Human Mitochondrial Leucyl-tRNA Synthetase Corrects Mitochondrial Dysfunctions Due to the tRNA\textsubscript{Leu(UUR)} A3243G Mutation, Associated with Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-Like Symptoms and Diabetes

Ronghua Li, Emory University
Min-Xin Guan, Cincinnati Children's Hospital Medical Center

Journal Title: Molecular and Cellular Biology
Volume: Volume 30, Number 9
Publisher: American Society for Microbiology | 2010-05-01, Pages 2147-2154
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1128/MCB.01614-09
Permanent URL: https://pid.emory.edu/ark:/25593/rr611

Final published version: http://dx.doi.org/10.1128/MCB.01614-09

Copyright information:
© 2010, American Society for Microbiology

Accessed February 17, 2018 9:08 PM EST
Mutations in mitochondrial tRNA genes are associated with a wide spectrum of human diseases. In particular, the tRNA_{Leu(UUR)}^{A3243G} mutation causes mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS) and 2% of cases of type 2 diabetes. The primary defect in this mutation was an inefficient aminoacylation of the tRNA_{Leu(UUR)}^{A3243G}. In the present study, we have investigated the molecular mechanism of the A3243G mutation and whether the overexpression of human mitochondrial leucyl-tRNA synthetase (LARS2) in the cytoplasmic hybrid (cybrid) cells carrying the A3243G mutation corrects the mitochondrial dysfunctions. Human LARS2 localizes exclusively to mitochondria, and LARS2 is expressed ubiquitously but most abundantly in tissues with high metabolic rates. We showed that the alteration of aminoacylation of tRNA_{Leu(UUR)} caused by the A3243G mutation led to mitochondrial translational defects and thereby reduced the aminoacylated efficiencies of tRNA_{Leu(UUR)} as well as tRNA_{Ala} and tRNA_{Met}. We demonstrated that the transfer of human mitochondrial leucyl-tRNA synthetase into the cybrid cells carrying the A3243G mutation improved the efficiency of aminoacylation and stability of mitochondrial tRNAs and then increased the rates of mitochondrial translation and respiration, consequently correcting the mitochondrial dysfunction. These findings provide new insights into the molecular mechanism of maternally inherited diseases and a step toward therapeutic interventions for these disorders.

Human Mitochondrial Leucyl-tRNA Synthetase Corrects Mitochondrial Dysfunctions Due to the tRNA_{Leu(UUR)}^{A3243G} Mutation, Associated with Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-Like Symptoms and Diabetes

Ronghua Li^1 and Min-Xin Guan^1,2*

Division of Human Genetics, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio,^1 and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio^2

Received 16 December 2009/Returned for modification 22 January 2010/Accepted 22 February 2010

MATERIALS AND METHODS

Cell lines and culture conditions. 143B.TK is a human osteosarcoma-derived cell line (ATCC CRL 8303). The 43B cybrid cell line carrying the nearly homoplasmic A3243G mutation and an isogenic control cybrid cell line harboring the homoplasmic wild-type version of tRNA_{Leu(UUR)} were rescued by overexpression of the translation elongation factor EFTu (11) and mitochondrial leucyl-tRNA synthetase (6). Thus, it is anticipated that the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation would improve the efficiency of aminoacylation of tRNA_{Leu(UUR)}, 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 LARS2 cDNA into a cybrid cell line carrying the nearly homoplasmic A3243G mutation and an isogenic control cybrid cell line harboring the homoplasmic wild-type version of tRNA_{Leu(UUR)}. These stable transfectants were analyzed for the aminoacylation capacity of tRNAs, the stability of the tRNA_{Leu(UUR)}, and the rates of mitochondrial translation and respiration as well as RNA processing. Furthermore, human LARS2 was further characterized by examining the gene expression in different tissues and subcellular locations.

* Corresponding author. Mailing address: Division of Human Genetics, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. Phone: (513) 636-3337. Fax: (513) 636-2261. E-mail: min-xin.guan@cchmc.org.

