A deafness-associated tRNA mutation caused pleiotropic effects on the m\(^1\)G37 modification, processing, stability and aminoacylation of tRNA\(^{\text{Ile}}\) and mitochondrial translation

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ABSTRACT

Defects in the posttranscriptional modifications of mitochondrial tRNAs have been linked to human diseases, but their pathophysiology remains elusive. In this report, we investigated the molecular mechanism underlying a deafness-associated tRNA\(^{\text{Ile}}\) 4295A\(\rightarrow\)G mutation affecting a highly conserved adenosine at position 37, 3’ adjacent to the tRNA’s anticodon. Primer extension and methylation activity assays revealed that the m.4295A\(\rightarrow\)G mutation introduced a tRNA methyltransferase 5 (TRMT5)-catalyzed m\(^1\)G37 modification of tRNA\(^{\text{Ile}}\). Molecular dynamics simulations suggested that the m.4295A\(\rightarrow\)G mutation affected tRNA\(^{\text{Ile}}\) structure and function, supported by increased melting temperature, conformational changes and instability of mutated tRNA. An in vitro processing experiment revealed that the m.4295A\(\rightarrow\)G mutation reduced the 5’ end processing efficiency of tRNA\(^{\text{Ile}}\) precursors, catalyzed by RNase P. We demonstrated that cybrid cell lines carrying the m.4295A\(\rightarrow\)G mutation exhibited significant alterations in aminoacylation and steady-state levels of tRNA\(^{\text{Ile}}\). The aberrant tRNA metabolism resulted in the impairment of mitochondrial translation, respiratory deficiency, decreasing membrane potentials and ATP production, increasing production of reactive oxygen species and promoting autophagy. These demonstrated the pleiotropic effects of m.4295A\(\rightarrow\)G mutation on tRNA\(^{\text{Ile}}\) and mitochondrial functions. Our findings highlighted the essential role of deficient posttranscriptional modifications in the structure and function of tRNA and their pathogenic consequence of deafness.

INTRODUCTION

Posttranscriptional modifications of transfer RNA (tRNA) affect all aspects of tRNA structure and function, including the proper processing, stability, folding and decoding properties of tRNAs, fidelity and efficiency of translation (1–3). Defects in the posttranscriptional modifications of mitochondrial tRNAs have been linked to an array of human diseases, including neurological and muscular disorders, cardiovascular diseases, diabetes and deafness (3–9). In human mitochondria, 18 types of nucleotide modifications are present in the 137 positions of 22 tRNA species, encoded by mitochondrial DNA (mtDNA) (10–12). The nucleotide modifications of tRNAs were catalyzed by tRNA modifying enzymes, such as TRMU, GTPBP3 and MTO1, encoded by nuclear genome (13–17). Of core modifications, the pseudouridine (Ψ) at position 55 is required for the proper folding and stability of tRNAs (18). The loss of pseudouridylation at position 55 at the TΨC...
loop of tRNA^{Glu} perturbed the tRNA biogenesis and translation and caused maternally inherited deafness and diabetes (8). The anticodon loop modifications of tRNAs, including nucleotides at positions 34 and 37, impact the stabilization of anticodon structure, fidelity and efficiency of translation (19–23). The deficient modifications at U34 of tRNA^{Leu}_{(CUN)} and tRNA^{Glu}_{(CUN)} caused by deficiencies of tRNA modifying enzymes GTPBP3, MTO1 and TRMU, were associated with several human diseases including deafness (24–27). However, pathophysiology underlying the deficient tRNA modifications remains poorly understood.

The nucleotides at position 37 (A or G) of mammalian mitochondrial tRNAs contain a diverse range of complex modifications including N^6-threonylcarbamoyladenosine (t^6A), N^6-isopentenyladenosine (i^6A), 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A), and 1-methylguanosine (m^1G) (10,28). However, nucleotide modification at this position is absent in five mitochondrial tRNAs such as tRNA{Pro} and tRNA{Gln}, the m^1G37 modification is synthesized by YRDC and OSGPEP1 (10,14). In contrast, the biosynthesis of t^6A37 present in the tRNA{ile}, tRNA{Lys}, tRNA{Ser(AGY)}, tRNA{Asn} and tRNA{Thr} was catalyzed by tRNA dimethylallyltransferase (TRIT1) (10,14,28,29). Deficiencies in the YRDC/OSGEP1 or TRMT5 perturbed the t^6A37 or m^1G37 modifications leading to several clinical presentations (29, 30). Furthermore, mitochondrial tRNA mutations at position 37 including the tRNA{asp}^Ap G mutation and tRNA{ile}^G mutation were associated with hypertension, diabetes, visual loss, and deafness (31–37). In particular, the tRNA{asp}^Ap G mutation and tRNA{ile}^G mutation introduced the new modified nucleotide m^1G37 and altered the structure and function of corresponding tRNA (31,32). The tRNA{ile}^G mutation was identified in several French and Chinese pedigrees with maternal transmission of deafness (35,36). It was hypothesized that the substitution of A with G at position 37 of the tRNA{ile}^G may replace the t^6A37 synthesized by YRDC/OSGEP1 with the m^1G37 modification catalyzed by TRMT5, thereby altering the structure and function of tRNA{ile}^G. Especially, the m.4295A>G mutation impacts the processing of tRNA precursors, aminoacylation capacity and stability of tRNA{ile}^G and mitochondrial translation. It was also anticipated that impairment of mitochondrial translation caused by the m.4295A>G mutation altered the respiration, production of ATP and reactive oxygen species (ROS). To further investigate the molecular mechanism of m.4295A>G mutation, cybrid cell lines were constructed by transferring mitochondria from lymphoblastoid cell lines derived from an affected matrilineal relative in a Chinese family bearing the m.4295A>G mutation and from a control individual lacking the mtDNA mutation, into human mtDNA-less (ρ−) cells (38–39). In the present study, we first examined if the m.4295A>G mutation introduced the m^1G37 modification of tRNA{ile}^G by using primer extension and in vitro methylation assays. These resultant cybrid cell lines were then assessed if the m.4295A>G mutation on the aminoacylation capacity and stability of this tRNA, mitochondrial translation, respiration, and the production of ATP and oxidative reactive species (ROS), mitochondrial membrane potential and autophagy.

**Materials and Methods**

**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 (40). One hearing-impaired Chinese Han pedigree for this study was ascertained at the Otology Clinic of the Children’s Hospital, Zhejiang University School of Medicine. 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 School of Medicine. Audiological and neurological examinations of hearing impairment were performed as detailed previously (41,42). All available members of the pedigree and 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.

