Nuclear modifier genes have been proposed to modify the phenotypic expression of mitochondrial DNA mutations. Using a targeted exome-sequencing approach, here we found that the p.191Gly>Val mutation in mitochondrial tyrosyl-tRNA synthetase 2 (YARS2) interacts with the tRNA\textsubscript{Ser(UCN)}\textsuperscript{7511A>G} mutation in causing deafness. Strikingly, members of a Chinese family bearing both the YARS2 p.191Gly>Val and m.7511A>G mutations displayed much higher penetrance of deafness than those pedigrees carrying only the m.7511A>G mutation. The m.7511A>G mutation changed the A4:U69 base-pairing at the aminoacyl acceptor stem of tRNA\textsubscript{Ser(UCN)} and perturbed tRNA\textsubscript{Ser(UCN)} structure and function, including an increased melting temperature, altered conformation, instability, and aberrant aminoacylation of mutant tRNA. Using lymphoblastoid cell lines derived from symptomatic and asymptomatic members of these Chinese families and control subjects, we show that cell lines harboring only the m.7511A>G or p.191Gly>Val mutation revealed relatively mild defects in tRNA\textsubscript{Ser(UCN)} or tRNA\textsubscript{Tyr} metabolism, respectively. However, cell lines harboring both m.7511A>G and p.191Gly>Val mutations displayed more severe defective aminoacylations and lower tRNA\textsubscript{Ser(UCN)} and tRNA\textsubscript{Tyr} levels, aberrant aminoacylation, and lower levels of other tRNAs, including tRNA\textsubscript{Thr}, tRNA\textsubscript{Ile}, tRNA\textsubscript{Leu(UUB)}, and tRNA\textsubscript{Ser(AGY)}, than those in the cell lines carrying only the m.7511A>G or p.191Gly>Val mutation. Furthermore, mutant cell lines harboring both m.7511A>G and p.191Gly>Val mutations exhibited greater decreases in the levels of mitochondrial translation, respiration, and mitochondrial ATP and membrane potentials, along with increased production of reactive oxygen species. Our findings provide molecular-level insights into the pathophysiology of maternally transmitted deafness arising from the synergy between tRNA\textsubscript{Ser(UCN)} and mitochondrial YARS mutations.

Defects of mitochondrial tRNA metabolisms have been associated with both syndromic deafness (hearing loss with other medical problems, such as diabetes) and nonsyndromic deafness (where hearing loss is the only obvious medical problem) (1–5). In humans, mitochondrial genomes (mtDNA) encode 13 subunits of the oxidative phosphorylation system (OXPHOS), two tRNAs and 22 tRNAs required for translation (6, 7). The formation of functional tRNA molecules used for protein synthesis requires the transcription, nucleolytic processing, posttranscriptional nucleotide modifications, and aminoacylation (4–9). These proteins involved in the tRNA maturation processing, especially mitochondrial tRNA synthetases, encoded by nuclear genes, were synthesized in the cytosol and subsequently imported into mitochondria (7, 9–11). These deafness-associated tRNA mutations have structural and functional consequences for corresponding tRNAs (1, 12). These included the aberrant processing of 3′ end tRNA\textsubscript{Ser(UCN)} pre-cursor, caused by m.7445A>G mutation (13, 14), instability of the folded secondary structure of tRNA\textsubscript{Glu} due to m.14692A>G mutation (15), deficient m'G37 modification of tRNA\textsubscript{Asp} caused by m.7551A>G mutation (16), and defective aminoacylation of tRNA\textsubscript{Glu} resulting from m.12201T>C muta-

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This article contains Tables S1–S3 and Figs. S1 and S2.

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Moreover, nonsyndromic deafness in some families was caused associated with syndromic deafness, respectively (18–21). Moreover, nonsyndromic deafness in some families was caused associated with syndromic deafness, respectively (18–21). The coexistence of the 12S rRNA m.1555A>G mutation and p.Ala10Ser mutation in TRMU responsible for the biosynthesis of tRNALys (22, 23). However, the pathophysiology underlying deafness-linked aberrant tRNA metabolisms remains poorly understood.

As shown in Fig. 1A, the deafness-associated tRNA<sub>Ser(UCN)</sub> m.7511A>G mutation converted the A4-U69 base-pairing into a G4-U69 base-pairing at the aminoacyl acceptor stem of this tRNA (24–27). This base-pairing may play an important role in the stability and identity of tRNA (24, 25). We therefore hypothesized that the m.7511A>G mutation perturbed both structure and function of tRNA<sub>Ser(UCN)</sub>. The m.7511A>G mutation was identified in several families from different ethnic groups, with varying expressivity and penetrance of deafness (27–30). In particular, 9 of 10 matrilineal relatives in a three-generation Chinese pedigree carrying the m.7511A>G mutation exhibited hearing impairment, in contrast with only a small portion of hearing-impaired matrilineal relatives in two French pedigrees and one Japanese family carrying the same mtDNA mutation (27–30). These findings suggest that the nuclear modifier genes, especially those involved in mitochondrial tRNA metabolism, contributed to the phenotypic expression of m.7511A>G mutation. By target exome sequencing (genes encoding 20 mitochondrial tRNA synthetases and 25 tRNA-modifying enzymes), we identified the known variant (c.572G>T, p.191Gly>Val) in the YARS2 gene encoding the mitochondrial tyrosyl-tRNA synthetase (31, 32) that interacted with the m.7511A>G mutation to cause hearing loss in a three-generation Chinese family with extremely high penetrance of hearing loss (Table S2). In the present study, we further investigated the impact of the m.7511A>G mutation on the structure and function of tRNA<sub>Ser(UCN)</sub>. The effects of YARS2 p.191Gly>Val and m.7511A>G mutations on mitochondrial functions were first assessed for the tRNA metabolism, including aminoacylation capacities and stability of tRNA, through the use of lymphoblastoid mutant cell lines derived from members of the Chinese family (individuals carrying only the m.7511A>G mutation, only the YARS2 p.191Gly>Val mutation or both m.7511A>G and heterozygous or homozygous p.191Gly>Val mutations), and genetically unrelated control subjects lacking these mutations. These cell lines were further evaluated for an effect on mitochondrial translation, respiration, production of ATP, mitochondrial membrane potential, and reactive oxygen species (ROS).