Published ahead of print on 1 March 2010.
gene were isolated by transfer mitochondria from myoblasts of the same MELAS subject carrying the heteroplasmic A3243G mutation into human mtDNA-less p206 cells (51). The 143B.TK cells and cybrid cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The stable transfectants were grown in DMEM supplemented with 10% FBS and 15 ng/ml of phleomycin (Zeocin).

Isolation of human LARS2 cDNA. To construct the plasmid pLARS2 containing the entire coding region of LARS2 cDNA, reverse transcription-PCR (RT-PCR) was performed using Taq DNA polymerase (Promega) and total RNA isolated from 143B cells as template, with the primers 5'-ATGCTCTTGTTTGGCAGAC (nucleotides [nt] 186 to 205) and 5'-TGCTTTCTCTTCCTGC (nt 2980 to 2998) (GenBank accession no. NM_015340) (3). The predominant PCR product was purified by agarose gel electrophoresis and subsequently cloned into a PCR 2.1-Topo vector (Invitrogen). Nucleotide sequencing was done using a Dye Terminator cycle sequencing kit (Perkin-Elmer) and an ABI Prism 3700 genetic analyzer.

Northern blot analysis of LARS2 expression. A 12-base human multiple tissue RNA blot (Clontech) containing 2 μg poly(A)+ RNA/lane was used for this study. An 1,124-bp LARS2 cDNA fragment corresponding to the nucleotide positions 1309 to 2432 (3) was random prime labeled with [32P]dATP and hybridized with the RNA blots according to the manufacturer’s instructions. Membranes were then washed to a final stringency of 0.1× SSC (0.15 M NaCl plus 0.01 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 65°C for 40 min. As an internal control, the human RNA blots were stripped and rehybridized with a human actin probe, as described above. RNA blots were then stripped and rehybridized with a LARS2 RNA probe, as described above. RNA blots were then stripped and rehybridized with a human actin β subunit cDNA as a control.

Subcellular localization of human LARS2. The coding region of LARS2 cDNA lacking its natural stop codon was obtained by PCR using pLARS2 cDNA as template. Primers 5'-TGGCTCTGTTTGCGGAC (nt 186 to 205) and 5'-GGACTTTCATGGAATGGTT (nt 2870 to 2891) were used for the PCR amplification. PCR products were digested with EcoRI and cloned into pBluescript II KS(+) (Promega). After sequence determination, the inserts were subcloned into pEGFP-N1 (Clontech) to generate the pEGFP-N1-LARS2. Resultant constructs were transfected into 143B cells using the SuperFect transfection reagent (Qiagen, Inc.) according to the manufacturer’s protocol. Immunofluorescence analysis was performed as detailed elsewhere (29, 30, 49).

Construction of stable transfectants. The insert of pLARS2 was subcloned into pcDNA3.1/Zeocin (Invitrogen). The resultant constructs or vectors only were transfected into 43B and HSI cell lines using the SuperFect transfection reagent (Qiagen, Inc.) according to the manufacturer’s protocol. The stable transfectants were isolated by culturing cells in DMEM supplemented with 15% fetal bovine serum for 4 weeks. The expression level of the resulting 43B cell line, as the wild-type and mutated mtDNA was determined. In the subcellular fractionation of transfected 143B cells, double labeled with a monoclonal antibody specific for the GFP and mouse monoclonal antibody to COX1, a subunit of cytochrome c oxidase (COX) complex in the mitochondrial inner membrane. A typical mitochondrial staining pattern was observed with both antibodies, and superimposition of two panels showed complete overlap of the two patterns, demonstrating that human LARS2 localizes exclusively to mitochondria. This suggested that this protein, like other nucleus-encoding mitochondrial proteins such as MTO1, GTPBP3, and TRMU (29, 30, 49), was synthesized on cytoplasmic ribosomes and imported into and then functioned in the mitochondrion.

