A hypertension-associated mitochondrial DNA mutation introduces an m\(^{1}\)G37 modification into tRNA\(^{\text{Met}}\), altering its structure and function

Defective nucleotide modifications of mitochondrial tRNAs have been associated with several human diseases, but their pathophysiology remains poorly understood. In this report, we investigated the pathogenic molecular mechanism underlying a hypertension-associated m.4435A→G mutation in mitochondrial tRNA\(^{\text{Met}}\). The m.4435A→G mutation affected a highly conserved adenosine at position 37, 3’ adjacent to the tRNA’s anticodon, which is important for the fidelity of codon recognition and stabilization. We hypothesized that the m.4435A→G mutation introduced an m\(^{1}\)G37 modification of tRNA\(^{\text{Met}}\), altering its structure and function. Primer extension and methylation activity assays indeed confirmed that the m.4435A→G mutation created a tRNA methyltransferase 5 (TRMT5)–catalyzed m\(^{1}\)G37 modification of tRNA\(^{\text{Met}}\). We found that this mutation altered the tRNA\(^{\text{Met}}\) structure, indicated by an increased melting temperature and electrophoretic mobility of the mutated tRNA compared with the wildtype molecule. We demonstrated that cybrid cell lines carrying the m.4435A→G mutation exhibited significantly decreased efficiency in aminoacylation and steady-state levels of tRNA\(^{\text{Met}}\), as compared with those of control cybrids. The aberrant tRNA\(^{\text{Met}}\) metabolism resulted in variable decreases in mitochondrial DNA (mtDNA)-encoded polypeptides in the mutant cybrids. Furthermore, we found that the m.4435A→G mutation caused respiratory deficiency, markedly diminished mitochondrial ATP levels and membrane potential, and increased the production of reactive oxygen species in mutant cybrids. These results demonstrated that an aberrant m\(^{1}\)G37 modification of mitochondrial tRNA\(^{\text{Met}}\) affected the structure and function of its tRNA and consequently altered mitochondrial function. Our findings provide critical insights into the pathophysiology of maternally inherited hypertension, which is manifested by the deficient tRNA nucleotide modification.

Defects in nucleotide modifications of mitochondrial tRNAs have been associated with several clinical abnormalities including cancer, diabetes, neurological disorders, deafness, and hypertension (1–4). These nucleotide modifications of human 22 mitochondrial tRNAs encoded by its own genome were catalyzed by tRNA-modifying enzymes encoded by nuclear genome (5–8). To date, 15 types of modifications have been identified in 118 positions in different mammalian mitochondrial tRNAs (9, 10). These nucleotide modifications play a vital role in the structure and function of tRNAs (11–14). Core modifications including pseudouridinylation at position 55 at the TΨC loop primarily contributed to structural stability of tRNAs and in some cases, may affect the aminoacylation (2, 3). These were exemplified by our recent discovery that the loss of pseudouridinylation at position 55 at the TΨC loop of tRNA\(^{\text{Glu}}\) caused the maternally inherited deafness and diabetes (4). Indeed, the anticodon loop modifications including nucleotides at positions 34 and 37 regulate the stabilization of anticond structure, fidelity, and efficiency of translation (2, 3, 11, 15–17). The defective 5-taurinomethyluridine (rm\(^{5}\)U) at U34 of tRNA\(^{\text{Leu(UUR)}}\) was associated with mitochondrial encephalopathy lactic acidosis and stroke-like episodes, whereas the lack of 5-taurinomethyl-2-thiouridine (rm\(^{5}\)S\(^{2}\)U) at U34 of tRNA\(^{\text{Lys}}\) was responsible for myoclonus epilepsy associated with ragged red fibers (MERRF) (18–20). Furthermore, mutations in tRNA modifying enzymes TRMU, MTO1, GTPBP3, and NSUN3 involved in nucleotide modifications at position 34 of mitochondrial tRNAs have been associated several clinical phenotypes including deafness (21–25).

