gly</sup>, and tRNA<sup>Ser(AGY)</sup> using lymphoblastoid cell lines derived from an affected matrilineal relative carrying both A1555G and G15927A mutations, from an affected Chinese participant carrying only A1555G mutation, a hearing normal individual carrying only the G15927A mutation and from a married-in-control individual lacking those mtDNA mutations. Furthermore, it was implied that *TRMU* and

*GJB2* mutations modulated the phenotypic manifestation of hearing loss associated with the A1555G mutation [21,26]. To further examine the role of the *TRMU* and *GJB2* genes in the phenotypic expression of the A1555G mutation, we performed a mutational analysis of the *TRMU* and *GJB2* genes in the hearing-impaired and normal hearing participants of these families.

## Participants and methods

### Participants and audiological examinations

As the part of genetic screening program for hearing impairment, four Han Chinese families, as shown in Fig. 1, were ascertained through the Otology Clinic of Wenzhou Medical College. A comprehensive history and physical examination were performed to identify any syndromic findings, the history of the use of aminoglyco-sides, genetic factors related to the hearing impairment in members of these pedigrees. An age-appropriate audiological examination was performed and this examination included pure-tone audiometry and/or auditory brainstem response, immittance testing and distortion product otoacoustic emissions. The pure-tone audiometry was calculated from the sum of the audiometric thresholds at 500, 1000, 2000, 4000, and 8000 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. Informed consent was obtained from participants before their participation in the study, in accordance with the Cincinnati Children's Hospital Medical Center Institutional Review Board and Ethics Committee of Wenzhou Medical College. The 262 control DNA used for screening for the presence of mtDNA variants were obtained from a panel of unaffected participants from Han Chinese ancestry.

### Mutational analysis of mitochondrial genome

Genomic DNA was isolated from whole blood of participants using Puregene DNA Isolation kits (Gentra Systems, Minneapolis, Minnesota, USA). Participant's DNA fragments spanning the 12S rRNA gene were amplified by PCR using oligodeoxynucleotides corresponding to positions 618–635 and 1988–2007 [11]. For the detection of the A1555G mutation, the amplified segments were digested with a restriction enzyme *BsmAI* [11]. Equal amounts of various digested samples were then analyzed by electrophoresis through 1.5% agarose gel. The proportions of digested and undigested PCR product were determined by laser densitometry after ethidium bromide staining to determine if the A1555G mutation is in homoplasmy in these participants.

The entire mitochondrial genomes of four probands were PCR amplified in 24 overlapping fragments by use of sets of the light-strand and the heavy strand oligonucleotide primers, as described elsewhere [27]. Each fragment was purified and subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle (Applied Bio-systems, Foster City, California, USA) sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC\_001807) [28].

For the examination of the G15927A mutation, the first PCR segments (1122 bp) were amplified using genomic DNA as template and oligodeoxynucleotides corresponding to mtDNA at positions 14 856–15 978 to rule out the coamplification of possible nuclear pseudogenes [29]. Then, the second PCR product (138 bp) was amplified using the first PCR fragment as template and oligodeoxynucleotides corresponding to mtDNA at positions 15 822–15 960 and subsequently digested with restriction enzymes *HpaII* as the G15927A mutation abolished the site for this enzyme. Equal amounts of various digested samples were then analyzed by electrophoresis through 7% polyacrylamide gel. The proportions of digested and

undigested PCR product were determined by laser densitometry after ethidium bromide staining to determine if the G15927A mutation is in homoplasmy in these participants. The allele frequency of G15927A variant was determined by PCR amplification using the genomic DNA derived from 262 Han Chinese controls and subsequent restriction enzyme analysis of PCR products, as described above.

### Cell cultures

Lymphoblastoid cell lines were immortalized by transformation with the Epstein–Barr virus, as described elsewhere [30]. Cell lines derived from one affected individual (WZD32-III-7) carrying A1555G and G15927A mutations and a married-in-control (WZD32-III-1) in this Chinese family, an affected individual (WZD1-IV-2) from the other Chinese family carrying only A1555G mutation (Tang *et al.*, 2007) [25] and a hearing normal individual (A4) carrying only the G15927A mutation were grown in RPMI 1640 medium (Invitrogen, Carlsbad, California, USA), supplemented with 10% fetal bovine serum.

### Mitochondrial tRNA Northern analysis

Total mitochondrial RNA were obtained using TOTALLY RNA kit (Ambion, Austin, Texas, USA) from mitochondria isolated from lymphoblastoid cell lines ( $\sim 4.0 \times 10^8$  cells), as described previously [21,31]. Five microgram of total mitochondrial RNA were electrophoresed through a 10% polyacrylamide/7 mol/l urea gel in Tris–borate–EDTA buffer (after heating the sample at 65°C for 10 min), and then electroblotted onto a positively charged nylon membrane (Roche, Basel, Switzerland) for the hybridization analysis with oligodeoxynucleotide probes. For the detection of tRNA<sup>Thr</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Gly</sup>, and tRNA<sup>Ser(AGY)</sup>, the following nonradioactive digoxigenin (DIG)-labeled oligodeoxynucleotides specific for each RNA were used: 5'-CCTTGGAAAAG GTTTTCATCTC C-3' (tRNA<sup>Thr</sup>); 5'-TACTTTTATTTGGAGTTGCACC-3' [tRNA<sup>Leu(CUN)</sup>]; 5'-TCACTGTAAAGAGGTGTTGGTTCTCTTAATCTT-3' (tRNA<sup>Lys</sup>); 5'-TACTCTTTTTTGAATGTTGTC-3' (tRNA<sup>Gly</sup>); 5'-TTAGCAGTTCTTGTGAGCTTTCTC-3' [tRNA<sup>Ser(AGY)</sup>] [28]. DIG-labeled oligodeoxynucleotides were generated by using DIG oligonucleotide Tailing kit (Roche). The hybridization was carried out as detailed elsewhere [31]. Quantification of density in each band was made as detailed previously [32].

