Overexpression of mitochondrial histidyl-tRNA synthetase restores mitochondrial dysfunction caused by a deafness-associated tRNA\(^{\text{His}}\) mutation

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The deafness-associated m.12201T>C mutation affects the A5-U68 base-pairing within the acceptor stem of mitochondrial tRNA\(^{\text{His}}\). The primary defect in this mutation is an alteration in tRNA\(^{\text{His}}\) aminoaacylation. Here, we further investigate the molecular mechanism of the deafness-associated tRNA\(^{\text{His}}\) m.12201T>C mutation and test whether the overexpression of the human mitochondrial histidyl-tRNA synthetase gene (HARS2) in cytoplasmic hybrid (cybrid) cells carrying the m.12201T>C mutation reverses mitochondrial dysfunctions. Using molecular dynamics simulations, we demonstrate that the m.12201T>C mutation perturbs the tRNA\(^{\text{His}}\) structure and function, supported by decreased melting temperature, conformational changes, and instability of mutated tRNA. We show that the m.12201T>C mutation-induced alteration of aminoaacylation tRNA\(^{\text{His}}\) causes mitochondrial translational defects and respiratory deficiency. We found that the transfer of HARS2 into the cybrids carrying the m.12201T>C mutation raises the levels of aminoaacylated tRNA\(^{\text{His}}\) from 56.3 to 75.0% but does not change the aminoaacylation of other tRNAs. Strikingly, HARS2 overexpression increased the steady-state levels of tRNA\(^{\text{His}}\) and of noncognate tRNAs, including tRNA\(^{\text{Ala}}\), tRNA\(^{\text{Gln}}\), tRNA\(^{\text{Glu}}\), tRNA\(^{\text{Leu(UUR)}}\), tRNA\(^{\text{Asp}}\), and tRNA\(^{\text{Met}}\), in cells bearing the m.12201T>C mutation. This improved tRNA metabolism elevated the efficiency of mitochondrial translation, activities of oxidative phosphorylation complexes, and respiration capacity. Furthermore, HARS2 overexpression markedly increased mitochondrial ATP levels and membrane potential and reduced production of reactive oxygen species in cells carrying the m.12201T>C mutation. These results indicate that HARS2 overexpression corrects the mitochondrial dysfunction caused by the tRNA\(^{\text{His}}\) mutation. These findings provide critical insights into the pathophysiology of mitochondrial disease and represent a step toward improved therapeutic interventions for mitochondrial disorders.

Mitochondrial DNA (mtDNA)\(^2\)-dependent defects have been associated with hearing deficit, either the nonsyndromic form (where hearing loss is the only obvious medical problem) or the syndromic form (hearing loss with other medical problems, such as diabetes) (1–5). The majority of these deafness-linked mtDNA mutations are located in the mitochondrial 12S rRNA and tRNA genes (1–6). The m.1555A>G and m.1494C>T mutations in the 12S rRNA gene have been associated with both aminoglycoside-induced and nonsyndromic deafness in many families worldwide (1, 2, 7–9). Mitochondrial tRNAs are the hot spots for mutations associated with hearing loss (3, 10, 11). The syndromic deafness-associated mtDNA mutations are the MELAS-associated tRNA\(^{\text{Leu(UUR)}}\) 3243A>G mutation (12) and MERRF-associated tRNA\(^{\text{Ala}}\) 3444A>G mutation (13) and MDD-associated tRNA\(^{\text{Glu}}\) 14692A>G (14), whereas the nonsyndromic deafness-associated mtDNA mutations included the tRNA\(^{\text{Ser(UCC)}}\) 7445A>G, 7505T>C, and 7511T>C, tRNA\(^{\text{His}}\) 12201T>C, tRNA\(^{\text{Asp}}\) 7551A>G, and tRNA\(^{\text{Leu}}\) 4295A>G mutations (15–20). These tRNA mutations have structural and functional consequences, including the processing of the tRNA from the primary transcripts, stability of the folded secondary structure, the charging of the tRNA, or the

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This article contains Table S1 and Fig. S1.

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2 The abbreviations used are: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; DIG, digoxigenin; FBS, fetal bovine serum; OCR, oxygen consumption rate; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; nt, nucleotides.
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codon-anticodon interaction in the process of translation (14–19). Of these, the m.12201T>C mutation resided at the uridine at position 68 (U68) and abolished a base-pairing (5A-68U) on the acceptor stem of the tRNA\textsuperscript{His} that may play an important role in the stability and identity of tRNA (18, 21, 22) (Fig. 1A). The primary defect in this mutation was the aberrant aminoacylation of the tRNA\textsuperscript{His} (18). The deficient aminoacylation of tRNA\textsuperscript{His} mainly contributed to a shortage of tRNA\textsuperscript{His}, thereby causing the reduced rate of mitochondrial protein synthesis and respiration defects (18).

