Contribution of the tRNA\textsuperscript{Ile} 4317A>G mutation to the phenotypic manifestation of the deafness-associated mitochondrial 12S rRNA 1555A>G mutation

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Running title: A tRNA modifier for deafness expression of 12S rRNA mutation

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ABSTRACT
The 1555A>G mutation in mitochondrial 12S rRNA has been associated with aminoglycoside-induced and nonsyndromic deafness in many individuals worldwide. Mitochondrial genetic modifiers are proposed to influence the phenotypic expression of m.1555A>G mutation. Here, we report that a deafness-susceptibility allele (m.4317A>G) in the tRNA\textsuperscript{Ile} gene modulates the phenotype expression of m.1555A>G mutation. Strikingly, a large Han Chinese pedigree carrying both m.4317A>G and m.1555A>G mutations exhibited much higher penetrance of deafness than those carrying only
m.1555A>G mutation. The m.4317A>G mutation affected a highly conserved adenine at position 59 in the T-loop of tRNA^{ile}. We therefore hypothesized that the m.4317A>G mutation alters both structure and function of tRNA^{ile}. Using lymphoblastoid cell lines derived from members of Chinese families (three carrying both m.1555A>G and m.4317A>G mutations, three harboring only m.1555A>G mutation, and three controls lacking these mutations), we found that the cell lines bearing both m.4317A>G and m.1555A>G mutations exhibited more severe mitochondrial dysfunctions than those carrying only m.1555A>G mutation. We also found that the m.4317A>G mutation perturbed the conformation, stability, and aminoacylation efficiency of tRNA^{ile}. These m.4317A>G mutation-induced alterations in tRNA^{ile} structure and function aggravated the defective mitochondrial translation and respiratory phenotypes associated with m.1555A>G mutation. Furthermore, mutant cell lines bearing both m.4317A>G and m.1555A>G mutations exhibited greater reductions in the mitochondrial ATP levels and membrane potentials and increasing production of reactive oxygen species than those carrying only m.1555A>G mutation. Our findings provide new insights into the pathophysiology of maternally inherited deafness arising from the synergy between mitochondrial 12S rRNA and tRNA mutations.

INTRODUCTION

Mutations in mitochondrial DNA (mtDNA) have been associated with both syndromic deafness (hearing loss with other medical problems such as diabetes) and nonsyndromic deafness (hearing loss is the only obvious medical problem) (1-3). Human mtDNA encodes 13 polypeptides (essential components of oxidative phosphorylation complexes), 2 rRNAs and 22 tRNAs required for mitochondrial translation (4). The mitochondrial tRNA genes are the hot spots for deafness-associated mutations, including the tRNA^{Leu(UUR)} 3243A>G, tRNA^{Ser(UCN)} 7445A>G, 7511T>C, tRNA^{His} 12201T>C, tRNA^{Asp} 7551A>G, and tRNA^{Glu} 14692A>G mutations (5-10). The m.1555A>G and m.1494C>T mutations in the 12S rRNA gene have been associated with both aminoglycoside-induced and nonsyndromic deafness in many families worldwide (11-19). The administration of aminoglycosides induced or worsened hearing loss in these subjects carrying the m.1555A>G or m.1494C>T mutation. In the absence of aminoglycosides, matrilineal relatives within and among families carrying the m.1555A>G or m.1494C>T mutation exhibited a wide range of penetrance, severity and age-of-onset in hearing impairment (11,14,17,19-22). Functional characterization of cell lines derived from matrilineal relatives of Arab-Israeli and Chinese families demonstrated that the m.1555A>G or m.1494C>T mutation conferred mild mitochondrial dysfunctions and sensitivity to aminoglycosides (23-26). These findings strongly suggest that the m.1555A>G or m.1494C>T mutation is the primary causative event, but by itself is insufficient to produce the deafness phenotype. Modifier factors including aminoglycosides, nuclear and mitochondrial genetic modifiers are required for the phenotype expression of the m.1555A>G or m.1494C>T mutation (2,20,25,27). In previous investigations, we showed that MTO1, MSSI/GTPBP3, or MTO2/TRMU involved in the biosynthesis of the hypermodified nucleoside 5-methyl-aminomethyl-2-thio-uridine in the wobble position of several mitochondrial tRNAs were the potential nuclear modifier genes for the phenotypic expression of the m.1555A>G or m.1494C>T mutations (28-33). Genetic evidence suggests that several mtDNA variants, including tRNA^{Glu} 14693A>G, tRNA^{Thr} 15927G>A,
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15908T>C, tRNA_Arg 10454T>C, tRNA_Ser(UCN)
7444G>A and tRNA_Cys 5821G>A may act as mitochondrial modifiers to modulate the phenotypic manifestation of the m.1555A>G or m.1494C>T mutation (20,34-36). However, the role of these mitochondrial modifiers in the deafness expression remains poorly understood.

In this study, we investigated the pathophysiology of a deafness susceptibility allele (m.4317A>G) in the tRNA_Ile gene in the phenotypic expression by taking advantage of a large cohort of 2651 hearing-impaired Han Chinese probands (15,37,38). Among these, 105 pedigrees with nonsyndromic and aminoglycoside-induced deafness harbored the homoplasmic m.1555A>G mutation (20). These families exhibited a wide range of penetrance and expressivity of hearing impairment. The average penetrances of deafness among 105 pedigrees carrying m.1555A>G mutation were 29.5% and 17.6%, respectively, when aminoglycoside-induced hearing loss was included or excluded (20). Strikingly, the penetrance of deafness in the pedigree (WZD91) harboring both m.1555A>G and m.4317A>G mutations were 73.9% and 60.9%, respectively, when aminoglycoside-induced hearing loss was included or excluded (20,38). It was anticipated that the m.4317A>G mutation may further deteriorate the mitochondrial dysfunction associated with m.1555A>G mutation, therefore increasing the penetrance and risk of deafness expression. As shown in Figure 1A, the m.4317A>G mutation was localized at the highly conserved adenine (A59) of the T-loop in tRNA_Ile (39-40). The m.4317A>G mutation may introduce a new G-C (G59-C54) base pair to the stem of the T-loop and lead to the rearrangement of the T-arm region (41-43). This T-arm region is important for the 3’ end processing of tRNA_Ile precursor and subsequently CCA-addition process (42-44).

Therefore, it was hypothesized that the m.4317A>G mutation altered both structure and function of tRNA_Ile. In particular, the mutation may affect the aminoacylation capacity and stability of this tRNA. A failure in tRNA metabolism may lead to the impairment of mitochondrial translation and respiration (2,3). It was also proposed that mitochondrial dysfunctions caused by the tRNA mutation affect the production of ATP and reactive oxygen species (ROS). To further investigate the effect of the m.4317A>G mutation on mitochondrial function, lymphoblastoid cell lines were generated from three members of pedigree WZD91 carrying both m.1555A>G and m.4317A>G mutations, three individuals of pedigree WZD92 harboring only m.1555A>G mutation and three control subjects lacking two mutations belonging to the same mtDNA haplogroup B4. These cell lines were first assessed for the effect of the m.4317A>G mutation on the conformation, stability and aminoacylation capacity of tRNA_Ile. We then examined whether the m.4317A>G altered mitochondrial translation, enzymatic activities of electron transport chain complexes, the rate of oxygen consumption, ATP production, mitochondrial membrane potential and the generation of reactive oxygen species (ROS).