### Mitochondrial tRNA aminoacylation analysis

Total mitochondrial RNAs were isolated as above but under acid condition. The total mitochondrial RNA (2 mg) was electrophoresed at 41C through an acid 10% polyacrylamide/7 mol/l urea gel in a 0.1 mol/l sodium acetate (pH 5.0) to separate the charged and uncharged tRNA, as detailed elsewhere [33,34]. Then RNAs were electroblotted onto a positively charged membrane (Roche) and hybridized sequentially with the specific tRNA probes as above.

### Genotyping analysis of *TRMU* gene

The genotyping for the *TRMU* A10S variant in subjects from three pedigrees was PCR-amplified for exon 1 and was followed by digestion the 467-pb segment with the restriction enzyme Bsp1286I. The forward and reverse primers for exon 1 are 5'-ACAGCGCAGAAGAAGAGCAGT-3' and 5'-ACAACGCCACGACGGACG-3', respectively. The Bsp1286I-digested products were analyzed on 1.5% agarose gels [21]. The PCR segments were subsequently analyzed by direct sequencing in an ABI 3700 automated DNA sequencer using the Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the *TRMU* genomic sequence (GenBank accession number: AF448221) [21].

## Mutational analysis of *GJB2* gene

The DNA fragments spanning the entire coding region of *GJB2* gene were amplified by PCR using the following oligodeoxynucleotides: forward-5'TATGACACTCCCCAGCACAG3' and reverse-5'GGGCAATGCTTAACTGGC3'. PCR amplification and subsequent sequencing analysis were performed as detailed elsewhere [11]. The results were compared with the wild-type *GJB2* sequence (GenBank accession number: M86849) to identify the mutations.

## Results

### Clinical and genetic evaluations of four Chinese pedigrees

Four Han Chinese participants were diagnosed as aminoglycoside ototoxicity by the Otolaryngology Clinics at the Wenzhou Medical College. Mutational analysis of the 12S rRNA gene revealed that four participants harbored the A1555G mutation (data not shown). A comprehensive history and physical examination as well as audiological examination were performed to identify any syndromic findings, the history of the use of aminoglycosides, genetic factors related to the hearing impairment in all available members of these families. In fact, comprehensive family medical histories of those probands and other members of these Chinese families showed no other clinical abnormalities, including diabetes, muscular diseases, visual dysfunction, and neurological disorders. The restriction enzyme digestion and subsequent electrophoresis of available members in three pedigrees indicated that the A1555G mutation was indeed present in homoplasmic in matrilineal relatives but not other members of these families (data not shown).

Of these, the proband (III-1) of WZD31 family received gentamycin for high fever at the age of 3 years. She exhibited bilateral hearing impairment 1 month later after the drug administration. As illustrated in Fig. 2, she had profound hearing impairment (98 dB at both right and left ears, with a slope-shaped pattern). Of other members in this family, III-6 exhibited profound hearing loss after the administration of gentamycin at the age of 2 years. Of other matrilineal relatives, six participants exhibited hearing deficit, ranging from severe (I-2, II-4, III-2), to moderate (II-2, II-9, III-4), whereas others had normal hearing.

In the family WZD32, the proband IV-2 was a 17-year-old girl. She was treated with gentamycin for high fever at the age of 2 years. As shown in Fig. 2, she had bilateral severe hearing loss (75 dB at right ear, 84 dB at left ear; a flat-shaped pattern). Two matrilineal relatives (III-2, III-5) had profound hearing loss after administration of aminoglycosides. Other four matrilineal relatives without exposure to aminoglycosides suffer from hearing impairment as the sole clinical symptom, ranged from profound (III-7), to severe (II-2), to moderate (II-3, II-4, III-4) to normal hearing.

In family WZD33, the proband V-1, as shown in Fig. 2, was evaluated as profound hearing loss (102 dB at right ear, 99 dB at left ear; a slope-shaped pattern) by the Otolaryngology Clinic of Wenzhou Medical College at the age of 12 years. He developed hearing loss at the age of 2 years after administration of gentamycin for high fever. Of other family members, IV-4 exhibited severe hearing loss after an administration with gentamycin at the age of 10 years. Other matrilineal relatives, who did not have a history of exposure to aminoglycosides, had hearing loss, varying from profound (III-4, IV-15, IV-17), to severe (II-2, II-3, III-8, III-11, IV-11), to moderate (IV-2, IV-5, IV-9) and to mild (IV-14), and to normal hearing of others.

In the family WZD34, the proband III-2 came to otology clinic of Wenzhou Medical College at the age of 15 years. He received gentamycin for high fever at the age of 4 years. As illustrated in Fig. 2, he suffered from profound hearing loss (105 dB at right ear, 109 dB at left ear, with a slope-shaped pattern). His mother (II-2) exhibited profound hearing loss after administration

of gentamycin. Other matrilineal relatives, who were not exposed to aminoglycosides, had hearing loss, ranging from severe (II-4), to moderate (II-3, II-11, III-5, III-12), to mild (II-6, III-7, III-14), to normal hearing.

### Mutational analysis of mitochondrial genome

To assess the role of mtDNA variants in the phenotypic expression of the A1555G mutation, we performed a PCR amplification of fragments spanning entire mitochondrial genome and subsequent DNA sequence analysis in four probands. In addition to the identical A1555G mutation, as shown in Table 1, these participants exhibited distinct sets of mtDNA polymorphism. We failed to detect the presence of the secondary deafness-associated tRNA<sup>Glu</sup> A14693G, tRNA<sup>Thr</sup> T15908C, tRNA<sup>Arg</sup> T10454C, tRNA<sup>Ser(UCN)</sup> G7444A, and tRNA<sup>Cys</sup> G5821A variants in these probands. Of other nucleotide changes, there are 30 variants in the noncoding region D-loop, five known variants in 12S rRNA gene, two variants in the 16S rRNA gene, one novel variant (G5744A) in the noncoding L-strand origin, one known variant (G15927A) in the tRNA<sup>Thr</sup> gene, the previously identified CO2/tRNA<sup>Lys</sup> intergenic 9 bp deletion corresponding to mtDNA at positions 8271–8279 [14], 15 known and six novel silent variants in the protein encoding genes as well as 10 missense mutations (one novel and nine known) in the protein encoding genes (<http://www.mitomap.org>) [35]. These missense mutations are the G7269A (V456M) in the CO1 gene, the G8584A (A20T) and A8860G (T112A) in A6 gene, the A10398G (T114A) in the ND3 gene, the C12084T (S442F) in the ND4 gene, the A12361G (T8A) in the ND5 gene, and the C14766T (T7I), A15326G (T194A), A15662G (I306 V), and A15851G (I369 V) in the cyto b gene.

