Decreased CCA-addition in human mitochondrial tRNAs
bearing a pathogenic A4317G or A10044G mutation

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Running title: Decreased CCA-addition in pathogenic mutant tRNAs
Summary

Pathogenic point mutations in mitochondrial tRNA genes are known to cause a variety of human mitochondrial diseases. Reports have associated an A4317G mutation in the mitochondrial tRNA\textsubscript{Ile} gene with fatal infantile cardiomyopathy and an A10044G mutation in the mitochondrial tRNA\textsubscript{Gly} gene with sudden infant death syndrome. Here we demonstrate that both mutations inhibit in vitro CCA-addition to the respective tRNA by the human mitochondrial CCA-adding enzyme. Structures of these two mutant tRNAs were examined by nuclease probing. In the case of the A4317G tRNA\textsubscript{Ile} mutant, structural rearrangement of the T-arm region, conferring an aberrantly stable T-arm structure and an increased Tm value, was clearly observed. In the case of the A10044G tRNA\textsubscript{Gly} mutant, high nuclease sensitivity in both the T- and D-loops suggested a weakened interaction between the loops. These are the first reported instances of inefficient CCA-addition being one of the apparent molecular pathogeneses caused by pathogenic point mutations in human mitochondrial tRNA genes.
Introduction

Mitochondrial (mt) DNA mutations are known to be associated with a variety of human diseases. More than 100 different pathogenic mutations have so far been reported, 58% of which reside in tRNA genes (1-3). Functional analysis of mt tRNAs with such mutations will thus be necessary to clarify the molecular pathogenesis of mitochondrial diseases. If a mutation does not affect replication of mt DNA or transcription of the corresponding mt tRNA, its deleterious effect in the mt tRNA gene can be assigned to the post-transcriptional level — including maturation, modification, folding, stability, aminoacylation, association with translation factors, and/or various functions on the ribosome.

A novel taurine-containing uridine modification was recently identified (4), which was found to be absent in two mutant mt tRNAs: tRNA\textsuperscript{Leu(UUR)}\textsuperscript{A3243G} or tRNA\textsuperscript{Lys}U3271C with an A3243G point mutation and tRNA\textsuperscript{Lys}A8344G with an A8344G point mutation. These mutant tRNAs were respectively obtained from human pathogenic cells of two mitochondrial encephalomyopathic diseases — MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) (5) and MERRF (myoclonus epilepsy associated with ragged-red fibers) (6). The MERRF mt tRNA\textsuperscript{Lys} lacking the wobble modification was found to be incapable of translating cognate codons due to a complete loss of codon-anticodon pairing on the ribosome (7), strongly implicating deficient decoding arising from the modification defect as one of the main causes of the mitochondrial dysfunction, and representing the first known case of a human disease apparently resulting from the loss of a post-transcriptional modification.

CCA-addition at the 3’-terminus of tRNA is one of the essential steps for tRNA
maturation in mitochondria. In human mitochondrial DNA, the CCA sequence of tRNAs is not encoded in the tRNA genes but is post-transcriptionally synthesized by ATP (CTP):tRNA nucleotidyltransferase (CCA-adding enzyme). We recently cloned and characterized the human mt CCA-adding enzyme, and showed that it efficiently recognizes mt tRNAs with unusual structures (8). Bacterial and yeast CCA-adding enzymes have been shown to recognize the elbow-region of tRNA formed by D- and T-loop interaction (9-11). In contrast, human mt tRNAs have no consensus sequence in either the D- or T-loop. Although the tRNA recognition mechanism of the human mt CCA-adding enzyme is now under investigation, the T-arm has been found to be important for efficient CCA-addition (8).