**Results**

**The m.7511A>G mutation altered the stability and conformation of tRNA<sub>Ser(UCN)</sub>**

As shown in Fig. 1A, the m.7511A>G mutation changed the typical A4-U69 base-pairing into a noncanonical G4-U69 base-pairing at the acceptor stems. To experimentally test the effect of m.7511A>G mutation on the stability of tRNA<sub>Ser(UCN)</sub>, we examined the melting temperatures (T<sub>m</sub>) of WT (A4) and mutant (G4) tRNA<sub>Ser(UCN)</sub> transcripts. These T<sub>m</sub> values were determined by calculating the derivatives of the absorbance against a temperature curve. As shown in Fig. 1B, the T<sub>m</sub> values for WT (A4) and mutant (G4) tRNA<sub>Ser(UCN)</sub> transcripts were 41.7 and 51 °C, respectively. These data suggested that the tRNA<sub>Ser(UCN)</sub> with a G4:U69 bp may be more stable than the tRNA<sub>Ser(UCN)</sub> with an A4:U69 bp.

As shown in Fig. 1C, electrophoretic patterns showed that the mutant (G4) tRNA<sub>Ser(UCN)</sub> transcript migrated faster than the WT (A4) tRNA<sub>Ser(UCN)</sub> transcript under native conditions. However, there was no difference of migration pattern between WT (A4) and mutant (G4) tRNA<sub>Ser(UCN)</sub> transcripts under denaturing conditions. These data indicated that the m.7511A>G mutation resulted in the conformational change of tRNA<sub>Ser(UCN)</sub>.

**Clinical presentation of a hearing-impaired Han Chinese pedigree**

One Han Chinese hearing-impaired proband carrying the m.7511A>G mutation was identified among 2651 Chinese hearing-impaired probands but absent in 574 Chinese hearing-normal controls (28). As shown in Fig. S1A, the Chinese family exhibited extremely high penetrance of hearing loss. As shown in Fig. S2 and Table S1, 9 of 10 matrilineal relatives exhibited the variable degree of hearing impairment (two with mild hearing loss, six with moderate hearing loss, and one with severe hearing loss), whereas none of other members in this family had hearing loss. The age-at-onset of hearing loss ranged from 5 to 55 years old, with an average of 25 years old. There was no evidence that any of the other members of this family had any other causes to account for hearing loss. These matrilineal relatives showed no other clinical abnormalities, including cardiac failure, muscular diseases, visual failure, and neurological disorders. Further analysis showed that the m.7511A>G mutation was present in homoplasy in all matrilineal relatives but not in other members of this family (Fig. S1B).

**Targeting exome sequence analysis**

The higher penetrance of hearing loss in this Chinese family implied that nuclear modifier genes, especially for genes involved in mitochondrial tRNA metabolism, influence the phenotypic manifestation of m.7511A>G mutation. To test this hypothesis, we performed targeting exome-sequencing analyses of 45 genes encoding 20 mitochondrial tRNA synthetases and 25 tRNA-modifying enzymes (Table S2) among seven matrilineal relatives (II-5, II-7, III-3, III-4, III-5, III-6, and III-7) and two married-in controls (II-4 and II-5) of WZD200 pedigree carrying the m.7511A>G mutation. As a result, we identified the known (c.572G>T, p.191Gly>Val) mutation in the YARS2 gene encoding the mitochondrial tyrosyl-tRNA synthetase in six hearing-impaired matrilineal relatives but not in the hearing-normal matrilineal relative (III-7). We further analyzed the presence of the c.572G>T mutation in three symptomatic members and six asymptomatic subjects of this Chinese family and 13 symptomatic members and five asymptomatic
subjects of a Japanese family (30), by restriction fragment length polymorphism analysis, because the c.572G>T mutation disrupted a Tsp45I site (32). In the Chinese family, the symptomatic subjects (II-1 and II-5) and married-in control (I-1) carried the homozygous c.572G>T mutation, the symptomatic subjects (I-2, II-2, II-7, III-3, III-4, III-5 and III-6) harbored the heterozygous c.572G>T mutation, and the asymptomatic individual (III-7) and three married controls lacked the c.572G>T mutation (Fig. S1A and Table S1). However, this mutation was absent in the members of the Japanese family (30). These suggested that the c.572G>T mutation may increase the penetrance of hearing loss in the Chinese family.