A specific cognate amino acid is charged or aminoacylated to each tRNA catalyzed by aminoacyl tRNA synthetase (10, 11). Therefore, the deficient aminoacylation of tRNAs may be restored by the transfer of aminoacyl tRNA synthetase in cells carrying the tRNA\textsuperscript{mutation}. In the previous studies, the over-expression of human mitochondrial leucyl-tRNA synthetase in the cybrid cells carrying the tRNA\textsuperscript{Aeu(UUR)} 3243A>G mutation improved the efficiency of aminoacylation and stability of mitochondrial tRNA\textsuperscript{Leu(UUR)} and oxidative phosphorylation (23–26). The aberrant tRNA\textsuperscript{Ala} and tRNA\textsuperscript{Val} metabolisms were restored by overexpression of AARS2 and VARS2 in the cells carrying the tRNA\textsuperscript{Ala} 5565A>G or tRNA\textsuperscript{Val} 1624C>T mutation, respectively (27, 28). In fact, human mitochondrial histidyl-tRNA synthetase is a highly conserved enzyme composed of 506 amino acids with a mitochondrial signal sequence (29).

Thus, it is anticipated that the overexpression of human HARS2 in the cybrid cells carrying the m.12201T>C mutation would improve the aminoacylation capacity of tRNA\textsuperscript{His}, enhance the stability of tRNA, and then increase the rates of mitochondrial translation and respiration, consequently correcting the mitochondrial dysfunction. To test this hypothesis, stable transfectants were constructed by transferring a human HARS2 cDNA into a cybrid cell line carrying the m.12201T>C mutation and a control cybrid cell line harboring the WT version of tRNA\textsuperscript{His}. Human HARS2 was further characterized by examining subcellular locations. These stable transfectants were analyzed for the aminoacylation capacity of tRNAs, the stability of the tRNA\textsuperscript{His}, the rates of mitochondrial translation and respiration, the levels of mitochondrial ATP, and the mitochondrial membrane potential (ΔΨ\textsubscript{m}) as well as the production of reactive oxygen species (ROS).

Results

MD simulation analyses

To assess the impact of m.12201T>C mutation on the tertiary structure of tRNA\textsuperscript{His}, we carried out the molecular dynamics simulation using the acceptor stems (15 nt) of both WT and mutant tRNA\textsuperscript{His} by the 100-ns all-atom method. As shown in Fig. 1B, the root mean square deviation (RMSD) curve of the mutated acceptor stem fluctuated more significantly than those of the WT counterpart, suggesting that the mutated acceptor stem exhibited more instability than its WT counterpart. Using the cpptraj program Amber14, the U68 formed a canonical base pair with A5 in the WT tRNA\textsuperscript{His} through hydrogen bonds with an occupancy of 47 and 64%, respectively (Fig. 1, C and D). In contrast, the newly formed interaction between A5 and C68 in the mutant tRNA\textsuperscript{His} was decreased to one hydrogen bond with a lower occupancy of 38%. These data implied that the mutant tRNA\textsuperscript{His} molecule with the mismatch of 5A and C68 may be less stable than those in the WT counterpart.

Aberrant stability and conformation of tRNA\textsuperscript{His}

To experimentally test whether there was an effect of the m.12201T>C mutation on the stability of tRNA\textsuperscript{His}, we measured the melting temperatures (T\textsubscript{m}) of WT and mutant tRNA\textsuperscript{His} transcripts by calculating the derivatives of absorbance against a temperature curve. As shown in Fig. 2A, the T\textsubscript{m} values of WT (U68) and mutant (C68) transcripts were 47.0 ± 1.0 and 43.0 ± 1.7 °C, respectively. These data were in a good agreement with data of molecular stimulation, indicating that the m.12201T>C mutation led to the instability of tRNA\textsuperscript{His}.

These transcripts were then assessed for conformational change by PAGE analysis under denaturing and native conditions. As shown in Fig. 2B, electrophoretic patterns showed that the mutant (C68) tRNA\textsuperscript{His} transcript migrated faster than the WT (U68) tRNA\textsuperscript{His} transcript under native conditions. However, there were no differences in the migration pattern between WT (U68) and mutant (C68) tRNA\textsuperscript{His} transcripts under denaturing conditions. To further test whether the m.12201T>C mutation affected the conformation of tRNA\textsuperscript{His} in vivo, total RNAs isolated from mutant and control cybrids were electrophoresed through 10% native polyacrylamide gel in Tris-glycine buffer and then electroblotted onto a positively charged nylon membrane for hybridization analysis with digoxigenin (DIG)-labeled oligodeoxynucleotide probes for tRNA\textsuperscript{His} and tRNA\textsuperscript{Leu(UUR)}, respectively. As shown in Fig. 2C, electrophoretic patterns showed that the tRNA\textsuperscript{His} in mutant cybrids carrying the m.12201T>C mutation migrated much faster than control cybrids lacking this mutation.

