Biochemical Evidence for a Nuclear Modifier Allele (A10S) in TRMU (Methylaminomethyl-2-thiouridylate-methyltransferase) Related to Mitochondrial tRNA Modification in the Phenotypic Manifestation of Deafness-associated 12S rRNA Mutation

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Nuclear modifier gene(s) was proposed to modulate the phenotypic expression of mitochondrial DNA mutation(s). Our previous investigations revealed that a nuclear modifier allele (A10S) in TRMU (methylaminomethyl-2-thiouridylate-methyltransferase) related to tRNA modification interacts with 12S rRNA 1555A→G mutation to cause deafness. The A10S mutation resided at a highly conserved residue of the N-terminal sequence. It was hypothesized that the A10S mutation altered the structure and function of TRMU, thereby causing mitochondrial dysfunction. Using molecular dynamics simulations, we showed that the A10S mutation introduced the Ser10 dynamic electrostatic interaction with the Lys106 residue of helix 4 within the catalytic domain of TRMU. The Western blotting analysis displayed the reduced levels of TRMU in mutant cells carrying the A10S mutation. The thermal shift assay revealed the $T_m$ value of mutant TRMU protein, lower than that of the wild-type counterpart. The A10S mutation caused marked decreases in 2-thiouridine modification of U34 of tRNA\textsuperscript{Lys}, tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln}. However, the A10S mutation mildly increased the aminocytlated efficiency of tRNAs. The altered 2-thiouridine modification worsened the impairment of mitochondrial translation associated with the m.1555A→G mutation. The defective translation resulted in the reduced activities of mitochondrial respiration chains. The respiratory deficiency caused the reduction of mitochondrial ATP production and elevated the production of reactive oxidative species. As a result, mutated TRMU worsened mitochondrial dysfunctions associated with m.1555A→G mutation, exceeding the threshold for expressing a deafness phenotype. Our findings provided new insights into the pathophysiology of maternally inherited deafness that was manifested by interaction between mtDNA mutation and nuclear modifier gene.

The impairments in mitochondrial protein synthesis 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) (1–5). Human mitochondrial translation machinery composed of 2 rRNAs and 22 tRNAs, encoded by mitochondrial DNA (mtDNA),\textsuperscript{4} and more than 150 proteins (ribosomal proteins, ribosomal assembly proteins, aminoacyl-tRNA synthetases, tRNA-modifying enzymes, tRNA methylating enzymes, and initiation, elongation, and termination factors), encoded by nuclear genes and imported into mitochondrion (6, 7). Mutations in \textit{LARS2}, \textit{NARS2}, and \textit{KARS} encoding mitochondrial leucyl-tRNA synthetase, asparaginyl-tRNA synthetase, and lysyl-tRNA synthetase have been associated with deafness, respectively (8–10). The mitochondrial tRNA genes are the hot spots for deafness-associ-
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ated mutations, including the tRNA_{Leu(UUR)} 3243A→G, tRNA_{Ser(UCN)} 7445A→G, 7511T→C, tRNA_{Glu} 12201T→C, tRNA_{Asp} 7551A→G, and tRNA_{Glu} 14692A→G mutations (11–17). 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 (3, 4, 18–20). The m.1555A→G or m.1494C→T mutation is the primary causative evident, but modifier factors including aminoglycosides or nuclear modifier genes are required for the phenotypic manifestation of these mtDNA mutations (21–26). However, the role of these nuclear modifier genes remains poorly understood.