**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 tRNA{ile}^G genes were PCR amplified by use of oligodeoxynucleotides corresponding to mtDNA at 3796–4693 (12). 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) (12). The entire mtDNAs of proband WZD104 III-8 and one Chinese control subject (C59) were PCR amplified in 24 overlapping fragments using sets of the light (L) and heavy (H) strand oligonucleotide primers, as described previously (43). These sequence results were compared with the updated consensus Cambridge sequence, as described above. To quantify the m.4295A>G mutation, PCR segment (251 bp) spanning the tRNA{ile}^G gene was amplified and subsequently digested with restriction enzyme NlaIII, as the m.4295A>G mutation and mismatched oligodeoxynucleotides primer together created the site for this enzyme (44). Equal amounts of various digested samples were then analyzed by electrophoresis through 10% polyacrylamide/8 M urea gel. The proportions of digested and undigested PCR products were determined by ImageQuant program after ethidium bromide staining to determine if the m.4295A>G mutation is in homoplasmic in these subjects.

**Cell lines and culture conditions**

Immortalized lymphoblastoid cell lines were generated from one hearing-impaired matrilineal relative (III-8) of the Chinese family carrying the m.4295A>G mutation and one genetically unrelated Chinese control individual (C59) belonging to the same mtDNA haplogroup D4j but lacking the mutation (45). These cell lines were grown in RPMI
Molecular dynamics (MD) simulation

The anticodon stem and loop (ASL) of tRNA\textsubscript{Ile} containing 17 nucleotide bases (Figure 1A) were constructed as the model of simulations. The initial coordinates for wild type tRNA\textsubscript{Ile} molecule were obtained from the crystal structure of human mitochondrial tRNA\textsubscript{Ile}\_PheRS complex (Protein Data Bank entry 3TUP). Based on the anticodon stem and loop structure of wild type tRNA\textsubscript{Ile}, A37 was substituted to m\textsuperscript{1}G37 in Chimera to establish the initial coordinates of the mutant. For the wild type tRNA molecule, MD simulation were performed with Amber14 utilizing ff14SB force field parameters (47). For the mutant molecule, Antechamber was used to generate complete unused force field files of the modified nucleotide. Finally, 100 ns production simulations were carried out with a time step of 2 fs for both systems, as detailed previously (48).

Primer extension assays of in vivo m\textsuperscript{1}G37 modification

A primer extension experiment to analyze the m\textsuperscript{1}G37 modification of tRNA\textsubscript{Ile} was carried out as detailed elsewhere (31,32). Total mitochondrial RNAs were obtained using TOTALLY RNATM kit (Ambion) from mutant and control cybrids (~2.0 × 10\textsuperscript{5} cells) (49). A DNA primer (5′-TGTTAGATATATAGGATTAGCCCTATA-3′) complementary to the 3′ end of the tRNA\textsubscript{Ile} was the 5′ end labeled with non-radioactive digoxigenin (DIG). Primescript II 1st Strand cDNA Synthesis Kit (TAKARA) was used for reverse transcription with DIG-labeled oligodeoxynucleotide probes for tRNA\textsubscript{Ile} precursor. DIG-labeled probes were generated by using DIG-oligonucleotide Tailing kit (Roche). The hybridization and quantification of density in each band were performed as detailed previously (53).

UV melting assays

UV melting assays were carried out as described elsewhere (32). The wild type and mutant tRNA\textsubscript{Ile} transcripts were generated as described above. The tRNA\textsubscript{Ile} transcripts were diluted in the buffer including 50 mM sodium
Figure 1. MD simulations on the anticodon stem-loop of wild-type and mutated tRNA<sub>Leu</sub>. (A) Cloverleaf structure of human mitochondrial tRNA<sub>Leu</sub>. An arrow indicated the location of the m.4295A→m1G mutation. Nucleotides at the dashed box in the anticodon stem–loop of tRNA<sub>Leu</sub> were used for MD simulation. (B) The tertiary structures of the anticodon stem-loop for the wild-type (gray) and mutated (dodger blue) tRNA<sub>Leu</sub>. (C) Time evolution of the root mean square deviation (RMSD) values of all backbone atoms on the anticodon stem–loop for the wild-type (WT) (black curve) and mutant (MT) (red curve) of tRNA<sub>Leu</sub>. (D) RMSF curves calculated from the backbone atoms for the wild-type (black lines) and mutated (red lines) anticodon stem-loop of tRNA<sub>Leu</sub>.

Mitochondrial tRNA analysis

Total mitochondrial RNAs were obtained from mitochondria isolated from cybrid cell lines (~2.0 × 10<sup>8</sup> cells), as described previously (49). The tRNA Northern blot analysis was performed as detailed elsewhere (32). DIG-labeled oligodeoxynucleotide probes specific for tRNA<sub>Leu</sub>, tRNA<sub>Met</sub>, tRNA<sub>Asp</sub>, tRNA<sub>Glu</sub>, tRNA<sub>Lys</sub> and 12S rRNA were as detailed elsewhere (55). The hybridization and quantification of density in each band were carried out as detailed previously (53–55).

For the aminoacylation assays, total mitochondrial RNAs were isolated under acid conditions, and 4 μg of total mitochondrial RNAs were electrophoresed at 4<sup>°</sup>C through 10% polyacrylamide–8 M urea gel to separate the charged and uncharged tRNA as detailed elsewhere (32,56). To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated by being heated for 10 min at 60°C at pH 8.3 and then run in parallel (32,56). The gels were then electroblotted onto a positively charged nylon membrane (Roche) for the hybridization analysis with oligodeoxynucleotide probes as

phosphate (pH 7.0), 50 mM NaCl, 5 mM MgCl<sub>2</sub> and 0.1 mM EDTA. Absorbance against temperature melting curves were measured at 260 nm with a heating rate of 1°C/min from 25 to 95°C through Agilent Cary 100 UV Spectrophotometer.
described above. Quantification of density in each band was performed as detailed previously (32).

For the tRNA mobility shift assay, two μg of total mitochondrial 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 (32,48).

**Mitochondrial translation assays**

Pulse-labeling of the mutant and control cell lines for 30 min with [35S] methionine, [35S] cysteine in methionine-free DMEM in the presence of emetine, electrophoretic analysis of the translation products, and quantification of radioactivity in the whole-electrophoretic patterns or in individual well-resolved bands were carried out as detailed previously (57).