Reductions in the steady-state levels of mitochondrial tRNAs

To test if the m.7511A>G mutation affected the conformation of tRNA^{Ser(UCA)} ex vivo, total RNAs from mitochondria isolated from various cell lines were electrophoresed through 15% polyacrylamide gel (native condition) and then electroblotted onto a positively charged nylon membrane (Roche...
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A Northern blot analysis of tRNA under denaturing conditions. A Northern blot analysis of tRNA under denaturing conditions. 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 for the tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Glu</sub>, tRNA<sub>Asp</sub>, tRNA<sub>Met</sub>, tRNA<sub>Leu(UUR)</sub>, and tRNA<sub>Ser(AGY)</sub> in mutant cell lines carrying both m.7511A>G and homozygous c.572G>T mutations were decreased by 72.2, 58.9, 33.5, 69.6, 86.1, 13.5, and 21.2%, as compared with the average values in the control cell line (A61), respectively. Furthermore, the average steady-state levels of tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Glu</sub>, tRNA<sub>Asp</sub>, tRNA<sub>Met</sub>, tRNA<sub>Leu(UUR)</sub>, and tRNA<sub>Ser(AGY)</sub> in mutant cell lines carrying both m.7511A>G and heterozygous c.572G>T mutations were decreased by 75.8, 51.1, 35.5, 64.4, 71.1, 11.2, and 26.1%, as compared with the average values in the control cell line (A61), respectively.

Figure 3. Northern blot analysis of tRNA under denaturing conditions. A, Northern blot analysis of tRNA under denaturing conditions. 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 for the tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Glu</sub>, tRNA<sub>Asp</sub>, tRNA<sub>Met</sub>, tRNA<sub>Leu(UUR)</sub>, and tRNA<sub>Ser(AGY)</sub>, respectively. B, quantification of tRNA levels. Shown is average relative content of each tRNA per cell, normalized to the average content per cell of 5S rRNA in mutant cell lines harboring both the m.7511A>G and heterozygous YARS2 p.Gly191Val mutations, both the m.7511A>G and homozygous YARS2 p.Gly191Val mutations, only the homozygous YARS2 p.Gly191Val mutation, or only the m.7511A>G mutation and 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. Error bars, S.D.; p, significance, according to the t test, of the differences between mutant and control cell lines.

The aminoacylation capacities of tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Thr</sub>, tRNA<sub>Leu(UUR)</sub>, tRNA<sub>Leu(UUR)</sub>, and tRNA<sub>Ser(AGY)</sub> in various control and mutant cell lines were examined by using electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA, electroblotting and hybridizing with the above tRNA probes. As shown in Fig. 3A, the slower-migrating band (top band) represents the charged tRNA, and the faster-migrating band (bottom band) represents uncharged tRNA. The electrophoretic patterns revealed two stacked bands present for the WT tRNA<sub>Ser(UCN)</sub> and two well-separated bands for the mutant tRNA<sub>Ser(UCN)</sub>. Furthermore, either charged or uncharged tRNA<sub>Ser(UCN)</sub> migrated faster in all mutant cell lines carrying the m.7511A>G mutation than those in other cell lines lacking the mutation. To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated after heating for 10 min at 60 °C (pH 9.0) and then run in parallel. As shown in Fig. 3, the deacylated samples gave only one band (uncharged tRNA) in both mutant and control cell lines.

As shown in Fig. 3, ~10% decreases in the aminoacylation efficiency of tRNA<sub>Ser(UCN)</sub> in the cell line III-7, bearing only the m.7511A>G mutation, and 25.4% reductions in the aminoacylation efficiency of tRNA<sub>Tyr</sub> in the cell line I-1, carrying only the homozygous c.572G>T mutation, were observed, as compared with those in the control cell line (A61). Strikingly, cells harboring both m.7511A>G and c.572G>T mutations exhibited greater reductions in aminoacylated efficiencies of tRNA<sub>Ser(UCN)</sub> and tRNA<sub>Tyr</sub> as well as various reductions in those of other tRNAs (Fig. 3A). In particular, the aminoacylated efficiencies of tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Thr</sub>, tRNA<sub>Leu(UUR)</sub>, tRNA<sub>Ser(AGY)</sub>, and tRNA<sub>Ser(AGY)</sub> in mutant cell lines carrying both m.7511A>G and homozygous c.572G>T mutations were 37.9, 62.5, 88.6, 40.7, 65.1, and 74.3% of the average values in the control cell line (A61), respectively. Furthermore, the aminoacylated efficiencies of tRNA<sub>Ser(UCN)</sub>, tRNA<sub>Tyr</sub>, tRNA<sub>Thr</sub>, tRNA<sub>Leu(UUR)</sub>, tRNA<sub>Ser(AGY)</sub>, and tRNA<sub>Ser(AGY)</sub> in mutant cell lines carrying both m.7511A>G and heterozygous c.572G>T mutations were 44, 62.8, 111.2, 51.6, 99, and 88.6% of the average values in the control cell line (A61), respectively.
Decreases in the levels of mitochondrial proteins