We report here in vitro evidence that two pathogenic mitochondrial point mutations significantly inhibit CCA-addition to the corresponding tRNAs. The first mutation, A4317G in the mt tRNA^{Ile} gene, was described to be associated with severe cardiomyopathy in a 1-year-old infant who died from cardiac failure (12). The other, A10044G in the mt tRNA^{Gly} gene, was reported in several siblings of one family and appeared to be associated with sudden unexpected death (13). Although little is known of the molecular pathogenesis of these two point mutations, since they occur at similar positions in the respective T-loops of mt tRNA^{Ile} and tRNA^{Gly}, we speculated that they could modulate CCA-adding activity during the tRNA maturation. A kinetic analysis of CCA-addition and a structural investigation of the mutant tRNAs indicated the involvement of these pathogenic point mutations in the molecular pathogenesis of mitochondrial diseases.
Materials and methods

Materials

\[\alpha^{32}\text{P}]\text{ATP} (110 \ \text{TBq/mmol}) \text{ was obtained from Amersham Biosciences and } [5^\prime-^{32}\text{P}]-(\text{pCp}) (111 \ \text{TBq/mmol}) \text{ from NEN Life Science Products. Nuclease S1 and T4 RNA ligase were purchased from Takara Bio Inc., RNase V1 from Ambion Inc., and RNase U2 from Sigma.}

In vitro transcription of human mt tRNAs

Template plasmids for in vitro transcription of human mt tRNAs for Ile and Gly were constructed with synthetic DNAs. DNA fragments containing the class III promoter of T7 RNA polymerase directly connected upstream of human mt \text{tRNA}^{\text{Ile}} \text{ or mt \text{tRNA}^{\text{Gly}}} \text{ gene were synthesized by Klenow enzyme using two overlapping primers. The A1–U72 base pair of both tRNAs was replaced with G1–C72 to promote the efficiency of transcription by T7 RNA polymerase. The following DNA primers were used:}

mtIle1—AAAAGGGGAATTCTAATACGACTCACTATAGGAATATGTCTGATA AAAGAGTACTTTGGATAGA;

mtIle2—AAAAGGGGAAGCTTTGGAAATAAGGGGGTTTAAGCTCCTATATTTAC TCTATCAAAAGTAACTCTTT;

mtGly1—AAAAGGGGAATTCTAATACGACTCACTATAGCTCTTTTAGTATAAA TAGTACGTAAACTTCCAA;

mtGly2—AAAAGGGGAAGCTTTGCTCTTTTTGAATGTTGTCAAAACTAGTTA ATTGGAAGTTAACGGTACTA.

The synthetic DNA fragments were cloned into the \text{Eco RI/Hind III sites of pUC18}
and sequenced. The DNA fragments were amplified from the cloned plasmid to be used as templates for T7 transcription by PCR with the following primer sets:

GGGAATTCTAATACGACTCACATAGGAAAT (T7-Ile) and
TGGAAATAAGGGGGTTTAAGC for the wild-type tRNA_{ile}^{D}; T7-Ile and
GTGGAAATAAGGGGGTTTAAGC for the wild-type tRNA_{ile}^{DC}; T7-Ile and
GGTGGAATAAGGGGGCTTAAGC for the wild-type tRNA_{ile}^{DCC}; T7-Ile and
TGGAAATAAGGGGGCTTAAGC for the A4317G tRNA_{ile}^{D}; T7-Ile and
GTGGAAATAAGGGGGCTTAAGC for the A4317G tRNA_{ile}^{DC}; T7-Ile and
GGTGGAATAAGGGGGCTTAAGC for the A4317G tRNA_{ile}^{DCC}; T7-Ile and
GAATTTCTAATACGACTCACAATAGC (T7-Gly) and
TGCTCTTTTTGTAATGTGTGCTAAAA for the wild-type tRNA_{Gly}^{D}; T7-Gly and
GTGCTCTTTTTGTAATGTGTGCTAAAA for the wild-type tRNA_{Gly}^{DC}; T7-Gly and
GGTGCTCTTTTTGTAATGTGTGCTAAAA for the wild-type tRNA_{Gly}^{DCC}; T7-Gly and
GTGCTCTTTTTGTAACGTTGTCAAAAAACTAGT for the A10044G tRNA_{Gly}^{D}; T7-Gly and
GTGCTCTTTTTGTAACGTTGTCAAAAAACTAGT for the A10044G tRNA_{Gly}^{DC}; T7-Gly and
GGTGCTCTTTTTGTAACGTTGTCAAAAAACTAGT for the A10044G tRNA_{Gly}^{DCC}. In vitro run-off transcription was performed according to the literature (14). All transcripts obtained were purified by denaturing 12% polyacrylamide gels containing 7 M urea.

