Defect in modification at the anticodon wobble nucleotide of mitochondrial tRNA^{Lys} with the MERRF encephalomyopathy pathogenic mutation

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Received 3 December 1999; received in revised form 11 January 2000

Edited by Shozo Yamamoto

Abstract A mitochondrial tRNA^{Lys} gene mutation at nucleotide position 8344 is responsible for the myoclonus epilepsy associated with ragged-red fibers (MERRF) subgroup of mitochondrial encephalomyopathies. Here, we show that normally modified uridine at the anticodon wobble position remains unmodified in the purified mutant tRNA^{Lys}. We have reported a similar modification defect at the same position in two mutant mitochondrial tRNAs^{Leu}(UUR) in another subgroup, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), indicating this defect is common in the two kinds of tRNA molecules with the respective mutations of the two major mitochondrial encephalomyopathies. We therefore suggest the defect in the anticodon is responsible, through the translational process, for the pathogenesis of mitochondrial diseases.

Key words: Mitochondrial disease; Mitochondrial tRNA; Anticodon; Post-transcriptional modification; Cybrid

1. Introduction

Point mutations as well as large-scale deletions in mitochondrial DNA (mtDNA) are associated with a wide spectrum of human diseases caused by mitochondrial dysfunction, and most pathogenic mutant mtDNAs coexist with wild-type mtDNA in a heteroplasmic manner [1]. In particular, point mutations are frequently found in mitochondrial tRNA genes [2]. Among the tRNA point mutations reported so far, an A to G transition at nucleotide position (np) 8344 located in the TΨC arm of the mitochondrial tRNA^{Lys} gene is found in 90% of patients with myoclonus epilepsy associated with ragged-red fibers (MERRF) [3], which is one of the most well-studied mitochondrial encephalomyopathies [4]. In addition, a point mutation at either np 3243 or 3271 of the tRNA^{Leu}(UUR) gene is responsible for mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), another major subgroup of the mitochondrial encephalomyopathies [5–8], which is distinguishable from MERRF by its clinical features [4]. It remains unknown why the phenotypes are distinct, corresponding to particular tRNA genes with point mutations.

By constructing cybrid cells in which mutant mtDNA derived from patients was intercellularly transferred into human cells lacking mtDNA (ρ^0 cells), these three mutations (at np 8344, 3243 and 3271) have been demonstrated to be directly involved in either decreased respiratory activity or oxygen consumption without nuclear gene involvement [9–13]. However, just how a mutation in a tRNA gene causes the mitochondrial dysfunction that gives rise to mitochondrial disease is little understood. In the case of MERRF, mutant cybrid cells in which the ratio of mtDNA with the 8344 mutation exceeded 90% exhibited severely impaired mitochondrial protein synthesis and produced abortive polypeptides [13]. It has been proposed that an approximately 50% reduction in aminoacyl-tRNA^{Lys} accounts for these defects, resulting in decreased respiratory activity. Although this model is attractive, it is also plausible that abnormal functioning of tRNA molecules with a pathogenic mutation is involved in the mitochondrial disease.

Here, we demonstrate that the 8344 mutation in tRNA^{Lys} leads to lack of the modification in the wobble nucleotide. Recently, we purified the two mutant tRNAs^{Leu}(UUR) with the 3243 and the 3271 MELAS mutations from the respective cybrid cells and found that both were deficient in the modification of uridine at the first position of the anticodon [14]. The fact that the lack of the modification in the pathogenic mutant tRNA molecules is observed in both MERRF and MELAS suggests that this defect could be the cause of the pathogeneses of these two major mitochondrial encephalomyopathies. Since the modification is essential for accurate and efficient decoding in mitochondrial translation, investigation of the defect might shed light on the pathogenesis of MERRF at the translational level.

2. Materials and methods

2.1. Cybrid cell lines

A mutant cybrid cell line which possesses mtDNA with the A8344G

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Abbreviations: MERRF, myoclonus epilepsy associated with ragged-red fibers; np, nucleotide position; tRNA^{Leu}(UUR), leucine-specific tRNA corresponding to the codons UUR, where R means A or G; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; tRNA^{Lys}(A8344G), MERRF patient-derived mitochondrial tRNA^{Lys} harboring the A to G transition in TΨC-loop

PII: S0014-5793(00)01145-5

mutation was a kind gift from Dr. J.-I. Hayashi of Tsukuba University. This cybrid cell line was constructed by the intercellular transfer of patient mtDNA to ρ^0 HeLa cells (EB8) and isolation of cybrid clones as described [15,16]. The mutant mtDNA was accumulated by subcloning and the ME1-4 cell line exclusively possessing mtDNA with the A8344G mutation was obtained. The Ft2-11 cell line for use as a wild-type control was obtained by fusing EB8 cells with enucleated fetal human fibroblasts as described [16]. The cells were cultured in normal medium (Dulbecco's modified Eagle's medium/F-12 (1:1) (Gibco BRL), 10% fetal calf serum).

