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博士論文

Biogenesis of N^6–threonylcarbamoyladenosine in human mitochondrial tRNAs: implication for molecular pathogenesis of mitochondrial diseases

（ヒトミトコンドリアtRNAにおけるN^6–threonylcarbamoyladenosineの生合成とミトコンドリア病の発症機構）

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Introduction

In all living organisms, RNA molecules play essential roles in maintaining accurate and efficient cellular processes. Gene sequences in genomic DNA are transcribed to messenger RNAs (mRNAs) to be translated into specific proteins. Ribosomal RNAs (rRNAs) shape core structure of the ribosome, and play critical roles in protein translation. Transfer RNAs (tRNAs) are adaptor molecules that link codons and their cognate amino acids on the ribosome during translation. RNA molecules contain a number of modified nucleosides that are introduced post-transcriptionally. More than 100 species of RNA modifications are reported so far. Especially, 70% of them are found in tRNAs. tRNA modifications have been well-studied for more than half century, and are required for tRNA stability, accurate decoding, efficient aminoacylation, and so on.

N^6-threonylcarbamoyladenosine (t^6A) is an evolutionally conserved modification found in tRNAs which decode A-starting codons (ANN) (**Fig. 1**). t^6A is a modified adenosine conjugated with threonine through carbonyl group at N^6 position of adenine base. t^6A is present at the position 3'-adjacent to the anticodon (position 37). t^6A37 prevents base-paring between t^6A37 and U33 to stabilize the anticodon loop structure. The extended planar ring of t^6A conferred by the intramolecular hydrogen bond between N1 and N11 allows t^6A37 to stack with A38 in the anticodon loop and the first adenine base of the codon, strengthening anticodon-codon interaction (**Fig. 1**). There are some derivatives of t^6A including 2-methylthio t^6A (ms^2t^6A) and N^6-methyl t^6A (m^6t^6A). Our group reported that t^6A is further modified to cyclic t^6A (ct^6A) to ensure decoding ability in bacteria, fungi, plants and protists. t^6A is an essential modification in most bacteria, and also required for efficient growth of eukaryotic cells. t^6A plays various functions in decoding and protein synthesis. This modification is required for aminoacylation of tRNA, tRNA binding to the A-site codon, efficient translocation, reading frame maintenance. In addition, t^6A prevents leaky scanning of initiation codon and read through of stop codon.

Mitochondrion is an organelle that produces ATP by oxidative phosphorylation in eukaryotic cells. In human mitochondria, 22 species of tRNAs (mt tRNAs) encoded in mitochondrial DNA (mtDNA) are required to translate 13 essential subunits of the respiratory chain complexes. mt tRNAs contain 16 species of modified bases that are introduced by nuclear-encoded tRNA-modifying enzymes. These modifications are required for proper function of mt tRNAs, and lack of these modifications cause pathological consequences. Our laboratory previously discovered 5-taurinomethyluridine (τm^5U) found at the anticodon first position of mt tRNAs, and found absence of τm^5U in mt $tRNA^{Leu(UUR)}$ bearing pathogenic point mutations associated with mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Lack of τm^5U resulted in deficient decoding of UUG codon, leading to mitochondrial dysfunction. This is the

first reported instance of human disease caused by RNA modification disorder. Loss-of-function of tRNA-modifying enzymes can also cause human diseases. Recently, large scale exome analyses have identified a number of genes encoding tRNA-modifying enzymes responsible for mitochondrial tRNA modifications.

In human mitochondria, t^6A is present at position 37 of five species of mt tRNAs for Thr, Ile, Asn, Lys and Ser(AGY). On the basis of functional importance of this modification in most organisms, I aim to characterize biogenesis and function of t^6A in human mt tRNAs, and investigate pathogenic mutations in mt tRNAs that impair t^6A formation.