**Preparation of human mt CCA-adding enzyme**

Human mt CCA-adding enzyme was expressed in *E. coli* and purified as previously described (8).
Assays of CCA-adding enzyme

Assays were carried out as described previously (8). The 10-µl reaction mixtures contained 50 mM Tris–HCl (pH 8.5), 10 mM MgCl₂, 100 mM KCl, 0.1 mM CTP and/or ATP, 0.033 µM [α-³²P]CTP or [α-³²P]ATP, 1 µM substrate tRNA, and 10 ng purified recombinant CCA-adding enzyme. Reaction products were resolved by denaturing 10% PAGE and the radioactivity of the labeled bands was measured using an image analyzer (BAS-5000; Fuji Photo Film).

Determination of kinetic parameters for C-addition or A-addition

The 10-µl reaction mixtures for C-addition or A-addition contained 50 mM Tris–HCl (pH 8.5), 10 mM MgCl₂, 100 mM KCl, 10 ng CCA-adding enzyme, 0.5 mM CTP or ATP, 0.033 µM [α-³²P]CTP or [α-³²P]ATP, and 1–12 µM tRNA-DC or tRNA-DCC. After incubation for 5 min while the nucleotide incorporation rate was constant, reactions were stopped by adding 8 M urea. Mixtures were loaded onto denaturing 10% PAGE, and the gel was exposed to an imaging plate along with the standard dilution series of [α-³²P]CTP or [α-³²P]ATP electrophoresed on another gel. The amount of nucleotide incorporated was calculated by comparing the radioactivities of labeled tRNA with those of nucleotide standards. The initial velocities of nucleotide incorporation were then utilized in Lineweaver–Burk plots to determine the kinetic parameters.

Nuclease probing

The tRNAs were labeled at the 3’ terminal with [5’-³²P]-(pCp) by T4 RNA ligase and then purified by denaturing PAGE. Limited digestion under denaturing condition by
alkaline, RNase T1, and RNase U2 was performed according to the Donis-Keller’s method (15) to verify the sequence of tRNAs. Limited digestion under non-denaturing condition by nucleases S1, V1, and U2 was carried out at 25°C for 5 min in 50 mM NaOAc (pH 5.0), 20 mM MgCl₂, 300 mM NaCl, and 100 A₂₆₀ units/ml of a yeast tRNA mixture. For the digestion with nuclease S1, 1 mM ZnSO₄ was added. The enzyme concentrations were 0.04, 0.2 and 0.4 U/µl nuclease S1, 4×10⁻³, 8×10⁻³ and 1.6×10⁻² U/µl RNase V1, and 8×10⁻⁴, 4×10⁻³ and 8×10⁻³ U/µl RNase U2. Reactions were stopped by adding 8 M urea and quenching in liquid nitrogen. Mixtures were then loaded onto 50-cm-long denaturing 15% polyacrylamide gel containing 7 M urea and 10% glycerol. The gels were exposed to an imaging plate and analyzed with a bioimaging analyzer (FLA3000; Fuji Photo Film).

Conformational analysis of small RNA fragments

Short RNA fragments containing the T-arm region of the wild-type and A4317G tRNA^Ile were in vitro-transcribed (14) using the following DNA fragments as templates, and purified with super-denaturing PAGE (15% polyacrylamide, 7 M urea, 30% formamide, 1× TBE). Fragments: wild type

—TAGAAATAAGGGGTTTAAGCTCCTATAGTGAGTCGTATTAGAATAATACGA CTCACTATAGG; A4317G

—TAGAAATAAGGGGCTTAAGCTCCTATAGTGAGTCGTATTAGAATAATACGA CTCACTATAGG. The RNA fragments were then electrophoresed under a super-denaturing condition (15% polyacrylamide, 7 M urea, 30% formamide, 1× TBE) after denaturation in a super-denaturing buffer (50% formamide, 1× TBE) at 65°C for 15 min, the regular denaturing condition (15% polyacrylamide, 7 M urea, 1× TBE) after
being mixed with a denaturing buffer (7 M urea, 1× TBE), and a non-denaturing condition (15% polyacrylamide, 1× TBE) without denaturation.