2.2. Purification of $tRNA^{Lys}$ from cybrid cells by solid-phase probing

About 300–1000 A_{260} units (the actual number varied depending on the cell line) of total RNA were extracted from approximately 10^9 semi-confluent cultured cybrid cells by Isogen (Nippon Gene, Toyama, Japan). Total RNA was incubated at 37°C for 3 h in 50 mM Tris–HCl (pH 9.5) to discharge amino acids from the tRNAs. The deacylated total RNA preparation was adjusted to pH 7.5 and fractionated on a DEAE-Sepharose Fast Flow (Amersham Pharmacia Biotech) column (1×45 cm) with a linear gradient of NaCl and $MgCl_2$, between 250 and 500 mM and between 8 and 16 mM, respectively, in a buffer containing 20 mM Tris–HCl (pH 7.5). Fractions enriched with $tRNA^{Lys}$ were monitored by dot hybridization with an oligonucleotide probe specific for mitochondrial $tRNA^{Lys}$: 5′-TCACTGTAAAGAGGTGTTGG-3′ (not including the mutation position). $tRNA^{Lys}$ was purified by solid-phase probing using a 3′-biotinylated oligonucleotide with a sequence identical to that used for the dot hybridization, which was immobilized on streptavidin agarose (Gibco BRL) as described [17]. The $tRNA^{Lys}$ was further purified by gel electrophoresis.

2.3. Sequence determination of wild-type and mutant $tRNAs^{Lys}$ including modifications

Purified $tRNA^{Lys}$ with or without the 8344 mutation was sequenced by a combination of the methods of Donis-Keller [18] and Kuchino et al. [19]. For Donis-Keller's method [18], the homogeneous tRNA was labeled at the 5′-terminal with [γ-^{32}P]ATP (110 TBq/mmol, Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Toyobo, Osaka, Japan). The nucleotide-specific RNases used for restricted digestion of tRNA were RNase T_1 (Amersham Pharmacia Biotech), U_2 (Seikagaku Kogyo, Tokyo, Japan), PhyM (Amersham Pharmacia Biotech) and CL_3 (Boehringer Mannheim). For the method of Kuchino et al. [19], each nucleotide in the tRNA was analyzed by two-dimensional thin-layer chromatography (2D-TLC) using two different solvent systems. Solvent system A consisted of isobutyric acid/concentrated ammonia/H_2O (66:1:33 by volume) in the first dimension, and 2-propanol/HCl/H_2O (70:15:15 by volume) in the second. In solvent system B, the first dimension was the same as that used for system A, but 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/1-propanol (100 ml:60 mg:2 ml) was used for the second dimension.

2.4. Identification of $tRNA^{Lys}$ anticodon first letter by restrictive digestion with RNase H

Restrictive digestion at the anticodon first letter was done according to the literature [20]. Using a splint oligonucleotide primer composed of DNA and RNA (5′-rCUrCUUrArAUrCUUUrArArCUUrAr-AAAGGTTA-3′) and possessing a cleavage site between positions 33 and 34 (anticodon first letter) of $tRNA^{Lys}$, 0.005 A_{260} unit homogeneous wild-type $tRNA^{Lys}$ or mutant $tRNA^{Lys}$(A8444G) was incubated at 65°C for 10 min and then cooled down to 35°C for annealing in a buffer consisting of 40 mM Tris–HCl (pH 7.9), 1 mM DTT, 4% glycerol and 0.003% BSA. Magnesium chloride was added to the mixture up to a final concentration of 4 mM and the reaction was carried out at 30°C for 1 h by addition of RNase H (Takara Shuzo, Kyoto, Japan). A cleaved 3′-half fragment was obtained by gel electrophoresis purification. The fragment was labeled at the 5′-terminal with ^{32}P and digested completely by RNase P_1. The ^{32}P-labeled nucleotide (anticodon first letter nucleotide) derived from the tRNA fragment was analyzed by 2D-TLC using two different solvent systems according to the method of Kuchino et al. [19].