**Western blot analysis**

Western blotting analysis was performed as detailed previously (32,55). Twenty micrograms of total cell proteins obtained from various cell lines were denatured and loaded on sodium dodecyl sulfate (SDS) polyacrylamide gels. The gels were electrophobed onto a polyvinylidene difluoride (PVDF) membrane for hybridization. The antibodies used for this investigation were from Abcam [TOM20 oride (PVDF) membrane for hybridization. The antibodies used for this investigation were from Abcam [TOM20

**Blue native electrophoresis analysis**

Blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed by isolating mitochondrial proteins from mutant and control cell lines, as detailed previously (58,59). Samples containing 30 μg of total cellular proteins were separated on 3–12% Bis-Tris Native PAGE gel. The total OXPHOS Human WB Antibody Cocktail was applied as primary antibodies for this experiment. Peroxidase AffiniPure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) were used as secondary antibody and protein signals were detected using the ECL system (CW-BIO). Quantification of density in each band was performed as detailed elsewhere (55).

**Assays of activities of respiratory complexes**

The enzymatic activities of complex I, II, III and IV were assayed as detailed elsewhere (39,60). 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 5,5'-dithiobis-2-nitrobenzoic acid, 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 DCPIP by decylubiquinone 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). Complex I–IV activities were normalized by citrate synthase activity.

**Measurements of oxygen consumption**

The rates of oxygen consumption (OCR) in various hybrid cell lines were measured with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously (61,62). Cybrid cells were seeded at a density of 2 × 10^4 cells per well on Seahorse XF96 polystyrene tissue culture plates (Seahorse Bioscience). Inhibitors were used at the following concentrations: oligomycin (1.5 μM), carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) (0.8 μM), antimycin A (1.5 μM) and rotenone (3 μM).

**ATP measurements**

The CellTiter-Glo® Luminescence Cell Viability Assay kit (Promega) was used for the measurement of cellular and mitochondrial ATP levels, according to the modified manufacturer’s instructions (62). Briefly, the assay buffer and substrate were equilibrated at room temperature, transferred to and gently mixed with the substrate to obtain a homogeneous solution. After a 30 min equilibration of the cell plate at room temperature, 100 μl of the assay reagents were added into each well with 2 × 10^4 cells and the content was mixed for 2 min on an orbital shaker to induce cell lysis. After 10 min incubation in room temperature, the luminescence was read on a microplate reader (Synergy H1, BioTek).

**Assessment of mitochondrial membrane potential**

Mitochondrial membrane potential was assessed with JC-10 Assay Kit-Microplate (Abcam) following general manufacturer’s recommendations with some modifications, as detailed elsewhere (32,63). In brief, ∼2 × 10^6 cells of each cybrid cell line were harvested, resuspended in 200 μl of JC-10 Assay buffer and then incubated at 37°C for 30 min. Alternatively, harvested cells were preincubated with 10 μM of FCCP for 30 min at 37°C prior to staining with JC-10 dye. After washing with PBS twice, cells were resuspended in 200 μl PBS. The fluorescent intensities for both J-aggregates and monomeric forms of JC-10 were measured at Ex/Em = 490/530 and 490/590 nm with DB-LSR II flow cytometer system (Beckton Dickson, Inc.).
Measurement of ROS production

The levels of mitochondrial reactive oxygen species (ROS) generation were determined using MitoSOX assay as detailed previously (53,64). Briefly, approximate 2 × 10⁶ cells of each cell line were harvested, resuspended in PBS supplemented with 100 μM of 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) and then incubated at 37°C for 20 min. After washing with PBS twice, cells were resuspended in PBS in the presence of 2 mM freshly prepared H₂O₂ and 2% FBS and then incubated at room temperature for another 45 min. Cells were further washed with PBS and resuspended with 1 ml of PBS with 0.5% parafomaldehyde. Samples with or without H₂O₂ stimulation were analyzed by BD-LSR II flow cytometer system (Beckton Dickson, Inc.), with an excitation at 488 nm and emission at 529 nm. Ten thousand events were analyzed in each sample.

Autophagy assessment

The status of autophagy in cybrids were measured via CYTO-ID® Autophagy Detection Kit (Enzo), according to the modified manufacturer’s instructions (53). In brief, 5 × 10⁵ cells were trypsinized, washed with 1 × Assay buffer and resuspended with CYTO-ID® Green stain solution. After incubated at 37°C for 30 min in the dark, cells were washed and resuspended in 1 × Assay Buffer. Samples were analyzed by BD-LSR II flow cytometer system (Beckton Dickson, Inc.), with an excitation at 488 nm and emission at 529 nm. Ten thousand events were analyzed in each sample.

Statistical analysis

Statistical analysis was performed using the unpaired, two-tailed Student’s t-test contained in the Microsoft-Excel program (version 2017). Differences were considered significant at a P < 0.05.

RESULTS

Clinical presentations and derived cell lines of a hearing-impaired Chinese pedigree carrying the m.4295A>G mutation

One Han Chinese hearing-impaired proband carrying the m.4295A>G mutation was identified among 2651 Chinese hearing-impaired probands but absent in 574 Chinese normal hearing controls (35,40). As shown in Supplemental Figure S1, the Chinese pedigree exhibited the maternal inheritance of hearing loss. As shown in Supplemental Figure S2 and Supplemental Table S1, 9 of 14 matrilineal relatives exhibited the variable degree of hearing impairment (one with mild hearing loss, four with moderate hearing loss, three with severe hearing loss and one with profound hearing loss), whereas none of other members in this family had hearing loss. The age-at-onset of hearing loss ranged from 23 to 50 years, with the average of 35 years old. There was no evidence that any of other members of this family had any other causes to account for hearing loss. These matrilineal relatives showed no other clinical presentations. Further analysis showed that the m.4295A>G mutation was present in homoplasy in all matrilineal relatives but not in other members of this family (Supplemental Figure S1B, C).

Immortalized lymphoblastoid cell lines were derived from one affected matrilateral relative carrying the m.4295A>G mutation (III-8; male, 23 years) and from one genetically unrelated control subject lacking the m.4295A>G mutation belonging to the same mtDNA haplogroup D4 (C59, male, 20 years) (Supplemental Table S2). The lymphoblastoid cells were enucleated, and subsequently fused to a large excess of mtDNA-less human ρ0/206 cells, derived from the 143B.TK- cell line (38–39). These cybrid clones were isolated by growing the fusion mixtures in the selective DMEM medium, containing BrdU and lacking uridine and subsequently analyzed for the presence and levels of the m.4295A>G mutation (44). The results confirmed the absence of the mtDNA mutation in the control clones and its presence in homoplasy in all cybrids derived from the mutant cell line. Three cybrids derived from each donor cell line with similar mtDNA copy number and same karyotype were used for the biochemical characterization described below.