Furthermore, the probes specific for tRNA\textsuperscript{His}, tRNA\textsuperscript{Leu(UUR)}, and cytosol-tRNA\textsuperscript{His} were validated using total RNAs isolated from mutant (E1) and control (C9) cybrids as well as 143B cells and derived mtDNA-less ρ\textsuperscript{0}206 cell lines. As shown in Fig. 2D, mitochondrial tRNA\textsuperscript{His} and tRNA\textsuperscript{Leu(UUR)} were only absent in ρ\textsuperscript{0}206 cells, whereas the ct-tRNA\textsuperscript{His} were present in the ρ\textsuperscript{0}206 as well as 143B, C9, and E1 cells. These data suggested that the structural alterations by the m.12201T>C mutation caused the conformational change of tRNA\textsuperscript{His}.

Subcellular location of human HARS2

To determine the subcellular localization of human HARS2, pEGFP-N1-HARS2 expressing the HARS2-GFP fusion protein was transfected into the 143B cell line. Fig. 3A shows that the immunofluorescence pattern of transfected 143B cells was double-labeled with an mAb specific for the GFP and Mitotracker probes, which contain a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. A typical mitochondrial staining pattern was observed, and superimposition of two panels showed the complete overlap of two patterns, demonstrating that human HARS2 localizes exclusively at mitochondria.

Construction of stable transfectants expressing the human HARS2

A 1.6-kb human HARS2 cDNA expressed in a pCDH-puro vector or the vector only was transfected into the mutant cybrid
cell line (E1) carrying the m.12201T>C mutation and control cell line (C9) lacking this mutation (18). These stable transfectants were isolated by culturing cells in DMEM supplemented with 1 μg/ml puromycin and 10% FBS for 2 weeks.

The expression levels of the HARS2 cDNA in resultant stable transfectants were examined by Western blot analysis, as shown in Fig. 3B. The levels of exogenous HARS2 in transfectants C9 and E1 were more than 8-fold higher than those of our own HARS2. These four transfectants [C9V (vector only), C9H (exogenous HARS2), E1V (vector only), and E1H (exogenous HARS2)] were then used for further characterization.

To test whether HARS2 overexpression affected the expression of other mitochondrial aminoacyl-tRNA synthetases, the levels of LARS2 (leucyl-tRNA synthetase 2) and SARS2 (seryl-tRNA synthetase 2) were determined by Western blot analysis. As illustrated in Fig. 3B, the levels of LARS2 and SARS2 in C9H and E1H cell lines were comparable with those in four other cell lines lacking the exogenous HARS2 expression. This suggested that the overexpression of HARS2 did not affect the expression levels of these mitochondrial aminoacyl-tRNA synthetases.

Enhancing aminoacylation capacity of mitochondrial tRNA<sup>His</sup>

We investigated the effects of the transfer of human HARS2 on the aminoacylation of tRNA<sup>His</sup> in various cell lines <i>ex vivo</i>. The aminoacylation levels of tRNA<sup>His</sup> in these cell lines were determined by using electrophoresis in an acid polyacrylamide/urea gel system to separate uncharged and charged tRNA species, electroblotting and hybridizing with specific probes for tRNA<sup>His</sup> as well as tRNA<sup>Leu</sup>(UUR), tRNA<sup>Met</sup>, tRNA<sup>His</sup>, and tRNA<sup>Ser</sup>(AGY), respectively. To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of mitochondrial tRNAs were decacylated by being heated for 10 min at 60 °C at pH 8.3 and then run in parallel (30, 31). As shown in Fig. 4, the overexpression of HARS2 increased the aminoacylated...
levels of tRNA\textsuperscript{His} mutant cell line E1 but not those of control cell line C9. In particular, the proportions of aminoacylated tRNAs in the E1H cell lines were 75.0, 67.5, 61.6, 43.6, and 47.9% in the tRNA\textsuperscript{His}, tRNA\textsuperscript{Leu(UUR)}, tRNA\textsuperscript{Met}, tRNA\textsuperscript{Ile}, and tRNA\textsuperscript{Ser(AGY)} respectively, whereas 56.3% of tRNA\textsuperscript{His}, 67.6% of tRNA\textsuperscript{Leu(UUR)}, 59.6% of tRNA\textsuperscript{Met}, 44.3% of tRNA\textsuperscript{Ile}, and 47.8% of tRNA\textsuperscript{Ser(AGY)} were aminoacylated in the parental E1 cell lines. In contrast, there were no significant differences in the aminoacylated levels of these five tRNAs between the C9H cell lines and parental C9 cell lines.