The nucleotides at position 37 (A or G) of 17 mammalian mitochondrial tRNAs carry the diverse species of modifications, whereas no nucleotide modification at this position was detected in 5 tRNAs, such as tRNA\(^{\text{Met}}\) and tRNA\(^{\text{Arg}}\) (9). In fact, modifications of nucleotides A37 and G37 in human mitochondria...
Hypertension-linked mitochondrial tRNA\textsuperscript{Met} mutation

drial tRNAs were catalyzed by the modifying enzymes TRIT1 and TRMT5 encoded by nuclear genes, respectively (8, 26, 27). The modifications at position 37 contributed to the high fidelity of codon recognition and to the structural formation and stabilization of functional tRNAs (11, 15, 26, 27). The defective i\textsuperscript{A37} or m\textsuperscript{1G37} modifications caused by mutations in the TRIT1 or TRMT5 were responsible for mitochondrial dysfunction leading to clinical phenotypes (26, 27). Mutations in the nucleotides at position 37 including the tRNA\textsuperscript{Ile} 4295A→G, tRNA\textsuperscript{Asp} 7551A→G, and tRNA\textsuperscript{Met} 4435A→G mutations were associated with hypertension, diabetes, visual loss, and deafness (28–33). In particular, the tRNA\textsuperscript{Met} 4435A→G mutation was identified in four genetically unrelated Chinese families with maternal transmission of hypertension (31–33). Therefore, it was hypothesized that the substitution of A with G at position 37 of the tRNA\textsuperscript{Met} may introduce the m\textsuperscript{1G37} modification catalyzed by TRMT5, thereby altering the structure and function of tRNA\textsuperscript{Met}. In particular, the m.4435A→G mutation may affect the aminoaacetylation capacity and stability of tRNA\textsuperscript{Met} and then impair mitochondrial translation. It was also anticipated that defective mitochondrial translation caused by the m.4435A→G mutation alters the respiration, production of ATP and reactive oxygen species (ROS). To further investigate the pathogenic mechanism of the m.4435A→G mutation, cybrid cell lines were generated by transferring mitochondria from lymphoblastoid cell lines derived from an hypertensive matrilineal relative carrying the mutation and from a control subject lacking the mtDNA mutation but belonging to the same mtDNA haplogroup, into human mtDNA-less (ρ−) cells (34, 35). Using these cell lines, we investigated if the m.4435A→G mutation introduced the m\textsuperscript{1G37} modification of tRNA\textsuperscript{Met} by primer extension and methylation activity assays. These cell lines were then assessed for the effects of the m.4435A→G mutation on the stability and aminoaacetylation capacity of tRNA\textsuperscript{Met}, mitochondrial translation, enzymatic activities of electron transport chain complexes, the rate of O\textsubscript{2} consumption, ATP, and oxidative reactive species (ROS) as well as mitochondrial membrane potential.

Results

The m.4435A→G mutation created the m\textsuperscript{1G37} modification of tRNA\textsuperscript{Met}

Neither i\textsuperscript{A37} nor t\textsuperscript{A37} modification was detected in the mammalian mitochondrial tRNA\textsuperscript{Met} (9). To investigate if the m.4435A→G mutation introduced the m\textsuperscript{1G37} modification of tRNA\textsuperscript{Met}, we subjected mitochondrial RNAs from mutant and control cybrid cell lines to the reverse transcription with a digoxigenin (DIG)-labeled oligonucleotide probe specific for tRNA\textsuperscript{Met}. Arrows indicate the location of the m.4435A→G mutation. Solid lines represent the DIG-labeled oligonucleotide probe specific for tRNA\textsuperscript{Met}. Broken lines represented the potential stops of primer extension caused by the m\textsuperscript{1G37} modification. B, primer extension demonstrated the creation of m\textsuperscript{1G37} in the tRNA\textsuperscript{Met} carrying the m.4435A→G mutation. The primer extension termination products caused by m\textsuperscript{1G37} modification are showed.

* Methanocaldococcus jannaschii Trm5 (Mj-Trm5) catalyzed the m\textsuperscript{1G37} modification in mutant tRNA\textsuperscript{Met} transcripts

Trm5 is one of the tRNA (m\textsuperscript{G37})-methyltransferases that catalyzes the identical tRNA modification, m\textsuperscript{G37} (36, 37). To further examine if the m.4435A→G mutation introduced the m\textsuperscript{1G37} modification of tRNA\textsuperscript{Met}, we prepared wildtype and mutant tRNAs by in vitro transcription and evaluated the methylation activity catalyzed by the recombinant M. jannaschii Trm5 (Mj-Trm5) (36, 37). As shown in Fig. 2A, the mutant tRNA\textsuperscript{Met} transcripts (G37) were modified with m\textsuperscript{1G37} modification by the Mj-Trm5 but as less efficiently as cytoplasmic tRNA\textsuperscript{Leu}(CA\textsubscript{G}) transcripts (G37) (10). In contrast, the modification was not detected in the wildtype tRNA\textsuperscript{Met} transcripts (A37) in the presence of Mj-Trm5. As controls, the human cytoplasmic tRNA\textsuperscript{Leu}(CA\textsubscript{G}) transcripts (G37) were modified by the Mj-Trm5, whereas human cytoplasmic tRNA\textsuperscript{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\textsuperscript{1G37} modification of tRNA\textsuperscript{Met}.