Construction of stable transfectants expressing the human LARS2. A 2.8-kb human LARS2 cDNA was expressed in a pcDNA3.1 vector or the vector only was transfected into the 43B hybrid cell line carrying the A3243G mutation and the isogenic HSI cell line with the wild-type version of tRNALeu(UUR) (5). The HSI cell line carrying the homoplasmic wild-type version of the tRNALeu(UUR) gene was isogenic with its respective mutant 43B cell line, as the wild-type and mutated mtDNA were derived from the same heteroplasmic patient cells (5, 51). These stable transfectants were isolated by culturing cells in DMEM supplemented with 15 ng/ml of phleomycin and 10% FBS for 4 weeks. The expression level of the LARS2 cDNA in
resultant stable transfectants was examined by a Northern blot analysis, as shown in Fig. 2A. Here, the levels of exogenous LARS2 mRNA (2.8 kb) harboring only the coding region were more than 5-fold higher than those of endogenous LARS2 mRNA (4.2 kb) representing full-length mRNA in transfectants 43B-LARS2 and HSI-LARS2. To test if the transfer of LARS2 may change the proportion of mutated mtDNA molecules and mtDNA copy number in transfectant lines carrying the A3243G mutation, the presence and degree of the A3243G mutation in transfectants were examined by methods described elsewhere (34, 51). As shown in Fig. 2B, the presence and degree of the A3243G mutation in the tRNALeu(UUR) gene in transfectants and parental cybrids, PCR products around the A3243G mutation were digested with ApaI and analyzed by electrophoresis in a 6% polyacrylamide gel stained with ethidium bromide. The A3243G mutation creates the site for restriction enzyme ApaI (14). Transfectants and their parental cybrid cell lines are indicated.

Aminoacylation capacity of mitochondrial tRNAs. We first investigated the effects of the transfer of human LARS2 on the aminoacylation deficiency of tRNA^{Leu(UUR)} in transfectants 43B-LARS2 and 43B-V and their parental mutant cybrid cell line 43B as well as transfectants HSI-LARS2 and HSI-V and their parental wild-type cybrid cell line HSI in vivo. Total mitochondrial RNA was isolated from cell lines under acidic conditions to preserve the aminoacyl-tRNA linkage (46). The aminoacylated tRNAs were separated from nonaminoacylated tRNA species on acidic denaturing polyacrylamide-urea gels and then electroblotted and hybridized with specific probes for tRNA^{Leu(UUR)}, tRNA^{Lys}, tRNA^{Met}, tRNA^{Ser(UCN)}, and tRNA^{Ala}, respectively. To further distinguish nonaminoacylated tRNA from aminoacylated tRNA, samples of mitochondrial tRNAs were deacylated by being heated for 10 min at 60°C at pH 8.3 and then run in parallel.
Notably, the efficiencies of aminoacylated tRNA\textsubscript{Met} and tRNA\textsubscript{Ala}, in addition to those of tRNA\textsubscript{Leu(UUR)}, as shown in Fig. 3, were markedly reduced in mutant 43B cells, relative to wild-type HSI cells. In particular, the proportions of aminoacylated tRNAs in the 43B cells were 24.9%, 61.8%, 66.3%, 38.7%, 71.2%, and 56.1% in the tRNA\textsubscript{Leu(UUR)}, tRNA\textsubscript{Leu(CUN)}, tRNA\textsubscript{Lys}, tRNA\textsubscript{Met}, tRNA\textsubscript{Ser(U CN)}, and tRNA\textsubscript{Ala}, respectively, while 64.1% of tRNA\textsubscript{Leu(UUR)}, 66% of tRNA\textsubscript{Leu(CUN)}, 67.4% of tRNA\textsubscript{Lys}, 73.1% of tRNA\textsubscript{Met}, 66.1% of tRNA\textsubscript{Ser(UCN)}, and 73% of tRNA\textsubscript{Ala} were aminoacylated in the isogenic HSI cells. In contrast, the aminoacylated levels of these six tRNAs in the 43B-V cells and HSI-V cells carrying only the vector as well as HSI-LARS2 cells expressing the LARS2 cDNA only were comparable with those of 43B cells and HSI cells, respectively. Strikingly, the expression of LARS2 in the 43B cybrids led to an ~100% increase in the efficiency of aminoacylation of tRNA\textsubscript{Leu(UUR)} (from 24.9% to 50.5%), but the level was still below that of controls (64.1%). Furthermore, the efficiencies of aminoacylation of tRNA\textsubscript{Leu(CUN)}, tRNA\textsubscript{Lys}, tRNA\textsubscript{Met}, tRNA\textsubscript{Ser(UCN)}, and tRNA\textsubscript{Ala} in the 43B-LARS2 cell line expressing LARS2 were 106.6%, 111.6%, 133.33%, 98.5%, and 118% of those in parental 43B cell line, respectively. These suggested that the deficiencies of aminoacylation of tRNAs were partially restored by the overexpression of human LARS2 in the 43B cells.