**The increasing steady-state levels of tRNAs**

To evaluate whether the overexpression of HARS2 enhanced the levels of tRNA\textsuperscript{His} in transfectants carrying the m.12201T>C mutation, we subjected mitochondrial RNAs from various cell lines to Northern blot analysis and hybridized
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Figure 4. In vivo aminoacylation assays. A, 10 μg of total cellular RNAs purified from various cell lines under acid conditions were electrophoresed at 4 °C through an acid (pH 5.2) 10% polyacrylamide, 7 M urea gel, electroblotted, and hybridized with a DIG-labeled oligonucleotide probe specific for the tRNAHis. The blots were then stripped and rehybridized with tRNALeu(UUR), tRNAMet, tRNAIle, and tRNASer(AGY), respectively. Samples from control and mutant cell lines were deacylated (DA) by heating for 10 min at 60 °C at pH 8.3, electrophoresed, and hybridized with DIG-labeled oligonucleotide probes specific for the tRNAHis and tRNALeu(UUR). B, in vivo aminoacylated proportions of tRNAHis, tRNALeu(UUR), tRNAMet, tRNAIle, and tRNASer(AGY) in six cell lines. The calculations were based on three independent determinations. Error bars, S.D. values. p indicates the significance, according to the analysis of variance test, of the differences for various cell lines. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Northern blot analysis of mRNA and rRNAs

We then examined whether the m.12201T>C mutation affected the expression/processivity of polycistrionic transcripts. RNA transfer hybridization experiments were performed with total cellular RNAs from various mutant and control cell lines, using a set of DIG-labeled RNA probes: ND6 from L-strand transcripts; COX1, COX2, CYTB, 12S rRNA, and 16S rRNA from H-strand transcripts (32, 33); and β-actin as a control, respectively. As shown in Fig. 6, the levels of ND6, COX1, COX2, CYTB, 12S rRNA, and 16S rRNA in mutant cell lines, normalized with respect to those of β-actin mRNA, were comparable with those in the control cell lines. These results indicated that the m.12201T>C mutation did not affect the expression of these mRNAs and rRNAs.

Elevated levels of mtDNA-encoding proteins

To assess whether the overexpression of HARS2 affected the mitochondrial protein synthesis, a Western blot analysis was performed to examine the levels of eight mtDNA-encoding proteins in transfectants as well as their parental cell lines with Tom20 as a loading control. As shown in Fig. 7 (A and B), the overexpression of HARS2 significantly increased the levels of these mitochondrial proteins in the mutant cell line E1. Of these, the levels of ND1, ND4, ND5, and ND6 (subunits 1, 4, 5, and 6 of NADH dehydrogenase (complex I)); COX2 (cytochrome b of ubiquinone cytochrome c oxidoreductase (complex III)); CO2 (subunit 2 of cytochrome c oxidase (complex IV)); and ATP6 and ATP8 (subunits 6 and 8 of the H+-ATPase (complex V)) in E1 cell lines were 66.4, 100.9, 66.0, 97.6, 108, 53.3, 90.0, and 85.1%, with the average 77.4% of the control cell line C9, whereas those in E1 cell lines were 43.7, 55.6, 52.4.
103.7, 70.4, 35.6, 62.0, and 89.8% with the average 43.0% of the control cell line C9. In contrast, the overexpression of HARS2 did not significantly affect the levels of mitochondrial proteins in the control cell line C9. To test whether the m.12201T/H11022C-induced deficiency affected the mitochondrial proteostasis, we measured the levels of Clpp involved in mitochondrial ribosome assembly (36) and ATP family gene 3–like 2 (Afg3l2) proteases involved in the turnover of misfolded proteins markers for proteostasis stress (37), in the various cell lines. As shown in Fig. 7C and Fig. S1, there was no significant difference in the levels of Afg3l2 and Clpp among mutant and control cell lines. These data indicated that m.12201T/H11022C mutation may not affect the proteostasis stress.

The restoration of respiration deficiency

To evaluate whether the overexpression of HARS2 rescued the respiratory deficiency caused by the m.12201T>C mutation, we measured the activities of respiratory complexes by the use of isolating mitochondria of various cell lines. Complex I activity was measured by following the oxidation of NADH with ubiquinone as the electron acceptor (38, 39). The activity of complex II (succinate ubiquinone oxidoreductase) was exclusively encoded by the nuclear DNA was examined by the artificial electron acceptor DCCP (40, 41). Complex III (ubiquinone cytochrome c oxidoreductase) activity was measured as the reduction of cytochrome c (III) using D-ubiquinol-2 as the electron donor. The activity of complex IV was monitored by following the oxidation of cytochrome c (II). As shown in Fig. 8, the overexpression of HARS2 enhanced the activities of complex I, III, and IV but not complex II in cells carrying the m.12201T>C mutation. As shown in Table S1, the activities of complexes I, III, and IV in the E1H cell line were 150.2, 145.3, and 209.3% of those in the parental cell line E1, respectively. By contrast, the activities of complexes I, III, and IV in the C9H cell line were 89.4, 107.6, and 139.5% of those in the parental C9 cell line, respectively.