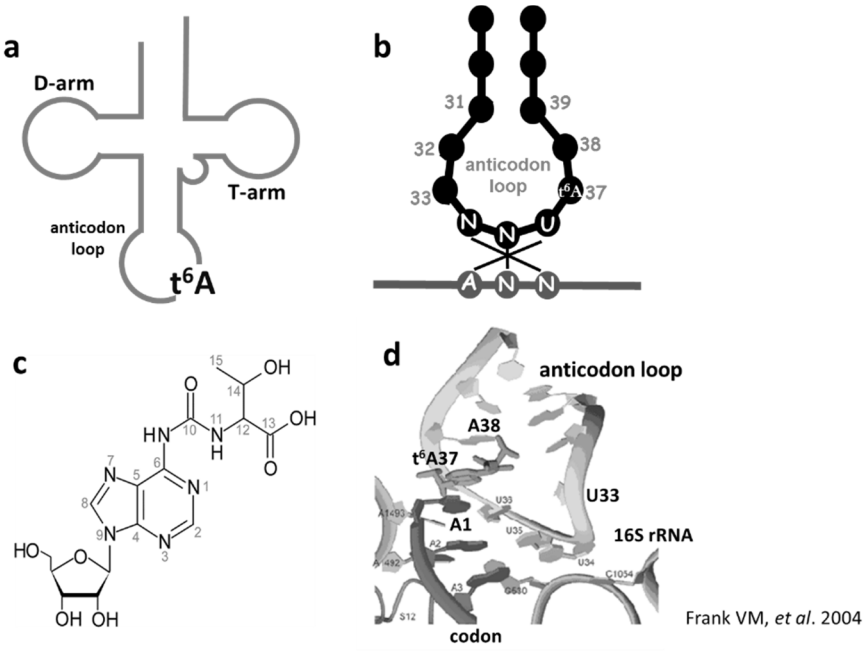

Figure. 1 Characteristics of t^6A37. Schematic depiction of tRNA **(a)** and anticodon stem loop with t^6A at position 37 **(b)**. **(c)** Chemical structure of t^6A. **(d)** Crystal structure of anticodon stem loop with t^6A37 and A-site codon complexed with 30S ribosome.

Results and discussion

Subcellular localization of YRDC and OSGEPL1

Biosynthesis of t^6A37 in *Escherichia coli* (*E. coli*) was well documented. Four enzymes, YrdC, YgjD, YeaZ and YjeE cooperatively synthesize t^6A37 in bacterial tRNAs from ATP, L-threonine and bicarbonate as substrates. The catalytic reaction consists of two consecutive steps. First, YrdC forms threonylcarbamoyl-adenylate (TC-AMP), an active intermediate for t^6A37 formation, from ATP, L-threonine and bicarbonate. Second, YgjD facilitates a nucleophilic attack of N^6-amino

group of A37 on the carbonyl group of TC-AMP to form t^6A37 with releasing AMP. In this step, YeaZ and YjeE serve as accessory proteins for YgjD.

YRDC and OSGEPL1, homologs of YrdC and YgjD, were predicted to be tRNA-modifying enzymes for t^6A37 formation in human mt tRNAs, but no direct evidence had been provided. Transient expression of YRDC and OSGEPL1 with FLAG-tag in HeLa cells was performed, and the proteins were immunoprecipitated from the cell lysates. Mass spectrometric analyses revealed that N-termini of both proteins are truncated, indicating that mitochondria targeting sequences (MTS) of both proteins are cleaved after importation to mitochondria. Immunostaining revealed that OSGEPL1 was clearly localized in mitochondria. In contrast, YRDC predominantly localizes to cytoplasm. However, I detected small fraction of YRDC in mitochondrial fraction by western blotting. Supporting to this finding, mitochondrial localization of YRDC was enhanced when some point mutations were introduced in MTS of YRDC, indicating that YRDC has a weak MTS, because a large fraction of YRDC localized in cytoplasm participates in t^6A37 formation in cytoplasmic tRNAs.