3. Results

3.1. Defect in the modification at the anticodon first position of $tRNA^{Lys}$ with the A8344G mutation

We purified the wild-type and mutant $tRNAs^{Lys}$ in amounts sufficient for structural analysis from mass cultures of cybrid cells and determined their nucleotide sequences, including modified nucleotides, by a combination of the methods of Donis-Keller [18] and Kuchino et al. [19]. Restrictive digestion with RNase H was also employed to analyze the anticodon wobble nucleotide modification [20]. Sequence analysis by Do-

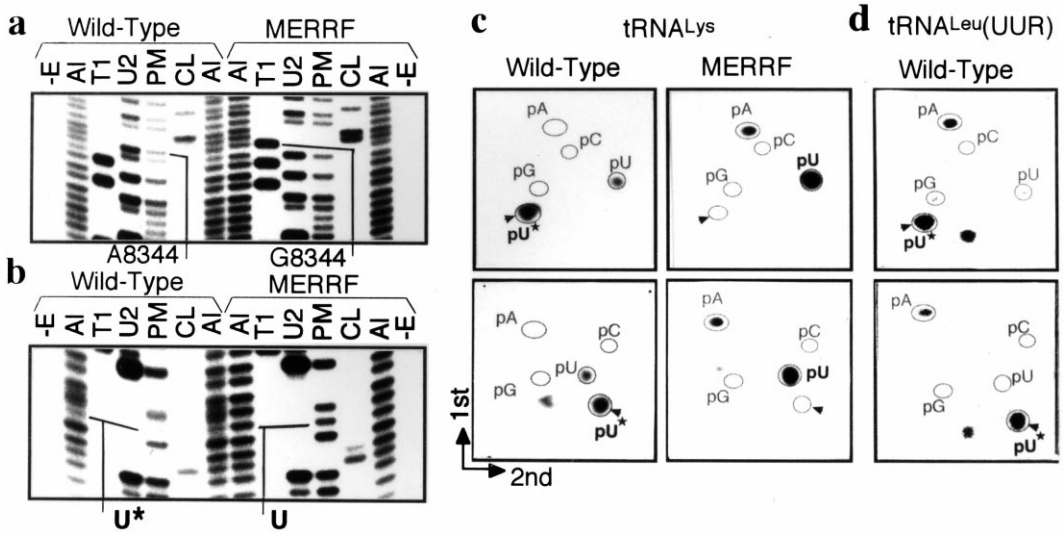

Fig. 1. Sequence analysis of human mitochondrial $tRNA^{Lys}$ and MERRF patient-derived $tRNA^{Lys}$ with the A to G 8344 mutation. (a, b) Sequencing ladders obtained by Donis-Keller's method [18] for wild-type and mutant $tRNAs^{Lys}$ from each cybrid clone in regions around the point mutation (a) and the anticodon loop (b). -E, Al, T1, U2, PM and CL indicate no treatment and digestion by alkali, RNase T_1 (specific for G), RNase U_2 (for A > G), RNase PhyM (for A and U) or RNase CL_3 (for C), respectively. In (b), U and U*, respectively, indicate unmodified and modified uridine. (c) 2D-TLC patterns of a ^{32}P-labeled nucleotide at the anticodon wobble position obtained from wild-type and mutant $tRNAs^{Lys}$ by restrictive digestion with RNase H as described in Section 2. The upper panels show the results with solvent system A and the lower panels those with solvent system B. pU, pA, pG, pC and pU* indicate the position of each nucleotide. Arrowheads show the position of the modified uridine (pU*). (d) 2D-TLC patterns of a ^{32}P-labeled nucleotide at the anticodon wobble position obtained from wild-type $tRNA^{Leu}$(UUR) [14]. $tRNA^{Leu}$(UUR) and $tRNA^{Lys}$ are seen to have the identical modified uridine at the anticodon first position.

nis-Keller's method [18] revealed that tRNA^{Lys} from ME1-4 cybrid cells harboring the MERRF 8344 mutant mtDNA had an A to G transition in the TΨC-loop (tRNA^{Lys}(A8344G)) (Fig. 1a), thereby demonstrating the existence of the mutant tRNA molecule in the mitochondria of the mutant cybrid cells. The sequencing ladders also indicate that the tRNA with the 8344 mutation was purified to homogeneity (Fig. 1a). The sequencing ladders in Fig. 1b clearly show that the first letter of the anticodon of the wild-type tRNA^{Lys} was resistant against RNase PhyM (A- or U-specific) digestion, and an irregular band pattern was observed at this position in the alkaline-treated lanes. These features suggest that the first nucleotide of the anticodon of wild-type tRNA^{Lys} is a modified uridine, as has been reported [21]. Recently, by means of 2D-TLC using two different solvent systems together with high-performance liquid chromatography/electrospray ionization mass spectrometry, we revealed that human and bovine mitochondrial tRNAs^{Leu}(UUR) have the same novel modified uridine at the wobble position [14,22]. In the present study, the nucleotide derived from the anticodon wobble position of the wild-type tRNA^{Lys} was obtained by restrictive digestion with RNase H as described in Section 2 [20], and was developed on 2D-TLC with the same solvent systems [19]. The mobility of this nucleotide differed from that of unmodified uridine or the uridine derivatives reported so far (Fig. 1c) [23], but was exactly the same as the nucleotide obtained from the wobble position of mitochondrial tRNA^{Leu}(UUR) (Fig. 1d). Therefore, both tRNA^{Lys} and tRNA^{Leu}(UUR) have the identical novel uridine modification.