To assess whether the c.572G>T mutation enhanced the defects in mitochondrial translation associated with m.7511A>G mutation, a Western blot analysis was carried out to examine the levels of seven mtDNA encoding polypeptides (of respiratory complex) in various cell lines with VDAC as a loading control. As shown in Fig. 4A, the levels of ND1, ND4, ND5, and ND6 (subunits 1, 4, 5, and 6 of NADH dehydrogenase); CYTB (apocytochrome b); CO1 (subunit 1 of cytochrome c oxidase); and ATP6 (subunit 6 of the H^+-ATPase) exhibited variable reductions in mutant cell lines, as compared with those of the control cell line. As shown in Fig. 4B, the average levels of ND1, ND4, ND5, ND6, CO1, CYTB, and ATP6 in mutant cell lines carrying only the homozygous c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 84.9, 77.7, 61.7, and 49.8% of those in the control cell line (A61), respectively. In particular, the levels of ND1, ND4, ND5, ND6, CO1, CYTB, and ATP6 in the cell line carrying only m.7511A>G mutation were 89.1, 73.9, 47.3, and 49.1% of those in the control cell line (A61). However, the activities of complex III in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 84.8, 68.3, 42.6, and 33.2% of the control cell line (A61), respectively. The activities of complex III in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 19.4, 52.7, 47.1, and 70% of those in control cell line (A61), respectively (Fig. 5B).

Reduced activities of respiratory complexes I, III, and IV

To examine whether the c.572G>T mutation worsened the respiratory deficiency caused by m.7511A>G mutation, we measured the activities of respiratory complexes by isolating mitochondria from mutant and control cell lines (33, 34). As shown in Fig. 6, the activity of complex I in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 84.9, 77.7, 61.7, and 49.8% of those in the control cell line (A61), respectively. In particular, the levels of ND1, ND4, ND5, ND6, CO1, CYTB, and ATP6 in the cell line carrying only m.7511A>G mutation were 35.4, 97.8, 70.2, 83.7, 52.8, 102.6, and 101.3% of those in control cell line (A61) (p < 0.05), respectively.

We then examined the levels of seven subunits (mtDNA-encoding CO2 and six nucleus-encoding proteins) of the phosphorylation system (OXPHOS) in control and mutant cell lines by Western blot analysis. As shown in Fig. 5A, the levels of NDUF3, NDUF8B (subunits of NADH:ubiquinone oxidoreductase), CO2, and COX10 (subunits of cytochrome c oxidase) were decreased in the mutant cell lines. By contrast, the levels of other mitochondrial proteins (ATP5A, UQCRCC2, and SDHB) in mutant cell lines were comparable with those in the control cell line. As illustrated in Fig. 5B, the average levels of NDUF3, NDUF8B, CO2, and COX10 in mutant cell lines carrying both m.7511A>G and heterozygous c.572G>T mutations were 42.2, 71.8, 50.3, and 70.5% of those in control cell line (A61). Furthermore, the activities of complex IV in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 19.4, 52.7, 47.1, and 70% of those in control cell line (A61), respectively. The activities of complex IV in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 42.2, 71.8, 50.3, and 70.5% of those in control cell line (A61) (p < 0.05), respectively.

Respiration defects in mutant cells

To further assess whether the m.7511A>G and c.572G>T mutations altered cellular bioenergetics, we examined the oxygen consumption rates (OCR) of various mutant and control
cell lines using a Seahorse Bioscience XF-96 extracellular flux analyzer (35, 36). In this system, a single experiment can measure all major aspects of mitochondrial coupling and respiratory control, including basal respiration, O$_2$ consumption attributed to ATP production, proton leak, maximum respiratory rate, reserve capacity, and nonmitochondrial respiration (Fig. 6B). As shown in Fig. 6C, the basal OCR in the mutant cell lines carrying only the c.572G>T mutation, the m.7511T>G mutation, or both the m.7511A>G and heterozygous or homozygous c.572G>T mutations were 92.5, 68.6, 47.0, and 36.9% of the mean values measured in the control cell lines ($p < 0.05$), respectively. To investigate which of the enzyme complexes of the respiratory chain was affected in the mutant cell lines, OCR was measured after the sequential addition of oligomycin (to inhibit the ATP synthase), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (to uncouple the mitochondrial inner membrane and allow for maximum electron flux through the electron transfer chain), rotenone (to inhibit complex I), and antimycin A (to inhibit complex III) (56). The differences between the basal OCR and the drug-insensitive
A nuclear modifier for deafness expression of tRNA mutation

OCR yielded the amount of ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and nonmitochondrial OCR. As illustrated in Fig. 6, the amounts of ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and nonmitochondrial OCR in mutant cell line carrying only homoygous c.572G>T mutation were 94.7, 82.1, 76.3, 63.2, and 106.4% of those in the control cell lines. The values in mutant cell line carrying only the m.7511A>G mutation were 77.6, 58.9, 62.9, and 44.8% of the control cell lines. Moreover, the levels of total cellular ATP production in the above mutant cell lines were 97.5, 84.3, 79.5, and 67.1%, relative to the mean value measured in the control cell lines, respectively.