RESULTS

Identification of the tRNA_Ile 4317A>G mutation in a large cohort of hearing-impaired subjects - The m.4317A>G mutation in tRNA_Ile gene was identified in only 1 proband (WZD91-III-3) carrying the m.1555A>G mutation among 2651 Chinese hearing-impaired probands (37,38) but absent in 574 Chinese control subjects. As shown in Figure 1, the m.4317A>G mutation affected the highly conserved adenine (A59) of the T-loop in tRNA_Ile (39-40). The m.4317A>G mutation may introduce a new G-C (G59-C54) base-pairing to the stem of the T-loop and lead to the rearrangement of the T-arm region (41-43).
Therefore, it was anticipated that the m.4317A>G mutation altered both structure and function of tRNA\(^{\text{ile}}\). The sequence analysis of entire mtDNA in the proband (WZD91-III-3), 3 symptomatic affected matrilineal relatives (III-1, III-9 and III-17) and 2 asymptomatic matrilineal relatives (IV-5, IV-10) (Figure 1B) exhibited the presence of both m.1555A>G and m.4317A>G mutations and a set of polymorphisms belonging to the Eastern Asian haplogroups B4 (Supplementary Table S1) (45). These variants included 16 variants in the D-loop region, 4 known variants in 12S rRNA gene, 3 variants in the 16S rRNA gene, the previously identified COII/tRNA\(^{\text{Lys}}\) intergenic 9 bp deletion corresponding to mtDNA at positions 8281–8289, 10 known and one novel silent variant in the protein encoding genes as well as a missense variant m.8573G>A (p.16G>D) in the ATP6 gene (46). The phylogenetic analyses showed that there were no other functionally significant variants in their mtDNAs. Further analysis showed that both m.4317A>G and m.1555A>G mutations were present in homoplasy in all matrilineal relatives of pedigree WZD91 but absent in other members of these families (Figure 1C).

Clinical presentation of two Chinese families and derived cell lines - All available members of two hearing-impaired Han Chinese families (pedigree WZD91 carrying both m.1555A>G and m.4317A>G mutations, and pedigree WZD92 harboring only m.1555A>G mutation), as shown in Figure 1D, underwent comprehensive evaluations of their medical histories and physical examination with the aim to identify any clinical abnormalities and genetic factors related to the deafness. The audiological examination was performed as detailed elsewhere (14). As shown in Figure 1D, 17 of 23 matrilineal relatives of pedigree WZD91 suffered from hearing impairment as the sole clinical symptom. Three matrilineal relatives with profound hearing loss had a history of exposure to aminoglycosides, and other 14 affected matrilineal relatives exhibited the variable degrees of hearing impairment (2 members with severe hearing loss, 8 subjects with moderate hearing loss and 4 individuals with mild hearing loss). In pedigree WZD92, 4 matrilineal relatives exhibited severe hearing loss due to administration with aminoglycosides, while other members of this family had normal hearing. There is no evidence that any member of these families had any other known cause to account for hearing impairment. Comprehensive family medical histories of these individuals showed no other clinical abnormalities, including diabetes, visual impairment, and neuro-muscular disorders.

Immortalized lymphoblastoid cell lines were generated from 3 matrilineal relatives harboring both m.1555A>G and m.4317A>G mutations (WZD91 III-1, male, 24 years; WZD91 II-3, male, 47 years and WZD91 III-7, male, 34 years), 3 individuals carrying only m.1555A>G mutation (WZD92 III-13, female, 35 years; WZD92 III-17, female, 28 years and WZD92 III-18, male, 31 years) belonging to the mtDNA haplogroup B4, and 3 control subjects lacking these mutations (A23, male, 33 years; A22, male, 33 years; A21, female, 32 years) belonging to the same mtDNA haplogroup B4. These nine cell lines were used for the biochemical characterization as described below.

Altered conformation of tRNA\(^{\text{ile}}\) - To test if the m.4317A>G mutation affected the conformation of tRNA\(^{\text{ile}}\), total mitochondrial RNA were electrophoresed through 10% polyacrylamide gel (native condition) in Tris-glycine buffer and then electroblotted onto a positively charged nylon membrane for hybridization analysis with digoxigenin (DIG)-labeled oligodeoxynucleotide probes for tRNA\(^{\text{ile}}\), tRNA\(^{\text{Ser(AGY)}}\) and tRNA\(^{\text{Met}}\), respectively. As shown in Figure 2A,
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electrophoretic patterns under native condition showed that the tRNA\textsubscript{Ile} in three mutant cell lines carrying both m1555A>G and m.4317A>G mutations migrated faster than those of three control cell lines. However, there were no obvious electrophoretic mobility differences between the mutant cell lines harboring only m1555A>G mutation and control cell lines. These data indicated that the m.4317A>G mutation changed the conformation of tRNA\textsubscript{Ile}.

Marked reduction in the steady state levels of tRNA\textsubscript{Ile} - To examine if the m.4317A>G mutation perturbed the metabolism of tRNA\textsubscript{Ile}, we subjected mitochondrial RNAs from mutant and control cell lines to Northern blots and hybridized them with DIG-labeled oligodeoxynucleotide probes for tRNA\textsubscript{Ile}, tRNA\textsubscript{Leu(UUR)}, tRNA\textsubscript{Val}, tRNA\textsubscript{Gln}, tRNA\textsubscript{Ala} and tRNA\textsubscript{Ser(UCN)}, respectively. As shown in Figure 2B, the average steady-state levels of tRNA\textsubscript{Ile} in the mutant cells were significantly decreased, as compared with the control cell lines. As shown in Figure 2C, the average levels of tRNA\textsubscript{Ile} in these mutant cell lines carrying both m.1555A>G and m.4317A>G mutations were 42% (\textit{P}=0.007), 48% (\textit{P}=0.002), 55% (\textit{P}=0.008), 58% (\textit{P}=0.011) and 51% (\textit{P}=0.004) of average values of three control cell lines after normalization to tRNA\textsubscript{Leu(UUR)}, tRNA\textsubscript{Val}, tRNA\textsubscript{Gln}, tRNA\textsubscript{Ala} and tRNA\textsubscript{Ser(UCN)}, respectively. Furthermore, the average levels of tRNA\textsubscript{Ile} in mutant cell lines carrying only m.1555A>G mutation were comparable to those of three control cell lines.