We then analyzed the binding affinities of tRNA\textsuperscript{Met} with Mj-Trm5 using an electrophoretic mobility shift assay. The shift representing the Trm5–tRNA complex occurred at an enzyme concentration of 0.25 μM. As shown in Fig. 2B, the mutant...
Hypertension-linked mitochondrial tRNA\textsuperscript{Met} mutation

Figure 2. Methylation activity assays. The unmodified human mitochondrial wildtype (A37) and mutant (G37) tRNA\textsuperscript{Met}, cytosolic tRNA\textsuperscript{Leu(UUR)}, and tRNA\textsuperscript{Thr} were generated from in vitro transcription. A, analysis for the m\textsuperscript{1}G37 modification of tRNA\textsuperscript{Met}. The unmodified tRNA transcripts were incubated with M.\textit{jannaschi} (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. Graph shows the results of a representative experiment. B, electrophoretic mobility shift assay. The unmodified mitochondrial wildtype (A37) and mutant (G37) tRNA\textsuperscript{Met} were incubated with various concentrations of enzymes Mj-Trm5. These samples were electrophoresed through 6% polyacrylamide gel and stained with ethidium bromide.

and wildtype tRNA\textsuperscript{Met} transcripts were fully shifted to the \textit{Mj-Trm5}–tRNA complex at enzyme concentrations of 1.5 and 4.0 \textmu M, with the calculated \(K_{d}\) value of 1.105 \pm 0.028 and 1.822 \pm 0.025 \textmu M, respectively. These data provided further evidence that the m.4435A→G mutation introduced the m\textsuperscript{1}G37 modification of tRNA\textsuperscript{Met}.

Altered conformation and stability of tRNA\textsuperscript{Met}

It was anticipated that the m.4435A→G mutation caused structural alteration and the instability of tRNA\textsuperscript{Met}. To test if the m.4435A→G mutation affected the conformation of tRNA\textsuperscript{Met}, total RNAs from mitochondria isolated from mutant and control cell lines were electrophoresed through a 10% native gel and then electroblotted onto a positively charged nylon membrane for hybridization analysis with oligodeoxynucleotide probes for tRNA\textsuperscript{Met} and tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Leu(CUN)} and tRNA\textsuperscript{Ser(AGY)}, respectively. As shown in Fig. 3A, electrophoretic patterns showed that the tRNA\textsuperscript{Met} in three mutant cybrid cell lines carrying the m.4435A→G mutation migrated faster than those of control cybrid cell lines lacking this mutation.

We then examined the stability of the wildtype and mutant tRNA\textsuperscript{Met} transcripts through the measurement of the melting temperatures (\(T_m\)) by calculating the derivatives of the absorbance against a temperature curve. As shown in Fig. 3B, the \(T_m\) values for wildtype (A37) and mutant (G37) transcripts were 43.52 \pm 0.71 and 46.02 \pm 0.41 °C, respectively. These results suggested that the m.4435A→G mutation affected the stability of tRNA\textsuperscript{Met}.

Marked decrease in the steady-state levels of tRNA\textsuperscript{Met}

To assess if the m.4435A→G mutation ablated the metabolism of tRNA\textsuperscript{Met}, we subjected mitochondrial RNAs from mutant and control cybrid cell lines to Northern blots and hybridized them with DIG-labeled oligodeoxynucleotide probes for tRNA\textsuperscript{Met}, tRNA\textsuperscript{Lys}, tRNA\textsuperscript{Thr}, and tRNA\textsuperscript{Leu(CUN)} derived from the heavy (H)-strand transcription unit and tRNA\textsuperscript{Ser(UCN)} and tRNA\textsuperscript{Glu} derived from the light (L)-strand transcription unit (38, 39). As shown in Fig. 4A, the steady-state level of tRNA\textsuperscript{Met} in the mutant cells was markedly decreased compared with those in control cells. For comparison, the average levels of tRNA\textsuperscript{Met} in the mutant cybrid cell lines were among ~74.4, 69.5, 73.3, 64.9, and 75.3% of average values of three control cybrids after normalization to tRNA\textsuperscript{Lys}, tRNA\textsuperscript{Thr}, tRNA\textsuperscript{Leu(CUN)}, tRNA\textsuperscript{Ser(UCN)}, and tRNA\textsuperscript{Glu} (\(p = 0.0001\) to 0.0003), respectively.