These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis of these variants and sequences from other organisms including mouse [36], bovine [37], and *Xenopus laevis* [38]. Variants in the polypeptides showed no evolutionary conservation. Of RNA variants, the G-to-A transition at position 15927 (G15927A) in the tRNA<sup>Thr</sup> gene of all probands (Fig. 3a) is of special interest. The G15927A mutation, as shown in Fig. 3b, locates at the fourth base in the anticodon stem, corresponding to conventional position 42 of the tRNA<sup>Thr</sup> [39]. In fact, a guanine at this position is a highly conserved base in sequenced threonine tRNA from bacteria to human mitochondria [40]. It is anticipated that the G15927A mutation disrupts a very conservative base pairing (28C-42G) on the anticodon stem of this tRNA<sup>Thr</sup>, thus causing a failure in tRNA metabolism. To determine whether the G15927A mutation is homoplasmic, the fragments spanning the tRNA<sup>Thr</sup> gene were PCR amplified and subsequently digested with *HpaII*. As shown in Fig. 3c, there was no detectable wild-type DNA in all available matrilineal relatives, indicating that the G15927A variant was homoplasmic in these matrilineal relatives. In addition, allele frequency analysis showed that two of the 262 Chinese control participants carried this G15927A variant.

### Mitochondrial tRNA analysis

To examine whether the G15927A mutation affects the stability of the tRNA<sup>Thr</sup>, we determined the steady state level of the tRNA<sup>Thr</sup> determined by isolating total mitochondrial RNA from lymphoblastoid cell lines, separating them on a 10% polyacrylamide, 7-mol/l urea gel, electroblotting and hybridizing with a nonradioactive DIG-labeled oligodeoxynucleotide probe specific for tRNA<sup>Thr</sup>. After the blots were stripped, the DIG-labeled probes, including tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, and tRNA<sup>Ser(AGY)</sup> as representatives of the whole H-strand transcription unit and tRNA<sup>Gly</sup> derived from the L-strand transcription unit [32], were hybridized with the same blots for normalization purposes. As shown in Fig. 4a, the amount of tRNA<sup>Thr</sup> in the mutant cell line derived from affected individual (WZD32-III-7) carrying A1555G and G15927A mutations and a hearing normal individual (A4) carrying only the G15927A mutation were markedly decreased, compared with those in the cell line derived from the married-in-control (WZD32-III-1) lacking those mtDNA mutations. For comparison,

the average levels of tRNA<sup>Thr</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup> in various control or mutant cell lines were normalized to the average levels in the same cell line for 16S rRNA, respectively. As shown in Fig. 4b, the average levels of tRNA<sup>Thr</sup> in the cell lines derived from WZD32-III-7 and A4 were 55 and 57% of the control after normalization to 16S rRNA, whereas the average levels of tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup> these two cell lines were comparable with those of the control after normalization to 16S rRNA. On the contrary, the levels of tRNA<sup>Thr</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup> in one cell line derived from an affected individual (WZD1-IV-2) carrying only A1555G mutation were comparable with those in the cell line derived from the married-in-control (WZD32-III-1).

### Analysis of aminoacylation of mitochondrial tRNA

To test whether the G15927A mutation affects the aminoacylation of tRNAs, the aminoacylation capacity of tRNA<sup>Thr</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup> in wild-type and mutant cell lines were examined by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA [33], electroblotting and hybridizing with tRNA probes, as above. As shown in Fig. 5, the upper band represented the charged tRNA, and the lower band was uncharged tRNA. Electrophoretic patterns showed that either charged or uncharged tRNA<sup>Thr</sup> in cell lines carrying the G15927A mutation migrated much slower than those of cell lines lacking this variant. However, there were no obvious differences in electrophoretic mobility of tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup> between the cell line carrying the G15927A variant and cell lines lacking this variant. To determine whether the lower band is uncharged tRNA and upper band is charged tRNA, a sample of mt tRNA, deacylated by heating 10 min at 60°C at pH 8.3 [33], was run in parallel. As shown in Fig. 3, the deacylated samples gave all cases a single hybridization band comigrating with lower band of the acid-purified tRNA samples. The proportions of aminoacylation of the tRNAs in cell line derived from the married-in-control individual (WZD32-III-1) lacking these mutations were 58, 42, 45, 45, and 39% in the tRNA<sup>Thr</sup>, tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Lys</sup>, tRNA<sup>Ser(AGY)</sup>, and tRNA<sup>Gly</sup>, respectively. The levels of other three cell lines were comparable with those of the married-in-control individual (WZD32-III-1).

### Mutational analysis of *TRMU* gene

Our earlier study showed that the *TRMU* A10S mutation modulated the phenotypic manifestation of the A1555G mutation in the Israeli/European pedigrees [21]. To assess whether the *TRMU* A10S variant also plays a role in phenotypic expression of the A1555G mutation in these Chinese families, we carried out a mutational screening of exon 1 in *TRMU* gene in three affected matrilineal relatives of each pedigree who did not have a history of exposure to aminoglycosides. We failed to detect any variant in *TRMU* exon 1 in these affected matrilineal relatives of these Chinese pedigrees.