Deficient aminoacylation of tRNA\textsubscript{Ile} - To evaluate whether the m.4317A>G mutation aberrated the aminoacylation of tRNA\textsubscript{Ile}, we examined the aminoacylation capacities of tRNA\textsubscript{Ile}, tRNA\textsubscript{Leu(UUR)}, tRNA\textsubscript{Val}, tRNA\textsubscript{Gln}, tRNA\textsubscript{Ala} and tRNA\textsubscript{Ser(UCN)} in control and mutant cell lines by the use of electrophoresis in an acidic polyacrylamide/urea gel system. As shown in Figure 3A, there was less acylated tRNA\textsubscript{Ile} in the double mutant cell lines than those in control cell lines. The upper band represented the charged tRNA, while the lower band represented uncharged tRNA. To further distinguish non-aminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated by being heated for 10 min at 60°C (pH 8.3) and then run in parallel. As shown in Figure 3B, only one band (uncharged tRNA) was present in both mutant and control cell lines after deacylation. As shown in Figure 3C, the efficiencies of aminoacylated tRNA\textsubscript{Ile} in mutant cell lines carrying only m.1555A>G mutation, both m.1555A>G and m.4317A>G mutations were 94% (\textit{P}=0.316) and 54% (\textit{P}=0.001) of those in control cell lines, respectively. However, the levels of aminoacylation in tRNA\textsubscript{Leu(UUR)}, tRNA\textsubscript{His}, tRNA\textsubscript{Met} and tRNA\textsubscript{Ser(AGY)} in mutant cell lines were comparable with those in the control cell lines.

Decreases in levels of mitochondrial proteins - To determine whether the m.4317A>G mutation alters mitochondrial translation, the Western blotting analysis was conducted to examine the levels of seven mtDNA encoding polypeptides in mutant and control cell lines with TOM20 as a loading control. As shown in Figure 4A, the levels of ND1, ND4 and ND5, ND6 (subunits 1, 4, 5 and 6 of NADH:ubiquinone oxidoreductase); CO2 (subunit II of cytochrome c oxidase) and CYTB (apocytochrome b) exhibited the variable reductions in the mutant cell lines, while the levels of ATP8 (subunit 8 of the H+-ATPase) in the mutant cell lines were comparable with those of control cell lines. As shown in Figure 4B, the overall levels of seven mitochondrial translation products in mutant cell lines carrying both m.1555A>G and m.4317A>G mutations were 65% (\textit{P}=0.001), relative to the mean value measured in the control cell lines. As shown in Figure 4C, the average levels of ND1,
ND4, ND5, ND6, CO2, ATP8, and CYTB in these mutant cells carrying both m.1555A>G and m.4317A>G mutations were 58%, 74%, 62%, 68%, 59%, 102% and 29% of the average values of control cells, respectively. Furthermore, the overall levels of 7 mitochondrial translation products in mutant cell line carrying only m.1555A>G mutation were 82% ($P = 0.011$) of the average values in control cell lines. The levels of polypeptides in mutants relative to that in controls did not significantly correlate with either the number of codons or proportion of isoleucine residues (Supplementary Table S2).

We then examined the levels of five subunits of phosphorylation system (OXPHOS) in control and mutant cell lines by Western blotting analysis. As shown in Figure 5, the average level of mtDNA encoded subunit CO2 of cytochrome c oxidase in the mutant cell carrying only m.1555A>G mutation, both m.1555A>G and m.4317A>G mutations were 86% ($P = 0.019$) and 63% ($P = 0.007$) of control cell lines, respectively. However, the levels of other four polypeptides (NDUFB8 of NADH:ubiquinone oxidoreductase; SDHB of succinate ubiquinone oxidoreductase; UQCRC2 of ubiquinol cytochrome c reductase and ATP5A of H+-ATPase), encoded by nuclear genes, in mutant cell lines were comparable with those in control cell lines.

**Reduced activities of complex I, III and IV** - To investigate the effect of the m.4317A>G mutation on the oxidative phosphorylation, we measured the activities of respiratory complexes by isolating mitochondria from mutant and control cell lines. Complex I (NADH: ubiquinone oxidoreductase) activity was determined by following the oxidation of NADH with ubiquinone as the electron acceptor (47-49). The activity of complex II (succinate ubiquinone oxidoreductase) exclusively encoded by the nuclear DNA was examined by the artificial electron acceptor DCPIP (50,51). Complex III (ubiquinone cytochrome c oxidoreductase) activity was measured as the reduction of cytochrome c (III) using D-ubiquinol-2 as the electron donor. The activity of complex IV (cytochrome c oxidase) was monitored by following the oxidation of cytochrome c (II). As shown in Figure 6, the activity of complex I in the mutant cells carrying only m.1555A>G mutation, both m.1555A>G and m.4317A>G mutations were 79% ($P = 0.016$) and 55% ($P = 0.001$) of the average values of three control cell lines, respectively. The activity of complex III in the mutant cell carrying only m.1555A>G mutation, both m.1555A>G and m.4317A>G mutations were 80% ($P = 0.017$) and 42% ($P < 0.001$) of control cell lines, respectively. Moreover, the activity of complex IV in the mutant cell carrying only m.1555A>G mutation, both m.1555A>G and m.4317A>G mutations were 85% ($P < 0.001$) and 59% ($P = 0.022$) of controls, respectively. However, the activities of complexes II in all mutant cell lines were comparable with those of control cell lines.

**Respiration deficiency** - To evaluate whether the m.4317A>G mutation affects cellular bioenergetics, we examined the oxygen consumption rates (OCRs) of mutant and control cell lines. OCRs values were expressed as the relative value normalized to protein content. As shown in Figure 7, the average basal OCRs in mutant cell lines carrying both m.1555A>G and m.4317A>G, and only m.1555A>G mutation were 52% ($P = 0.002$) and 80% ($P = 0.042$), relative to the mean value measured in the control cell lines, respectively. To investigate which of the enzyme complexes of the respiratory chain was affected in the mutant cell lines, OCR were measured after the sequential addition of oligomycin (inhibit the ATP synthase), FCCP (to uncouple the mitochondrial inner membrane and allow for maximum electron flux through the ETC),
rotenone (to inhibit complex I), and antimycin A (to inhibit complex III). The difference between the basal OCR and the drug-insensitive OCR yields the amount of ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and non-mitochondrial OCR. As shown in Figure 7B, the ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and non-mitochondrial OCR were 50% ($P=0.001$), 62% ($P=0.027$), 51% ($P=0.020$), 52% ($P=0.010$), and 69% ($P=0.123$) in the mutant cell lines carrying both m.1555A>G and m.4317A>G mutations, and 79% ($P=0.031$), 94% ($P=0.106$), 81% ($P=0.045$), 82% ($P=0.301$), and 82% ($P=0.740$) in the mutant cell lines carrying only m.1555A>G mutation.