Deficient aminoacylation of tRNA\textsuperscript{Met}

To evaluate if the m.4435A→G mutation affects aminoacylation of tRNA, we examined the aminoacylation level of tRNA\textsuperscript{Met} as well as tRNA\textsuperscript{Leu(UUR)}, tRNA\textsuperscript{Leu(CUN)}, tRNA\textsuperscript{His}, and tRNA\textsuperscript{His} by the use of electrophoresis in an acidic urea PAGE system to separate uncharged tRNA species from the corresponding charged tRNA, electroblotting and hybridizing with the tRNA probes described above. As shown in Fig. 5A, the upper band represents the charged tRNA, and the lower band represents uncharged tRNA. There were no obvious differences in electrophoretic mobility between the control and mutant cell lines. To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of tRNAs were deacylated by being heated for 10 min at 60 °C (pH 8.3) and then run in parallel. As shown in Fig. 5B, only one band (uncharged tRNA) was present in both mutant and control cell lines after deacylation. However, the efficiencies of aminoacylated tRNA\textsuperscript{Met} in the mutant cell lines varied from 61.6 to 83.2%, with the average of 69%, relative to the average values of control cell lines (\(p = 0.034\) (Fig. 5C). However, the levels of aminoacylation in tRNA\textsuperscript{Leu(UUR)}, tRNA\textsuperscript{Leu(CUN)}, tRNA\textsuperscript{His}, and tRNA\textsuperscript{His} in mutant cell lines were comparable with those in the control cell lines.

Decreases in the levels of mitochondrial proteins

To investigate whether the aberrant tRNA metabolism caused by the m.4435A→G mutation impaired mitochondrial translation, a Western blot analysis was carried out to examine the steady-state levels of seven oxidative phosphorylation subunits of (encoded by mtDNA) in mutant (5) and control cell lines, whereas Tom20 (encoded by nuclear gene) as a loading control. As shown in Fig. 6A, the overall levels of seven mitochondrial translation products in the mutant cell lines ranged from ~29.6 to 97.5%, with an average of 67.2% (\(p < 0.0001\)), relative to the mean value measured in the control cell lines. However, there were variable reductions in levels of ND3, ND4, and ND5, subunits 3, 4, and 5 of NADH dehydrogenase; ATP6, ATP8, subunits 6 and 8 of the \(F^+\)–ATPase; CYTB, apocytochrome \(b\); CO\textsubscript{2}, subunit II of cytochrome \(c\) oxidase in mutant cell lines. In particular, mutant cell lines exhibited marked reductions (49.4 to 70.4%) in the levels of ND3, ATP6, and CYTB harboring 3.9 to 7.0% methionine codons, whereas rela-
tively mild reductions (2.5 to 22.9%) of the levels of ND4, ND5, ATP8, and CO2 containing 4.3 to 8.7% methionine codons were observed in mutant cell lines. However, the levels of polypeptide synthesis in mutant cells, relative to those in control cells, showed no significant correlation with either the number of codons or the proportion of methionine (Table S1).

Reduced activities of respiratory chain complexes I, III, IV, and V

To evaluate the effect of the m.4435A→G mutation on the oxidative phosphorylation, we measured the activities of respiratory complexes by the use of isolating mitochondria from
three mutant and three control cell lines. The activity of Complex I (NADH ubiquinone oxidoreductase) was determined through the oxidation of NADH with ubiquinone as the electron acceptor (40–42). Complex II (succinate ubiquinone oxidoreductase) was the only respiratory complex that was encoded by the nuclear DNA, so we examined the activity of complex II through the artificial electron acceptor dichlorophenolindophenol (41, 42). 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). The activity of complex V (F1-ATP synthase) was explored through the NADH oxidation via conversion of phosphoenolpyruvate to lactate by a two-step reaction (41). As shown in Fig. 7, the activity of complexes I, III, IV, and V in the mutant cells carrying the m.4435A→G mutation were 78.8 (%/H11005 0.013), 71.6 (%/H11021 0.001), 81.1 (%/H11005 0.003), and 73.0% (%/H11005 0.018) of the mean value measured in three control cell lines, respectively, whereas the activity of complex II in the mutant cells carrying the m.4435A→G mutation was 99.5% of the mean value measured in three control cell lines (%/H11005 0.868), which was similar to the activity of complex II in the wildtype cell lines as expected.

Respiration deficiency

To evaluate if the m.4435A→G mutation alters cellular bioenergetics, we examined the oxygen consumption rates (OCR) of three mutant cell lines carrying the m.4435A→G mutation and three control cell lines (43). As shown in Fig. 8, the basal OCR in mutant cell lines was 57.8% (%/H11021 0.001) relative to the mean value measured in three control cell lines, whereas the activity of complex II in the mutant cells carrying the m.4435A→G mutation was 99.5% of the mean value measured in three control cell lines (%/H11005 0.880), relative to the mean value measured in the control cell lines, respectively.