In the previous investigations, we showed that MTO1, MISS1 (GTPBP3), or MT22 (TRML2) genes involved in the biosynthesis of the hypermodified nucleoside 5-methylaminomethyl-2-thio-uridine of several mitochondrial tRNAs were the potential modifier genes for the phenotypic expression of deafness-associated 12S rRNA 1555A→G mutation (25–30). These modified uridines at the wobble positions of tRNA^{A15}, tRNA^{Glu}, and tRNA^{Gln} have a pivotal role in the structure and function of tRNAs, including structural stabilization, aminoacylation, and codon recognition at the decoding site of small rRNA (31–33). In particular, TRMU is the tRNA 5-methylaminomethyl-2-thio-uridylic methyltransferase responsible for the 2-thiolation of tRNA^{Lys}, tRNA^{Glu}, and tRNA^{Gln} with mm^5s^2U in bacteria, mm^5s^2U_{34} in yeast, and m^5s^2U_{34} in human mitochondria (34–37). Using TRMU as a candidate gene for genotyping analysis, in combination with functional assays, we identified a nuclear modifier allele (G28T and A10S) in the TRMU gene, which interacts with the m.1555A→G mutation to cause deafness (25). The A10S mutation resided at a highly conserved residue of the N-terminal sequence of this polypeptide but did not affect importation of TRMU precursors into mitochondria (25). Therefore, the homozygous A10S mutation caused marked decreases in the steady state levels of mitochondrial tRNAs (25). Therefore, it was anticipated that the A10S mutation affected the structure and function of TRMU, thereby altering the mitochondrial function. The effect of A10S mutation on the stability of TRMU was assessed by the Western blotting and thermal shift assays. The primary defects by the mutation appeared to be the altered biosynthesis of 5-taurinomethyl-2-thioruridine (mm^5s^2U) nucleotides at the wobble position of mitochondrial tRNA^{Gln}, tRNA^{Glu}, and tRNA^{Lys} (25, 34, 38). Furthermore, the deficient synthesis of s^2U_{34} may alter the tRNA aminoacylation, because s^2U_{34} serves as a determinant for tRNA recognition by cognate aminoacyl-tRNA synthetases in bacteria (39). To further investigate the effect of the TRMU A10S mutation on mitochondrial function, we examined for the levels of tRNA modification, aminoacylation of tRNAs, translation, the rates of respiration, and the production of ATP and reactive oxygen species (ROS), through use of lymphoblastoid mutant cell lines derived from Arab-Israeli control subjects and from members of an Arab-Israeli family (two subjects carrying only m.1555A→G mutation (F12H and F6D), two individuals (F20C and F8A) harboring both m.1555A→G and heterozygous TRMU A10S mutations, and two individuals (F20A and F20D) carrying both m.1555A→G and homozygous TRMU A10S mutations) (25, 40).

Results

MD Simulation Analyses—We performed the molecular dynamics simulation to examine whether the A10S mutation alters the structure of TRMU (41). This method has been widely used for evaluating structural impact of disease-causing mutations (42). Based on the rational initial structure (43), both wild-type and mutated TRMU were evaluated by 300-ns all-atom molecular dynamics simulations. As shown in Fig. 1A, the A10S mutation did not affect the local structure around residue 10 and the overall structure of the TRMU protein. As shown in Fig. 1B, root mean square deviation curve of the mutated protein fluctuated more heavily than that of the wild-type protein, suggesting that the mutated protein exhibited unstable than its wild-type counterpart. Furthermore, we carried out root mean square fluctuation (RMSF) analysis on the two trajectories to analyze the mobility of different regions of the protein. As shown in Fig. 1C, the RMSF around the residue 10 in the two trajectories exhibited almost same value, indicating that the A10S mutation does not affect the stability of this region. However, the RMSF values of the helix 4 (residue 98–118) in mutated protein were much higher than that of the wild-type form (Fig. 1C), suggesting that the helix 4 in mutant protein was unstable than that of wild-type protein. Both the residue 10 and the helix 4 belong to the N-terminal catalytic domain, which is located on the opposite sides within the ATP binding pocket of the protein (43). In addition, it was anticipated that the A10S mutation could introduce new interactions between the residue 10 and helix 4, which may account for the less stability of the helix 4. As shown in Fig. 1D, the hydroxyl group of Ser^{10} in the mutant trajectory could form the dynamic electrostatic interaction with the Lys^{106} residue of helix 4, whereas no interaction occurred between Ala^{10} and helix 4 in the wild-type trajectory. Thus, the electrostatic attraction between Ser^{10} and Lys^{106} is quite dynamic, with H-bond occupany of only around 1%, so this dynamic interaction caused the instability to the protein but did not affect its overall 3D structure.

The A10S Mutation Caused the Instability of TRMU—To experimentally test the predicted effect of A10S mutation for TRMU, we analyzed the levels of TRMU by Western blotting in these mutant cell lines carrying only m.1555A→G mutation, both m.1555A→G and heterozygous or homozygous A10S mutations and two control cell lines. These blots were then hybridized with other nuclear encoding mitochondrial proteins MTO1 and NDUFB8, as well as VADC as a loading control. As shown in Fig. 2, the levels of TRMU in mutant cell lines carrying only m.1555A→G, both m.1555A→G and heterozygous or homozygous A10S mutations were 94.1, 66.3, and 51.9%, relative to the average values of control cell lines, respectively. By contrast, the levels of MTO1 and NDUFB8 in mutant cell lines were comparable with those in control cell lines. These results strongly supported the deleterious effect of A10S mutation on TRMU structure.