MD simulation analysis

As shown in Figure 1A, the m.4295A>G mutation affected the highly conserved adenine (A37) of the anticodon loop in tRNAile, and led to the substitution of t6A37 with m1G37 modification. To assess the impact of m.4295A>G mutation on the tertiary structure of tRNAile, MD simulations were carried out using the anticodon stem-loop (ASL) (17nt) of wild type and mutant tRNAile by 100-ns all-atom. Figure 1B shows the tertiary structures of ASL region in the wild type and mutant tRNAile after simulation. As illustrated in Figure 1C, the RMSD (root mean square deviation) values in the mutant ALS region were much less than those in wild type counterpart, indicating that mutant ASL structure of tRNAile was less flexible than that of wild type counterpart. Furthermore, RMSF (root mean square fluctuation) was calculated from the two trajectories to analyze how this mutation affected the stability of ASL structure in the tRNAile. As shown in Figure 1D, there were no significant differences of RMSF values between tRNAile with m1G37 and A37. However, the RMSF values of G34 and U36 in mutant tRNAile were much lower than those of wild type tRNAile, indicating that the induced m1G37 affected the stability of anticodon structure. These results implied that the m.4295A>G mutation affected the structure and function of tRNAile.

The m.4295A>G mutation substituted the t6A37 modification to m1G37 of tRNAile

To investigate if m.4295A>G mutation caused the substitution of t6A37 with m1G37 modification, we subjected mitochondrial RNAs from mutant and control cybrids to the reverse transcription with DIG-labeled oligonucleotide probe specific for tRNAile (Figure 2A). This resulted in a stop band one residue 3' to the methylation on 15% polyacrylamide gel. As shown in Figure 2B, the m1G37 modification was present in tRNAile derived from mutant cell line but absent in the tRNAile derived from control cell line.

To further examine if the m.4295A>G mutation introduced the m1G37 modification of tRNAile in vitro, we
The m.4295A>G mutation introduced the m^1G37 modification of tRNA^Ile. (A) Schematic of methylation shown in the cloverleaf structures of the human mitochondrial tRNA^Ile. An arrow denotes the location of the m.4295A>G mutation. Solid lines represent the DIG-labeled oligonucleotide probe specific for mt-tRNA^Ile. Broken lines represent the potential stops of primer extension and m^1G9 rm 2G. (B) Primer extension demonstrated the creation of m^1G37 in the tRNA^Ile carrying the m.4295A>G mutation. The primer extension termination products are showed as m^1G9, m^2G26 and m^1G37. (C) Methylation activity assays. The unmodified human mitochondrial wild type (A37) and mutant (G37) tRNA^Ile, cytosolic tRNA^Leu(CAG) and tRNA^Thr were generated from in vitro transcription. The unmodified tRNA transcripts were incubated with M. jannaschii (Mj-Trm5) in the presence of S-adenosyl-L-methionine. Samples were withdrawn and stopped after 2, 4, 6 or 8 min, respectively. The relative modification efficiency was calculated from the initial phase of the reaction. The calculations were based on three independent determinations.

Figure 2. The m.4295A>G mutation introduced the m^1G37 modification of tRNA^Ile. (A) Schematic of methylation shown in the cloverleaf structures of the human mitochondrial tRNA^Ile. An arrow denotes the location of the m.4295A>G mutation. Solid lines represent the DIG-labeled oligonucleotide probe specific for mt-tRNA^Ile. Broken lines represent the potential stops of primer extension and m^1G9. (B) Primer extension termination products are showed as m^1G9, m^2G26 and m^1G37. (C) Methylation activity assays. The unmodified human mitochondrial wild type (A37) and mutant (G37) tRNA^Ile, cytosolic tRNA^Leu(CAG) and tRNA^Thr were generated from in vitro transcription. The unmodified tRNA transcripts were incubated with M. jannaschii (Mj-Trm5) in the presence of S-adenosyl-L-methionine. Samples were withdrawn and stopped after 2, 4, 6 or 8 min, respectively. The relative modification efficiency was calculated from the initial phase of the reaction. The calculations were based on three independent determinations.

Impaired the 5' end processing of tRNA^Ile precursor

We explored an in vitro processing system to assess the effect of m.4295A>G mutation on 5' end processing of tRNA^Ile. As shown in Figure 3A, the wild type and mutant tRNA^Ile precursors corresponding to mtDNA at positions 4235 to 4350 were incubated with mitochondrial RNase P, which was reconstituted from purified recombinant proteins MRPP1, MRPP2 and MRPP3, at various time courses (53,55,65). No qualitative processing alterations of the mutant tRNA^Ile precursors were observed, but the processing efficiencies of the mutant tRNA^Ile transcripts were mildly decreased, as compared with those of wild type counterparts (Figure 3B). The processing efficiencies of mutant tRNA^Ile transcripts catalyzed by RNase P were 46.8% of those in their wild type counterparts (Figure 3C). These results demonstrated that the m.4295A>G mutation perturbed the 5' end processing of tRNA^Ile precursors.

Perturbed flexibility and conformation of tRNA^Ile

To experimentally test the impact of m.4295A>G mutation on the stability of tRNA^Ile, we measured the melting temperatures (T_m) of wild type and mutant tRNA^Ile transcripts by calculating the derivatives of absorbance against a temperature curve. As shown in Figure 4A, the T_m values for wild-type (A37) and mutant (unmodified G37, m^1G37) transcripts were 41.67 ± 0.56°C, 45.05 ± 0.06°C and 45.08 ± 0.03°C, respectively. These data were in a good agreement with MD data, indicating that the m.4295A>G mutation caused the less flexibility of tRNA^Ile.

To examine if the m.4295A>G mutation affected the conformation of tRNA^Ile, total mitochondrial RNAs from mutant and control cell lines were electrophoresed through 10% native polyacrylamide gel in Tris–glycine buffer and then electroblotted onto a positively charged nylon membrane for hybridization analysis with DIG-labelled probes for tRNA^Ile, tRNA^Met and tRNA^Ser(AGY), respectively. As shown in Figure 4B, electrophoretic patterns showed that the tRNA^Ile in three mutant cybrid cell lines carrying the m.4295A>G mutation migrated faster than those of control cybrid cell lines lacking this mutation. These data suggested that the structural alterations by the prepared with wild type and mutant tRNAs by in vitro transcription and evaluated the methylation activity catalyzed by the recombinant M. jannaschii Trm5 (Mj-Trm5) (32,51,52). As shown in Figure 2C, the mutant tRNA^Ile transcripts (G37) were modified with m^1G37 modification by the Mj-Trm5 but less efficiently as cytoplasmic tRNA^Leu(CAG) transcripts (G37). In contrast, the modification was not detected in the wild type tRNA^Ile transcripts (A37) in the presence of Mj-Trm5. As controls, human cytoplasmic tRNA^Leu(CAG) transcripts (G37) were modified by the Mj-Trm5, while human cytoplasmic tRNA^Thr transcripts (A37) were not modified in the presence of Mj-Trm5. These results indicated that the substitution of A to G at position 37 introduced the m^1G37 modification of tRNA^Ile.
m.4295A>G mutation caused the conformation change of tRNA\textsuperscript{Ile}.