In striking contrast, PhyM digestion of the mutant tRNA^{Lys}-(A8344G) gave a discrete band at the wobble position and alkaline-treated ladders were normal around the position (Fig. 1b), suggesting that uridine at this position was not modified in the mutant cybrid cells. Lack of the modification at the anticodon wobble nucleotide in the mutant tRNA^{Lys} was demonstrated as described above (Fig. 1c), clearly proving that the major spot corresponds to genuine uridine [23]. All the nucleotides in tRNA^{Lys} were analyzed by 2D-TLC using the method of Kuchino et al. [19] to identify those that were modified, which were found to be the following already known nucleosides: 1-methyladenosine (m^{1}A), 2-methylguanosine (m^{2}G), pseudouridine (Ψ) and N^6-threoninocarbonyladenosine (t^{6}A) (Fig. 2) [23]. No differences were observed in respect of the other modified nucleotides of the wild-type and mutant tRNAs^{Lys}, indicating a specific lack of the modification at the wobble position of the mutant tRNA^{Lys}.

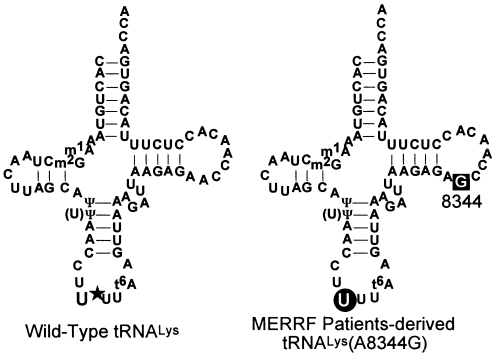

Fig. 2. Cloverleaf structures of human mitochondrial tRNAs^{Lys} from wild-type cells (left) and from MERRF patient-derived cells with the A8344G mutation (right). U* indicates the modified uridine and U on a round black background, unmodified uridine. G on a square black background represents the point mutation. The other modified nucleosides were determined according to the methods of Donis-Keller [18] and Kuchino et al. [19]: 1-methyladenosine (m^{1}A), 2-methylguanosine (m^{2}G), pseudouridine (Ψ) and N^6-threoninocarbonyladenosine (t^{6}A).

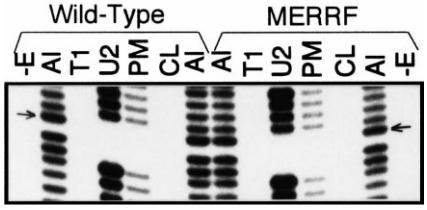

Fig. 3. Confirmation of the presence of modified uridine at the wobble position of mitochondrial tRNA^{Leu}(UUR) in the mutant ME1-4 cybrid clone. Sequencing ladders obtained by Donis-Keller's method [18] of tRNAs^{Leu}(UUR) from mutant cells and wild-type cells in the region around the anticodon loop. Unusual upper shifts of bands in the alkaline ladders and resistance against RNase PhyM digestion were similarly observed at the wobble position of the tRNA^{Leu}-(UUR) from the mutant cells and that from the wild-type cells, indicating that tRNAs^{Leu}(UUR) from both the mutant and wild-type cells have modified uridine at the position. The wobble positions are indicated by arrows.

Since mitochondrial tRNA^{Lys} and tRNA^{Leu}(UUR) in the wild-type cells have the identical modified uridine at the wobble position, we analyzed the modification in the tRNA^{Leu}-(UUR) that occurs in 8344 mutant ME1-4 cybrid cells. In the sequencing ladders in Fig. 3, the first letter of the anticodon of tRNA^{Leu}(UUR) purified from the mutant cells, as well as that of the wild-type cells, is resistant to RNase PhyM digestion and there is unusual mobility in the alkaline-treated lanes at this position. These features strongly suggest that modification of the uridine residue normally occurs at the wobble position of the tRNA^{Leu}(UUR) even in the mutant cells. Therefore, lack of the modification in the mutant tRNA^{Lys} is apparently due to the direct effect of the point mutation, and not to the indirect effect of mitochondrial dysfunction.