**Decreased levels of mitochondrial ATP production** - The capacities of oxidative phosphorylation in mutant and control cell lines were measured by examining the levels of cellular and mitochondrial ATP using a luciferin/luciferase assay. Populations of cells were incubated in the media in the presence of glucose, and 2-deoxy-D-glucose with pyruvate (8). As shown in Figure 8A, in the presence of glucose (total cellular levels of ATP), the average levels of ATP production in mutant cells carrying both m.1555A>G and m.4317A>G mutations and only m.1555A>G mutation were 92% ($P=0.729$) and 98% ($P=0.243$), relative to the mean value measured in the control cell lines, respectively. In the presence of pyruvate and 2-deoxy-D-glucose to inhibit the glycolysis (mitochondrial levels of ATP), as shown in Figure 8B, the levels of mitochondrial ATP production in mutant cell lines carrying both m.1555A>G and m.4317A>G mutations and only m.1555A>G mutation were 56% ($P<0.001$) and 81% ($P=0.001$) of the mean value measured in the control cell lines, respectively.

**Reductions in mitochondrial membrane potential** - To examine whether the m.4317A>G mutation affects mitochondrial membrane potential ($\Delta \Psi_m$), a fluorescence probe JC-10 assay system was used to measure the $\Delta \Psi_m$ in mutant and control cell lines. The ratio of fluorescence intensities Ex/Em=490/590 and 490/530 nm (FL590/FL530) were recorded to delineate the $\Delta \Psi_m$ of each sample. The relative ratios of FL590/FL530 geometric mean between mutant and control cell lines were calculated to represent the level of $\Delta \Psi_m$, as described elsewhere (8). As shown in Figure 9, the levels of $\Delta \Psi_m$ in mutant cell lines carrying both m.1555A>G and m.4317A>G mutations and only m.1555A>G mutation were 59% ($P<0.001$) and 76% ($P=0.001$) of the mean value measured in the control cell lines, respectively. By contrast, the levels of $\Delta \Psi_m$ in mutant cell lines in the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, to dissipate the mitochondrial membrane potential) were comparable to those of control cell lines.

**The increase in mitochondrial ROS production** - The levels of mitochondrial ROS (mitoROS) among the lymphoblastoid cells were determined using a MitoSOX assay via flow cytometry (52). Geometric mean intensity was recorded to measure and delineate the rate of ROS of each sample. As shown in Figure 10, the levels of ROS generation in the mutant cell lines cells carrying both m.1555A>G and m.4317A>G mutations ranged from 165% to 181%, with an average 171% ($P<0.001$) of the mean value measured in the control cell lines. Moreover, levels of ROS generation in the mutant cell lines carrying only m.1555A>G mutation was 133% ($P=0.001$) of the mean value measured in control cell lines.

**DISCUSSION**
Mitochondrial genetic modifiers were proposed to increase the susceptibility to deafness-associated m.1555A>G or m.1494T>C mutation (2,53). Using genetic and molecular approaches, in
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Combination with functional assays, we demonstrated that a deafness susceptibility allele (m.4437A>G mutation) in the tRNA<sup>Ile</sup> gene modulated the phenotypic expression of deafness-associated 12S rRNA 1555A>G mutation. The m.4317A>G mutation (conventional position 59 of tRNA) affected the highly conserved adenine of the T-loop in tRNA<sup>Ile</sup> (39-40). We hypothesized that the anticipated alteration of the tertiary structure of tRNA<sup>Ile</sup> by the m.4317A>G mutation led to a failure in tRNA metabolism. In vitro assays showed that the m.4317A>G mutation impaired the 3’ end processing of tRNA<sup>Ile</sup> precursor and led to the decreased CCA-addition of tRNA<sup>Ile</sup> (41-43). Alternatively, the aberrant structure of T-arm makes mutant tRNA<sup>Ile</sup> more unstable, inefficiently aminoacylated and finally subject to degradation (39,40). The instability of mutant tRNA<sup>Ile</sup> was evidenced by the altered conformation observed in mutant tRNA<sup>Ile</sup> derived from cell lines carrying the m.4317A>G mutation and in mutant tRNA<sup>Ile</sup> transcript (G59). Moreover, the altered tertiary structure caused by the m.4317A>G mutation may affect the aminoacylated efficiency of tRNA<sup>Ile</sup> (42). In the present study, 46% decrease in aminoacylated tRNA<sup>Ile</sup> was observed in mutant cell lines carrying both m.4317A>G and m.1555A>G mutations. These results were consistent with a slightly but significantly decreased aminoacylated efficiency of tRNA<sup>Ile</sup> in vitro transcripts (42). In particular, mutant cell lines bearing both m.4317A>G and m.1555A>G mutations exhibited ~50% decreases in the steady-state level of tRNA<sup>Ile</sup>, while there was no significant reduction in the level of tRNA<sup>Ile</sup> in mutant cell lines carrying only m.1555A>G mutation. This level of total tRNA<sup>Ile</sup> in mutant cell lines is, however, above a proposed threshold level, which is 30% of the control level of tRNA, to support the normal rate of mitochondrial translation (5,6,8,54). These data strongly indicate that the m.4317A>G mutation alone is insufficient to produce a deafness phenotype, as in the case of deafness-associated tRNA<sup>Glu</sup> 14692A>G, tRNA<sup>Glu</sup> 7551A>G, 12S rRNA 1555A>G and 1494C>T mutations (9,10,23,26).

The altered tRNA<sup>Ile</sup> metabolism led to a defect in mitochondrial translation. Alternatively, the mutant tRNA<sup>Ile</sup> may interact faultily with translation machinery, thereby affecting the mitochondrial translation (55,56). In fact, the mtDNA encoded 13 polypeptides in the complexes of the oxidative phosphorylation system (ND1-6, ND4L of complex I, CYTB of complex III, CO1, CO2, CO3 of complex IV, and ATP6 and ATP8 of complex V) (4,46). In this investigation, 35% reductions in the average levels of 7 mtDNA encoded proteins were observed in mutant cell lines carrying both m.4317A>G and m.1555A>G mutations, respectively. In contrast, the cell lines carrying only m.1555A>G mutation exhibited 18% reduction in the average levels of 7 mtDNA encoded proteins. However, both m.4317A>G and m.1555A>G mutations did not reduce the levels of four subunits (NDUF8 of NADH:ubiquinone oxidoreductase; SDHB of succinate ubiquinone oxidoreductase; UQRC2 of ubiquinol cytochrome c reductase and ATP5A of H+-ATPase), encoded by nuclear genes. These data were consistent with our previous data that 27% and 58% decreases in the levels of 7 mtDNA encoding proteins were observed in the lymphoblastoid cell lines carrying only m.1555A>G mutation or both m.1555A>G and TRMU A10S mutations, derived from matrilineal relatives of an Arab-Israeli family, respectively (33). Notably, there were variable decreases in the levels of 7 mtDNA-encoded polypeptides in mutant cell lines carrying the both m.1555A>G and m.4317A>G mutations, ranging from 29 % to 102 % of the average values of control cell lines. As shown in Supplementary Table S2, cell lines
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harboring both m.1555A>G and m.4317A>G mutations exhibited marked reductions (71%) in the levels of CYTB, mild reductions (26% to 42%) in the level of ND1, ND4, ND5, ND6 and CO2, but a comparable level in the level of ATP8, as compared with those in control cell lines. In contrast to what was previously shown in cells carrying the tRNA^{Lys} 8344A>G mutation (56) and tRNA^{Ser(UCN)} 7445A>G mutation (5), polypeptides levels in mutant cell lines, relative to those in control cell lines, did not significantly correlate with the number or proportion of isoleucine codons. Hence, the impaired synthesis of ND1, ND4 and ND5, ND6, subunits of complex I, CYTB, subunit of complex III and CO2, subunits of complex IV may perturb the activities of complex I, III and complex IV and then worsen the defects in mitochondrial translation and respiratory phenotypes associated with m.1555A>G mutation. In this study, there were more severe decreases of complexes I, III, and IV activities observed in cell lines carrying both m.1555A>G and m.4317A>G mutations than those bearing only m.1555A>G mutation. In addition, impairment of mitochondrial translation resulted in the decreased rates in the basal OCR, or ATP-linked OCR, reserve capacity and maximal OCR among the control and mutant cell lines. These data highlighted that aberrant tRNA metabolism played a critical role in producing their respiration defects, as in the cases of cells carrying deafness-associated m.7511T>C, m.7551A>G and m.14692A>G mutations (6,9,10).