Decreases in mitochondrial membrane potentials

The mitochondrial membrane potential (ΔΨm) generated by proton pumps (complexes I, III, and IV) is an essential component in the process of energy storage during oxidative phosphorylation (37). To examine the capacity of oxidative phosphorylation, we measured the levels of ΔΨm in the mutant and control cell lines using a fluorescence probe JC-10 assay system. The ratios of fluorescence intensity excitation/emission = 490/590 and 490/530 nm (FL590/FL530) were recorded to reflect the ΔΨm level of each sample. As shown in Fig. 8, the ΔΨm levels of mutant cell lines harboring only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homoygous c.572G>T mutations were 97.5, 71.1, 67.7, and 64.1% of the mean values measured in the control cell lines, respectively. In contrast, the ΔΨm levels in mutant cell lines in the presence of FCCP were comparable with those measured in the control cell lines.

Increase of ROS production

Respiratory deficiency can increase the production of ROS (38, 39). In this study, we measured the levels of ROS generation in mutant and control cell lines with flow cytometry under normal and H2O2-stimulated conditions. To detect the capacity of reaction upon increasing levels of ROS under oxidative stress, we calculated the ratio of geometric mean intensity between unstimulated and stimulated with H2O2 in each cell line. As shown in Fig. 9, the levels of ROS generation in the mutant cell lines harboring only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homoygous c.572G>T mutations were 119.3, 109.6, 127.3, 140.6% of the mean values measured in the control cell lines, respectively. In the present study, we further investigated the molecular mechanism of the deafness-associated m.7511A>G mutation. Indeed, the occurrence of the m.7511A>G mutation in several hearing-impaired families from different ethnic backgrounds strongly indicated that this mutation is involved in the pathogenesis of deafness (26–30). The m.7511A>G mutation caused the substitution of the A4:U69 base-pairing with G4:U69 base-pairing at the aminoacyl acceptor stem of tRNASer(UCN) (12, 26, 27). In fact, this A4:U69 base-pairing may play an important role in the stability and identity of tRNA (12, 24, 25, 39–42).

Figure 5. Western blot analysis of 7 OXPHOS subunits. A, 5 μg of total mitochondrial proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted, and hybridized with an antibody mixture specific for subunits of each OXPHOS complex and with GAPDH as a loading control. Body mixture specific for subunits of each OXPHOS complex and with GAPDH was used to detect the capacity of oxidative phosphorylation, we measured the levels of cellular and mitochondrial ATP production using a luciferin/luciferase assay. Populations of cells from various mutant and control cell lines were incubated in the medium in the presence of glucose (total cellular ATP production) or 2-deoxy-D-glucose with pyruvate (mitochondrial ATP production). As shown in Fig. 6, the levels of mitochondrial ATP production in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homoygous c.572G>T mutations were 77.6, 58.9, 62.9, and 44.8% of the control cell lines. Moreover, the levels of total cellular ATP production in the above mutant cell lines were 97.5, 84.3, 79.5, and 67.1%, relative to the mean value measured in the control cell lines, respectively.

Reduced levels in mitochondrial ATP production

To examine the capacity of oxidative phosphorylation, we measured the levels of cellular and mitochondrial ATP production using a luciferin/luciferase assay. Populations of cells from various mutant and control cell lines were incubated in the medium in the presence of glucose (total cellular ATP production) or 2-deoxy-D-glucose with pyruvate (mitochondrial ATP production). As shown in Fig. 6, the levels of mitochondrial ATP production in mutant cell lines carrying only the c.572G>T mutation, the m.7511A>G mutation, or both the m.7511A>G and heterozygous or homoygous c.572G>T mutations were 77.6, 58.9, 62.9, and 44.8% of the control cell lines. Moreover, the levels of total cellular ATP production in the above mutant cell lines were 97.5, 84.3, 79.5, and 67.1%, relative to the mean value measured in the control cell lines, respectively.
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**A**

| m.7511T>C | YARS2 Gly191Val |
|-----------|-----------------|
| +         | +/-             |
| +         | +/-             |
| -         | +/-             |
| -         | +/-             |

Relative level of enzyme activity (%)

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

**B**

| m.7511T>C | YARS2 Gly191Val |
|-----------|-----------------|
| +         | +/-             |
| +         | +/-             |
| +         | +/-             |
| -         | +/-             |
| -         | +/-             |
| -         | +/-             |

OCR (pMoles/min)

- Oligomycin
- FCCP
- Antimycin/Rotenone

**C**

| m.7511T>C | YARS2 Gly191Val |
|-----------|-----------------|
| +         | +/-             |
| +         | +/-             |
| -         | +/-             |
| -         | +/-             |

OCR (pMoles/min)

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

(p<0.001)
Therefore, it was hypothesized that m.7511A>G mutation led to structural and functional consequences for tRNAser(UCN), including the processing of RNA precursors, stability, and aminoacylation of tRNAser(UCN). In particular, the substitution A4:U69 base-pairing with G4:U69 base-pairing caused by the m.7511A>G mutation may restrict the accessible conformation space of tRNAser(UCN) (43–45). Here, the altered structure of tRNAser(UCN) caused by the m.7511A>G mutation was evidenced by the increased melting temperature and electrophoretic mobility of mutated tRNA with respect to the WT molecule in vitro or ex vivo. The instability of mutant tRNA was further supported by marked reductions in the steady-state level of tRNAser(UCN) in the cybrid mutant cell lines (26) and lymphoblastoid cell lines carrying the m.7511A>G mutation in the present study.