Analysis of TRMU Stability Using Differential Scanning Fluorimetry (DSF)—The thermal stability of mutant TRMU was also assessed using the DSF, a fluorescence method that was used to monitor solution phase protein stability (44). The technique
involves subjecting a protein to heat denaturation under continuous fluorescence monitoring in the presence of the environmental sensitive fluorescent dye (Thermal-shift dye). We used DSF to determine the melting temperature ($T_m$) of TRMU, the temperature at which the concentration of folded protein is equivalent to unfolded protein. The fluorescence changes of the dye orange occurred in the presence of 1/9262 wild-type and mutated TRMU over a temperature range from 25 to 95 °C. The thermal stability of mutant protein was compared with that of wild-type protein. As shown in Fig. 3, the $T_m$ value of wild-type TRMU was 49.1 °C, whereas the $T_m$ value of mutant TRMU was 45.9 °C. The lower thermal stability of mutant TRMU protein than wild-type protein further supported that the A10S mutation led to the instability of TRMU protein.

Decreases in the Thiolation of tRNAGln, tRNAGlu, and tRNALys—To further investigate whether the TRMU A10S mutation affected the 2-thio-uridine modification at position 34 in tRNAs, the 2-thiouridylation levels of tRNAs were determined by isolating total mitochondrial RNAs from eight lymphoblastoid cell lines, purifying tRNAs, qualifying the 2-thiouridine modification by the retardation of electrophoresis mobility in polyacrylamide gel containing 0.05 mg/ml (N-acryloylamino)phenyl) mercuric chloride (APM) (45–47), and hybridizing digoxigenin (DIG)-labeled probes for tRNAGlu, tRNAGlu, and tRNALys. In this system, the mercuric compound can specifically interact with the tRNAs containing the thiocarbonyl group-such as tRNAGln, tRNAGlu, and tRNALys, thereby retarding tRNA migration. As shown in Fig. 4, the 2-thiouridylation levels of tRNAGln, tRNAGlu, and tRNALys were reduced significantly in mutant cells carrying the homozygous TRMU A10S mutation, compared with control cells. In particular, the 2-thiouridylation levels of tRNAGln, tRNAGlu, and tRNALys in mutant cells carrying both homozygous A10S and m.1555A→G mutations were 52, 62, and 50%, relative to those of control cell lines, respectively. Furthermore, the 2-thiouridylation of tRNAGln, tRNAGlu, and tRNALys in mutant cells carrying both heterozygous A10S and m.1555A→G mutations were 76, 74, and 77%, relative to those of control cells, respectively. However, the levels of 2-thiouridylation of tRNAGln, tRNAGlu, and tRNALys in mutant cell lines carrying only m.1555A→G mutation were comparable with those in control cell lines.
Analysis of Aminoacylation of tRNAs—We tested whether the deficient thio-uridylation of tRNALys, tRNAGlu, and tRNA-Gln caused by the TRMU A10S mutation affects the aminoacylation of above tRNA as well as other tRNA. Indeed, our previous investigation also showed that the TRMU A10S mutation caused the reductions in the steady state levels of other tRNAs (25). The aminoacylation capacities of tRNALys, tRNATyr, tRNALeu(CUN), and tRNASer(AGY) in control and mutant cell lines were examined by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA, electroblotting, and hybridizing with above tRNA probes (15, 48, 49). As shown in Fig. 5, the upper band represented the charged tRNA, and the lower band was uncharged tRNA. Electrophoretic patterns showed that either charged or uncharged tRNASer(AGY) in all mutant cell lines migrated slower than control cell lines. The conformation change may be due to the presence of the m.12236G→A mutation in the tRNASer(AGY) gene in the mutant cell lines (18) (supplemental Table S1). However, there were no obvious differences in electrophoretic mobility of tRNALys, tRNATyr, and tRNALeu(CUN) between mutant and control cell lines. Notably, the efficiencies of aminoacylated tRNALys in cell lines carrying only m.1555A→G mutation, both m.1555A→G and heterozygous or homozygous TRMU A10S mutations were 103, 137, and 137% of those in control cell lines. Moreover, the efficiencies of aminoacylated tRNALeu(CUN), and tRNASer(AGY) in control and mutant cell lines were examined by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA, electroblotting, and hybridizing with above tRNA probes (15, 48, 49). As shown in Fig. 5, the upper band represented the charged tRNA, and the lower band was uncharged tRNA. Electrophoretic patterns showed that either charged or uncharged tRNA in all mutant cell lines migrated slower than control cell lines. The conformation change may be due to the presence of the m.12236G→A mutation in the tRNA in the mutant cell lines (18) (supplemental Table S1). However, there were no obvious differences in electrophoretic mobility of tRNA in cell lines carrying only m.1555A→G mutation, both m.1555A→G and heterozygous or homozygous TRMU A10S mutations were 103, 137, and 137% of those in control cell lines. Moreover, the efficiencies of aminoacylated tRNA in cell lines harboring homozygous (→−), heterozygous (+/−), or wild-type (+/+), TRMU mutations are indicated. Cell lines carrying the m.1555A→G (−) or wild type (+) are indicated.