The respiratory deficiency may lead to the uncoupling of the oxidative pathway for ATP synthesis, oxidative stress and subsequent failure of cellular energetic process (57). In this investigation, ~19% and 44% decreases in mitochondrial ATP production were observed in these mutant cell lines carrying only m.1555A>G or both m.1555A>G and m.4317A>G mutations, respectively. However, 44% reduction of mitochondrial ATP production in mutant cell lines harboring both m.1555A>G and m.4317A>G mutations were comparable with those in lymphoblastoid cell lines bearing both m.1555A>G and homozygous TRMU A10S mutations (33). As a result, cochlear hair cells bearing both m.1555A>G and m.4317A>G mutations may be particularly sensitive to increased ATP demand (1,2,58). Furthermore, the deficient activities of respiratory chain complexes often impaired mitochondrial membrane potentials, which is a key indicator of cellular viability (8,10). Indeed, mitochondrial membrane potentials reflect the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation (49,59). In this study, 24% and 41% reductions in mitochondrial membrane potential in lymphoblastoid cell lines carrying the only m.1555A>G mutation and both m.1555A>G and m.4317A>G mutations was much lower than those in cell lines carrying the m.12201T>C mutation (8). The abnormal oxidative phosphorylation and mitochondrial membrane potential led to the increased production of reactive oxygen species and the subsequent failure of cellular energetic processes in mutant cells carrying m.1555A>G and m.4317A>G mutations. In particular, the overproduction of ROS can establish a vicious cycle of oxidative stress in the mitochondria, thereby damaging mitochondrial and cellular proteins, lipids and nuclear acids and increasing apoptotic signaling (60,61). Hair cells and neurons in the cochlea may be preferentially affected, because they are somehow exquisitely sensitive to subtle imbalances in the cellular redox state or increased level of free radicals (58,62). This would lead to the dysfunction or death of hair cells in the cochlea carrying both m.1555A>G and m.4317A>G mutations.
In summary, our study demonstrated the role of a deafness susceptibility allele (m.4317A>G mutation) in the tRNA^{Ile} gene in the phenotypic manifestation of deafness-associated m.1555A>G mutation. The m.4317A>G mutation altered both structure and function of tRNA^{Ile}. The aberrant tRNA metabolism further deteriorated the defective mitochondrial translation associated with the 12S rRNA 1555A>G mutation. These alterations resulted in the respiratory deficiency, decreasing ATP production and increasing ROS production. These biochemical defects lead to the high penetrance and occurrence of deafness in these Chinese families carrying both m.1555A>G and m.4317A>G mutations. Therefore, the tRNA^{Ile} 4317A>G mutation acts in synergy with 12S rRNA 1555A>G mutation, modulating phenotypic manifestation.

**EXPERIMENTAL PROCEDURES**

**Subjects and audiological examinations** - A total of 2651 genetically unrelated Han Chinese subjects with hearing impairment and 574 normal hearing Han Chinese control subjects for this study were described elsewhere (37). Two hearing-impaired Chinese Han pedigrees for this study were ascertained at the Otology Clinic of the First Affiliated Hospital, Wenzhou Medical University. This study was in compliance with the Declaration of Helsinki. Informed consent, blood samples, and clinical evaluations were obtained from all participants and families, under protocols approved by Ethic Committees of Zhejiang University and Wenzhou Medical University. Audiological and neurotological examinations of hearing impairment were performed as detailed previously (14). All available members of two pedigrees and three control subjects were evaluated at length to identify both of personal and family medical history of hearing loss, the history of the use of aminoglycosides and other clinical abnormalities.

**Mutational analysis of mitochondrial DNA** - Genomic DNA was isolated from whole blood of participants using QIAamp DNA Blood Mini Kit (Qiagen, No.51104). The subject’s DNA fragments spanning the mitochondrial 12S rRNA and tRNA^{Ile} genes were PCR amplified by use of oligodeoxynucleotides corresponding to mtDNA at positions 618 to 2007 and 3796 to 4693, respectively (4). Each fragment was purified and then analyzed by direct sequencing. These sequence results were compared with the updated consensus Cambridge sequence (GenBank accession number: NC_012920) (4). The entire mtDNAs of two probands WZD91 III-3 and WZD92 II-8 and three Chinese control subjects (A23, A22 and A21) were PCR amplified in 24 overlapping fragments using sets of the light (L) and heavy (H) strand oligonucleotide primers, as described previously (63). These sequence results were compared with the updated consensus Cambridge sequence, as described above. To quantify the m.4317A>G mutation, PCR segment (898 bp) spanning the tRNA^{Ile} gene was amplified and subsequently digested with restriction enzyme AfII, as the m.4317A>G mutation created the site for this enzyme. Equal amounts of various digested samples were then analyzed by electrophoresis through 3% agarose gel. The proportions of digested and undigested PCR products were determined by Image-Quant program after ethidium bromide staining to determine if the m.4317A>G mutation is in homoplasmy in these subjects.

**Cell lines and culture conditions** - Lymphoblastoid cell lines derived from members of two Chinese families [three subjects harboring only m.1555A>G mutation (WZD92 III-13, WZD92 III-17 and WZD92 III-18), three individuals (WZD91 III-1, WZD91 II-3 and WZD91 III-7) carrying both m.1555A>G and m.4317A>G mutations] and three control
individuals (A21, A22 and A23) lacking both mutations belonging to the same mtDNA haplogroup were immortalized by transformation with the Epstein-Barr virus, as described elsewhere (64). Lymphoblastoid cells were cultured in RPMI 1640 medium supplemented with 10% FBS.