**Reductions in the steady state levels of tRNA\textsuperscript{Ile}**

To further evaluate the effect of m.4295A>G mutation on the stability of tRNA\textsuperscript{Ile}, we subjected mitochondrial RNAs from mutant and control cybrids to Northern blots under a denaturing condition and hybridized them with DIG-labeled oligodeoxynucleotide probes for tRNA\textsuperscript{Ile}, tRNA\textsuperscript{Met}, tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Lys} and 12S rRNA as loading control, respectively. As shown in Figure 4C, the steady-state levels of tRNA\textsuperscript{Ile} in the mutant cybrids were markedly decreased as compared to those in control cybrids. For comparison, the average levels of each tRNA in the various control or mutant cybrid cell lines were normalized to the average levels of 12S rRNA in the same cell lines for reference. As shown in Figure 4D, the average levels of tRNA\textsuperscript{Ile} in the mutant cybrid cell lines were 36.8% (P < 0.001) of mean values of three control cybrids. However, the average levels of tRNA\textsuperscript{Met}, tRNA\textsuperscript{Asp}, tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Lys} in the mutant cybrid cell lines were comparable with those in control cell lines, respectively.

**Aberrant aminoacylation of tRNA\textsuperscript{Ile}**

To investigate whether the m.4295A>G mutation affected the aminoacylation of tRNA\textsuperscript{Ile}, we examined the aminoacylation levels of tRNA\textsuperscript{Ile} as well as tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Lys}, tRNA\textsuperscript{Met} and tRNA\textsuperscript{Ser(AGY)} by the use of electrophoresis in an acidic urea PAGE system to separate uncharged tRNA species from the corresponding charged tRNA. As shown in Figure 5A, the upper and lower bands represented the charged and uncharged tRNA, respectively. The electrophoretic mobility of either charged or uncharged tRNA\textsuperscript{Ile} in mutant cell lines migrated faster than those of control cell lines. To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated by being heated for 10 min at 60°C at pH 8.3 and then run in parallel. As shown in Figure 5B, only one band (uncharged tRNA) was present in both mutant and control cell lines after deacylating. Notably, the efficiencies of aminoacylated tRNA\textsuperscript{Ile} in the mutant cell lines were 85.6% of the average values of control cell lines (P < 0.001) (Figure 5C). However, there were no obvious differences in the electrophoretic mobility and levels of tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Lys},
tRNA<sub>Met</sub> between mutant and control cell lines. These results suggested that the deficient nucleotide modification at position 37 affected the aminoacylation properties of tRNA.

Impairment of mitochondrial translation

To investigate whether the aberrant tRNA metabolism caused by m.4295A>G mutation impaired mitochondrial translation, mutant and control cybrids were labeled for 30 min with [35S]methionine–[35S]cysteine in methionine-free regular DMEM medium in the presence of 100 μg/ml of emetine to inhibit cytosolic protein synthesis (57). Figure 6A showed typical electrophoretic patterns of mitochondrial translation products of mutant and control cell lines. Patterns of the mtDNA-encoded polypeptides of the cybrids harboring the m.4295A>G mutation were qualitatively identical to those of the control cells, in terms of electrophoretic mobility of the various polypeptides. However, cell lines carrying the m.4295A>G mutation displayed significant decrease in the total rate of labeling of mitochondrial translation products relative to those of the control cell line. Figure 6B showed a quantification of the results of a large number of labeling experiments and electrophoretic runs. In particular, the overall levels of 13 mitochondrial translation products in the mutant cell lines were 72.5% (P < 0.001), relative to the mean values measured in the control cell lines. The average levels of ND1, ND2, ND3, ND4, ND4L, ND5, ND6, CYTB, CO1, CO2, CO3, ATP6 and ATP8 in the mutant cells were 66.3%, 63.5%, 84.3, 53.0%, 105.6%, 70.3%, 76.9%, 61.4%, 72.5%, 76.9%, 70.0%, 64.7% and 103.2% of those in the control cell lines, respectively.

Furthermore, a Western blot analysis was carried out to examine the levels of seven mtDNA encoded OXPHOS subunits in mutant and control cell lines with TOM20 as a
Figure 5. In vivo aminoacylation assays. (A) Various amounts of total mitochondrial RNA purified from six 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 a DIG-labeled oligonucleotide probe specific for the mt-tRNAIle. The blots were then stripped and rehybridized with mt-tRNAThr, tRNALys, tRNAMet and tRNAAGY, respectively. (B) The samples from one control (C59.32) and mutant (III-8.46) cell lines were decyalted (DA) by heating for 10 min at 60°C at pH 8.3 and electrophoresed as above. (C) Qualification of aminoacylated proportions of tRNAs in the mutant and control cell lines. The calculations were based on three independent determinations. Graph details and symbols are explained in the legend to the Figure 4.

loading control. As shown in Figure 6C and D, the overall levels of seven mtDNA encoded proteins in the mutant cybrids were 71.7% (P < 0.001), relative to the mean values measured in the control cybrids. The average levels of ND1, ND4, ND5, ND6, CO2, ATP8 and CYTB in the mutant cells were 65.3%, 31.8%, 68.5%, 69.7%, 79.1%, 102.1% and 85.3% of those in the control cell lines after normalization to TOM20, respectively. However, the levels of polypeptide synthesis in mutant cells, relative to those in control cells, showed no significant correlation with either the number or the proportion of the codons for isoleucine (Supplemental Table S3).

We then examined the levels of seven subunits (mtDNA-encoded CO2 and six nucleus-encoding proteins) of phosphorylation system (OXPHOS) in control and mutant cell lines by Western blotting analysis. As shown in Supplemental Figure 3, the average level of CO2 in the mutant cybrids was 76.0% (P = 0.001) of control cybrids. However, the levels of other six polypeptides, NDUFB8, NDUFS3, SDHB, UQCRCC2, CO10 and ATP5A, encoded by nuclear genes, in mutant cell lines were comparable with those in control cell lines.