Loss of OSGEPL1 causes mitochondrial dysfunction

To demonstrate that OSGEPL1 is responsible for t^6A37 formation in mt tRNAs, I knocked out *OSGEPL1* in HEK293T cells using the CRISPR/Cas9 system, and obtained two knockout (KO) cell lines in which both alleles have frameshift mutations. Lack of OSGEPL1 protein in the KO cell was confirmed by western blotting. Then, I isolated t^6A37-bearing mt tRNAs from *OSGEPL1* KO cells. Mass spec analyses (RNA-MS) revealed no t^6A37 in these tRNAs. I also confirmed that KO of *OSGEPL1* did not affect t^6A37 in cytoplasmic tRNA, demonstrating that OSGEPL1 is a t^6A37-modifying enzyme specific to mt tRNAs. Next, I examined mitochondrial activity of *OSGEPL1* KO cells. These KO cells grew well in glucose medium, but did not grow in galactose medium efficiently, indicating that *OSGEPL1* KO cells have little mitochondrial activity, because galactose is a non-fermentable carbon source, efficient mitochondrial activity is required to survive in this medium. Steady-state levels of mitochondrial protein subunits of respiratory chain complexes in isolated mitochondria from WT and KO strains were compared by western blotting. Remarkable reduction in ND2 and ND5 and slight reduction of COI were observed, showing that lack of t^6A37 in mt tRNAs impairs mitochondrial protein synthesis. To confirm this result, I conducted pulse labelling experiment to estimate rate of mitochondrial translation. Compared to WT cells, severe reduction of mitochondrial protein synthesis was observed in the KO cells. I also observed little oxygen consumption, reduced complex I activity, and low ATP production in *OSGEPL1* KO cells. In addition, aminoacylation level of mt $tRNA^{Lys}$ was reduced in *OSGEPL1* KO, indicating that t^6A37 is required for efficient lysylation of mt $tRNA^{Lys}$ in mitochondria. Taken together, t^6A37 in mt tRNAs is required for efficient translation and respiratory activity of mitochondria.

In vitro reconstitution of t^6A37 and kinetic measurement

For *in vitro* reconstitution of t^6A in mt tRNAs, I obtained recombinant YRDC and OSGEPL1 expressed in *E. coli*. As substrates, five species of t^6A-bearing mt tRNAs were transcribed *in vitro*. As expected, YRDC and OSGEPL1 successfully introduced t^6A37 in all five species of mt tRNAs in the presence of L-threonine, bicarbonate, and ATP as substrates. However, in my reaction condition, different efficiencies of t^6A formation were observed between five mt tRNAs. Full activity of t^6A formation was seen in mt $tRNA^{Thr}$ and mt $tRNA^{Asn}$, whereas t^6A was partially introduced in mt $tRNA^{Ile}$ and mt $tRNA^{Ser(AGY)}$. For mt $tRNA^{Ile}$, some other modifications are necessary for efficient t^6A37 formation. For mt $tRNA^{Ser(AGY)}$, some adaptor protein or metabolite that specifically recognizes this tRNA might be involved in efficient t^6A37 formation in mitochondria.

I next measured the kinetic parameters for mitochondrial t^6A37 formation on mt-$tRNA^{Thr}$, and determined apparent Km values for each substrate. The Km value for mt-$tRNA^{Thr}$ was sub-micromolar (0.42 μM), indicating that OSGEPL1 had sufficient affinity for the tRNA substrate. By contrast, the Km values for ATP and L-Thr were 76 μM and 39 μM, respectively. Strikingly, the Km value (31 mM) for bicarbonate was quite high, but within the range of bicarbonate concentration (10–40 mM) in human mitochondria, indicating that mitochondrial t^6A37 formation could be regulated by cellular bicarbonate or CO_2 concentration. I also found high Km value for TC-AMP formation catalyzed by YRDC, demonstrating that mitochondrial TC-AMP formation also requires a high cellular concentration of CO_2/bicarbonate.

Pathogenic mutations impair mitochondrial t^6A37 formation

Pathogenic mutations in mtDNA are associated with mitochondrial disorders. Among them, about 200 mutations reside in tRNA genes. Curiously, pathogenic mutations in each tRNA are linked to different symptoms of mitochondrial diseases. Given that t^6A37 plays critical roles in mitochondrial translation, I speculated that some pathogenic point mutations in mt-tRNA genes impair t^6A37 formation, in turn leading to mitochondrial dysfunction. From among the previously described pathogenic mutations, I prepared 21 mutant mt-tRNAs for Thr, Lys, and Asn by *in vitro* transcription, and then examined their ability to undergo t^6A37 modification mediated by YRDC and OSGEPL1. Collectively, two target-site mutations and nine mutations strongly affected t^6A37 formation, indicating that loss of t^6A37 is a major cause of mitochondrial disorders. Multiple mutations that reduced t^6A37 reside in the anticodon arm of mt-tRNAs, suggesting that OSGEPL1 primarily recognizes the anticodon stem-loop structure to introduce t^6A37.