Measurement of RNA melting profiles

Melting profiles were measured by a Gilford Response II spectrophotometer using 0.1 or 0.2 A_{260} units of RNA samples in 400 µl of a buffer consisting of 50 mM sodium cacodylate (pH 7.0), 10 mM MgCl₂, and 200 mM NaCl as previously described (16).

Results

Effects of pathogenic A4317G and A10044G mutations in mt tRNAs on CCA-addition

We previously found that the human mt CCA-adding enzyme requires the T-arm region of mt tRNAs for efficient CCA-addition (8), prompting us to hypothesize that some mitochondrial diseases associated with pathogenic point mutations in tRNA genes may result from an absence or an inefficiency of CCA-addition to mt tRNA during maturation. Because two pathogenic point mutations, A4317G and A10044G, were found at similar positions in the respective T-loop of mt tRNA^{Ile} and mt tRNA^{Gly}, we focused our attention on evaluating these mutations (Fig. 1).

To examine the effects of the mutations, we prepared wild-type and mutant tRNAs without the 3′ terminal CCA sequence (tRNA-D) by in vitro transcription, and evaluated their capability of incorporating CCA catalyzed by the recombinant human mt CCA-adding enzyme (8). As shown in Fig. 2a, in vitro-transcribed wild-type mt tRNAs for Ile and Gly were repaired by the human mt CCA-adding enzyme as efficiently as
canonical yeast tRNA^{Phe}. In contrast, both mutants exhibited significantly lower levels of CCA-incorporation, with remarkable reduction in the case of the A4317G mt tRNA^{Ile} mutation (Fig. 2a).

Since the CCA-adding reaction consists of C-addition and A-addition, we prepared wild-type and mutant tRNAs without the 3′ terminal CA sequence (tRNA-DC) and 3′ terminal A (tRNA-DCC), and investigated the efficiency of C-addition to tRNA-DC and A-addition to tRNA-DCC. Both C- and A-addition to mutant tRNAs were reduced (Fig. 2b), not as remarkably as the entire CCA-addition (Fig. 2a), suggesting that the reduced reaction at each step of C- and A-addition accumulatively results in significant decrease of whole CCA-addition (Fig. 2a).

The kinetic parameters of C-addition to tRNAs-DC and A-addition to tRNAs-DCC for wild-type and mutant tRNAs were determined as shown in Table I. The $k_{cat}$ values for both mutant tRNAs were severely reduced, whereas $K_m$ values were slightly affected in either C- or A-adding step. This observation suggests that these two mutations inhibit both C- and A-adding steps by mainly hindering the catalytic process without affecting the substrate recognition.