Furthermore, the substitution A4:U69 base-pairing with G4:U69 base-pairing induced by the m.7511A>G mutation may result in the faulty interaction of tRNAser(UCN) with mitochondrial seryl-tRNA synthetase, thereby altering the aminoacylation properties of tRNAser(UCN) (39, 43–46). Indeed, all human AlaRS mischarged to noncognate tRNAs, such as tRNACys and tRNAAsp, with the G4:U69 bp (45, 46). Therefore, mutant tRNAser(UCN) with G4:U69 bp can be mischarged with other amino acids. In this study, the possible mischarging to noncognate tRNAs of mutant tRNAser(UCN) may account for the improperly aminoacylated tRNAser(UCN), as suggested by the aberrantly aminoacylated tRNAser(UCN) in the mutant cell lines and faster electrophoretic mobility of mutated tRNA with re-
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Figure 9. Measurement of ROS. Shown is the ratio of geometric mean intensity between the levels of ROS generation in the vital cells with or without 

By contrast, mutant cell lines harboring both the m.7511A>G and homozygous p.191Gly>Val mutations revealed 70% decreases in the level of tRNA_{Ser(UCN)} and 59% reductions in the level of tRNA_{Tyr} as well as various decreases in the levels of other tRNAs, including tRNA_{Glu}, tRNA_{Asp}, tRNA_{Met}, tRNA_{Lys}, and tRNA_{Leu(UUR)}.

The resultant mitochondrial dysfunctions would lead to the dysfunction or death of cochlear cells, thereby contributing to the development of hearing loss.

The YARS2 p.191Gly>Val mutation enhanced the phenotypic manifestation of the m.7511A>G mutation

Genetic modifiers involved in mitochondrial tRNA metabolism modulate the phenotypic manifestation of the deafness-associated 125 rRNA mutations (22, 23, 52). In this study, the penetrances of hearing loss in this Chinese family harboring both the m.7511A>G and YARS2 p.191Gly>Val mutations were significantly higher than those in the French and Japanese families carrying only the m.7511A>G mutation (29, 30). Furthermore, cell lines bearing both p.191Gly>Val and m.7511A>G mutations exhibited greater mitochondrial dysfunctions than those carrying only p.191Gly>Val or m.7511A>G mutation. Strikingly, mutant cell lines harboring both m.7511A>G and p.191Gly>Val mutations exhibited not only more decreases in the aminoacylation efficiencies of tRNA_{Ser(UCN)} and tRNA_{Tyr} but also deficient aminoacylation of tRNA_{Thr}, tRNA_{Lys}, tRNA_{Leu(UUR)}, and tRNA_{Ser(AGY)} as compared with those in the cell lines carrying only the p.191Gly>Val or m.7511A>G mutation. The aberrantly aminoacylated tRNA makes the mutant tRNA metabolically less stable and more subject to degradation, thereby lowering the level of the tRNA in mutant cell lines (17, 26, 40). In the present study, mutant cell lines bearing only the m.7511A>G mutation exhibited 48% reductions in the level of tRNA_{Ser(UCN)}, and mutant cell lines harboring only the p.191Gly>Val mutation displayed 33.7% decreases in the level of tRNA_Tyr, respectively. By contrast, mutant cell lines harboring both the m.7511A>G and homozgyous p.191Gly>Val mutations revealed 70% decreases in the level of tRNA_{Ser(UCN)} and 59% reductions in the level of tRNA_{Tyr} as well as various decreases in the levels of other tRNAs, including tRNA_{Glu}, tRNA_{Asp}, tRNA_{Met}, tRNA_{Lys}, and tRNA_{Leu(UUR)}.

Notably, ~70% reductions in the steady-state levels of tRNA_{Ser(UCN)} in the cells carrying both p.191Gly>Val and m.7511A>G mutations were in good agreement with the 75% decrease in the levels of tRNA_{Ser(UCN)} in those in the mutant cybrid cells bearing the m.7511A>G, tRNA_{Ala}5655A>G, and ND1 3308T>C mutations (26). These data strongly suggested that the synergic interaction between the YARS2 p.191Gly>Val and m.7511A>G mutations mediated mitochondrial tRNA metabolisms, especially exacerbating the defects of tRNA_{Ser(UCN)} and tRNA_{Tyr} metabolisms. Notably, mutations in the TRMU involved in biosynthesis of m^5s^2U at the wobble position of tRNA_{Glu}, tRNA_{Glu}, and tRNA_{Lys} affected the metabolism of not only tRNA_{Lys}, tRNA_{Glu}, tRNA_{Glu}, but also other mitochondrial tRNA (22, 53).

Both shortage of and aberrant aminoacylation of tRNAs led to impairments of mitochondrial translation. In this investigation, 50% decreases in the levels of mtDNA encoding proteins observed in the mutant cells carrying both the m.7511A>G and p.191Gly>Val mutations are below the proposed threshold level (50%) to produce a clinical phenotype associated with a mtDNA mutation (23, 47, 48, 54). The defects of mitochondrial translation were responsible for the respiratory deficiency, uncoupling of the oxidative pathway for ATP synthesis, diminished mitochondrial membrane potentials, and overproduction of ROS (7, 48, 52, 55). In particular, more drastic decreases of oxygen consumption rates, mitochondrial ATP production, and mitochondrial membrane potentials and increases of ROS production were observed in the cell lines carrying both the p.191Gly>Val and m.7511A>G mutations than those in cell lines carrying only the p.191Gly>Val or m.7511A>G mutation. These mitochondrial dysfunctions yielded a preferential effect on the hair cells and neurons in the cochlea, because cochlear functions depend on a very high rate of ATP production (56–58). This would result in the dysfunction or death of hair cells and neurons in the cochlea carrying both the p.191Gly>Val and m.7511A>G mutations, thereby producing a phenotype of hearing loss.