**Mitochondrial RNA processing.** We then tested if the transfer of human LARS2 restores the defects in mitochondrial RNA processing in the 43B cell line carrying the A3243G mutation. For this purpose, RNA transfer hybridization experiments were performed with total mitochondrial RNA from transfectants and parental cybrids using the nonradioactive DIG-labeled ND1 RNA probe (16, 30). After the blots were stripped, a set of DIG-labeled RNA probes (ND4, ND6, 12S rRNA, and 16S rRNA) were rehybridized with the same blots for normalization purposes. As shown in Fig. 5, RNA19 comprising the 12S rRNA plus tRNA\textsubscript{Val} plus 16S rRNA plus tRNA\textsubscript{Leu(UUR)} plus ND1 was accumulated in the 43B cell line, confirming the defects in mitochondrial RNA processing caused by the A3243G mutation (22, 24, 41). Interestingly, the reduction in the levels of RNA19, ND1, ND4, and ND6 mRNA was observed in the 43B-LARS2 transfectant, while the
levels of 16S rRNA plus tRNA\textsubscript{Leu(UUR)} plus ND1 as well as 12S rRNA appeared to be unchanged in this transfectant compared with those of the parental 43B cell line.

**Mitochondrial protein synthesis.** To assess if the overexpression of LARS2 enhanced the rate of overall mitochondrial protein synthesis in the transfectant carrying the A3243G mutation, samples of cultures of transfectants as well as their parental cybrid cell lines were labeled with short \([^{35}\text{S}]\)methionine pulses in the presence of emetine to inhibit the cytosolic protein synthesis (4). As shown in Fig. 6A, the patterns of the mtDNA-encoded polypeptides of parental cybrid cell lines and their transfectants were qualitatively identical, in terms of electrophoretic mobility of the various polypeptides, to those of the isogenic control cybrid cell line HSI. However, the 43B cells exhibited a decrease in the total rate of labeling of mitochondrial translation products, relative to the control cybrid. In contrast, the overexpression of LARS2 apparently increased the total rate of labeling of mitochondrial translation products in the 43B-LARS2 transfectant line. Figure 6B illustrates a quantification of the results of three labeling experiments and three electrophoretic runs, which was carried out by Image-Quant program analysis of appropriate exposures of the fluorograms and normalization to the data obtained for the HSI sample included in each gel. The overall rates of labeling of the mitochondrial translation products in the 43B cell line and 43B-V and 43B-LARS2 transfectants were 32.3%, 31.1%, and 48.3%, respectively, of the mean value measured in the control HSI cell line. This translated to a 51% increase of the rate of mitochondrial translation in 43B-LARS2 cells, compared to the parental cybrid cell line 43B. In contrast, the rates of mitochondrial translation of HSI-LARS2 and HSI-V transfectants were comparable with that of the parental HSI cell line.

**Analysis of respiration.** To examine if the overexpression of LARS2 rescued the respiratory deficiencies caused by the A3243G mutation, the endogenous respiration rates of transfectants and their parental cell lines were measured by determining the \(O_2\) consumption rate in intact cells, as described previously (23). As can be seen in Fig. 7, the rate of total \(O_2\) consumption in 43B cell lines was 24%, relative to the mean value measured in the control HSI cell line. However, the rate of total \(O_2\) consumption in the 43B-LARS2 transfectant cell line expressing LARS2 was 142% of that of the parental 43B cell line, while the rate of total oxygen consumption
of HSI-LARS2 was 92% of that of the parental HSI cell line. However, rates for 43B-V and HSI-V transfectants were comparable with those of parental 43B and HSI cell lines. These data demonstrated that the increase of the rate of mitochondrial translation products by overexpression of LARS2 indeed enhanced the rate of respiration in the transfectant 43B-LARS2 carrying the A3243G mutation.