Blue native gel analysis of OXPHOS complexes

To investigate whether the m.4295A>G mutation induced alterations affected the stability of OXPHOS complexes, we measured the steady-state levels of five OXPHOS complexes by Blue-native polyacrylamide gel electrophoresis (58,66,67). As shown in Figure 7A and B, the levels of complex I (CI), complex III (CIII), complex IV (CIV) and complex V (CV) in the mutant cybrids were 45.94% (P < 0.001), 82.08% (P < 0.001), 68.79% (P < 0.001) and 60.54% (P < 0.001) of those in the control cybrids after normalization to TOM20, respectively. However, the levels of complex II (CII) in mutant cell lines were comparable with those in control cell lines. The lower levels of the respiratory complexes I, III and IV
Figure 6. Analysis of mitochondrial translation. (A) Representative gel for electrophoretic patterns of the mitochondrial translation products of six cybrid cell lines labeled for 30 min with [35S]methionine in the presence of 100 μg/ml of emetine and corresponding Coomassie Brilliant Blue stained gel used as loading control. Samples containing equal amounts of protein (30 μg) were run in SDS/polyacrylamide gradient gels. CO1, CO2 and CO3 indicate subunits I, II, and III of cytochrome c oxidase; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6, subunits 1, 2, 3, 4, 4L, 5 and 6 of the respiratory chain reduced nicotinamide-adenine dinucleotide dehydrogenase; A6 and A8, subunits 6 and 8 of the H+-ATPase; and CYTB, apocytochrome b. (B) Quantification of the rates of the mitochondrial translation labeling. The rates of mitochondrial protein labeling were expressed as percentages of the value for average values of three control cybrids in each gel, with error bars representing two SDs. A total of three independent labeling experiments and three electrophoretic analyses of each labeled preparation were performed on cell lines. (C) Twenty micrograms of total cellular proteins from various cell lines were electrophoresed through a denaturing polyacrylamide gel, electroblotted and hybridized with 7 mtDNA encoded subunits in mutant and control cells with TOM20 as a loading control. (D) Quantification of mitochondrial protein levels. Average relative ND1, ND4, ND5, ND6, CO2, ATP8 and CYTB content per cell, were normalized to the average content per cell of TOM20 in three mutant cell lines carrying the m.4295A>G mutation and three control cell lines lacking the mutation.

may be due to the misfolded and/or misassembled these complexes.

Deficient activities of respiratory chain complexes

To evaluate the effect of the m.4295A>G mutation on the oxidative phosphorylation, we measured the activities of respiratory complexes by the use of isolating mitochondria from mutant and control cell lines. The activity of complex I (NADH ubiquinone oxidoreductase) was determined through the oxidation of NADH with ubiquinone as the electron acceptor. The activity of complex II (succinate ubiquinone oxidoreductase) was examined through the artificial electron acceptor DCPIP. The activity of complex III (ubiquinone cytochrome c oxidoreductase) was measured through the reduction of cytochrome c (III) by using D-ubiquinol-2 as the electron donor. The activity of complex IV (cytochrome c oxidase) was monitored through the oxidation of cytochrome c (II). As shown in Figure 8A, the average activities of complexes I, III, and IV in three mutant cell lines carrying the m.4295A>G mutation were 49.9% (P < 0.001), 88.1% (P < 0.001) and 70.2% (P < 0.001) of the mean values measured in three control cell lines, respectively. The activity of complex II in three mutant cell lines carrying the m.4295A>G mutation was 99.7% (P = 0.905) of the mean values measured in three control cell lines.

The effect of m.4295A>G mutation on cellular bioenergetics was further evaluated by measurement of the oxygen consumption rates (OCR) of mutant and control cell lines.
Figure 7. Blue-native gel analysis of OXPHOS complexes. (A) The steady-state levels of five OXPHOS complexes by Blue-Native gel electrophoresis. Thirty microgram of mitochondrial proteins from mutant and control cell lines were electrophoresed through a Blue-Native gel, electroblotted and hybridized with antibody cocktail specific for subunits of each OXPHOS complex as well as Tom20 and Coomassie staining as a loading control. (B) Quantification of levels of complexes I, II, III, IV and V in mutant and control cell lines. The calculations were based on three independent experiments. Graph details and symbols are explained in the legend to Figure 4.

using a Seahorse Bioscience XF-96 Extracellular Flux Analyzer (61). As shown in Figure 8B, the basal OCR in three mutant cell lines was 64.8% ($P < 0.001$) relative to the mean values measured in three control cell lines. To further investigate which complex of the respiratory chain was affected in the mutant cell lines, oligomycin (to 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) were added sequentially while measuring OCR. 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. The ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity and non-mitochondrial OCR in mutant cell lines were 57.7% ($P < 0.001$), 91.7% ($P = 0.306$), 56.7% ($P < 0.001$), 48.0% ($P < 0.001$) and 95.9% ($P = 0.081$), relative to the mean values measured in the control cell lines, respectively (Figure 8C).

Decreases in mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta \Psi_m$) generated by proton pumps (complexes I, III and IV) is an essential component in the process of energy storage during oxidative phosphorylation (63). Together with the proton gradient ($\Delta \Psi$), $\Delta \Psi_m$ forms the transmembrane potential of hydrogen ions which is harnessed to make ATP (66). As shown in Figure 9B and Supplemental Figure S4, the $\Delta \Psi_m$ of three mutant cell lines carrying the m.4295A $>$ G mutation ranged from 63.2% to 64.6%, with an average 63.7% ($P < 0.001$) of the mean values measured in three control cell lines. In contrast, the levels of $\Delta \Psi_m$ in mutant cell lines in the presence of FCCP were comparable to those in the control cell lines.

The increase of mitochondrial ROS production

Mitochondrial ROS are gradually recognized as important signaling mediators in wide range of cellular processes, such as metabolic adaptation, adaptive responses to hypoxia, cellular differentiation, autophagy and regulation of innate or adaptive immunity (68). The levels of mitochondrial ROS among mutant and control cybrid cell lines were determined using MitoSOX assay via flow cytometry under normal conditions and then following $\text{H}_2\text{O}_2$ stimulation. Geometric mean intensity was recorded to measure the production rate of ROS of each sample. As shown in Figure 10A and B, the levels of ROS generation in the mutant cell lines harboring the m.4295A $>$ G mutation ranged from 131.6% to 161.7%, with an average 142.9% ($P < 0.001$) of the mean values measured in three control cell lines under unstimulated conditions. As illustrated in Figure 10C and D, the levels of ROS generation in the mutant cell lines varied from 210.1% to 228.0%, with an average 220.5% ($P < 0.001$) of the mean values measured in the control cell lines under stimulation conditions.

Reduced levels in mitochondrial ATP production

Luciferin/luciferase assay was carried out to examine the capacity of oxidative phosphorylation in mutant and wild-type cell lines. Populations of cells were incubated in the media in the presence of glucose, and 2-deoxy-D-glucose with pyruvate. As shown in Figure 9A, the levels of ATP production in mutant cell lines in the presence of glucose (total cellular levels of ATP) were comparable to those measured in control cell lines. In contrast, the levels of ATP production in mutant cell lines, in the presence of 2-deoxy-D-glucose and pyruvate to inhibit the glycolysis (mitochondrial levels of ATP), varied from 64.0% to 64.5%, with an average of 64.2% relative to the mean values measured in the control cell lines ($P < 0.001$).
To test whether the m.4295A>G mutation-induced mitochondrial ROS production affected the expression of antioxidant systems, we examined the levels of three antioxidant enzymes: SOD2 in mitochondrion, SOD1 and catalase in cytosol in the various cell lines (48,68). As shown in Figure 10E and F, the mutant cell lines revealed marked increases in the levels of SOD2 (182.9%), SOD1 (248.6%) and catalase (201.7%), as compared with those in the control cell lines.