FIGURE 2. The A10S mutation caused the reduced levels of TRMU. A, scheme for the multiple sequence alignment of the TRMU homologues. The position of A10S mutation is marked with an arrow. B, Western blotting analysis of six mutant and two control cell lines. 20 μg of total cellular proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with TRMU, MTO1, and NDUFB8, respectively, and with VDAC as a loading control. Quantifications of TRMU levels were determined as described elsewhere (15). The values for the mutant cell lines are expressed as percentages of the average values for the control cell lines. Cell lines harboring homozygous (→−), heterozygous (+/−), or wild-type (+/+), TRMU mutations are indicated. Cell lines carrying the m.1555A→G (−) or wild type (+) are indicated.

FIGURE 3. Thermal stability of wild-type and mutant TRMU. The thermal denaturation was induced heating wild-type (solid line) and mutant (dashed line) TRMU proteins from 25 to 95 °C. Relative fluorescence curves were generated with the equation \( (F_T - F_{\text{min}}) / (F_{\text{max}} - F_{\text{min}}) \), where \( F_T \) indicates fluorescence at temperature \( T \), \( F_{\text{min}} \) indicates the minimum fluorescence, and \( F_{\text{max}} \) indicates the maximum fluorescence. \( \Delta T_m \) indicates the difference of \( T_m \) value between wild-type and mutant TRMU. The calculations were based on three to four determinations.

Analysis of Aminoacylation of tRNAs—We tested whether the deficient thio-uridylation of tRNALys, tRNAGlu, and tRNA-Gln caused by the TRMU A10S mutation affects the aminoacylation of above tRNA as well as other tRNA. Indeed, our previous investigation also showed that the TRMU A10S mutation caused the reductions in the steady state levels of other tRNAs (25). The aminoacylation capacities of tRNALys, tRNATyr, tRNALeu(CUN), and tRNASer(AGY) in control and mutant cell lines were examined by the use of electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged tRNA species from the corresponding charged tRNA, electroblotting, and hybridizing with above tRNA probes (15, 48, 49). As shown in Fig. 5, the upper band represented the charged tRNA, and the lower band was uncharged tRNA. Electrophoretic patterns showed that either charged or uncharged tRNA in all mutant cell lines migrated slower than control cell lines. The conformation change may be due to the presence of the m.12236G→A mutation in the tRNASer(AGY) gene in the mutant cell lines (18) (supplemental Table S1). However, there were no obvious differences in electrophoretic mobility of tRNA in cell lines carrying only m.1555A→G mutation, both m.1555A→G and heterozygous or homozygous TRMU A10S mutations were 103, 137, and 137% of those in control cell lines. Moreover, the efficiencies of aminoacylated tRNA in cell lines harboring homozygous (→−), heterozygous (+/−), or wild-type (+/+), TRMU mutations are indicated. Cell lines carrying the m.1555A→G (−) or wild type (+) are indicated.
Reductions in the Level of Mitochondrial Proteins—To further determine whether the TRMU A10S mutation alters mitochondrial translation, the Western blotting analysis was carried out to examine the levels of seven mtDNA encoding polypeptides in mutant and control cells with VDAC as a loading control. As shown in Fig. 6A, the levels of CO2 (subunit II of cytochrome c oxidase); ND1, ND4, ND5, and ND6 (subunits 1, 4, 5, and 6 of NADH dehydrogenase); A6 (subunit 6 of the H+/H11001-ATPase); and CYTB (apocytochrome b) were decreased in mutant cell lines, as compared with those control cell lines. As shown in Fig. 6B, the overall levels of seven mitochondrial translation products in mutant cell lines carrying both m.1555A>G and homozygous TRMU A10S mutations were 42%, relative to the mean value measured in the control cell lines. Notably, the average levels of ND1, ND4, ND5, ND6, CO2, A6, and CYTB in these mutant cells carrying both m.1555A>G and homozygous TRMU A10S mutations were 42%, 40%, 53%, 24%, 54%, 51%, and 36%, respectively, and the overall levels of seven mitochondrial proteins in cell lines carrying both m.1555A>G and heterozygous TRMU A10S mutations were 55%, relative to those in controls. Moreover, the overall levels of seven mitochondrial translation products in mutant cell line carrying only m.1555A>G mutation were 73% of control cell lines. However, the levels of synthesis of polypeptides in mutants relative to that in controls did not correlate with either the number of codons or proportion of glutamic acid, glutamine, and lysine residues (supplemental Table S2).