In summary, we demonstrated that the pathophysiology of maternally inherited deafness was manifested by aberrant
A nuclear modifier for deafness expression of tRNA mutation

tRNA metabolisms due to the combination of YARS2 p.191Gly>Val with tRNA<sup>Ser(UCN)</sup> m.7511A>G mutations. The m.7511A>G mutation altered both the structure and function of tRNA<sup>Ser(UCN)</sup>. The p.191Gly>Val mutation deteriorated the aberrant tRNA metabolisms associated with the m.7511A>G mutation. The aberrant tRNA metabolisms resulted in defective mitochondrial translation, respiratory deficiency, decreasing ATP production, and increasing ROS production. These biochemical defects led to the high penetrance and occurrence of deafness in the Chinese family carrying both the m.7511A>G and p.191Gly>Val mutations. Our findings provide new insights into the pathophysiology of maternally inherited deafness, manifested by the synergetic interaction between mitochondrial and nuclear gene products underlying aberrant tRNA metabolism.

Experimental procedures

Subjects

One Han Chinese family (WZD200), as shown in Fig. S1A, was recruited from the Otology Clinics of Wenzhou Medical University (Zhejiang, China), as described previously (28). Comprehensive history-taking, physical examination, and audiological examination were performed to identify any syndromic findings, history of exposure to aminoglycosides, and genetic factors related to hearing impairment in all available members of this Chinese pedigree, as detailed previously (59, 60). The 574 control subjects were from a panel of unaffected subjects of Han Chinese ancestry from the same region. This study followed the principles of the Declaration of Helsinki. Informed consent was obtained from the participants prior to their participation in the study, under protocols approved by the Ethics Committees of Zhejiang University and the Wenzhou Medical University.

Mitochondrial DNA-sequencing analysis

Genomic DNA was isolated from whole blood of participants using the QIAamp DNA Blood Mini Kit (Qiagen, catalog no. 51104). The entire mtDNAs of the family members of WZD200 (I-1, I-2, II-2, III-5, and III-7) and one Chinese control subject (A61) were PCR-amplified in 24 overlapping fragments using sets of the light (L) and heavy (H) strand oligonucleotide primers, as described previously (61). These sequence results were compared with the updated consensus Cambridge sequence (GenBank<sup>TM</sup> accession number NC_012920) (6). For the analysis for the presence and level of the m.7511A>G mutation, the PCR DNA fragments (117 bp) spanning the tRNA<sup>Ser(UCN)</sup> gene were amplified using genomic DNA as the template and the oligodeoxynucleotides 5’-CCCCATGGGCTCATGTTT-TAAAA-3’ and 5’-TACCTGCGCTGATGCCCCATTAA-GAT-3’. The resultant 117-bp segments were digested with the restriction enzyme DraI and analyzed by electrophoresis through a 14% polyacrylamide gel. After ethidium bromide staining, the ImageQuant program was used to determine the proportions of digested and undigested PCR product to ascertain whether the m.7511A>G mutation was present in homoplasm in these subjects (Fig. S1B).

Target exome sequencing

A panel of exome sequencings (genes encoding 20 mitochondrial tRNA synthetases and 25 tRNA-modifying enzymes, Table S1) of seven matrilineal relatives (II-5, II-7, III-3, III-4, III-5, III-6, and III-7) carrying the m.7511A>G mutation and two married-in controls (II-4 and II-6) of WZD200 pedigree were performed by BGI (Shenzhen, China). High-quality genomic DNA (3 μg) was captured by hybridization using the SureSelect XT Human All Exon 50Mb kit (Agilent Technologies). Samples were prepared according to the manufacturer’s instructions. Each captured library was run on a HiSeq 2000 instrument, and sequences were generated as 90-bp pair-end reads. An average of 82 million paired reads were generated per sample, the mean duplication rate was 6.37%, and 98% of the targeted region was covered by at least 50 × mean depth. All sequencing reads were mapped to the human reference genome (GRCh37) at UCSI. The software SOAPsnp was used to assemble the consensus sequence and call genotypes in target regions. GATK (Indel Genotyper version 1.0) was used for indel detection. The threshold for filtering SNPs included the following criteria. SNP quality score should be ≥20; sequencing depth should be between 4 and 200; estimated copy number should be no more than 2; and the distance between two SNPs should be larger than 5.