**DISCUSSION**

In the present study, we have further investigated the molecular pathogenesis of the MELAS-associated tRNALeu(UUR) A3243G mutation and whether the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation corrects the mitochondrial dysfunctions. The A3243G mutation changes the A to G at conventional position 14 (A14) of the mitochondrial tRNALeu(UUR) (13) and thus alters the structure and function of tRNALeu(UUR). In particular, the tRNALeu(UUR) carrying the A3243G mutation was less charged to a lesser extent by the leucyl-tRNA synthetase, thereby altering aminoacylation. Alteration in aminoacylation of tRNALeu(UUR) may result in stalling of ribosomes at leucine codons, mistranslation of UUR codons, or translational shifting (1, 21, 42). This was evidenced by the disproportionate decrease of translation in some polypeptides (42). The idea that an inefficient aminoacylation of tRNALeu(UUR) is the primary defect in MELAS was further supported by the recent isolation of a suppressor mutation in the anticodon of the mitochondrial tRNALeu(CUN) gene in an A3243G lung carcinoma cell line (8, 9). Furthermore, the A3243G mutation disturbed the processing of the tRNALeu(UUR) precursor (22, 24, 41) as well as the posttranscriptional modification of uridine at the first position of the anticodon in the tRNALeu(UUR) (19, 26, 50). The mutant tRNALeu(UUR) may be metabolically less stable and subject to turnover, thereby lowering the steady-state level of tRNALeu(UUR). As a result, a failure in tRNA metabolism is responsible for defective mitochondrial protein synthesis and an impaired oxidative phosphorylation. In this investigation, it is interesting that the lowered efficiencies of aminoacylated mitochondrial tRNAMet and tRNA Ala were also observed in the cybrid cell line carrying the A3243G mutation. It is likely that the reduced ratio of charged to uncharged tRNALeu(UUR) may mediate mitochondrial tRNA metabolism, thereby reducing the aminoacylated efficiencies of other mitochondrial tRNAs. Indeed, the mutation in TRMU, encoding a highly conserved 5-methylaminomethyl-2-thioridylate-methyltransferase responsible for the biosynthesis of 5-taurinomethyl-2-thiouridine (tm5s2U) of mitochondrial tRNAAsp, tRNAGlu, and tRNA in the wobble position, not only lowered the steady-state level of tRNAAsp, tRNAGlu, and tRNA but also reduced the steady-state level of other
tRNAs such as tRNA$^{\text{Leu(UUR)}}$, tRNA$^{\text{Ser(UCN)}}$, tRNA$^{\text{Met}}$, and tRNA$^{\text{His}}$ (17).