Respiration Defects—To evaluate whether the TRMU A10S mutation affects cellular bioenergetics, we examined the oxygen consumption rates (OCRs) of mutant and control cell lines (50). As shown in Fig. 7, the average basal OCRs in mutant cell lines carrying both m.1555A>G and homozygous or heterozygous TRMU A10S mutations were 54, 76, and 88%, 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
FIGURE 5. **In vivo aminoacylation assays.** A, 2 μg of total mitochondrial RNAs purified from eight cell lines under acid conditions were electrophoresed at 4 °C through an acid (pH 5.2) 10% polyacrylamide with 7 M urea gel, electroblotted, and hybridized with a DIG-labeled oligonucleotide probe-specific for the tRNALys, tRNATyr, tRNALeu(CUN), and tRNAser(AGY), respectively. B, in vivo aminoacylated proportions of tRNALys, tRNATyr, tRNALeu(CUN), and tRNAser(AGY) in the mutant and controls. The calculations were based on three independent determinations. Graph details and symbols are explained in the legend to Fig. 4.

FIGURE 6. **Western blotting analysis of mitochondrial proteins.** A, 20 μg of total cellular proteins from lymphoblastoid cell lines were electrophoresed through a SDS-polyacrylamide gel, electroblotted, and hybridized with seven respiratory complex subunits in mutant and control cells with VADC as a loading control. CO2, subunit II of cytochrome c oxidase; ND1, ND4, ND5, and ND6, subunits 1, 4, 5, and 6 of the reduced nicotinamide-adenine dinucleotide dehydrogenase; A6, subunit 6 of the H+–ATPase; and CYTB, apocytochrome b. B, quantification of mitochondrial protein levels. Average content of CO2, ND1, ND4, ND5, ND6, A6, and CYTB per cell, normalized to the average content of VADC per cell in mutant cell lines and controls. The values for the mutant cell lines are expressed as percentages of the average values for the control cell lines. The horizontal dashed lines represent the average value for each group. The calculations were based on three independent determinations. Graph details and symbols are explained in the legend to Fig. 4.
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 Fig. 6, the ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and non-mitochondrial OCR were 51, 53, 53, 63, and 67% in the mutant cell lines carrying both m.1555A>G and homozygous TRMU A10S mutations and 68, 78, 78, 85, and 84% in the mutant cell lines carrying both m.1555A>G and heterozygous TRMU A10S mutations relative to the control cell lines, respectively. Moreover, the above five kinds of OCR levels in mutant cell line carrying only m.1555A>G mutation were 86, 92, 89, 91, and 88% of control cell lines.

Reduced Levels in Mitochondrial ATP Production—The capacity of oxidative phosphorylation in mutant and wild-type cells was examined by measuring 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 (15). As shown in Fig. 8, 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 homozygous or heterozygous TRMU A10S mutations and only m.1555A>G mutation were 93, 95, and 85%, 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 Fig. 8B, the levels of ATP production in mutant cell lines carrying both m.1555A>G and homozygous or heterozygous TRMU A10S mutations and only m.1555A>G mutation were 48, 61, and 83% of the mean value measured in the control cell lines, respectively.

The Increase of ROS Production—It was anticipated that respiration defects increase the production of ROS. The levels of
the ROS generation in the vital cells derived from six mutant cell carrying the m.1555A→G mutation with or without TRMU A10S mutation and two control cell lines lacking both mutations were measured with flow cytometry under normal and H2O2 stimulation (51, 52). Geometric mean intensity was recorded to measure the rate of ROS of each sample. The ratio of geometric mean intensity between unstimulated and stimulated with H2O2 in each cell line was calculated to delineate the reaction upon increasing level of ROS under oxidative stress. As shown in Fig. 9, the levels of ROS generation in the mutant cell lines cells carrying both m.1555A→G and homozygous TRMU A10S mutations ranged from 155 to 167%, with an average 161% of the mean value measured in the control cell lines. Moreover, ROS generation levels of cell lines carrying both m.1555A→G and heterozygous TRMU A10S mutations and mutant cells carrying only m.1555A→G mutation were 132 and 120% of controls, respectively.