### Mutational analysis of *GJB2* gene

To examine the role of *GJB2* gene in phenotypic expression of the A1555G mutation, we performed the mutational screening of *GJB2* gene in three affected matrilineal relatives of each pedigree who did not have a history of exposure to aminoglycosides. None of variants in *GJB2* gene was found in these affected matrilineal relatives of these Chinese pedigree. Indeed, the absence of variant in the *GJB2* gene in those participants with hearing impairment indicates that the *GJB2* gene may not be a modifier of the phenotypic effects of the A1555G mutation in those participants.

## Discussion

In this study, we investigated the role of modifiers in the phenotypic manifestation of the A1555G mutation in four Chinese pedigrees with aminoglycoside-induced and nonsyndromic hearing loss. Hearing loss as a sole clinical phenotype was only present in the maternal lineage of those pedigrees. These Chinese families exhibit much higher penetrance and expressivities of hearing loss than in other 27 Chinese pedigrees carrying the A1555G mutation [14,23,25]. As shown in Table 2, the penetrances of hearing loss (affected matrilineal relatives/total matrilineal relatives) in WZD31, WZD32, WZD33, and WZD34 pedigrees were 66.7, 66.7, 51.9, and 50%, respectively, when aminoglycoside-induced deafness was included. When the effect of aminoglycosides was excluded, the penetrances of hearing loss in these pedigrees were 50, 40.7, 44.4, and 39%, respectively. The penetrances of hearing loss in these Chinese pedigrees are comparable with the previously identified five Chinese pedigrees with a high penetrance of hearing loss, with the average of 54 and 31.7%, respectively, when aminoglycoside-induced deafness was included or excluded [15,22,24]. In contrast, the average penetrances of hearing loss in the other 27 pedigrees carrying the A1555G mutation were 9.3 and 1.7%, respectively, when aminoglycoside-induced deafness was included or excluded [14,23,25]. Furthermore, as shown in Table 2, when aminoglycoside-induced deafness was excluded, matrilineal relatives in these four Chinese families developed hearing loss at the average of 14, 13, 16, and 15 years of age, respectively, whereas the average age-of-onset for hearing loss were from 18 to 21 years from matrilineal relatives of 11 Chinese pedigrees carrying the A1555G mutation but lacking secondary mtDNA mutations, respectively [14,25]. Thus, matrilineal relatives in these four families had earlier age-onset of hearing loss than those in the other 11 Chinese families carrying the A1555G mutation. To assess whether the differences in penetrance and age-at-onset of hearing loss without aminoglycosides differ based on the presence and absence of additional mtDNA variant, a statistical analysis was performed by the unpaired, two-tailed Student's *t*-test contained in Microsoft Excel. The penetrances and age-at-onset of hearing loss among Chinese pedigrees carrying the additional mtDNA variants showed significantly higher than those pedigrees lacking significant mtDNA variants, when aminoglycoside-induced deafness was excluded ( $P < 0.0001$  and  $P = 0.001$ ).

The phenotypic variability of matrilineal relatives within and among these Chinese families indicated the involvement of modifier factors such as aminoglycosides, nuclear and mitochondrial genetic modifiers in the phenotypic manifestation of the A1555G mutation. Eight matrilineal relatives of these pedigrees exhibited severe or profound hearing loss after an administration with aminoglycosides, demonstrating the modifier role of aminoglycosides in deafness expression. The absence of *TRMU* A10S variant and *GJB2* mutation ruled out their involvement in the phenotypic expression of the A1555G mutation in these Chinese families, similar to other Chinese families carrying the A1555G mutation [22,25]. Hence, other nuclear modifier genes may contribute to the phenotypic variability in these Chinese families. Furthermore, the secondary mtDNA mutations may act as modifiers influencing the phenotypic expression of the primary mtDNA mutations. The haplogroup J specific variants T4216C and G13708A may increase the penetrance of vision loss associated ND4 G11778A mutation [41] or hearing loss associated with tRNA<sup>Ser(UCN)</sup> A7445G mutation [32], whereas the haplogroup L1b specific variants ND1 T3308C and tRNA<sup>Ala</sup> T5655C likely caused higher penetrance of deafness in an African pedigree than Japanese and French families carrying the T7511C mutation [31,42]. Furthermore, variants tRNA<sup>Glu</sup> A14693G, tRNA<sup>Thr</sup> T15908C, tRNA<sup>Arg</sup> T10454C, tRNA<sup>Ser(UCN)</sup> G7444A, and tRNA<sup>Cys</sup> G5821A may contribute to higher penetrance of hearing loss in five Chinese pedigrees carrying the A1555G mutation [15,22, 24].



Mitochondrial genomes in these four Chinese pedigrees harbored the identical A1555G and G15927A mutations as well as distinct sets of mtDNA variants, belonging to Eastern Asian haplogroup B5b1 [43]. Of mtDNA variants, the 961insC mutation may influence the phenotypic manifestation of hearing loss in pedigrees WZD33 and WZD34, as in the case of a Chinese pedigree carrying both A1555G and 961insC mutations [12]. The homo-plasmic G15927A variant (conventional position 42 of tRNA), however, occurred at a highly evolutionarily conserved nucleotide of tRNA<sup>Thr</sup> [39,40]. In fact, the G15927A variant disrupted a very conservative base pairing (28C-42G) on the anticodon stem of this tRNA<sup>Thr</sup>. An abolished base pair at the same position in tRNA<sup>Leu</sup> by the A4300G mutation associated with hypertrophic cardiomyopathy altered this tRNA metabolism [44]. Thus, the alteration of the tertiary structure of the tRNA<sup>Thr</sup> by the G15927A mutation may lead to a failure in this tRNA metabolism. Approximately 45 and 43% reductions in the level of tRNA<sup>Thr</sup> were observed in cells carrying both A1555G and G15927A mutations and cells carrying only the G15927A mutation, respectively, as compared with control cells lacking those mutations, while there was no significant reduction in the level of tRNA<sup>Thr</sup> in cells carrying only A1555G mutation. This level of total tRNA<sup>Thr</sup> in mutant cells is, however, above a proposed threshold, which is 30% of the control level of tRNA, to support a normal rate of mitochondrial translation [31,32]. Furthermore, an altered electrophoretic mobility of tRNA<sup>Thr</sup> was observed in cell lines carrying the G15927A mutation, as compared with those lacking this variant. In contrast, the G15927A mutation did not affect the aminoacylation efficiency of tRNA<sup>Thr</sup>. The lower level and altered electrophoretic mobility of tRNA<sup>Thr</sup> in cells carrying the G15927A mutation most probably results from the alteration of the tertiary structure of this tRNA by the G15927A mutation. The homoplasmic form, mild biochemical defect and presence of two of 262 Han Chinese controls indicated that the G15927A mutation itself is insufficient to produce a clinical phenotype. A failure in mitochondrial tRNA metabolism, caused by the G15927A mutation, may impair mitochondrial protein synthesis, thereby worsening mitochondrial dysfunctions altered by the A1555G mutation. Therefore, the G15927A mutation may have a potential modifier role in increasing the penetrance and expressivity of the deafness-associated 12S rRNA A1555G mutation in these Chinese pedigrees.