Structural rearrangement of T-arm in mt tRNA^{Ile} with the A4317G mutation

Suspecting that the reduction in the $k_{cat}$ value might arise from structural alteration caused by the pathogenic point mutation, we carried out nuclease probing using double-stranded specific RNase V1 and single-stranded specific cleavages by nuclease S1 and RNase U2, and compared the cleavage profiles with those of the wild type. The nuclease cleavage pattern in Fig. 3a indicates that while the A4317G mutant appears to retain the global secondary structure of the wild-type mt tRNA^{Ile}, some significant
differences are evident in the T-arm region. C54 and C62 of the A4317G mutant became sensitive to double-strand ed specific RNase V1 on the 5′ side, while the 5′ side of A49 exhibited high resistibility to this nuclease (Fig. 3b). This finding suggests structural rearrangement of the T-arm region caused by the A4317G mutation, as was predicted by Tanaka et al. (12) when they first reported the mutation. They proposed the T-stem "slippage" model as shown in the inset of Fig. 3b, in which the T-arm has a stem of six base pairs with two A–C mismatches and a 4-base loop. The RNase V1 cleavages at C54 and C62 in mutant tRNA are likely to result from two Watson–Crick base pairs, G53–C60 and C54–G59. An absence of cleavage at A49 can be explained by the A49–C64 mismatch. In addition, the 5′ side of U56 of the mutant was less sensitive to RNase V1 cleavage than that of the wild type. Because the base pairs between T-loop and D-loop are known to accommodate cleavage sites for RNase V1 (17), tertiary interaction between the T- and D-loops involving U56 is assumed to be impaired by the structural rearrangement.

Aberrantly stable secondary structure of T-arm in mt tRNA^{lle} induced by the A4317G mutation

The structural rearrangement by the A4317G mutation is supported by the abnormal migration of RNA fragments containing the T-arm region on the alkaline ladder (Fig. 3a). The 3′ part of the RNA fragment cleaved at the 3′ side of A49 of the mutant tRNA migrates faster than the corresponding fragment of the wild type, while normal migration of a one-nucleotide-short RNA fragment (cleaved at the 3′ side of G50) is observed. This extraordinary mobility of the RNA fragment of the mutant tRNA, in spite of the electrophoresis of the denaturing gel at 55°C, suggests the formation of
aberrantly stable secondary structure in the T-arm region caused by the A4317G mutation. Similar faster migration is observed in the cases of RNA fragments corresponding to positions 45–48, indicating that the stable secondary structure of the T-arm region is retained in the whole tRNA molecule with the A4317G mutation.

To characterize the structural rearrangement resulting from the A4317G mutation, the 3’ parts of RNA fragments from G50 to A73 with or without the mutation were synthesized by T7 RNA polymerase (Fig. 4a). Figure 4b shows that the RNA fragment with the A4317G mutation migrated faster than the wild type in gels under the non-denaturing or regular denaturing condition (as in Fig. 3a), while no discernable difference in the mobility was observed in the super-denaturing gel (see Materials and methods for details of each condition). Faster migration in the gel is indicative of a stable, compact structure of the RNA fragment with the A4317G mutation.

The melting profiles of the wild type and mutant fragments are plotted in Fig. 5a. Only the mutant fragment exhibits an evident melting curve with higher Tm value, verifying its stable secondary structure. This also explains the fact that the Tm value of mt tRNA\textsuperscript{Ile} with the A4317G mutation (59°C) was higher than that of the wild-type mt tRNA\textsuperscript{Ile} (57.5°C) (Fig. 5b).

Taken together, these results demonstrate that the strong inhibition of CCA-addition to the mt tRNA\textsuperscript{Ile} A4317G mutant is caused by the structural rearrangement of the T-arm region induced by the mutation.

*Structural change in mt tRNA\textsuperscript{Gly} with the A10044G mutation*

Considering that position 10044 in the secondary structure of mt tRNA\textsuperscript{Gly} is identical to that of 4317 in mt tRNA\textsuperscript{Ile} (Fig. 1b), the tertiary structure of the mt tRNA\textsuperscript{Gly}
A10044G mutant was also evaluated by nuclease probing (Fig. 6a). As shown in Fig. 6b, strong cleavages were observed at both the 5′ and 3′ sides of A55/A56 in the T-loop of the mutant tRNA by nuclease S1 and RNase U2, respectively. In addition, residues in the D-loop show slightly higher sensitivity to nuclease S1, with decreased accessibility at the 5′ side of U20/A21 by RNase V1. These results suggest that tertiary interaction between the T- and D-loops is weakened by the A10044G mutation, whereas the global secondary structure is conserved.