Alteration in autophagy

To investigate if the m.4295A>G mutation affected the autophagy, we evaluated the mitophagic states of mutant and control cell lines by flow cytometry using Cyto-ID, a cationic amphilic tracer dye that labeled mitophagic compartments (69,70). Geometric mean intensity was measured for assessment of autophagy in each sample. As shown in Figure 11A and B, the average levels of autophagy in three mutant cell lines was 129.0% ($P < 0.001$) of the

Figure 8. Respiration assays. (A) 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. (B) An analysis of O$_2$ consumption in the various cell lines using different inhibitors. The rates of O$_2$ (OCR) were first measured on $2 \times 10^4$ cells of each cell line under basal condition and then sequentially added to oligomycin (1.5 nM), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazine (FCCP) (0.5 nM), rotenone (1 nM) and antimycin A (1 nM) at indicated times to determine different parameters of mitochondrial functions. (C) 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 or 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. The average of 4 determinations for each cell line is shown. Graph details and symbols are explained in the legend to Figure 4.

Figure 9. Measurement of mitochondrial ATP levels and membrane potential analysis. (A) Measurement of mitochondrial and cell ATP levels. Cells 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 for 2 h. Average rates of mitochondrial and cellular ATP level per cell line are shown. (B) 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 (FL$_{590}$/FL$_{530}$) were recorded to delineate the ΔΨm level of each sample. Relative ratios of JC-10 fluorescence intensities at Ex/Em = 490/525 and 490/590 nm in the absence and presence of 10 μM of FCCP in three control cell lines and three mutant cell lines were shown. The average of 3–4 determinations for each cell line is shown. Graph details and symbols are explained in the legend to Figure 4.
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 FlowJo software. (A, C) Flow cytometry histogram showing MitoSOX-Red fluorescence of control cybrids (C59.32) (Green) and mutant cybrids (III-8.48) (Red) with or without H₂O₂ stimulation. (B, D) Relative ratios of MitoSOX-Red fluorescence intensity of control cybrids (C59.32) and mutant cybrids (III-8.48) with or without H₂O₂ stimulation. (E) Western blotting analysis of antioxidative enzymes SOD1, SOD2 and catalase in six cell lines with β-actin as a loading control. (F) Quantification of SOD1, SOD2 and catalase. Average relative values of SOD1, SOD2 and catalase were normalized to the average values of β-actin in various cell lines. The values for the latter are expressed as percentages of the average values for the control cell lines. The average of three independent determinations for each cell lines is shown. Graph details and symbols are explained in the legend to Figure 4.

mean values measured in three control cell lines. The status of autophagy in mutant and control cell lines was further examined using Western blot analysis with two markers: microtubule-associated protein 1A/1B light chain 3B (LC3) and sequestosome 1 (SQSTM1/p62). During autophagy, the cytoplasmic form (LC3-I) is processed into a cleaved and lipidated membrane-bound form (LC3-II), which is essential for membrane biogenesis and closure of the membrane. LC3-II is recleaved by cysteine protease (Atg4B) following completion of the autophagosome, and recycled. SQSTM1/p62, one of the best-known autophagic substrates, interacts with LC3 to ensure the selective delivery of these proteins into the autophagosome (71,72). As shown in Figure 11C, the increased levels of LC3 and re-
Figure 11. Assessment of autophagy. (A) Histogram of flow cytometric analysis of mutant cybrids (III-8.48) and control cybrids (C59.32) using CYTOID® Autophagy Detection Kit. Three control and three mutant cybrids were incubated with DMEM in the absence and presence of rapamycin (inducers of autophagy) and chloroquine (lysosomal inhibitor) at 37°C for 18 h, added to CYTO-ID®-Green dye and analyzed using a Novocyte flow cytometer (ACEA Biosciences). (B) Relative ratios of Cyto-ID fluorescence intensity from three mutant and three control cell lines. Three independent determinations were done in each cell line. (C) Western blot analysis for autophagic response proteins: LC3-I/II and p62. Twenty micrograms of total cellular proteins from various cell lines were electrophoresed, electroblotted and hybridized with LC3, p62 and with β-actin as a loading control. (D) Quantification of autophagy markers LC3 I/II and p62 in mutant and control cell lines were determined as described elsewhere (70). Three independent determinations were done in each cell line. G. Graph details and symbols are explained in the legend to Figure 4.

A reduced level of p62 were observed in mutant cybrids carrying the m.4295A>G mutation, compared to those in control cybrids. In particular, the average levels of LC3-II/(LC3-I+II) and p62 in three mutant cell lines carrying the m.4295A>G mutation were 153.8% (P = 0.001) and 27.3% (P < 0.001) of the mean values measured in three control cell lines, respectively (Figure 11D). These data suggested that the m.4295A>G mutation promoted autophagy in these cybrids.