**Mitochondrial tRNA analysis** - Total mitochondrial RNAs were obtained from mitochondria isolated from various cell lines (~2.0x10^8 cells) using TOTALLY RNATM kit (Ambion), as described previously (65). For the tRNA Northern blot analysis, 2 μg of total mitochondrial RNA were electrophoresed through a urea denaturing 10% polyacrylamide gel electrophoresis (PAGE) with 8 M urea in Tris-borate-EDTA buffer. The gels were electroblotted onto a positively charged nylon membrane (Roche) for the hybridization analysis with DIG-labeled oligodeoxynucleotide probes for tRNA^Ile^, tRNA^Leu(UUR)^, tRNA^Val^, tRNA^Glu^, tRNA^Ala^ and tRNA^Ser(UCN)^, as detailed previously (8-10,66,67). DIG-labeled oligodeoxynucleotides were generated by using DIG oligonucleotide Tailing kit (Roche). The hybridization and quantification of density in each band were performed as detailed previously (8-10).

For the aminoacylation assays, total mitochondrial RNAs were isolated under acid conditions, and 2 μg of RNAs was electrophoresed at 4°C through an acid (pH 5.0) 10% polyacrylamide-8 mM urea gel to separate the charged and uncharged tRNA as detailed elsewhere (56,68). To further distinguish non-aminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated by being heated for 10 min at 60°C (pH 8.3) and then run in parallel (9,68). The gels were then electroblotted onto a positively charged nylon membrane (Roche) for the hybridization analysis with oligodeoxynucleotide probes as described above. Quantification of density in each band was performed as detailed previously (68).

For the tRNA mobility shift assay, two μg of RNAs were electrophoresed through a 10% polyacrylamide native gel at 4°C with 50 mM Tris-glycine buffer. After electrophoresis, the gels were treated according to the Northern blot analysis as described above (66).

**Western blot analysis** - Western blotting analysis was performed as detailed previously (8-10). Five micrograms of total proteins obtained from lysed mitochondria were denatured and loaded on sodium dodecyl sulfate (SDS) polyacrylamide gels. The gels were electroblotted onto a polyvinylidene difluoride (PVDF) membrane for hybridization. The antibodies used for this investigation were from Abcam [TOM20 (ab56783), ND1 (ab74257), ND5 (ab92624), and CO2 (ab110258), Total OXPHOS Human WB Antibody Cocktail (ab110411)], Santa Cruz Biotechnology [ND4 (sc-20499-R) and ND6 (sc-20667)] and Proteintech [(CYTB (55090-1-AP) and ATP8 (26723-1-AP)]. Peroxidase Affini Pure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) were used as a secondary antibody and protein signals were detected using the ECL system (CWBIO). Quantification of density in each band was performed as detailed previously (8-10).

**Enzymatic Assays** – The enzymatic activities of complex I, II, III and IV were measured as detailed elsewhere (47-51). In brief, Citrate synthase activity was analyzed by the reduction of 5,5′-dithiobis-2-nitrobenzoic acid at 412 nm in the assay buffer containing 0.1 mM DTNB, 50 μM acetyl coenzyme A, and 250 μM oxaloacetate. Complex I activity was determined with 10 μg/ml antimycin A and 2 mM KCN by following the decrease in the absorbance due to the NADH oxidation at 340 nm in assay buffer. The activity of complex II was analyzed by tracking the
secondary reduction of 2,6-dichlorophenolindophenol (DCPIP) by DB at 600 nm in the assay buffer. Complex III activity was determined in the presence of 2 μg/ml antimycin A and 2 mM KCN by measuring the reduction of cytochrome c at 550 nm with reduced decylubiquinone in the assay buffer. Complex IV activity was measured by monitoring the oxidation of reduced cytochrome c as a decrease of absorbance at 550 nm in the assay buffer. All assays were performed by using Synergy H1 (Biotek, Winooski, VT, United States). Complex I-IV activities were normalized by citrate synthase activity.

Measurements of oxygen consumption - The rates of oxygen consumption in lymphoblastoid cell lines were assayed with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously (8,69). The protein content of each well was then measured to normalize OCR values.

ATP measurements - The Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega) was used for the measurement of cellular and mitochondrial ATP levels, following the modified manufacturer's instructions (8,66).

Assessment of mitochondrial membrane potential - Mitochondrial membrane potential was assessed with JC-10 Assay Kit-Microplate (Abcam) according to general manufacturer's recommendations with some modifications, as detailed elsewhere (8,66).

ROS measurements - ROS measurements were conducted as detailed previously (52,59).

Computer analysis - Statistical analysis was carried out using the unpaired, two-tailed Student's t-test contained in the Microsoft Excel program or Macintosh (version 2007). Differences were considered significant at a P<0.05.

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CONFLICT OF INTEREST
All authors have no proprietary or commercial interest in any of materials discussed in this article.

AUTHOR CONTRIBUTIONS
M.X.G and Q.S. designed the experiments, monitored the project progression, data analysis, and interpretation. F.M., Z.H., X.T. and J.Z. performed the experiments and contributed to data analysis in Figures 1-2 and 6-8, X.R., S.G. and M.Z. performed the performed the experiments and contributed to data analysis in Figures 2-4 and 9-10, F.M., and X.J. performed the experiments and contributed to data analysis in Figures 4-5, Y.Z., J.Z. and J.Q.M. performed the clinical evaluation. F.M., Z.H. and M.W. performed data analysis. F.M. prepared the initial draft of the manuscript. M.X.G. made the final version of the manuscript. All authors reviewed the manuscript.

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**FIGURE LEGENDS**

**Figure 1.** Identification and qualification of m.4317A>G mutation. (A) Cloverleaf structure of human mitochondrial tRNA\textsuperscript{Ile}. An arrow denotes the location of the m.4317A>G mutation. (B) Partial sequence electropherograms of the tRNA\textsuperscript{Ile} gene from the lymphoblastoid cell lines of an affected individual (WZD91 III-1) and a Han Chinese control (A21), respectively. Arrows indicate the locations of the base changes at position 4317. (C) Quantification of the m.4317A>G mutation by PCR-RFLP. PCR products were digested with AfIII and analyzed by electrophoresis in a 7% polyacrylamide gel stained with ethidium bromide. Mutant cell lines carrying m.4317A>G mutation and control cell lines are indicated. (D) Two Han Chinese pedigrees with aminoglycoside-induced and nonsyndromic hearing impairment.
A tRNA modifier for deafness expression of 12S rRNA mutation

Hearing impaired individuals are indicated by filled symbols. Arrowhead denotes individuals that the lymphoblastoid cells derived from. Asterisks denote individuals who had a history of exposure to aminoglycosides.