Reduced level in mitochondrial ATP production

We used the luciferin/luciferase assay to examine the capacity of oxidative phosphorylation in mutant and wildtype cell lines. Populations of cells were incubated in the media in the presence of glucose, and 2-deoxy-D-glucose with pyruvate (44). As shown in Fig. 9A, the levels of ATP production in mutant cell lines in the presence of glucose (total cellular levels of ATP) were comparable with those measured in control cell lines. In contrast, as shown in Fig. 9B, 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 66.0 to 73.1%, with an average of 69.8% relative to the mean value measured in the control cell lines (%/H11005 0.001).
Decrease in mitochondrial membrane potential

The mitochondrial membrane potentials (ΔΨm) were measured through the fluorescence probe JC-10 assay system in three mutant and three control cell lines (45). The ratio of fluorescence intensities excitation/emission = 490/590 and 490/530 nm (FL590/FL530) were recorded to delineate the ΔΨm of each sample. The relative ratios of FL590/FL530 geometric mean between mutant and control cell lines were calculated to represent the level of ΔΨm, as described elsewhere (44). As shown in Fig. 10, the ΔΨm of three mutant cell lines carrying the m.4435A→G mutation ranged from 62.8 to 64.0%, with an average 63.4% (p < 0.001) of the mean value measured in three control cell lines. In contrast, the levels of ΔΨm in mutant cell lines in the presence of FCCP were comparable with those of control cell lines (p = 0.290).

The increase of ROS production

The levels of mitochondrial ROS among the cybrids derived from three mutant cybrid cell lines carrying the m.4435A→G mutation and three control cybrid cell lines lacking the mutation were determined using MitoSOX assay via flow cytometry under normal conditions and then following H2O2 stimulation (46–48). Geometric mean intensity was recorded to measure the production rate of ROS of each sample. As shown in Fig. 11, the ΔΨm of three mutant cell lines carrying the m.4435A→G mutation ranged from 62.8 to 64.0%, with an average 63.4% (p < 0.001) of the mean value measured in three control cell lines. In contrast, the levels of ΔΨm in mutant cell lines in the presence of FCCP were comparable with those of control cell lines (p = 0.290).

Discussion

In the present study, we investigated the pathogenic mechanism of the hypertension-associated m.4435A→G mutation in the tRNAMet gene. The occurrences of the m.4435A→G mutation in four genetically unrelated Chinese families affected by hypertension strongly indicate that this mutation is involved in the pathogenesis of this disease (31–33). The m.4435A→G mutation affected a highly conserved adenosine (A37), adjacent to the 3’ end of the anticodon of tRNAMet (3, 7). The nucleotides at position 37 (A or G) of tRNAs are often modified, by such processes as thiolation and methylthiolation (3, 15). However, nucleotides at this position in 5 of 22 mammalian mitochondrial tRNAs including tRNAMet and tRNAAsp were not modified (9, 28). In particular, neither i6A37 nor t6A37 modification was detected in the mammalian mitochondrial tRNAMet (9). Therefore, it was hypothesized that the substitution of A37 with G37 caused by the m.4435A→G mutation created the m1G37 modification of tRNAMet, catalyzed by TRMT5.

In vitro assays showed that the m.4435A→G mutation impaired the f5C formation mediated by NSUN3 (49). In this study, the primer extension experiment revealed that the m.4435A→G mutation introduced the m1G37 modification of tRNAMet. This hypothesis was further supported by the fact that an archaea M. jannaschii Trm5 catalyzed the m1G37 modification in the unmodified mutant (G37) but not wildtype (A37) tRNAMet transcripts. Furthermore, an electrophoretic mobility shift assay showed that M. jannaschii Trm5 had higher affinity with the mutant tRNA Met transcripts (G37) than the wildtype tRNAMet transcript (A37). These data demonstrated that the m.4435A→G mutation created the m1G37 modification of tRNAMet, catalyzed by TRMT5.