DISCUSSION

In the present study, we investigated the pathophysiology underlying deafness-associated tRNA^Ile 4295A>G mutation. This mutation was only present in the matrilineal relatives of the Chinese family with maternal inheritance of deafness. The occurrence of m.4295A>G mutation in several hearing-impaired families from different ethnic backgrounds strongly indicated that this mutation is involved in the pathogenesis of deafness (35,36). The m.4295A>G mutation affected a highly conserved adenosine (A37), adjacent to the 3’ end of the anticodon of tRNA^Ile, where the position is important for the stability and identity of tRNA, fidelity, and efficiency of translation (18–23). Here, we demonstrated that the m.4295A>G mutation-induced m1G37 had wide-ranging and significant ramifications for the structure and function of tRNA^Ile in mitochondrial translation, and remarkable effects on mitochondrial function. The creation of m1G37 modification caused by the m.4295A>G mutation was evidenced by the primer extension and enzymatic assays that M. jannaschii Trm5 catalyzed the m1G37 modification in the unmodified mutant (G37) but not wild type (A37) tRNA^Ile transcripts. In fact, t6A37 modification is responsible for codons starting with A (ANN codons), while the m1G37 modification decodes CNN codons (2,73). In particular, the t6A37 enhances the anticodon-codon base-pairing by cross-strand base-stacking of the t6A37 base with the first position of the codon, and stabilizes the structure of anticodon loop by preventing across the loop base-pairing between U33-.
A37, as well as maintains the efficiency and accuracy of translation (74–76). In contrast, the m1G37 modification stabilizes the anticodon of tRNA to prevent +1 ribosomal frameshift errors (19,22). MD stimulation revealed that mutant ASL structure of tRNAile with m1G37 were less flexible than that in wild type counterpart, thereby causing the instability of tRNAile. The altered structure and stability of tRNAile caused by the m.4295A>G mutation were further supported by the increasing melting temperature and faster electrophoretic mobility of mutated tRNA with respect to the wild-type molecule, as in the case of tRNAMet 4435A>G mutation (32). The m.4295A>G mutation resulted in the aberrant 5’ end processing of tRNAile precursors, catalyzed by RNase P, and also reduced the 3’ end processing efficiency of tRNAile precursors, mediated by RNase Z (76). Moreover, the deficient nucleotide modifications of A37 or G37 often perturbed the aminoacylation properties of tRNA (32,77,78). In this study, the cell lines bearing the m.4295A>G mutation displayed reduced aminoacylation efficiency and faster electrophoretic mobility of tRNAile, with respect to the wild-type cell lines, comparable with those with deficient tPA37 modification of tRNAThr (64). As a result, the pleiotropic effects of m.4295A>G mutation on tRNAile metabolism including the processing of tRNA precursors, stability and aminoacylation of tRNAile contributed to marked reductions in the steady-state level of tRNAile, in contrast with mild reductions in tRNAasp and tRNAmet observed in the cell lines bearing the m.7551A>G and m.4435A>G mutations (31,32). Notably, the reduced levels of tRNAile in mutant cells harboring the m.4295A>G mutation approached the proposed threshold level, which is 30% of the control levels of tRNA, to support the normal rate of mitochondrial translation (62,79–81).

The m.4295A>G mutation-induced ramifications of tRNAile metabolisms including the creation of m1G37 modification, shortage and inefficient aminoacylation impaired the mitochondrial translation and subsequently caused oxidative phosphorylation deficiency. Both pulse-labeling mitochondrial translation and Western blot assays showed ~30% reductions in the levels of mitochondrial proteins in the cells harboring the m.4295A>G mutation. Notably, the mutant cell lines carrying the m.4295A>G mutation exhibited the variable reductions in the levels of 13 mtDNA-encoded polypeptides, ranging from marked reductions in the levels of ND4 with high content of isoleucine codons, to no reduction in the levels of ATP8 with extremely low content of isoleucine codons. However, the reduced levels of these polypeptides in mutant cybrids were not significantly correlated with the numbers or proportions of isoleucines (Supplemental Table S3), in contrast with what was previously shown in cells carrying the tRNASer(UCA) 7445A>G and tRNA lys 8344A>G mutations (81,82). Alternatively, the reduced fidelity of translation may make these mitochondrial proteins to be metabolically less stable. Hence, these alterations in mitochondrial protein synthesis perturbed the stabilities and activities of complex I, III, IV and V in the cells bearing the m.4295A>G mutation. In particular, the defects in the synthesis of ND1, ND2, ND4, ND5 and ND6 resulted in the pronounced effects on the assembly and activity of complex I, as in the cases of mutations in the genes encoding complex I subunits (39,70,83). Furthermore, the impairment of mitochondrial translation yielded the respiratory and oxidative phosphorylation deficiencies, including decreased rates in the basal OCR, ATP-linked OCR, reserve capacity and maximal OCR as well as significant decreases in the level of mitochondrial ATP in the cell lines carrying the m.4295A>G mutation, as revealed by those in cell lines carrying the tRNAasp 7551A>G and tRNAmet 4435A>G mutations (31,32). As a result, the defective oxidative phosphorylation diminished mitochondrial membrane potentials and elevated the production of reactive oxygen species and the subsequent failure of cellular energetic processes (84). In particular, the effect of ROS overproduction on cellular functions was evidenced by the increasing expression of anti-oxidant enzymes SOD1, SOD2 and catalase observed in the cells bearing m.4295A>G mutation (85,86). Furthermore, the m.4295A>G mutation-induced alterations may affect the mitophagic removal of damaged mitochondria (87,88). Using flow cytometry with Cyto-ID, a cationic amphilic tracer dye, the mutant cybrids bearing the m.4295A>G mutation displayed the increasing levels of autophagy. The effect of the m.4295A>G mutation on autophagy were further evidenced by the decreased levels of p62 which indicated the preferential reduction of autophagic substrates, and increased levels of LC3-II that implied the increasing generation of autophagosome in the mutant cybrids. These data indicated that the m.4295A>G mutation promoted the autophagic degradation of ubiquitinated proteins. These mitochondrial dysfunctions may lead to the dysfunction or death of hair cells and neurons in cochlea, thereby contributing to the development of hearing loss (89,90).

However, the incomplete penetrance of deafness and relatively mild biochemical defects indicated that the m.4295A>G mutation was necessary evident but not sufficient to produce a clinical phenotype. The other genetic or epigenetic factors may contribute to the development of clinical phenotype in the subjects carrying the m.4295A>G mutation (15,24,80). In particular, the hearing specific phenotypes of this tRNA mutation may be attributed to the tissue-specificity of OXPHOS via tRNA modification or involvement of nuclear modifier genes (91–93). However, the m.4295A>G mutation is also associated with progressive tissue-specific pathologies, including hypertrophic cardiomyopathy (37) and occipital stroke (94). Changes in tRNA methylation profiles may specify cellular metabolic states and efficiently adapt protein synthesis rates to cell stress in the different tissues (95). Alternatively, the tissue heterogeneity may arise from differential expression of tRNA genes, variable activation of the integrated stress response pathway, metabolic changes and the ability of certain tissues to respond to impaired mitochondrial translation (96–98).

In summary, our findings convincingly demonstrate the pathogenic mechanism underlying the tRNA modification deficiency caused by the deafness-associated tRNAile 4295A>G mutation. The m.4295A>G mutation caused the substitution of t6A37 with the m1G37 modification, and impacted the tRNAile structure and function, including the processing of tRNA precursor, stability and aminoacylation capacity of tRNAile. The aberrant tRNA metabolism
resulted in the defects in mitochondrial translation, respiratory deficiency, decreasing membrane potentials and ATP production, and finally increasing ROS production and promoting autophagy. As a result, mitochondrial dysfunctions caused by the m.4295A>G mutation manifested hearing loss. However, the tissue-specificity of this pathogenic mtDNA mutation is likely due to the involvement of nuclear modifier genes or tissue-specific differences in tRNA metabolism. Thus, our findings highlighted the essential role of deficient tRNA modifications in mitochondrial biogenesis and their pathogenic consequence of hearing loss. Furthermore, the approach utilized in this study provides a paradigm for understanding the pathogenic effects of other mitochondrial tRNA mutations.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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