Figure 2. Northern blot analysis of tRNA under a native or denaturing condition. (A) The analysis of tRNAIle conformation. Two microgram of total mitochondrial RNA from various cell lines were electrophoresed through native polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAIle, tRNASer(AGY) and tRNA Met, respectively. (B) Northern blot analysis of tRNA under a denaturing condition. Two micrograms of total mitochondrial RNA from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAIle, tRNA Leu(UUR), tRNA Val, tRNA Gln, tRNA Ala and tRNA Ser(UCN), respectively. (C) Quantification of tRNA levels. Average relative tRNAIle content per cell, normalized to the average content per cell of tRNALeu(UUR), tRNA Val, tRNA Gln, tRNA Ala and tRNA Ser(UCN) in three mutant cell lines carrying only m.1555A>G, three mutant cell lines harboring both m.1555A>G and m.4317A>G mutations and three control cell lines lacking these mutations. The values for the mutant cell lines are expressed as percentages of the average values for the control cell lines. The calculations were based on three independent experiments. The error bars indicate standard deviation. P indicates the significance, according to the t-test, of the differences between mutant and control cell lines.

Figure 3. In vivo aminoacylation assays. (A) Two micrograms of total mitochondrial RNA purified from various cell lines under acid conditions were electrophoresed at 4°C through an acid (pH 5.0) 10 % polyacrylamide-8 M urea gel, electroblotted and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAIle, tRNA Leu(UUR), tRNA His, tRNA Met and tRNA Ser(AGY), respectively. (B) The samples from control and mutant cell lines were deacylated (DA) by heating for 10 min at 60°C at pH 8.3, electrophoresed and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAIle and tRNA Leu(UUR). (C) Quantification of aminoacylated proportions of tRNAIle, tRNA Leu(UUR), tRNA His, tRNA Met and tRNA Ser(AGY) in the mutant and controls. The calculations were based on three independent experiments. Graph details and symbols are explained in the legend to Figure 2.

Figure 4. Western blot analysis of mitochondrial proteins. (A) Five micrograms of total mitochondrial proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with antibodies specific for ND1, ND4, ND5, ND6, CO2, CYTB and ATP8 and with TOM20 as a loading control, respectively. (B) Quantification of total mitochondrial protein levels. The levels of mitochondrial proteins in mutant and control cell lines were determined as described elsewhere (8). (C) Quantification of 7 polypeptides. The levels of ND1, ND4, ND5, ND6, CO2, CYTB and ATP8 in mutant and control cell lines were determined as described elsewhere (8-10). Graph details and symbols are explained in the legend to Figure 2.

Figure 5. Western blot analysis of OXPHOS subunits. Five micrograms of total mitochondrial proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with antibody cocktail specific for subunits of each OXPHOS complex and with TOM20 as a loading control. (B) Quantification of the levels of ATP5A, UQRC2, SDHB, CO2 and NDUFB8 in mutant and control cell lines were determined as described elsewhere (8-10). Graph details and symbols are explained in the legend to Figure 2.
Figure 6. Enzymatic activities of respiratory chain complexes. The activities of respiratory complexes were investigated by enzymatic assay on complexes I, II, III, and IV in mitochondria isolated from various cell lines. The calculations were based on 3-5 independent experiments. Graph details and symbols are explained in the legend to Figure 2.

Figure 7. Respiration assays. (A) An analysis of O₂ consumption in the various cell lines using different inhibitors. The rates of O₂ (OCR) were first measured on 2×10⁴ cells of each cell line under basal condition and then sequentially added to oligomycin (1.5 µM), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (0.5 µM), rotenone (1 µM) and antimycin A (1 µM) at indicated times to determine different parameters of mitochondrial functions. (B) Graphs presented the ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity and non-mitochondrial OCR in mutant and control cell lines. Non-mitochondrial OCR was determined as the OCR after rotenone/antimycin A treatment. Basal OCR was determined as OCR before oligomycin minus OCR after rotenone/antimycin A. ATP-linked OCR was determined as OCR before oligomycin minus OCR after oligomycin. Proton leak was determined as Basal OCR minus ATP-linked OCR. Maximal was determined as the OCR after FCCP minus non-mitochondrial OCR. Reserve Capacity was defined as the difference between Maximal OCR after FCCP minus Basal OCR. OCR values were expressed in picomoles of oxygen/minute/microgram of protein. The average values of 3 determinations for each cell line were shown. Graph details and symbols are explained in the legend to Figure 2.

Figure 8. Measurement of cellular and mitochondrial ATP levels. Mutant and control cell lines were incubated with 10 mM glucose or 5 mM 2-deoxy-D-glucose plus 5 mM pyruvate to determine ATP generation under mitochondrial ATP synthesis. Average rates of ATP level per cell line in mitochondria are shown: (A) ATP level in total cells; (B) ATP level in mitochondria. Four to five determinations were made for each cell line. Graph details and symbols are explained in the legend to Figure 2.

Figure 9. Mitochondrial membrane potential analysis. The mitochondrial membrane potential (ΔΨm) was measured in mutant and control cell lines using a fluorescence probe JC-10 assay system. The ratio of fluorescence intensities Ex/Em=490/590 nm and 490/530 nm (FL₅₉₀/FL₅₃₀) were recorded to delineate the ΔΨm level of each sample. The relative ratios of FL₅₉₀/FL₅₃₀ geometric mean between mutant and control cell lines were calculated to reflect the level of ΔΨm. Flow cytometry images of a control cell line A22, WZD92 III-18 carrying only m.1555A>G mutation and WZD 91 II-3 carrying both m.4317A>G and m.1555A>G mutations without (A) and with (B) FCCP. Relative ratio of JC-10 fluorescence intensities at Ex/Em = 490/525 and 490/590 nm in the absence (C) and presence (D) of 10 µM of FCCP in three control cell lines, three mutant cell lines carrying only m.1555A>G mutation and three mutant cell lines bearing both m.1555A>G and m.4317A>G mutations. The average of 3-4 determinations for each cell line is shown. Graph details and symbols are explained in the legend to Figure 2.