Oxygen consumption rate (OCR) is an indicator of mitochondrial respiration. Using a Seahorse Bioscience XF-96 extracellular flux analyzer, we can measure mitochondrial respiratory control, including basal respiration, O2 consumption...
attributed to ATP production, proton leak, maximum respiratory rate, reserve capacity, and nonmitochondrial respiration (42, 43). As shown in Fig. 9, the basal OCR in the E1H cell line increased 47.9% compared with those in the parental cell line E1, whereas the basal OCR of C9H was comparable with those in the C9 cell line. The drug-insensitive OCRs were then measured after the sequential addition of oligomycin (to inhibit the ATP synthase), carbonyl cyanide $p$-trifluoromethoxyphenylhydrazone (FCCP) (to uncouple the mitochondrial inner membrane and allow for maximum electron flux through the ETC), rotenone (to inhibit complex I), and antimycin A (to inhibit complex III). As shown in Fig. 9, the ATP-linked OCR, maximal OCR and reserve capacity OCR in the E1H cell line significantly increased 67.4, 89.5, and 144.7%, as compared with those in the parental cell line E1, respectively. However, basal OCR, ATP-linked OCR, maximal OCR, and reserve capacity OCR in the E1H cell line were significantly lower than those in the C9H cell line. By contrast, the OCRs for C9H cell line were comparable with those of the parental C9 cell line. These data indicated that the respiration of the E1H cell line was significantly improved but not restored to the levels of WT cell line.

Enhancement of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta \Psi_m$) generated by complexes I, III, and IV is an essential component in the process of energy storage during oxidative phosphorylation (44). The $\Delta \Psi_m$ levels in various cell lines were measured using a fluorescence probe JC-10 assay via flow cytometry. As shown in Fig. 11, the $\Delta \Psi_m$ of the C9V, C9H, E1, E1V, and E1H cell lines was 101.0, 93.5, 53.6, 60.4, and 86.5% relative to the mean values in the control cell line C9, respectively. In contrast, the populations with a normal level of $\Delta \Psi_m$ in these six cell lines were comparable with those in the presence of FCCP.

Overexpression of HARS2 reduced the production of mitochondrial ROS

The levels of mitochondrial ROS among these cells were determined using a MitoSOX assay via flow cytometry (45). Geometric mean intensity was recorded to measure and delineate the rate of ROS of each sample. As shown in Fig. 12 (A and B), the levels of mitochondrial ROS production in the cell lines C9V, C9H, E1, E1V, and E1H were 90.5, 87.0, 163.3, 147.7, and 109.2% relative to the mean values in the control cell line C9, respectively.

To test whether the m.12201T>C mutation–induced mitochondrial ROS production affected the antioxidant systems, we examined the levels of three antioxidant enzymes, SOD2 in the mitochondrion and SOD1 and catalase in the cytosol (46), in the various cell lines. As shown in Fig. 12C, the mutant cell lines were 103% of those in the parental C9 cell line, respectively. On the contrary, the overexpression of HARS2 did not significantly change the levels of total cellular ATP (the presence of glucose) in the mutant and control cell lines.

Figure 6. Northern blot analysis of mitochondrial RNAs. A, 5 $\mu$g of total cellular RNA from various mutant and control cell lines were electrophoresed through a 1.5% agarose-formaldehyde gel; transferred onto a positively charged membrane; and hybridized with DIG-labeled RNA probes for ND6 (from L-strand transcript), COX1, COX2, CYTB, 12S rRNA, and 16S rRNA (from H-strand transcript), and $\beta$-actin as a control, respectively. B, average relative levels of above mRNAs and rRNAs per cell were normalized to the average level per cell of $\beta$-actin in three control cell lines and three mutant cell lines. The values for the latter are expressed as percentages of the average values for the control cell lines. Three independent determinations were used in the calculations. Graph details and symbols are explained in the legend to Fig. 4. Error bars, S.D.
E1 and E1V revealed marked increases in the levels of SOD2 and mild increases in the levels of SOD1 and catalase, as compared with those in the WT cell line C9. Notably, overexpression of HARS2 in the mutant cell line led to pronounced reductions in the level of SOD2 but relatively mild decreases in the levels of SOD1 and catalase.