Discussion

The nuclear modifier genes were proposed to modulate the phenotypic manifestation of deafness-associated 12S rRNA mutations (3, 4, 53). In the present study, we further characterized the nuclear modifier allele (A10S) in the TRMU, which interacts with m.1555A→G mutation to cause deafness. Human TRMU encodes a highly conserved 5-methylamino-

methyl-2-thiouridylate-methyltransferase responsible for the biosynthesis of 5-taurinomethyl-2-thiouridine (m5s2U) nucleo-

tides at the wobble position of mitochondrial tRNA\[^{Glu}\],
tRNA\[^{Glu}\], and tRNA\[^{Lys}\] (30, 31). The highly conserved Ala\(^{10}\) residue locates at the N-terminal region of this polypeptide. In all available TRMU/MnmA protein sequences, residue 10 is highly conserved, either Ala or Gly. Based on our MD simulation results, either Ala\(^{10}\) or Gly\(^{10}\) does not interact with helix 4 of the protein (43). The change of alanine 10 residue with serine introduces the Ser\(^{10}\) dynamic electrostatic interaction with the Lys\(^{106}\) residue within the catalytic domain of TRMU. Thus, it was hypothesized that the A10S mutation altered the stability and catalytic activity of TRMU. A Western blotting analysis showed markedly reduced levels of TRMU in cell lines carrying the A10S mutation. Furthermore, the thermal shift assay revealed that the Tm value of mutant TRMU protein was lower than those of wild-type TRMU. These data are strong evidences that A10S mutation caused the instability of TRMU.

The primary defect in the A10S mutation was the deficient 2-thiouridine modification of \(U_{34}\) of tRNA\[^{Lys}\], tRNA\[^{Glu}\], and tRNA\[^{Lys}\]. In the present study, 48, 38, and 50% decreases in 2-thiouridine modification of \(U_{34}\) of tRNA\[^{Lys}\], tRNA\[^{Glu}\], and tRNA\[^{Glu}\] were observed in mutant cell lines carrying both m.1555A→G and homozygous A10S mutations, as compared with controls. These results were consistent with the fact that the small interfering RNA down-regulation of or other mutations of TRMU led to the defects in 2-thiouridylation in mitochondrial tRNA\[^{Lys}\], tRNA\[^{Glu}\], and tRNA\[^{Glu}\] (34, 54, 55). Furthermore, in vitro assays showed that the deficient synthesis of s\(^2\)U\(^{34}\) altered the tRNA aminoacylation, because s\(^2\)U\(^{34}\) serves as a determinant for tRNA recognition by cognate aminoacyltRNA synthetases in bacteria (39, 56). However, in vivo assays revealed that the lack of 5-methyl-aminomethyl group did not affect the charging levels for tRNA\[^{Lys}\], tRNA\[^{Glu}\], and tRNA\[^{Glu}\] in bacteria (56). In the present study, the efficiencies of aminoacylated tRNA\[^{Lys}\], tRNA\[^{Leu(CUN)}\], and tRNA\[^{Ser(AGY)}\] in cell lines carrying both m.1555A→G and homozygous TRMU A10S mutations were 137, 135, and 129% of those in control cell lines. These data suggested that the TRMU A10S mutation may increase the charging levels for tRNA\[^{Lys}\], tRNA\[^{Leu(CUN)}\], and tRNA\[^{Ser(AGY)}\]. An increase in aminoacylation of tRNAs in mutant cell lines may be due to the instability of the mutant tRNA, where aminoacylation may provide some levels of stabilization by compensatory effect (15, 57, 58). Alternatively, these tRNAs may be mischarged with a noncognate amino acids, because anticodon modifications act as antideterminants (59). Therefore, an inefficient modification of tRNAs caused by the TRMU mutations may then make these tRNAs to be metabolically less stable and more subject to degradation, thereby lowering the level of the tRNAs (25, 35, 54, 55).

A failure in the tRNA metabolism caused by the TRMU A10S mutation should be responsible for the impairment of mitochondrial translation. In particular, the mischarged tRNAs may cause the global protein misfolding (32, 59). 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, and CO3 of complex IV; and ATP6 and ATP8 of complex V) (6, 60). In the present study, 58, 45, and 27% reductions in the levels of mitochondrial proteins were observed in mutant cell lines carrying both m.1555A→G and homozygous or heterozygous TRMU A10S mutations and only m.1555A→G mutation, respectively. These results were comparable with the in vivo pulse-labeling mitochondrial protein synthesis assay (21, 25). Notably, variable decreases in the levels of seven mtDNA-encoded polypeptides were observed in mutant cell lines. In particular, cell lines carrying both
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m.1555A→G and homozygous TRMU A10S mutations exhibited marked reductions (from 46 to 76%) in the levels of seven polypeptides. However, the levels of synthesis of polypeptides in mutants relative to that in controls did not correlate with either the number of codons or proportion of glutamic acid, glutamine, and lysine residues. These data were not fully comparable with the case of MERRF-associated m.8344A→G mutation in tRNA^Glx gene (61). The impairment of mitochondrial protein synthesis was apparently responsible for the reduced rates in the basal OCR or ATP-linked OCR reserve capacity and maximal OCR among the control and mutant cell lines. In particular, the 12, 34, and 46% decreases in basal OCR were observed in cell lines harboring only m.1555A→G mutation, both m.1555A→G and heterozygous or homozygous TRMU A10S mutations, respectively. This correlation is clearly consistent with the importance that a failure in tRNA metabolism plays a critical role in producing their respiration defects in deafness patients carrying the m.1555A→G mutation.