Modification at position 37 contributes to the high fidelity of codon recognition and the structural formation and stabilization of functional tRNAs (3, 15, 50, 51). In Escherichia coli, the deficient modification of A37 decreased the activity of the corresponding tRNA (52) and increased +1 frameshifts for tRNA^Phe (53), whereas the A to G substitution at position 37 led to a 10-fold reduction in the section of tRNAs at the A-site (54). Therefore, the m1G37 modification introduced...
by the m.4435A→G mutation altered the structure and function of tRNA^{Met}, as in the case of m.7551A→G mutation in the tRNA^{Asp} (28). Here, the altered structure of tRNA^{Met} caused by the m.4435A→G mutation was evidenced by the increased melting temperature and electrophoretic mobility of mutated tRNA with respect to the wildtype molecule. Furthermore, the m.4435A→G mutation caused 31% reduction in aminoacylation efficiency of tRNA^{Met} in mutant cell lines, in contrast to the increasing efficiency of aminoacylation in several tRNAs caused by TRMU mutation (22). Both altered structure and improper aminoacylation of tRNA^{Met} made the mutant tRNA^{Met} to be metabolically less stable and more subject to degradation, thereby lowering the level of this tRNA, as in the case of tRNA^{Asp} 7551A→G mutation (28). In the present study, 40% reductions in the steady-state level of tRNA^{Met} observed in mutant cell lines were consistent with the previous observations in the lymphoblastoid cell lines bearing the m.4435A→G mutation (30, 31). However, the reduced levels of tRNA^{Met} in mutant cells harboring the m.4435A→G mutation were indeed above the proposed threshold, which is 30% of the control levels of tRNA, to support the normal rate of mitochondrial translation (39, 44, 55, 56), indicating that the m.4435A→G mutation itself is insufficient to produce a clinical phenotype.

The inefficient aminoacylation and shortage of tRNA^{Met}, or the faulty interaction between mutant tRNA^{Met} and the translational machinery, contributed to the defective mitochondrial translation (57, 58). In the present study, reduced levels of mitochondrial proteins (an average decrease of ~33%) were compa-
Hypertension-linked mitochondrial tRNAMet mutation

ATP synthesis, oxidative stress, and subsequent failure of the cellular energetic process (59). In this investigation, 32% decreases in mitochondrial ATP production in the cell lines carrying the m.4435A→G mutation, which may be caused by the defective activity of complexes I, III, IV, and V, was comparable with those in cell lines carrying the tRNA^{Glu} 14692T→C, tRNA^{Asp} 7551A→G, and tRNA^{Ala} 5655A→G mutations (4, 28, 35). Furthermore, the deficient oxidative phosphorylation often affected mitochondrial membrane potentials, which reflect the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation (44, 46). In this study, 37% reduction in mitochondrial membrane potential in cybrid cell lines carrying the m.4435A→G mutation was comparable with those in cell lines carrying the tRNA^{Glu} 14692T→C and tRNA^{Asp} 7551A→G (4, 28). The abnormal oxidative phosphorylation and mitochondrial membrane potential enhanced the production of reactive oxygen species and the subsequent failure of cellular energetic processes in mutant cells carrying the m.4435A→G mutation. In particular, the overproduction of ROS can establish a vicious cycle of oxidative stress in the mitochondria, thereby damaging mitochondrial and cellular proteins, lipids, and nucleic acids and increasing apoptotic signaling (60, 61). The skeletal and vascular smooth muscles may be preferentially involved because they were exquisitely sensitive to inefficient metabolism, subtle imbalance in cellular redox state, and increased level of free radicals (62, 63). An inefficient metabolism caused by the mitochondrial dysfunction in skeletal and vascular smooth muscles may lead to the elevation of systolic blood pressure and therefore be involved in hypertension. The relative mild mitochondrial dysfunction caused by the m.4435A→G mutation suggested that the mutation is an inherited risk factor necessary for the development of hypertension but may by itself be insufficient to produce a clinical phenotype. The nuclear genetic or epigenetic factors and lifestyles may contribute to the development of clinical phenotypes in subjects bearing the m.4435A→G mutation (64, 65). In particular, the tissue-specific effect of this tRNA mutation may be attributed to the tissue-specific RNA metabolism or the involvement of nuclear modifier genes (66–69).

In summary, our findings suggested the pathogenic mechanism leading to an impaired oxidative phosphorylation in cells carrying the hypertension-associated m.4435A→G mutation in the tRNA^{Met} gene. The m.4435A→G mutation altered the structure and function of tRNA^{Met}. The aberrant tRNA metabolism resulted in the defects in mitochondrial translation, respiratory deficiency, decreasing membrane potentials and ATP production, and finally increasing ROS production. As a result, mitochondrial dysfunction caused by the m.4435A→G mutation manifests hypertension. 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 may provide new insights into the pathophysiology of hypertension, which was manifested by the deficient modification of mitochondrial tRNA^{Met}.
Figure 11. Measurement of ROS. Ratio of geometric mean intensity between levels of the ROS generation in the vital cells with or without H₂O₂ stimulation. The rates of production in ROS from three mutant cell lines and three control cell lines were analyzed by BD-LSR II flow cytometer system using MitoSox (5 μM) either without (A) or with (C) H₂O₂ stimulation. The relative ratio of intensity (stimulated or unstimulated with H₂O₂) was calculated. B and D, the average of three independent determinations for each cell lines is shown. Graph details and symbols are explained in the legend to Fig. 4.