**Discussion**

The objective of this study was to further elucidate the molecular pathogenesis of the deafness-associated tRNAHis 12201T>C mutation and to test whether human HARS2 overexpression in the hybrid cells carrying the m.12201T>C mutation reverses the mitochondrial dysfunctions. The m.12201T>C mutation destabilized the canonical A5-U68 base-pairing within the aminoacyl acceptor stem of this mitochondrial tRNAHis (18, 21, 47, 48). Thus, we hypothesized that the m.12201T>C mutation led to the structural and functional effects on this tRNA, including the alteration of conformation, thermal stability, and aminoacylation. MD studies indicated that the U68 formed a canonical base pair with A5 of WT tRNAHis through hydrogen bonds with an occupancy of 47 and 64%, respectively, whereas the newly formed interaction between A5 and C68 in the mutant tRNAHis was decreased to one hydrogen bond with a lower occupancy of 38% (49). These findings indicated that the mutant tRNAHis molecule with the mismatch of 5A and C68 may be less stable than those in the WT counterpart. In fact, the T_m in mutant tRNAHis molecule was 4 °C lower than those in the WT counterpart. The instability of the mutant tRNA molecule was further evidenced by the drastically reduced level of tRNAHis in the mutant cell lines carrying the
m.12201T>C mutation (18). Furthermore, the m.12201T>C mutation caused the conformational change of tRNA\(^{\text{His}}\), as suggested by faster electrophoretic mobility of mutated tRNA with respect to the WT molecule \textit{in vitro} or \textit{ex vivo}, consistent with the conformational changes of tRNAs carrying the m.4435A>G and m.3253T>C mutations (50, 51). However, the m.12201T>C mutation did not affect the expression/processivity of polycistronic transcripts, in contrast with the aberrant processing of the polycistronic transcripts observed in the cell lines carrying the tRNA\(^{\text{Leu(UUR)}}\) 3243A>G and tRNA\(^{\text{Ser(UCN)}}\) 7445A>G mutations and the m.4401A>G mutation in the precursor of tRNA\(^{\text{Met}}\)/tRNA\(^{\text{Gln}}\) mutations (15, 24, 52). Moreover, the abolishment of A5:U68 base-pairing substitution may affect the tRNA\(^{\text{His}}\) interaction with mitochondrial histidyl-tRNA synthetase, thereby altering the aminoacylation properties of tRNA\(^{\text{His}}\) by either charging inefficiently or mischarging with mitochondrial histidyl-tRNA synthetase (53, 54). In this study, the cell line bearing the m.12201T>C mutation displayed increasing levels of aminoacylated tRNA\(^{\text{His}}\) and faster electrophoretic mobility of mutated tRNA with respect

Figure 9. Respiration assays. A, analysis of O\(_2\) consumption in the various cell lines using different inhibitors. OCRs were first measured on 2 × 10\(^6\) cells of each cell line under basal conditions, and then sequentially oligomycin (1.5 μM), FCCP (0.5 μM), rotenone (1 μM), and antimycin A (1 μM) were added at the indicated times to determine different parameters of mitochondrial functions. B, graphs present the ATP-linked OCR, proton leak OCR, maximal OCR, reserve capacity, and nonmitochondrial OCR in six cell lines. Nonmitochondrial OCR was determined as the OCR after rotenone/antimycin A treatment. Basal OCR was determined as OCR before oligomycin minus OCR after rotenone/antimycin A. ATP-linked OCR was determined as OCR after 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 nonmitochondrial OCR. Reserve capacity was defined as the difference of maximal OCR after FCCP minus basal OCR. The data were based on three determinations for each cell line. Graph details and symbols are explained in the legend to Fig. 4. Error bars, S.D.
Aminoacylated tRNA\text{\textsubscript{His}} in the WT cybrids, suggesting that 50% aminoacylated tRNA\text{\textsubscript{His}} from 56.3 to 75.0% but did not change cybrids bearing the m.12201T/HARS2 gene may provide a step toward therapeutic interventions for these disorders.

In summary, our results demonstrated that overexpression of HARS2 corrected the mitochondrial dysfunction caused by the deafness-associated tRNA\text{\textsubscript{His}} m.12201T>C mutation. The biochemical phenotypes manifested by interplay between tRNA\text{\textsubscript{His}} m.12201T>C mutation and HARS2 gene may provide new insights into the pathophysiology of maternally inherited deafness. The restoration of m.12201T>C mutation--induced mitochondrial dysfunctions by overexpression of HARS2 may be a step toward therapeutic interventions for these disorders.

**Experimental procedures**

**Cell lines and culture conditions**

The 143B.TK\textsuperscript{-} cell line, the mutant cybrid cell lines (E1) carrying the m.12201T>C mutation, and control cybrid cell lines (C9) belonging to the same mtDNA haplogroup Z3 but lacking the mutation (H7) were grown in DMEM (containing 4.5 mg of glucose and 0.11 mg of pyruvate per ml), supple-
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Figure 11. Mitochondrial membrane potential analysis. A, \( \Delta \Psi_m \) was measured in six cell lines by a BD-LSR II flow cytometer system using a fluorescence probe JC-10 assay system. The ratios of fluorescence intensity excitation/emission = 490/590 and 490/530 nm were recorded to delineate the \( \Delta \Psi_m \) level of each sample. Represented are flow cytometry images of cell lines E1V and E1H with and without 10 \( \mu \)M FCCP. B, the ratios of the population with a normal level of \( \Delta \Psi_m \) relative to the control cell line C9 were calculated to reflect the level of \( \Delta \Psi_m \). The average of three determinations for each cell line is shown. Graph details and symbols are explained in the legend to Fig. 4. Error bars, S.D.

Figure 12. Assays for mitochondrial ROS production. A, ratio of geometric mean intensity between levels of the ROS generation in the vital cells. The rates of production in ROS from six cell lines were analyzed by a BD-LSR II flow cytometer system using MitoSox (5 \( \mu \)M). B, the relative ratio of intensity was calculated. The average of three independent determinations for each cell line is shown. C, Western blot analysis of antioxidative enzymes Sod2, Sod1, and catalase in six cell lines with \( \beta \)-actin as a loading control. D, quantification of Sod2, Sod1, and catalase. Average relative values of Sod2, Sod1, and catalase were normalized to the average values of \( \beta \)-actin in various cell lines. The values for the latter are expressed as percentages of the average values for the control cell line C9. The average of three independent determinations for each cell line is shown. Graph details and symbols are explained in the legend to Fig. 4. Error bars, S.D.