Hypomodification of t^6A37 in mt-tRNA in patient cells

To verify the loss of t^6A37 in mutant mt-tRNAs in patient cells, I obtained fibroblasts and myoblasts harboring the A15923G mutation from a 12-year-old female patient with symptoms of myoclonic epilepsy with ragged-red fibers (MERRF), lactic acidosis, short stature, and hearing loss. I isolated mt-$tRNA^{Thr}$ from the patient's cells and analyzed their modification status by RNA-MS. I observed low levels of t^6A37 in the mutant tRNAs isolated from myoblasts (5.7%) and fibroblasts (5.4%). This finding is consistent with our *in vitro* mutation studies, and demonstrates that pathogenic point mutations in mtDNA impaired t^6A37 formation of mt-tRNA in patient cells, strongly indicating that the lack of t^6A37 contributes to molecular pathogenesis of mitochondrial disease.

Regulation of mitochondrial t^6A37 formation by sensing CO_2/bicarbonate

To determine whether t^6A37 frequency is regulated by CO_2/bicarbonate concentration, I cultured HEK293T cells in a medium without sodium bicarbonate in the absence of 5% CO_2 (air) for 6 days. Subsequently, five species of mt-tRNAs, along with cyto-$tRNA^{Ile}$, were isolated and subjected to RNA-MS. The t^6A37 frequencies of mt-$tRNA^{Ser(AGY)}$ and mt-$tRNA^{Ile}$ decreased markedly, whereas the frequency decreased slightly in the other three mt-tRNAs; no change was observed in cyto-$tRNA^{Ile}$. As expected, when sodium bicarbonate was added back to the medium the t^6A37 frequencies of the two mt-tRNAs increased gradually as a function of sodium bicarbonate concentration. The different responses of t^6A37 formation in the five mt-tRNAs to bicarbonate concentration might be due differences in efficiency of t^6A37 formation.

Rapidly growing tumors frequently fall into hypoxia due to low oxygen availability. Given that mitochondrial CO_2/bicarbonate is largely provided by TCA cycle, I asked whether mitochondrial t^6A37 could be affected by hypoxic conditions in solid tumors. For these experiments, I prepared solid tumor xenografts by subdermally injecting colorectal adenocarcinoma HT-29 cells into nude mice, and then isolated mt-$tRNA^{Ser(AGY)}$ and mt-$tRNA^{Ile}$ for RNA-MS. I clearly detected hypomodification of t^6A37 in mt-$tRNA^{Ser(AGY)}$ and mt-$tRNA^{Ile}$ isolated from the tumor, whereas both mt-tRNAs isolated from cell culture were fully modified. This finding suggests that t^6A37 frequency can be modulated by hypoxic conditions in solid tumors.

Conclusions

In this study, I showed that YRDC and OSGEPL1 are required for t^6A37 formation in five mt-tRNAs and that t^6A37 plays critical roles in protein synthesis and respiratory activities in mitochondria. Kinetic studies revealed that mitochondrial t^6A37 formation is dynamically regulated by cellular CO_2/bicarbonate concentration. In support of this finding, I observed

hypomodification of t^6A37 in two mt-tRNAs isolated from human cells cultured with non-bicarbonate medium, as well as hypoxic solid tumors, implying codon-specific regulatory translation under hypoxic conditions. I also identified pathogenic point mutations that impaired t^6A37 formation in mt-tRNAs and confirmed low levels of t^6A37 in mt-$tRNA^{Thr}$ with the A15923G mutation isolated from a patient with MERRF-like symptoms, indicating that the lack of t^6A37 has pathological consequences.

Publication

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4. Lin, H., *et al*., submitted