Figure 10. Mitochondrial membrane potential analysis. ΔΨₘ was measured in three mutant and three control cell lines using a fluorescence probe JC-10 assay system. The ratio of fluorescence intensities excitation/emission = 490/590 and 490/530 nm (FL590/FL530) were recorded to delineate the ΔΨₘ level of each sample. Represented flow cytometry images of cell lines II-9.2 and C59.8 with (A) and without (B) 10 μM FCCP. Relative ratio of JC-10 fluorescence intensities at excitation/emission = 490/530 and 490/590 nm in the absence (C) and presence (D) of 10 μM FCCP. The average of three to five determinations for each cell line are shown. Graph details and symbols are explained in the legend to Fig. 4.
Hypertension-linked mitochondrial tRNAMet mutation

Experimental procedures

Cell lines and culture conditions

Immortalized lymphoblastoid cell lines were generated from one hypertensive matrilineal relative (II-9) of a Chinese family carrying the m.4435A→G mutation (33) and one genetically unrelated Chinese control individual (C59) belonging to the same mtDNA haplogroup B5 but lacking the mutation (Table S2) (70). These cell lines were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS). The bromodeoxyuridine (BrdU)-resistant 143B.TK− cell line was grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies) (containing 4.5 mg of glucose and 0.11 mg of pyruvate/ml), supplemented with 100 μg of BrdU/ml and 5% FBS. The mtDNA-less ρo206 cell line, derived from 143B.TK− (34, 35, 71) was grown under the same conditions as the parental line, except for the addition of 50 μg of uridine/ml. Transformation by cytoplasts of mtDNA-less ρo206 cells using one affected subject (II-9) carrying the m.4435A→G mutation and one control individual (C59) was performed as described elsewhere (34, 35, 71). All cybrid cell lines constructed with enucleated lymphoblastoid cell lines were maintained in the same medium as the 143B.TK− cell line. An analysis for the presence and level of the m.4435A→G mutation was carried out as described previously (Fig. S1) (31). Quantification of the mtDNA copy number from different cybrids was performed as detailed elsewhere (47). Three mutant cybrids (II-9.2, II-9.4, and II-9.5) carrying the m.4435A→G mutation and three control cybrids (C59.8, C59.10, and C59.12) lacking the mutation with similar mtDNA copy numbers and the same karyotype were used for the biochemical characterization described below.

Primer extension assay

A primer extension experiment to analyze the m1G37 modification of tRNAMet was carried out by a modified procedure, as described elsewhere (72, 73). Total mitochondrial RNAs were obtained from mitochondria isolated from mutant and control cell lines (~2.0 × 10^6 cells) using the TOTALLY RNATM kit (Ambion), as described previously (74). A DNA primer (5'-TGGTGTACGGGAAAGGTTAAACACATT-3') complementary to the 3' end of the tRNAMet was 5' end labeled with DIG. Two μg of total mitochondrial RNA as templates and 1 μM DIG-labeled oligodeoxynucleotide probe specific for the tRNAMet were used in the PrimeScript II 1st Strand cDNA Synthesis Kit (TAKARA) for reverse transcription. Extension reactions were carried out as detailed previously (72, 73). The samples were applied onto 15% PAGE without (native gel) or with (denature gel) 8M urea. The fragments spanning tRNAMet (corresponding to mtDNA at positions 4402–4469) were PCR-amplified genomic DNAs from one hypertensive subject (II-9) carrying the m.4435A→G mutation and control subject (C59) and cloned into the HindIII/BamHI sites of pUC19. The plasmid carrying the human cytoplasmic tRNALeu(CAG) and tRNAThr genes were the gifts from Dr. En-Duo Wang (75, 76). The unmodified tRNAs were generated by in vitro transcription using T7 RNA polymerase, as detailed elsewhere (77). The purifications of archaean Trm5 (aTrm5) from M. jannaschii (Mj-Trm5) was performed as detailed previously (36, 37). The methylation reaction contained 200 μM [3H]SAM, 50 mm Tris-HCl (pH 7.0), 100 mm KCl, 10 mm MgCl2, 100 μg/ml of bovine serum albumin (BSA), 5 mm DTT, and 5 μM transcribed wildtype or mutant tRNAMet. The reaction was initiated by the addition of 2 μM Mj-Trm5. Reactions were performed under identical conditions at 37 °C, at time intervals ranging between 2 and 8 min, aliquots of 5 μl were removed, absorbed on paper discs, and precipitated in trichloroacetic acid.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described elsewhere (78, 79). A range of 0.25–8 μM Mj-Trm5 was incubated with 100 nm tRNA in the presence of a 30-μl reaction volume including 50 mm Tris-HCl (pH 8.0), 100 mm KCl, 5 mm MgCl2, and 20% glycerol at 37 °C for 10 min. After incubation, each sample was loaded onto a 6% polyacrylamide native gel immediately after adding loading buffer. The gel was stained with ethidium bromide.