The respiratory deficiency then affects the efficiency of mitochondrial ATP synthesis. In this investigation, the m.1555A→G mutation caused 17% reduction of mitochondrial ATP production in lymphoblastoid cell lines, as in the cases of cell lines bearing the LHON-associated m.11778G→A mutations (62, 63). By contrast, the ~52% drop in mitochondrial ATP production in lymphoblastoid cell lines bearing both m.1555A→G and homozygous TRMU A10S mutations may result from the defective activities of respirations caused by both m.1555A→G mutation and altered tRNA metabolism associated with TRMU A10S mutation. These data are consistent with the fact that 20 and 51% reductions in mitochondrial ATP production were observed in lymphoblastoid cell lines carrying the only m.11778G→A and both m.11778G→A and homozygous YARS2 p.191Gly→Val mutations (62). Alternatively, the reduction in mitochondrial ATP production in mutant cells was likely a consequence of the decrease in the proton electrochemical potential gradient of mutant mitochondria (64). As a result, the hair cells carrying the mtDNA mutation may be particularly sensitive to increased ATP demand (3, 4, 65). The impairment of oxidative phosphorylation can lead to more electron leakage from electron transport chain and, in turn, elevate the production of ROS in mutant cells (66), thereby damaging mitochondrial and cellular proteins, lipids, and nucleic acids (67). However, a 61% increase of ROS production in cells carrying both m.1555A→G and homozygous TRMU A10S mutations was the consequence of the altered activities of respiration. The hair cells and cochlear neurons may be preferentially involved because they are somehow exquisitely sensitive to subtle imbalance in cellular redox state or increased level of free radicals (68–70). This would lead to dysfunction or apoptosis of hair cells and cochlear neurons carrying both m.1555A→G and TRMU A10S mutations, thereby producing a phenotype of deafness.

In summary, our study demonstrated the role of the first nuclear modifier allele (A10S) in the TRMU gene in the phenotypic manifestation of deafness-associated m.1555A→G mutation. The A10S mutation altered the structure and function of TRMU. The mutated TRMU caused the deficient thiolation of tRNA^Glu, tRNA^Glu, and tRNA^Lys but increased the aminoclylation of tRNAs. The failures in tRNA metabolism led to impairment of mitochondrial translation, respiratory phenotype, defects in mitochondrial ATP production, and increasing ROS production. The resultant biochemical defects aggravate the mitochondrial dysfunction associated with m.1555A→G mutation, below the threshold for normal cell function, thereby expressing the deafness phenotype. Therefore, the mutated TRMU, acting as a nuclear modifier, triggers the deafness in individuals harboring the m.1555A→G mutation.

Experimental Procedures

MD Simulations—Simulation systems. The starting coordinates of wild-type TRMU were taken from the crystal structure of TRMU-tRNA^Glu complex (Protein Data Bank entry 2DER) (44). The coordinates of A10S mutation mutated protein were generated from the wild-type TRMU coordinates through PyMOL (Schrödinger). All terminal residues adopted the neutral state. Each system was solvated in a cubic box of TIP3P water with an extension of at least 10 Å from each side. Approximately 50 mM NaCl were added to the solvent in addition to the neutralizing Na⁺ or Cl⁻. This leads to a wild-type system of 109422 atoms and a mutant system of 109426 atoms.

Simulation protocol. MD simulations were carried out with the GROMACS 4.5.5 package 2 (42). The CHARMM36 force field with CMAP modification 4 was applied for protein (43). Energy minimizations were performed to relieve unfavorable contacts, followed with equilibration steps to fully equilibrate the solvent. Each system was equilibrated in the NPT ensemble at 310 K and 1 bar in periodic boundary condition, and the time step was set to 1 fs. Positional restraints were first applied on all the heavy atoms of protein for 50 ps, then main chain atoms for 50 ps, and then α-atoms for 500 ps. After equilibrations, the production simulation was carried out with a time step of 2 fs, and each system was run up to 300 ns. Electrostatic interactions were calculated with the particle mesh Ewald algorithm (71). SETTLE constraint was applied on hydrogen-involved covalent bonds in water, and LINCS constraint was applied on the hydrogen-involved covalent bonds in those molecules other than waters in the system (72).