In the present investigation, we have shown that the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation corrected the mitochondrial dysfunctions. In particular, the overexpression of LARS2 in the 43B cybrid cell line carrying the A3243G mutation raised the percentage of aminoacylated tRNA$^{\text{Leu(UUR)}}$ from 24.9% to 50.5%. Despite ~100% increasing efficiency of charged tRNA$^{\text{Leu(UUR)}}$, the partial function of tRNA$^{\text{Leu(UUR)}}$ in the 43B cybrid cell line would cause the level of aminoacylated tRNA$^{\text{Leu(UUR)}}$ in 43B cell lines expressing LARS2 to be below that of control cell line HSI. The fact that overexpression of LARS2 did not elevate the level of aminoacylated tRNA$^{\text{Leu(UUR)}}$ in cells appeared to be the maximum threshold level to maintain the normal function. On the other hand, the improvement of aminoacylation of tRNA$^{\text{Leu(UUR)}}$ likely modulated the uncharged/charged ratios of other mitochondrial tRNAs, thereby enhancing the efficiency of aminoacylation of other tRNAs, including tRNA$^{\text{Met}}$ and tRNA$^{\text{Ala}}$. Furthermore, the high-level expression of LARS2 increased the steady-state level of tRNA$^{\text{Leu(UUR)}}$ in the 43B cells from 36.1% to 65.2% of the level in wild-type cybrid cells. The overexpression of LARS2 may stabilize metabolically unstably mutant tRNA$^{\text{Leu(UUR)}}$ by increasing the proportion of aminoacylated tRNA in the cells, as in the cases of overexpression of yeast LARS2 in the mitochondrial tRNA$^{\text{Leu}}$ mutant cells (6) and human VARS2L in a cybrid cell carrying the tRNA$^{\text{Val}}$ C1624T mutation (40). In addition, the modified human FARS2 displayed significantly improved aminoacylation efficiency for the mitochondrial tRNA$^{\text{A\text{Pho}}}$ G611A mutation (32). Alternatively, an increasing LARS2 expression in 43B cells may facilitate the correct folding and stabilization of the hypomodified tRNA$^{\text{Leu(UUR)}}$ (39). Moreover, an improvement of aminoacylation of tRNA$^{\text{Leu(UUR)}}$ by high levels of LARS2 appeared to lead to more efficient processing of the longer RNA precursors (22, 24), evidenced by the fact that a reduction in the RNA19 was observed in the 43B-LARS2 cell line compared with the parental 43B cell line.

In the present study, we have shown that increasing expression of LARS2 in the mutant 43B cybrid cell line raised 50% of rates of mitochondrial translation in the mutant 43B cybrid cell line but not in the wild-type HSI cybrid cell line. The improved translation by overexpression of LARS2 could be the result of more efficient charging of tRNA$^{\text{Leu(UUR)}}$ and more efficient processing of the longer RNA precursors in mutant cells. The restoration of mitochondrial translation by overexpression of LARS2 appeared to be more efficient than that by overexpression of EF-Tu and EF-G2 in cybrid cells carrying the A3243G mutation (42). In fact, EF-Tu mediates the entry of the aminoacylated tRNA into the A site of the ribosome and then promotes the addition of amino acids to the polypeptide chain. Thus, the overexpression of EF-Tu may enhance the overall mitochondrial translational efficiency, while overexpression of LARS2 improves the efficiency of aminoacylation of tRNA$^{\text{Leu(UUR)}}$ and then increases the efficiency and fidelity of mitochondrial translation. Subsequently, the increasing expression of LARS2 in the mutant 43B cell line improved the respiration capacity even though it was still far short of the respiration rate in the wild-type HSI cybrid cell line, as in the case of overexpression of PGC-1α/β in the cybrid cell line carrying the A3243G mutation (44). Indeed, the degree of increase in the rate of oxygen consumption in 43B-LARS2 cells correlated well with the increase in the rate of mitochondrial protein synthesis, suggesting that the improvement of mitochondrial translation was responsible for the increasing oxidative phosphorylation. However, this result was not in good agreement with the observation that the full recovery of respiration chain function occurred by means of overexpression of LARS2 in a mutant cybrid cell line carrying the A3243G mutation (39). This discrepancy may be attributed to different nuclear backgrounds, as in the case of cell lines carrying other mtDNA mutations (7, 15). In conclusion, we demonstrated that the overexpression of human mitochondrial leucyl-tRNA synthetase corrected the mitochondrial dysfunction caused by the MELAS-associated tRNA$^{\text{Leu(UUR)}}$ A3243G mutation. These findings will provide new insights into the pathophysiology of maternally inherited diseases and a step toward therapeutic interventions for these disorders.

**ACKNOWLEDGMENTS**

This paper is dedicated to the memory of Giuseppe Attardi, a pioneer in mitochondrial genetics and biomedicine.

We are grateful to Anne Chomyn (California Institute of Technology) for cell lines and Linda Spremulli (University of North Carolina) for the human LARS2 cDNA plasmid.

This work was supported by Public Health Service grants RO1NS44015 from the National Institute of Neurological Disorders and Stroke and RO1DC05230 and RO1DC07696 from the National Institute on Deafness and Other Communication Disorders to M.X.G.

**REFERENCES**