UV melting assays

UV melting assays were carried out as previously described (4, 80). The wildtype and mutant tRNAMet transcripts were generated as described above. The tRNAMet transcripts were diluted in the buffer including 50 mm sodium phosphate (pH 7.0), 50 mm NaCl, 5 mm MgCl2, and 0.1 mm EDTA. Absorbance against temperature melting curves were measured at 260 nm with a heating rate of 0.5 °C/min from 25 to 95 °C through Agilent Cary 100 UV Spectrophotometer.

Mitochondrial tRNA analysis

For the tRNA Northern blot analysis, 2 μg of total mitochondrial RNAs were electrophoresed through a 10% polyacrylamide gel without (native gel) or with (denature gel) 8 μl urea. The gels were then electroblotted onto a positively charged nylon membrane (Roche), and the membranes were washed with 20 ml of washing buffer twice and equilibrated with detection buffer (0.1M Tris-HCl, 0.3% (v/v) Tween 20 (pH 7.5)), incubated for 30 min in blocking solution (dilute blocking reagent (Roche) in 0.1 μL maleic acid, 0.15 μL NaCl, 0.3% (v/v) Tween 20 (pH 7.5)), and then incubated for 30 min in Antibody solution (dilute anti-digoxigenin-AP Fab fragments (Roche), 1:10,000, in blocking solution). Anti-digoxigenin-AP Fab fragments for binding to the hybridized probes were used for the immunological detection. After incubation, the membranes were washed with 20 μl of washing buffer twice and equilibrated with detection buffer (0.1 μL Tris-Cl, 0.1 μL NaCl (pH 9.5)). CDP-Star, ready-to-use solution (Roche) was used to soak the membrane evenly before exposure and signals were detected using the ECL system (CWBIO). After exposure, the membranes were washed with sterile diethyl pyrocarbon-
ate-treated distilled water, incubated for 2 × 60 min at 80 °C in stripping buffer (50 mM Tris-HCl (pH 7.5), 50% formamide, 5% SDS), then washed for 2 × 5 min in 2 × SSC (300 mM NaCl, 30 mM sodium citrate (pH 7.0)) before prehybridization and hybridization with the next probe. To reduce the loss of tRNA during stripping, fresh stripping buffers were used for each time under sterile RNase-free conditions. The DIG-labeled oligodeoxynucleotides were generated by using a DIG oligonucleotide tailing kit (Roche). The hybridization and quantification of denoxynucleotides were generated by using a DIG oligonucleotide under sterile RNase-free conditions. The DIG-labeled oligodeoxynucleotide mitochondrial RNAs was electrophoresed at 4 °C through an acid (Promega) was used for the measurement of cellular and mitochondrial ATP levels, following the modified manufacturer’s instructions (44, 46).

**Assessment of mitochondrial membrane potential**
Mitochondrial membrane potential was assessed with a JC-10 Assay Kit-Microplate (Abcam) according to the general manufacturer’s recommendations with some modifications, as detailed elsewhere (44, 45).

**Measurement of ROS production**
ROS measurements were performed as detailed previously (29, 35, 47, 48).

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


**Acknowledgments**—We are grateful to Dr. En-Duo Wang (Shanghai Institute of Biochemistry and Cell Biology) for the pTrc99B-tRNA Lys (CAG) and pTrc99B-tRNA Thr plasmids and Dr. Shigeyuki Yokoyama (RIKEN Structural Biology Laboratory) for the pET26b-aTrm5 plasmid. We thank Dr. Gilbert Eriani (Institut de Biologie Moléculaire et Cellulaire) for the critical comments of this manuscript.

**References**
Hypertension-linked mitochondrial tRNA^{Met} mutation


Hypertension-linked mitochondrial tRNA<sup>Met</sup> mutation

drial DNA mutation alters the tertiary interaction and function of tRNALeu<sup>UUR</sup>. *J. Biol. Chem.* **292**, 13934–13946 CrossRef Medline