Discussion

In the initial step of mammalian mitochondrial tRNA maturation process, the 5′-end leader and 3′-end trailer are removed by RNase P and 3′-tRNAse, respectively, followed by the addition of 3′ CCA terminus by the CCA-adding enzyme (18,19). It is possible to consider that some pathogenic mutations may have deleterious effect on the tRNA maturation step. Here, we have shown that both the A4317G and A10044G mutations in mt tRNAs significantly inhibit the CCA-addition by the human mt CCA-adding enzyme. Both C- and A-addition were inhibited by these mutations, not as remarkably as the entire CCA-addition, suggesting that the reduced reaction at two C-adding steps and one A-adding step accumulatively results in significant decrease of whole CCA-addition. The CCA sequence of mt tRNAs is not encoded in the tRNA genes but is post-transcriptionally synthesized by mt CCA-adding enzyme. Immature tRNAs without complete CCA sequence cannot be aminoacylated nor protected by elongation factors, resulting in their instability in mitochondria. Thus, the significant decrease of entire CCA-addition by A4317G and A10044G mutations (Fig. 2a) might be a direct cause for mitochondrial dysfunction.
The A4317G mutation in mt tRNA\textsubscript{Ile} induced a structural rearrangement of the T-arm region, while the A10044G mutation in mt tRNAGly weakened the T-loop/D-loop interaction. It has been considered that the CCA-adding enzyme recognizes the elbow region of tRNA formed by the D- and T-loops (9,10,20,21), and we previously showed that the mt CCA-adding enzyme has a lower substrate specificity than the \textit{E. coli} enzyme — presumably to enable it to recognize mt tRNAs with unusual structures (8). Our finding that both the A4317G and A10044G mutations in human mt tRNAs had small effect on the $K_m$ values for CCA-addition indicates that these mutations do not inhibit recognition of the tRNA elbow-region by the mt CCA-adding enzyme. Thus, the decreases observed in the $k_{cat}$ values might result from an inappropriate positioning of the tRNA substrate in the enzyme during the catalytic process.

The sequences of the T- and D-loops are highly conserved in cytoplasmic tRNAs, but mammalian mt tRNAs have unusual structures without consensus sequences in either of the loops (22). Hence, it is difficult to predict the conformational influence of a single mutation in mt tRNAs because of their abnormality. When Tanaka et al. first reported the A4317G mutation in mt tRNA\textsubscript{Ile}, they deduced that it would cause structural rearrangement of the T-arm region (Fig. 3b)(12). Our findings based on nuclease probing and RNA fragment analysis have clearly demonstrated their proposed structural rearrangement. We previously analyzed another pathogenic point mutation — A4269G in mt tRNA\textsubscript{Ile} associated with fatal cardiomyopathy — and found that this mutation destabilizes the whole tRNA structure with a lower Tm, which explains the rapid decay of the A4269G mutant tRNA observed in cybrid cells (16). In contrast, the A4317G mutation stabilizes tRNA by forming an aberrantly stable secondary structure in the T-arm region, thereby increasing its Tm value. It is plausible that this structural
rearrangement affects various steps in the tRNA maturation process, including CCA-addition. In the case of the A10044G mutation in mt tRNA$_{\text{Gly}}$, such structural rearrangement was not observed, but instead a weakened interaction between the T- and D-loops was suggested. In mitochondria, high sensitivity to nucleases in either the T- or D-loop can lead to instability of the tRNA.

The A4317G mutation was reported to significantly decrease isoleucylation (23,24). Thus, it can be assumed that the pathogenic A4317G mutation at least causes defects in both CCA-addition and the following aminoacylation. In addition, because certain mutations in the T-loop were demonstrated to decrease the tRNA processing activity of Drosophila RNase P and 3′-tRNAse (25), it can be speculated that both the A4317G and A10044G mutations also affect the 5′- and 3′-processing of tRNA. We cannot define which step in tRNA maturation process is the most damaged in vivo, since neither of the cell lines carrying these mutations is available at the present time (Tanaka et al., personal communication). Even if the 5′- and 3′-processing proceeds inefficiently by these mutations, the following CCA-addition and aminoacylation are still considered to be crucial steps for tRNA function.