Mutation analysis of YARS2 gene

Five pairs of primers for PCR-amplifying exons and their flanking sequences, including splicing-donor and acceptor-consensus sequences of YARS2, were used for this analysis, as described previously (32). Fragments spanning five exons and flanking sequences from seven matrilineal relatives (II-5, II-7, III-3, III-4, III-5, III-6, and III-7) and three married-in controls (I-1, II-4, and II-6) carrying the m.7511A>G mutation in the Chinese family and two genetically unrelated Chinese controls were PCR-amplified, purified, and subsequently analyzed by Sanger sequencing. These sequence results were compared with the YARS2 genomic sequence (RefSeq NC_000012.12). Genotyping for the c.572G>T mutation in other subjects was PCR-amplified for exon 1 and followed by digestion of the 626-bp segment with the restriction enzyme Tsp45I. The forward and reverse primers for exon 1 are 5’-GACTCGCTT-CATGTTGGTCAAT-3’ and 5’-CGAGGCGCAGCAACT-ACAATC-3’, respectively. The Tsp45I-digested products were analyzed on 10% polyacrylamide gel (Fig. S1C).

Cell lines and culture conditions

Lymphoblastoid cell lines were immortalized by transformation with the Epstein–Barr virus, as described elsewhere (62). Cell lines derived from five members of the Chinese family (hearing-impaired subjects II-2 and III-4 harboring both m.7511A>G and heterozygous c.572G>T mutations, II-1 and II-5 carrying both m.7511A>G and homozygous c.572G>T mutations, a hearing-normal individual (I-1) bearing only the homozygous c.572G>T mutation, and one hearing-normal subject (III-7) carrying only the m.7511A>G mutation) and two genetically unrelated control individuals (A61 and A62) lacking these mutations (Table S3) were grown in the RPMI.
UV melting assays

UV melting assays were carried out as described previously (50, 63). The WT and mutant tRNA\(^{\text{Ser(UCN)}}\) transcripts were generated as detailed elsewhere (64). The transcripts were diluted in buffer including 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, 5 mM MgCl\(_2\), and 0.1 mM EDTA. Absorbance against temperature melting curves were measured at 260 nm with a heating rate of 0.5 °C/min from 25 to 95 °C through an Agilent Cary 100 UV spectrophotometer.

Mitochondrial tRNA analysis

Total mitochondrial RNAs were obtained from mitochondria isolated from lymphoblastoid cell lines (∼2.0 × 10⁸ cells), as described previously (65). The tRNA Northern blot analysis was performed as detailed elsewhere (63). Oligodeoxynucleotide probes for tRNA\(^{\text{Ser(UCN)}}\), tRNA\(^{\text{Tyr}}\), tRNA\(^{\text{Lys}}\), tRNA\(^{\text{Met}}\), tRNA\(^{\text{Leu(UUR)}}\), tRNA\(^{\text{Leu(CUN)}}\), tRNA\(^{\text{Amp}}\), tRNA\(^{\text{Glu}}\), and 55 rRNA were as detailed elsewhere (55). The hybridization and quantification of density in each band were performed as detailed previously (63).

The aminoacylation assays were carried out as detailed previously (63, 66). To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, total RNAs were treated by heat shock for 10 min at 60 °C at pH 9.0 and then run in parallel (63, 66). DIG-labeled oligodeoxynucleotide probes for tRNA\(^{\text{Ser(UCN)}}\), tRNA\(^{\text{Tyr}}\), tRNA\(^{\text{Ser(AGY)}}\), tRNA\(^{\text{Leu(UUR)}}\), tRNA\(^{\text{Amp}}\), and tRNA\(^{\text{Thr}}\) were as described above. Quantification of density in each band was performed as detailed previously (63, 66).

For the tRNA mobility shift assay, 2 µg of total mitochondrial RNAs were electrophoresed through a 10% polyacrylamide native gel at room temperature in 50 mM Tris-glycine buffer. After electrophoresis, the gels were treated according to the procedure for the tRNA Northern blot analysis described above.

Western blot analysis

Western blot analysis was performed as detailed previously (17, 32). The antibodies used for this investigation were from Abcam (GAPDH (ab8245), ND1 (ab74257), ND5 (ab92624), ND6 (ab81212), CO1 (ab14705), ATP6 (ab101908), NDUF53 (ab14711), and total OXPHOS human WB antibody mixture (ab110411)), Novus (ND4 (NB2-47365)), and Proteintech (VDAC (10866-1-AP), CYTB (55090-1-AP), and COX10 (10611-2-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 (CWBIIO). Quantification of density in each band was performed as detailed previously (17, 32).

Assays of activities of respiratory complexes

The enzymatic activities of complex I, II, III, and IV were assayed as detailed elsewhere (33, 67, 68). Briefly, complex I (NADH ubiquinone oxidoreductase) activity was determined by following the oxidation of NADH with ubiquinone as the electron acceptor. 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).

Measurements of oxygen consumption

OCR in lymphoblastoid cell lines were measured with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously (17, 35, 36).

ATP measurements

The Cell Titer-Glo® luminescent cell viability assay kit (Promega) was used for the measurement of cellular and mitochondrial ATP levels, according to the modified manufacturer’s instructions (17, 68).

Assessment of mitochondrial membrane potential

The JC-10 Assay Kit-Microplate (Abcam) was used to assess the mitochondrial membrane potential, according to a modification of the manufacturer’s instructions (37).

Measurement of ROS production

ROS measurements were performed following the procedures detailed previously (40, 50, 69).

Computer analysis

Statistical analysis was performed using the unpaired, two-tailed Student’s test contained in the Microsoft Excel program (version 2017). Differences were considered significant at \( p < 0.05 \).

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