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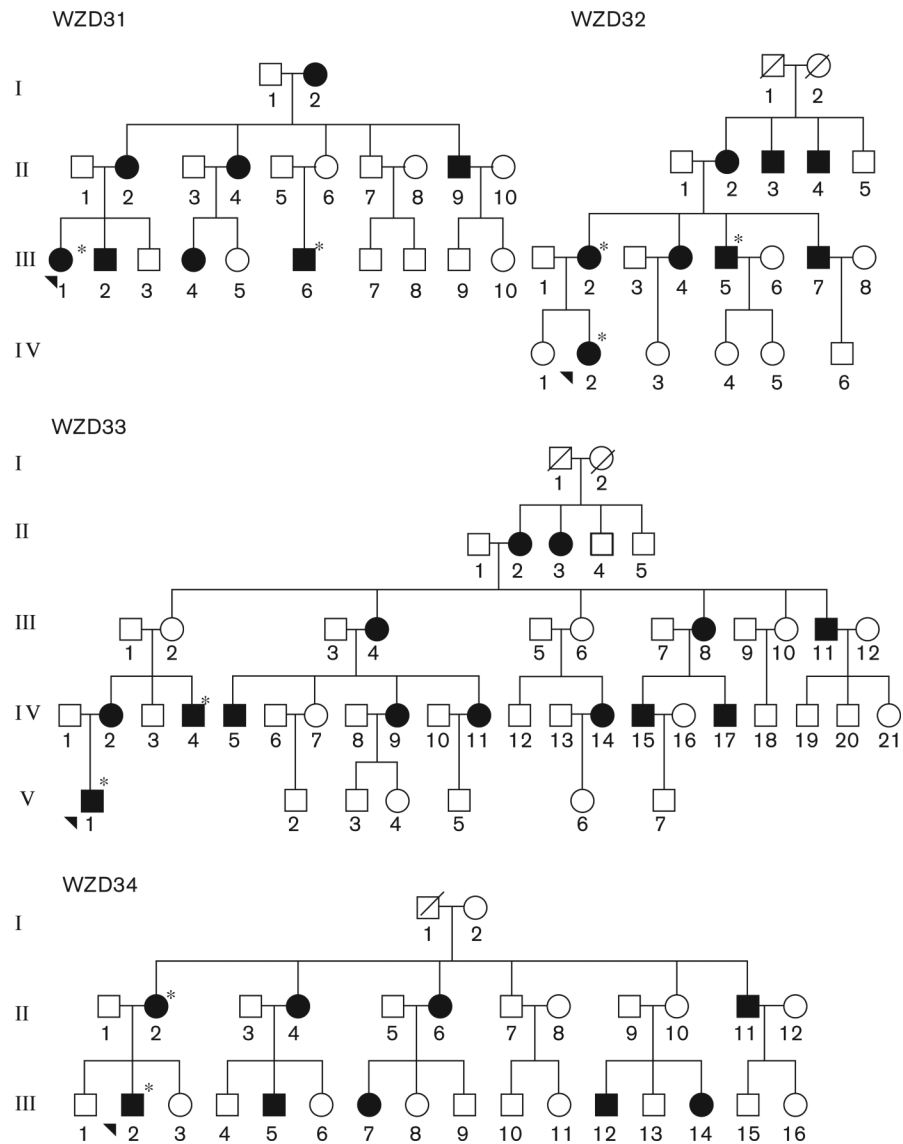
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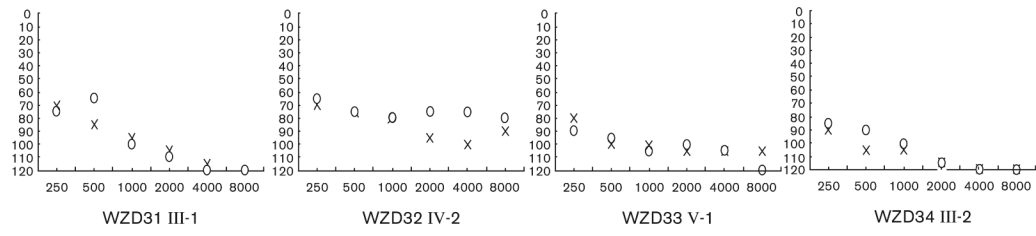
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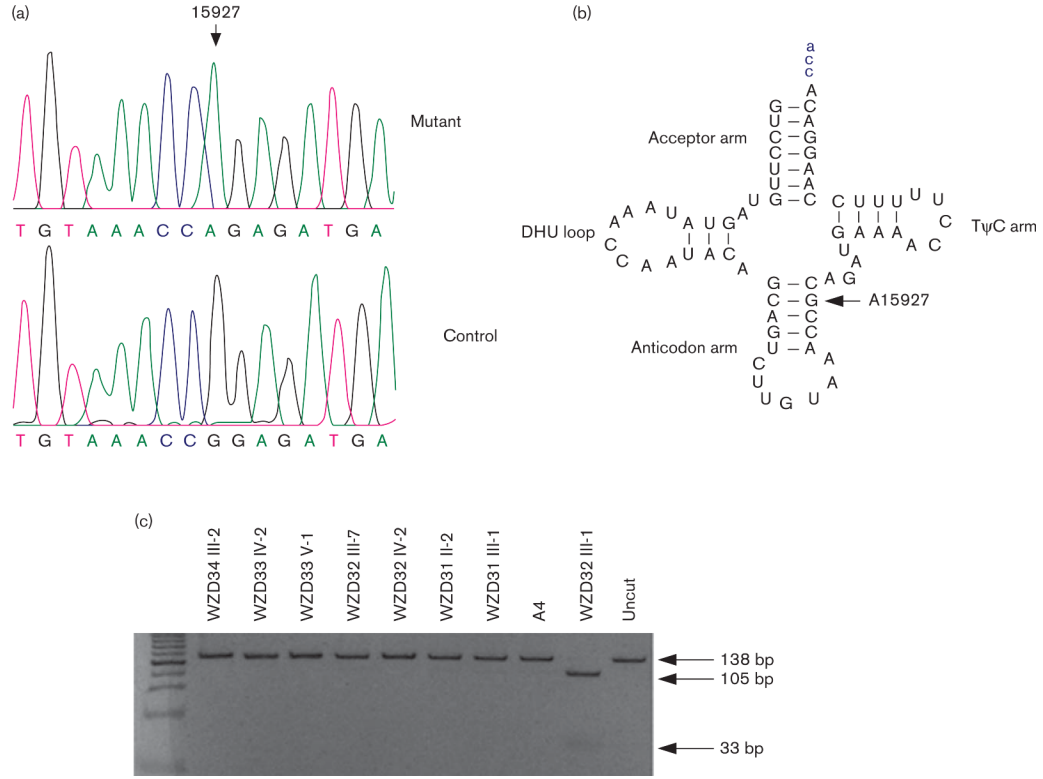