Figure 10. Measurement of mitochondrial ROS production. The levels of ROS generation by mitochondria in living cells from mutant and control cell lines were determined using the mitochondrial superoxide indicator MitoSOX-Red. Fluorescence was measured using a FACS Calibur instrument (BD Biosciences), with excitation at 488 nm and emission at 580 nm. The data were analyzed with Flow Jo software. (A) Flow cytometry histogram showing MitoSOX-Red fluorescence of A22 (Red), WZD92 III-18 (Green) and WZD 91 II-3 (Blue). (B) Relative ratios of MitoSOX-Red fluorescence intensity. The average of 4 determinations for each cell line is shown. Graph details and symbols are explained in the legend to Figure 2.
legend to Figure 2.
Figure 2

**A**

| A23 | A22 | WZD92 II-17 | WZD92 II-13 | WZD91 II-1 | A21 | WZD92 II-18 | WZD91 II-7 |
|-----|-----|-------------|-------------|-------------|-----|-------------|-------------|

- tRNA\text{\textsuperscript{Ile}}
- tRNA\text{\textsuperscript{Ser(AGY)}}
- tRNA\text{\textsuperscript{Met}}

**B**

- m.1555A>G
- m.4317A>G

**C**

- p=0.078–0.921
- p=0.002–0.011
Figure 3

A

|   | + | + | - | - | - | - | + | - |
|---|---|---|---|---|---|---|---|---|
|   | A23 | A22 | WZD92 III-17 | WZD92 III-13 | WZD91 III-1 | WZD91 I-3 | A21 | WZD92 I-18 | WZD91 I-7 |

m.1555A>G  
m.4317A>G

B

|   | + | + | - | - | + | + | + | + |
|---|---|---|---|---|---|---|---|---|
|   | A21 | DA-A21 | WZD92 III-18 | WZD92 III-13 | WZD91 III-18 | WZD91 III-7 | DA-WZD92 | DA-WZD91 |

Ile-tRNA<sup>ile</sup>  
tRNA<sup>ile</sup>

Leu-tRNA<sup>Leu(UUR)</sup>  
tRNA<sup>Leu(UUR)</sup>

His-tRNA<sup>His</sup>  
tRNA<sup>His</sup>

Met-tRNA<sup>Met</sup>  
tRNA<sup>Met</sup>

Ser-tRNA<sup>Ser(AGY)</sup>  
tRNA<sup>Ser(AGY)</sup>

C

|   | 100 | 80 | 60 | 40 | 20 | 0 |
|---|-----|----|----|----|----|---|
|   | P=0.316 | P=0.859~0.597 | P=0.398~0.789 | P=0.755~0.817 | P=0.641~0.878 |
|   | A23 | A22 | A21 | WZD92 III-17 | WZD92 III-13 | WZD91 III-18 | WZD91 III-7 | WZD91 I-3 | WZD91 III-7 |

Aminoacylation of tRNAs (%)
Figure 4

A

Relative level of seven mitochondrial proteins (%)

B

Relative level of mitochondrial proteins (%)

C

Relative level of mitochondrial proteins (%)
Figure 5

A

\[
\begin{array}{ccccccc}
+ & + & + & - & - & - & - \\
+ & + & + & + & + & - & - \\
\end{array}
\]

m.1555A>G  
m.4317A>G

\[
\begin{array}{ccccccc}
ATP5A (Complex V) & UQCRC2 (Complex III) \\
SDHB (Complex II) & CO2 (Complex IV) & NDUFB8 (Complex I) \\
\end{array}
\]

B

| Protein     | m.1555A>G | m.4317A>G |
|-------------|----------|----------|
| ATP5A       | 100      | 90       |
| UQCRC2      | 110      | 105      |
| SDHB        | 85       | 80       |
| CO2         | 95       | 90       |
| NDUFB8      | 120      | 115      |

Relative level of complex proteins (%)

\[
\begin{array}{cccccc}
\text{ATP5A} & \text{UQCRC2} & \text{SDHB} & \text{CO2} & \text{NDUFB8} \\
\text{Relative level} & 100 & 110 & 85 & 95 & 120 \\
\end{array}
\]

KDa

55
40
25
15
15
25
40
55

A23A22A21
WZD92 II-17
WZD92 II-18
WZD92 II-13
WZD91 II-1
WZD91 II-3
WZD91 II-7

SDHB
CO2
NDUFB8 (Complex I)

TOM 20
Figure 6

Relative level of enzyme activity (%)

- Complex I
- Complex II
- Complex III
- Complex IV

Genetic variants:
- m.1555A>G
- m.4317A>G

Comparisons:
- p=0.001
- p=0.660
- p=0.017
- p<0.001
- p=0.016
- p=0.501
- p<0.001
- p=0.022

Legend:
- Controls
- m.1555A>G
- m.1555A>G + m.4317A>G
Figure 7

A

Oligomycin  FCCP  Antimycin/Rotenone

OCR (pMols/min/µg)

B

OCR (pMols/min/µg)

Controls  m.1555A>G  m.4317 A>G

p=0.042  p=0.002
p=0.031  p=0.001
p=0.106  p=0.027
p=0.045  p=0.020
p=0.301  p=0.010
p=0.740  p=0.123

Basal  ATP-Linked  Proton leak  Maximal  Reserve Capacity  Non-Mitochondrial
Figure 8

A

| m.1555A>G | + | + | + | - | - | - | - | - | - | - | - | - |
| m.4317A>G | + | + | + | + | + | + | - | - | - | - | - | - |

P = 0.729

B

| A23 | A22 | A21 | WZD91 III-17 | WZD91 III-18 | WZD91 III-13 | WZD91 II-3 | WZD91 III-7 |
|-----|-----|-----|---------------|---------------|---------------|-------------|-------------|

P = 0.001

P < 0.001
Figure 9

A Controls m.1555A>G m.1555A>G + m.4317A>G

A22 WZD92 III-18 WZD91 II-3

101 103 105 107.2

101 103 105 107.2

85% 65% 49%

14% 34% 51%

Membrane potential (%)

(relative ratio of FL590/FL530)

B FCCP

4% 4% 4%

96% 96% 96%

A22 WZD92 III-18 WZD91 II-3

C m.1555A>G + + + — — —
m.4317A>G + + + + + +

P<0.001 P<0.001

Membrane potential (%)

(relative ratio of FL590/FL530)

D FCCP

P=0.945 P=0.737

Membrane potential (%)

(relative ratio of FL590/FL530)
Relative intensity of MitoSox Red fluorescence (%)

A22  WZD92 III-18
A22  WZD91 II-3

Counts (%)

0  20  40  60  80  100

10^{1.5}  10^{3}  10^{4}  10^{5.5}

m.1555A>G  + + +
m.4317A>G  + + +

Figure 10

A

B

Counts (%)

0  20  40  60  80  100

10^{1.5}  10^{3}  10^{4}  10^{5.5}

Relative intensity of MitoSox Red fluorescence (%)

A23  A22  A21  WZD92 II-17  WZD92 II-18  WZD92 II-13  WZD91 III-1  WZD91 III-3  WZD91 III-7

Counts (%)

0  40  80  120  160  200

10^{1.5}  10^{3}  10^{4}  10^{5.5}

m.1555A>G  + + +
m.4317A>G  + + +

P=0.001  P<0.001

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Contribution of the tRNA\textsuperscript{Ile} 4317A>G mutation to the phenotypic manifestation of the deafness-associated mitochondrial 12S rRNA 1555A>G mutation
Feilong Meng, Zheyun He, Xiaowen Tang, Jing Zheng, Xiaofen Jin, Yi Zhu, Xiaoyan Ren, Mi Zhou, Meng Wang, Shasha Gong, Jun Qin Mo, Qiang Shu and Min-Xin Guan

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