The mtDNA-less \( \rho^0 \)206 cell line, derived from 143B.TK \( ^{-} \) (57) was grown under the same conditions as the parental line, except for the addition of 50 \( \mu \)g of uridine/ml. The stable transfectants were grown in DMEM supplemented with 10% FBS and 1 \( \mu \)g/ml puromycin.
**Molecular dynamics simulation procedure**

The acceptor stem of tRNAHis containing 15 nucleotide bases spanning the A5-68U/C base-pairing (Fig. 1A) was constructed as the model for simulations. The initial coordinates for WT were extracted from the crystal structure of human mitochondrial tRNAAsp-PheRS complex (Protein Data Bank entry 3TUP). The coordinates of the backbones were maintained, and nucleotide bases were substituted with the mitochondrial tRNAHis sequence by Chimera (58). In the mutated structure, the coordinates of U to C substitution at position 68 were generated by Chimera using the established WT model. The acceptor stems of WT and mutant tRNAHis were simulated using the same methods. The ff14SB force field parameters were employed in MD simulations with Amber14 (48). The coordinates of U to C substitution at position 68 were extracted from the crystal structure of human mitochondrial tRNAHis sequence by Chimera (58). In the mutated structure, the coordinates of U to C substitution at position 68 were generated by Chimera using the established WT model. The acceptor stems of WT and mutant tRNAHis were simulated using the same methods. The ff14SB force field parameters were employed in MD simulations with Amber14 (59). The initial models were surrounded by TIP3P water molecules and ions. The simulation systems were maintained in a solution containing 50 mM NaCl, made by the addition of 3Na+ and 3Cl−, and then 13 Na+ for neutralization. The SHAKE algorithm was used to constrain hydrogen atoms (60). Alternately, we minimized and equilibrated the whole system to relieve all unfavorable interactions of the initial model. The procedure of equilibration in NVT ensemble was heated to 300 K and was not subjected to pressure. NPT ensemble was applied to equilibrate the solvent at the final equilibration step, in which the temperature was kept at 300 K and pressure at 1 bar in periodic boundary conditions. Subsequently, 100-ns production simulations were performed for both systems with a time step of 2 fs. The MD trajectory of each system was observed with VMD (61). Extraction and analysis of trajectories were performed using the cpptraj program in Amber14. Distance cutoff for hydrogen bond and angle cutoff were set to 3.0 Å and 135°, respectively.

**Measurement of melting temperature**

UV melting assays were carried out as described previously (50, 51). The WT and mutant tRNAHis transcripts were produced using in vitro transcription by T7 RNA polymerase according to previous protocols (62). The tRNAHis transcripts were dissolved in 50 mM sodium phosphate buffer (pH 7.0), containing 50 mM NaCl, 5 mM MgCl2, and 0.1 mM EDTA. Absorbance against melting temperature curves was measured at 260 nm with a heating rate of 1 °C/min from 25 to 95 °C via an Agilent Cary 100 UV spectrophotometer.

**Isolation of human HARS2 cDNA**

To construct the plasmid pHARS2 containing the entire coding region of HARS2 cDNA, RT-PCR was performed by using TaqDNA polymerase (Promega) and total RNA isolated from 143B cells as template, with the primers 5’-ATAATGTCCTGA-CCGCCCTCCCTTT-3’ (nt 159–176) and 5’-CTCTTGGTGGCT-AGTTGTGTTGTA (nt 1705–1720) (GenBank™ accession no. NM_001278732.1). The predominant PCR product was purified by agarose gel electrophoresis and subsequently cloned into a pGEM-T vector (Promega). Nucleotide sequence was determined by Sanger sequencing.

**Subcellular localization of human HARS2**

The coding region of HARS2 cDNA lacking its natural stop codon was obtained by PCR using pHARS2 cDNA as the template. Primers 5’-CCGCTGAGCCGGCCTCGCGC (nt 204–220) and 5’-CCGACCGGTATCGACAGAG (nt 2870–2891) were used for the PCR amplification. PCR products were digested with AgeI and XhoI and then cloned into pEGFP-N1. After sequence determination, Resultant constructs were transfected into 143B cells using the jetPRIME™ transfection reagent (Polyplus Transfection) according to the manufacturer’s protocol. Immunofluorescence analysis was performed as detailed elsewhere (63, 64).

**Construction of stable transfectants**

The insert of pHARS2 was subcloned into pCDH-puro (Invitrogen). The resultant constructs or vector only were transfected into E1 and C9 cell lines using the jetPRIME™ transfection reagent (Polyplus Transfection) according to the manufacturer’s protocol. The stable transfectants were isolated by culturing cells in DMEM supplemented with 1 μg/ml puromycin and 10% FBS for 2 weeks. The resultant clones were examined for the expression of HARS2 by Western blot analysis.