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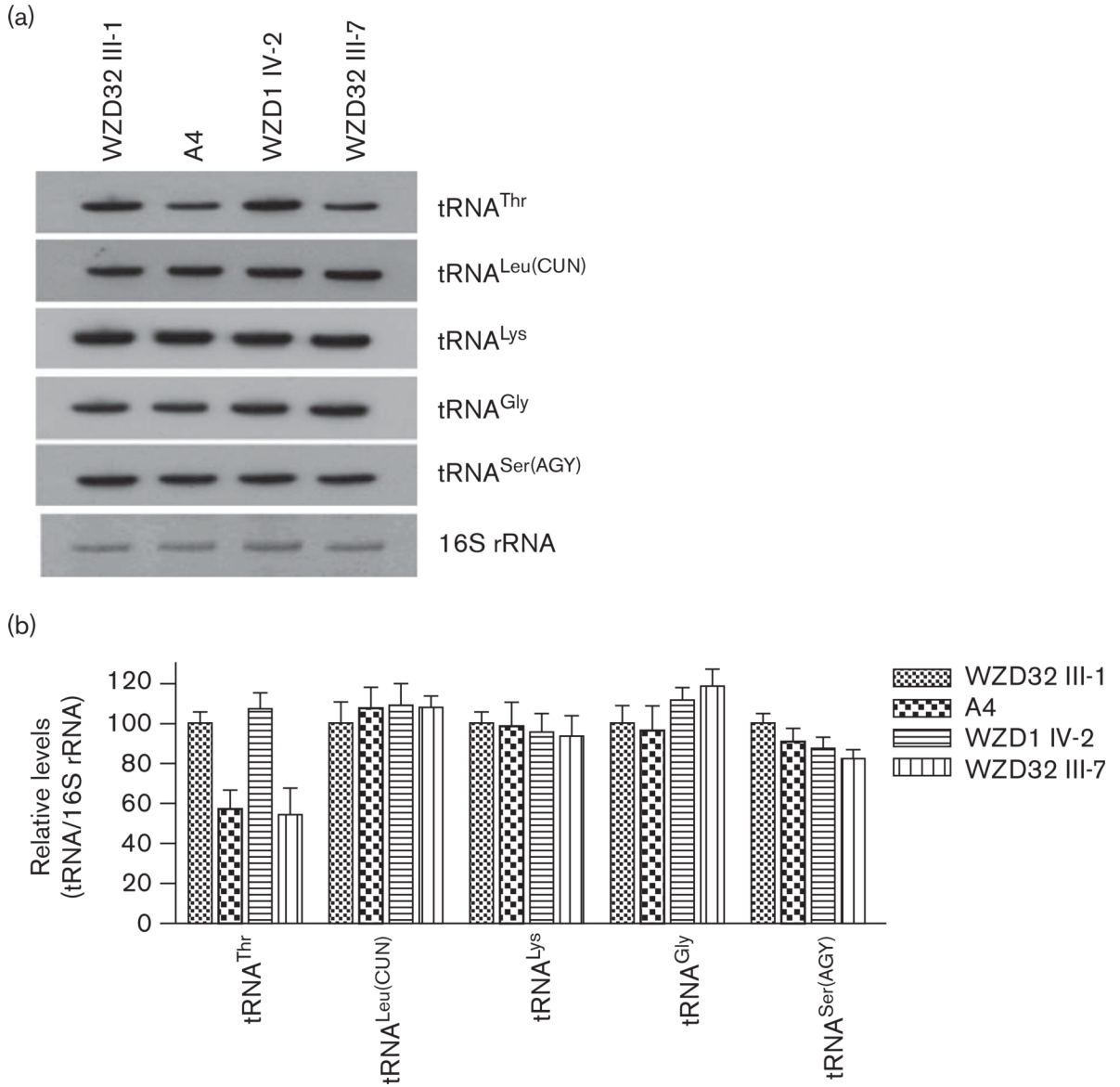
**Fig. 1.** Four Han Chinese pedigrees with aminoglycoside-induced and nonsyndromic hearing impairment. Hearing impaired individuals are indicated by filled symbols. Arrowhead denotes probands. Asterisks denote individuals who had a history of exposure to aminoglycosides.