Cell Lines and Culture Conditions—Eight human immortalized lymphoblastoid cell lines derived from members of an Arab-Israeli family (two subjects carrying only m.1555A→G mutation (F12H and F6D), two individuals (F20C and F8A) harboring both m.1555A→G and heterozygous TRMU A10S mutations, two individuals (F20A and F20D) carrying both m.1555A→G and homozygous TRMU A10S mutations, and two control individuals (F7A and F7E) lacking both mutations) were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate and 10% FBS (15, 21).

Differential Scanning Fluorimetry—The wild-type and mutant human TRMU cDNAs were amplified by PCR and cloned in-frame with a C-terminal His tag into pET-28a vector (25). Recombinant wild-type and mutant TRMU were produced as His tag fusion proteins in Escherichia coli Rosetta (DE3) as detailed elsewhere (73). The proteins were purified using a nickel-nitrilotriacetic acid column (Qiagen). The stability of proteins was assessed using a protein thermal shift dye kit (Life Technologies) unfolding temperature (T_m) test performed on a
Mitochondrial tRNA Aminoacylation Analysis—Total mitochondrial RNAs were obtained using a Totally RNA™ kit (Ambion) from mitochondria isolated from mutant and wild-type cell lines, as described previously (74). The presence of the thiouridine modification in the tRNAs was verified by the retardation of electrophoretic mobility in a polyacrylamide gel that contains 0.05 mg/ml APM (45–47). 2 µg of total mitochondrial RNA was separated by polyacrylamide gel electrophoresis and blotted onto positively charged membranes (Roche Applied Science). Each mRNA fraction was detected with a specific non-radioactive DIG oligodeoxynucleotide probe at the 3’ termini according to the method as described elsewhere (15, 25, 47). Oligonucleotide probes for tRNA^1^Gln, tRNA^Glu^, and tRNA^Gin^ were detailed previously (15, 25). DIG-labeled oligodeoxynucleosides were generated by using the DIG oligonucleotide tailing kit (Roche). APM gel electrophoresis and quantification of 2-thiouridine modification in tRNAs were conducted as detailed (25).

Mitochondrial tRNA Thiolation Analysis—Total mitochondrial RNAs were isolated under acidic condition. 2 µg of total mitochondrial RNA was electrophoresed at 4 °C through an acrid (pH 5.2) 10% polyacrylamide gel with 7 M urea gel to separate the charged and uncharged tRNA as detailed elsewhere (48). The gels were then electroblotted onto a positively charged nylon membrane (Roche) for the hybridization analysis with oligodeoxynucleotide probes of tRNA^1^Gln, tRNA^Glu^, tRNA^Leu(CUN)^, and tRNA^Ser(AGY)^, as described elsewhere (25, 48–49). DIG-labeled oligodeoxynucleotides were generated by using a DIG oligonucleotide tailing kit (Roche). The hybridization and quantification of density in each band were carried out as detailed elsewhere (48, 49).

Western Blotting Analysis—Western blotting analysis was performed as detailed previously (15, 49). The antibodies used for this investigation were from Abcam (TRMU (ab50895), VDAC (ab14734), NDUBB (ab110411), ND1 (ab74257), ND5 (ab29624) and A6 (ab101908), and CO2 (ab110258)), Santa Cruz Biotechnology (ND4 (sc-20499-R), ND6 (sc-20667)), MTO1 (sc-398760), and Proteintech (CYTB (55901-AP)). Peroxidase Affini Pure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) were used as secondary antibodies, and protein signals were detected using the ECL system (CWBIO). Quantification of density in each band was performed as detailed previously (15, 49).

Measurements of Oxygen Consumption—The rates of oxygen consumption in cybrid cell lines were measured with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously (15, 50).

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 (15, 49, 62).

Measurement of ROS Production—ROS measurements were performed following the procedures detailed previously (51–52).

Author Contributions—F. M. performed the experiments and contributed to data analysis in Figs. 2, 3, and 6–9. X. C. and Z. Z. carried out the MD simulations. Y. P. performed the aminoacylation experiment. R. L. led the thiolation analysis. F. L. and Q. F. contributed to the Western blotting analysis. A. S. G. performed the statistical analysis. N. F.-G. provided the cell lines. M.-X. G. designed the experiments. A. S. G. and X. Z. monitored the project progression, data analysis, and interpretation. F. M. prepared the initial draft of the manuscript. M.-X. G. made the final version of the manuscript.

References

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variants in 2651 Han Chinese subjects with hearing loss. *Mitochondrion* 23, 17–24


42. Wang, X., Yan, Q., and Guan, M. X. (2010) Combination of the loss of cmG<sub>18</sub>U<sub>18</sub> with the lack of s<sub>18</sub>U<sub>18</sub> modifications of tRNA<sup>Glu</sup> and tRNA<sup>Gln</sup> altered mitochondrial biogenesis and respiration. *J. Mol. Biol.* 395, 1038–1048

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