**Mitochondrial tRNA analysis**

For the tRNA Northern blot analysis, the isolation of total cellular RNAs, gel electrophoresis, electrolotting, and hybridization were as detailed elsewhere (18, 24, 45, 65–67). DIG-labeled probes of tRNAHis, tRNAAla, tRNACys, tRNAGlu, tRNAleu(UUR), tRNAlys, tRNAMet, and 5S rRNA for hybridization were as described elsewhere (18, 24, 45, 52). Quantification of density in each band was made as detailed previously (18, 24, 45).

For the tRNA mobility shift assay, 2 or 5 μg of RNAs were electrophoresed through a 10% polyacrylamide native gel at 4 °C with 50 mM Tris-glycine buffer. After electrophoresis, the gels were treated according to the Northern blot analysis as described above (50, 51).

The aminoacylation assays including the isolation of total cellular and gel electrophoresis were as detailed elsewhere (30, 45). The gels were then electrolotted onto a positively charged nylon membrane (Roche Applied Science) for the hybridization analysis with oligodeoxynucleotide probes as described above. Quantification of the density in each band was performed as detailed previously (30, 45).

tRNA half-life measurements were performed as detailed elsewhere (35). Briefly, various cell lines were incubated in fresh medium containing 250 ng/ml EtBr for the times indicated in Fig. 5C. Ten micrograms of total cellular RNAs, extracted as above, were subjected to Northern blot analysis as described above.

**Mitochondrial RNA Northern blot analysis**

Eight micrograms of total cellular RNAs were fractionated by electrophoresis through a 1.5% agarose-formaldehyde gel, transferred onto a positively charged membrane (Roche Applied Science), and hybridized with DIG-labeled RNA probes.
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probes: ND6, COX1, COX2, CYTB, 12S rRNA, 16S rRNA, and β-actin as a control, respectively. Probes were synthesized on the corresponding restriction enzyme linearized plasmid using a DIG RNA-labeling kit (Roche Applied Science). The plasmids used for RNA probes were constructed by PCR-amplifying fragments of ND6 (positions 14343–14618), COX1 (positions 7146–7425), COX2 (positions 7823–8156), CYTB (positions 14824–15208), 12S rRNA (positions 1201–1235), 16S rRNA (positions 2245–2635), and β-actin (positions 69–618, NM 001101.5) and cloning these fragments into the pCRII-TOPO vector (16, 52, 68).

Western blot analysis

Western blot analysis was carried out as detailed previously (18, 67, 69, 70). Twenty micrograms of total proteins obtained from lysed mitochondria were denatured and loaded onto SDS-polyacrylamide gels. The gels were electroblotted onto a polyvinylidene difluoride membrane for hybridization. The antibodies used for this investigation were from Abcam (TOM20 (ab56783), ND1 (ab74257), ND5 (ab92624), A6 (ab101908), and CO2 (ab110258)), Santa Cruz Biotechnology, Inc. (ND4 (sc-20499-R) and ND6 (sc-20667)), Proteintech (CTYT (55090-1-AP), ATP8 (26723-1-AP), HARS2 (11301-1-AP), LASR(S2 (17097-1-AP), SARS2 (17258-1-AP), AFG3L2 (14631-1-AP), and CLPP (15698-1-AP)), and Cell Signaling Technology (SOD2 (13141), SOD1 (4266), and catalase (12980)). Peroxidase AffiniPure goat anti-mouse IgG and goat anti-rabbit IgG (Jackson) were used as secondary antibodies, and protein signals were detected using the ECL system (Millipore). Quantification of density in each band was performed as detailed previously (18, 50).

Enzymatic assays

The enzymatic activities of complexes I–IV were measured as detailed elsewhere (38–40, 67).

Measurements of oxygen consumption

The rates of oxygen consumption in lymphoblastoid cell lines were assayed with a Seahorse Bioscience XF-96 extracellular flux analyzer (Seahorse Bioscience), as detailed previously (42, 43, 67). The protein content of each well was then measured to normalize OCR values.

Measurements of ATP levels

The Cell Titer-Glo® luminescent cell viability assay kit (Promega) was used for the measurement of cellular and mitochondrial ATP levels, following the modified manufacturer’s instructions (18, 51).

Assessment of mitochondrial membrane potential

Mitochondrial membrane potential was assessed with the JC-10 assay kit-microplate (Abcam) according to the manufacturer’s general recommendations with some modifications, as detailed elsewhere (44, 51).

ROS measurements

ROS measurements were conducted as detailed previously (45, 51).

Statistical analysis

Statistical analysis was performed by the analysis of variance test contained in the StatView program SAS (version 9.4) (SAS Institute) and entering individual replicate values. Unless indicated otherwise, a p value < 0.05 was considered statistically significant.

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