**Fig. 2.** Air conduction audiogram of four affected participants with the A155G mutation. X, left ear; O, right ear.

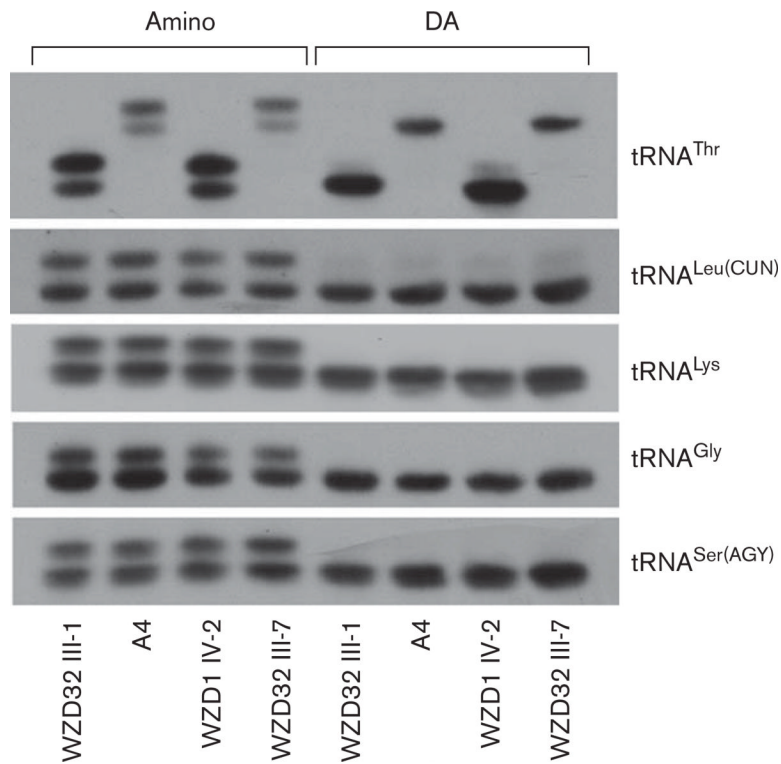


**Fig. 3.** Identification and qualification of the G15927A mutation in the mitochondrial tRNA<sup>Thr</sup> gene. (a) Partial sequence chromatograms of tRNA<sup>Thr</sup> gene from an affected individual (WZD32-III-2) and a married-in-control (WZD32-III-1). An arrow indicates the location of the base changes at position 15927. (b) The location of the G15927A mutation in the mitochondrial tRNA<sup>Thr</sup>. Cloverleaf structure of human mitochondrial tRNA<sup>Thr</sup> is derived from the study of Florentz *et al.* [39]. Arrow indicates the position of the G15927A mutation. (c) Quantification of G15927A mutation in the tRNA<sup>Thr</sup> gene of mutants and controls derived from the Chinese families. PCR products around the G15927A mutation were digested with *Hpa*II and analyzed by electrophoresis in a 7% polyacrylamide gel stained with ethidium bromide.



**Fig. 4.** Northern blot analysis of mitochondrial tRNA. (a) Equal amounts (5  $\mu$ g) of total mitochondrial RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with digoxigenin (DIG)-labeled oligonucleotide probes specific for the tRNA<sup>Thr</sup>. The blots were then stripped and rehybridized with DIG-labeled tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Ser(AGY)</sup>, tRNA<sup>Lys</sup>, and tRNA<sup>Gly</sup>, respectively. (b) Quantification of mitochondrial tRNA levels. Average relative tRNA<sup>Thr</sup> content per cell, normalized to the average content per cell of 16S rRNA in cells derived from an affected individual (WZD32-III-7) carrying A1555G and G15927A mutations, a hearing normal individual (A4) carrying only the G15927A mutation, an individual (WZD1-IV-2) carrying only A1555G mutation, a married-in-control (WZD32-III-1) lacking those mitochondrial DNA mutations. The values for the latter are expressed as percentages of the average values for the control cell line (WZD32-III-1). The calculations were based on three independent determinations of each tRNA content in each cell line and three determinations of the content of reference RNA marker in each cell line.





**Fig. 5.** In-vivo aminoacylation assays for mitochondrial tRNA. Equal amounts (2  $\mu$ g) of total mitochondrial RNA purified from various cell lines under acid conditions were treated with electrophoresis at 4°C through an acid (pH 5.1) 10% polyacrylamide/7 mol/l urea gel, electroblotted onto a positively charged nylon membrane, and hybridized with digoxigenin (DIG)-labeled oligonucleotide probes specific for mitochondrial tRNA<sup>Thr</sup>. Samples were also deacylated (DA) by heating 10 min at 60°C at pH 8.3. The blots were then stripped and rehybridized with DIG-labeled probes for tRNA<sup>Leu(CUN)</sup>, tRNA<sup>Ser(AGY)</sup>, tRNA<sup>Lys</sup>, and tRNA<sup>Gly</sup>, respectively.

Table 1

mtDNA variants in four Han Chinese families with hearing loss

Gene	Position	Replacement	Conservation (H/B/M/Y/G)	CRS	WZD31	WZD32	WZD33	WZD34	Previously reported <sup>b</sup>
D-Loop	73	A to G		A	G	G	G	G	Yes
	103	G to A		G	A	A	A	A	Yes
	131	T to C		T	C	C			Yes
	200	A to G		A			G	G	Yes
	204	T to C		A	C	C	C	C	Yes
	207	G to A		G			A	A	Yes
	263	A to G		A	G	G	G	G	Yes
	297	A to C		A	C	C			No
	303	C to CC		C	CC	CC			Yes
	310	T to CTC/TC		T	TC	TC	CTC	TC	Yes
	481	C to T		C	T				No
	514	C Del		C	C del				Yes
	515	A Del		A	A del				Yes
	523	A Del		A	A del	A del	A del	A del	Yes
	524	C Del		C	C del	C del	C del	C del	Yes
	16111	C to T		C	T	T			Yes
	16140	T to C		T	C	C	C	C	Yes
	16182	A to C		A	C	C			Yes
	16183	A to C		A	C	C	C	C	Yes
	16189	T to CC/C		T	CC	CC	C	CC	Yes
	16194	A to C		A			C	C	Yes
	16195	T to A		T			A	A	Yes
	16196	G to TG		G			TG	TG	Yes
	16234	C to T		C	T	T			Yes
	16243	T to C		T	C	C	C	C	Yes
	16291	C to T		C	T	T			Yes
	16344	C to T		C	T				Yes
	16355	C to T		C			T	T	Yes
	16463	A to G		A	G	G			Yes