4. Discussion

Enriquez et al. made a detailed analysis of the MERRF np 8344 mutation [13]. They surmised that the approximately 50% decrease in aminoacyl-tRNA^{Lys} observed in mutant cells caused a severe reduction in mitochondrial protein synthesis and the production of aberrant translation products, resulting in the respiratory chain defect. In the cases of the 3243 and 3271 mutations in the tRNA^{Leu}(UUR), however, we estimated the total amount of leucyl-tRNA^{Leu}(UUR) with the mutation to be less than 30% that of the wild-type [14], and no significant decrease in protein synthesis was observed [12,24]. Also, in the case of chronic progressive external ophthalmoplegia, Hayashi et al. found that progressive inhibition of mitochondrial translation as well as a reduction in cytochrome *c* oxidase activity occurred in cybrid cells only when Δ-mtDNA (mutant mtDNA with large-scale deletions, including five tRNA genes) accumulated to over 60% of the total mtDNA, i.e. there was a 60% reduction in the five tRNAs and more than 60% of the five respective aminoacyl-tRNAs was missing [16]. Taking these findings together, a 50% decline in only one aminoacyl-tRNA seems inadequate to completely explain the biochemical pathology of MERRF. Furthermore, we observed neither instability nor a decrease in the steady-state

amount of the $tRNA^{Lys}$ with the MERRF mutation as compared with its wild-type counterpart in the respective cybrid cells (data not shown).

Since the uridine at the anticodon first position of $tRNA^{Leu}$(UUR) in mitochondria of mutant MERRF cybrid cells was confirmed to undergo the modification, we consider that the lack of the modification in the $tRNA^{Lys}$ with the 8344 mutation is not caused by a defect in or loss of the modification enzyme(s) in the ME1-4 cybrid cells but by the point mutation in the TΨC arm of the MERRF mutant $tRNA^{Lys}$.

In view of our previous discovery that the nucleotide modification at the first letter of the anticodon is deficient in the two mutant mitochondrial $tRNAs^{Leu}$(UUR) derived from patients with MELAS [14], we considered that a similar modification abnormality was likely to occur in $tRNA^{Lys}$ with the MERRF mutation. To confirm this, we purified the wild-type and mutant $tRNAs^{Lys}$ to homogeneity in amounts sufficient for structural analysis from mass cultures of cybrid cells grown in a complete and non-isotopic medium. Sequencing of the tRNAs revealed that $tRNA^{Lys}$ with the 8344 mutation is in fact deficient in the modification at the anticodon wobble position. The modified uridine is identical to that at the anticodon first position of $tRNA^{Leu}$(UUR). This is the first account of a common defect in the two kinds of tRNA molecules with the respective pathogenic mutations of two major mitochondrial encephalomyopathies. The fact that the three mutant tRNAs possess this common defect in their post-transcriptional modification at the wobble position leads us to speculate that the defect is a strong candidate for being at the root of both MERRF and MELAS pathogeneses.

Modifications at the anticodon wobble position play an important role in the decoding of cognate codons. One possible explanation is that mutant $tRNA^{Lys}$ lacking the modified base cannot decode its cognate codons (AAA and AAG) efficiently, consequently disturbing natural protein synthesis in mitochondria. As codon-anticodon base pairing of the mitochondrial $tRNA^{Lys}$ consists of A-U and G-U pairs which are relatively weak as compared to the G-C pair, the modified uridine at the wobble position might contribute to the stability of the codon-anticodon base pairing. If this is the case, it could explain the reported decrease in protein synthesis and the production of aberrant polypeptides [13] even without a reduction in the amount of $tRNA^{Lys}$. Another possibility, which is based on the mitochondrial wobble rule [25–29] and in vitro translation experiments [30,31], is that an unmodified uridine at the wobble position of the mutant tRNA can recognize all four bases at the third letter of the codon (AAN; N = A, G, U and C), leading to misincorporation. Whatever the mechanism, lack of the mutant $tRNA^{Lys}$ anticodon modification could significantly affect the mitochondrial translation process even when the mutation ratio does not exceed the previously proposed threshold [11,13]. Our proposal does not contradict the fact that patients express clinical symptoms when the proportion of the mutant mtDNA in a tissue, such as muscle, is substantially less than the threshold proposed previously, which leads us to speculate that the modification defect in $tRNA^{Lys}$ with the 8344 mutation plays a significant role in the pathogenesis of MERRF.

Acknowledgements: The authors would like to express their thanks to Dr. J.-I. Hayashi of Tsukuba University for providing a mutant cybrid cell line possessing mtDNA with the A8344G mutation. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (Japan).

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