Our findings presented here indicate the probability that the CCA-addition disorder of the pathogenic A4317G and A10044G mutants is involved in mitochondrial dysfunction. Furthermore, they suggest an approach to understanding the mechanism of tRNA recognition by the mt CCA-adding enzyme, which is currently under investigation.

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References


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Figure Legends

Fig 1. Pathogenic mutations in human mt tRNAs. The mutation positions are indicated as outline characters. The base alterations to promote the efficiency of transcription are shown in boxes. Bases are numbered according to the numbering rule proposed by Sprinzl et al. (26).

Fig 2. a: Effects of pathogenic point mutations on CCA-addition. The CCA-repairing efficiency of human mt CCA-adding enzyme was compared using yeast tRNA^Phc-D, wild type (WT) and A4317G mutant of mt tRNA^ile-D, and WT and A10044G mutant of mt tRNA^Gly-D, in the presence of CTP and [$\alpha$-32P]ATP. b: Effects of pathogenic point mutations on C-addition and A-addition. The C-adding efficiency to WT and mutant of tRNA-DC (left), and A-adding efficiency to WT and mutant of tRNA-DCC (right) were compared. Above, tRNA^ile; below, tRNA^Gly.

Fig 3. a: Nuclease cleavage patterns of wild-type and mutant tRNA^ile. The abbreviations –, Al, T1, and U2 indicate no treatment and treatment by alkaline digestion, RNase T1 (Specific for G), or RNase U2 (for A>G), respectively. The structure lanes contained tRNA partially digested by nuclease S1, V1, or U2 under the non-denaturing condition. b: Structure of wild-type and mutant tRNA^ile. Secondary structure models of the wild-type and mutant tRNA^ile and a summary of the nuclease probing results are depicted. Black arrows indicate positions where the mutant is more sensitive to the nuclease than the wild type; gray arrows show where it is less sensitive. Outline characters indicate the mutation points. Inset: T-stem "slippage" model proposed by
Fig 4. a: The sequence of short RNA fragments containing the T-arm region of the wild-type and A4317G tRNA\text{Ile}. b: Migration of wild-type and A4317G RNA fragments in super-denaturing, regular denaturing, and non-denaturing gels.

Fig 5. a: Melting curves of short RNA fragments containing the T-arm region of the wild-type and A4317G tRNA\text{Ile}. Unfilled circles indicate the wild type and filled circles the A4317G mutant. b: Melting curves of wild-type and A4317G tRNA\text{Ile}. Unfilled squares indicate the wild type and filled squares the A4317G mutant.

Fig 6. a: Nuclease cleavage pattern of wild-type and mutant tRNA\text{Gly}. The abbreviations --, Al, T1, and U2 indicate no treatment and treatment by alkaline digestion, RNase T1 (Specific for G), or RNase U2 (for A>G), respectively. The structure lanes contained tRNA partially digested by nuclease S1, V1, or U2 under the non-denaturing condition. b: Structure of wild-type and mutant tRNA\text{Gly}. Secondary structure models of the wild-type and mutant RNA\text{Gly} and a summary of the nuclease probing results are depicted. Black arrows indicate positions where the mutant is more sensitive to the nuclease than the wild type; gray arrows show where it is less sensitive. Outline characters indicate the mutation points.
Table I

Kinetic parameters of wild-type and mutant tRNAs for C-addition and A-addition

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Fig 1

Fig 2a
**tRNA\textsuperscript{Ile}**

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**tRNA\textsuperscript{Gly}**

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Fig 2b
Fig3a
Fig 3b
Wild type

A4317G

Alkaline cleavage

Fig. 4
**Fig. 5**

A

![Graph A](image)

B

![Graph B](image)
Fig 6a
Wild type

A10044G Mutant

Fig 6b
Decreased CCA-addition in human mitochondrial tRNAs bearing a pathogenic A4317G or A10044G mutation
Yukihide Tomari, Narumi Hino, Takashi Nagaike, Tsutomu Suzuki and Takuya Ueda

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