Gene	Position	Replacement	Conservation (H/B/N/M/X) <sup>a</sup>	CRS	WZD31	WZD32	WZD33	WZD34	Previously reported <sup>b</sup>
	16519	T to C		T	C	C	C	C	Yes
12S rRNA	709	G to A	G/G/A/	G	A	A	A	A	Yes
	750	A to G	A/G/G/	A				G	Yes
	961	C to CC	T/A/T/A	C		CC	CC	CC	Yes
	1438	A to G	A/A/A/G	A	G	G	G	G	Yes
	1555	A to G	A/A/A/A	A	G	G	G	G	Yes
	1598	G to A	G/A/T/T	G	A	A	A	A	Yes
16S rRNA	2706	A to G	A/G/A/A	A	G	G	G	G	Yes
	3107	C Del	C/T/T/T	C	C Del	C Del	C Del		Yes
ND1	4161	C to T		C	T				No
ND2	4769	A to G		A	G	G	G	G	Yes
	4895	A to G		A	G	G			Yes
	4985	G to A		G	A	A	A	A	Yes
NC4	5744	G to A		G	A	A			No
CO1	6446	G to A		G	G		A	A	Yes
	7028	C to T		C	T	T	T	T	Yes
	7269	G to A (Val to Met)	V/M/M/I	G			A		No
NC7	8271-79	9-bp del			Del	Del	Del	Del	Yes
ATPase6	8584	G to A (Ala to Thr)	A/V/V/I	G	A	A	A	A	Yes
	8784	A to G		A		G			Yes
	8829	C to T		C	T	T	T	T	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G	G	G	Yes
CO3	9947	G to A		G	A	A			Yes
	9950	T to C		T	C	C	C	C	Yes
ND3	10398	A to G (Thr to Ala)	T/T/T/A	A	G	G	G	G	Yes
ND4	11101	A to G		A	G				No
	11335	T to C		T	C	C			Yes
	11437	T to C		T	C	C			No
	11719	G to A		G	A	A	A	A	Yes
	11893	A to G		A			G	G	Yes
	12084	C to T (Ser to Phe)	S/L/I/I	C		T			Yes
ND5	12361	A to G (Thr to Ala)	T/S/I/M	A	G	G	G	G	Yes

Gene	Position	Replacement	Conservation (H/B/M/X) <sup>a</sup>	CRS	WZD31	WZD32	WZD33	WZD34	Previously reported <sup>b</sup>
	14148	A to G		A			G		No
ND6	14560	G to A		G	A	A			No
Cyt b	14766	T to C (Thr to Ile)	T/S/I/S	T			C		Yes
	15223	C to T		C	T	T	T		No
	15326	A to G (Thr to Ala)	T/M/I/I	A	G	G	G		Yes
	15508	C to T		C	T	T	T		Yes
	15662	A to G (Ile to Val)	I/L/F/L	A	G	G	G		Yes
	15734	G to A		G		A			Yes
	15850	T to C		T	C	C			Yes
	15851	A to G (Ile to Val)	I/A/S/M	A	G	G	G		Yes
tRNA	15927	G to A	G/G/G/G	G	A	A	A	A	Yes

CRS, consensus Cambridge sequence; mtDNA, mitochondrial DNA.

<sup>a</sup>Conservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X).

<sup>b</sup>See the online mitochondrial genome database <http://www.mitomap.org>

**Table 2**  
Summary of clinical and molecular data for 21 Chinese families carrying the A1555G mutation

Pedigree	Number of matrilineal relatives	Penetrance (including the use of drugs) (%) <sup>a</sup>	Penetrance (excluding the use of drugs) (%)	Average age-at-onset (excluding the use of drugs)	Second mtDNA mutations	mtDNA haplogroup
WZD31	12	66.7	50	14	tRNA <sup>Thr</sup> G15927A	B5b1
WZD32	12	66.7	41.7	13	tRNA <sup>Thr</sup> G15927A	B5b1
WZD33	27	51.9	44.4	16	tRNA <sup>Thr</sup> G15927A	B5b1
WZD34	19	50	39	15	tRNA <sup>Thr</sup> G15927A	B5b1
WZD1 [25]	17	5.9	0		None	B4C1C1
WZD2	21	9.5	4.7	19	None	D4a
WZD3	16	12.5	0		None	D5a2
WZD4	24	29.2	16.7	20	None	F1a1
WZD5	31	3.2	0		None	D52b
WZD6	8	25	12.5	19	None	D4b2b
WZD7	30	10	3.3	21	None	D5a2
BJ101 [14]	14	5	0		None	F3
BJ102	13	13	8	18	None	N9a1
BJ103	20	5	0		None	D4a
BJ104	11	4	0		None	D4b2b
BJ105 [15]	15	67	45		tRNA <sup>Cys</sup> G5821A	F3
BJ106 [24]	18	33	33		None	M7b
BJ107 [22]	34	35	24		tRNA <sup>Thr</sup> T15908C	N
BJ108	16	63	38		tRNA <sup>Glu</sup> A14693G	F
BJ109	9	67	44		tRNA <sup>Arg</sup> T10454C	M
BJ110 [14]	17	59	5.9		CO1/tRNA <sup>Ser</sup> (UCN) G7444A	D4a

mtDNA, mitochondrial DNA.

<sup>a</sup> Affected matrilineal relatives/total matrilineal relatives.