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Molecular Diagnostics and Genetics

Quantitative PCR Measurement of tRNA 2-Methylthio Modification for Assessing Type 2 Diabetes Risk

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BACKGROUND: Genetic variants in the human *CDKAL1* (CDK5 regulatory subunit associated protein 1–like 1) gene have been associated with reduced insulin secretion and type 2 diabetes (T2D). CDKAL1 is a methylthiotransferase that catalyzes 2-methylthio (ms^2) modification of the adenine at position 37 (A37) of cytoplasmic $tRNA^{Lys}$(UUU). We investigated the ms^2-modification level of $tRNA^{Lys}$(UUU) as a direct readout of CDKAL1 enzyme activity in human samples.

METHOD: We developed a quantitative PCR (qPCR)-based method to measure ms^2 modification. $tRNA^{Lys}$(UUU) was reverse-transcribed with 2 unique primers: Reverse primer r1 was designed to anneal to the middle of this tRNA, including the nucleotide at A37, and reverse primer r2 was designed to anneal to the region downstream (3′) of A37. Subsequent qPCR was performed to detect the corresponding transcribed cDNAs.

RESULTS: The efficiency of reverse transcription of $tRNA^{Lys}$(UUU) was ms^2-modification dependent. The relative difference in threshold cycle number obtained with the r1 or r2 primer yielded the ms^2-modification level in $tRNA^{Lys}$(UUU) precisely as predicted by an original mathematical model. The method was capable of measuring ms^2-modification levels in $tRNA^{Lys}$(UUU) in total RNA isolated from human peripheral blood samples, revealing that the ms^2-modification rate in $tRNA^{Lys}$(UUU) was decreased in individuals carrying the *CDKAL1* genotype associated with T2D. In addition, the ms^2-modification level was correlated with insulin secretion.

CONCLUSIONS: The results point to the critical role of ms^2 modification in T2D and to a potential clinical use of a simple and high-throughput method for assessing T2D risk.

Nucleotides in tRNAs are intensively modified after transcription. These posttranscriptional modifications are necessary for the structural integrity and decoding activity of tRNA *(1–3)*. Defects in tRNA modifications can lead to such pathologies as type 2 diabetes (T2D)[3] and mitochondrial diseases *(4–6)*. Recent genomewide association studies have that shown single-nucleotide polymorphisms (SNPs) in an intron region of *CDKAL1* (CDK5 regulatory subunit associated protein 1–like 1)[4] are associated with T2D *(7, 8)*. Individuals carrying the risk allele of *CDKAL1* have decreased insulin secretion *(7)*. We recently reported that CDKAL1 is a tRNA-modifying enzyme that catalyzes 2-methylthio (ms^2) transformation of N^6-threonylcarbamoyladenosine (t^6A) to 2-methylthio-N^6-threonylcarbamoyladenosine (ms^2t^6A) at position 37 of cytoplasmic $tRNA^{Lys}$(UUU) *(9)*. The ms^2 modification is required for accurate decoding of the lysine codon *(4)*. In pancreatic β-cell–specific *Cdkal1* knockout (KO) mice, the deficiency in ms^2 modification of $tRNA^{Lys}$(UUU) produces aberrant translation of the lysine codon in proinsulin, which leads to the development of T2D symptoms, including reduced insulin secretion and impaired blood glucose control *(4)*. These results not only suggest that a decrease in the ms^2-modification level in $tRNA^{Lys}$(UUU) is critical to the development of T2D but also raise the possibility for the use of ms^2-modification rates in assessing T2D risk.

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[3] Nonstandard abbreviations: T2D, type 2 diabetes; SNP, single-nucleotide polymorphism; ms^2, 2-methylthio; t^6A, N^6-threonylcarbamoyladenosine; ms^2t^6A, 2-methylthio-N^6-threonylcarbamoyladenosine; KO, knockout (mice); qPCR, quantitative PCR; qPCR-MtR, qPCR-based method for detecting methylthiolation of tRNA; WT, wild-type; Ct, threshold cycle; Ct_{r1}, Ct number obtained from a qPCR with a cDNA template generated by primer r1; dCt_{r2r1}, $Ct_{r2} - Ct_{r1}$; T_m, melting temperature; CIR_{30}, corrected insulin response at 30 min; A37, adenine at position 37.

[4] Genes: *CDKAL1*, CDK5 regulatory subunit associated protein 1–like 1; *Cdkal1*, *Mus musculus* gene CDK5 regulatory subunit associated protein 1–like 1.

We therefore aimed to measure ms^2-modification status as a direct marker for investigating CDKAL1 enzyme activity in human samples and its association with T2D risk; however, current methods of measuring site-specific tRNA modifications, such as mass spectrometry and the primer-extension method *(6, 10)*, are not applicable to clinical samples, for several reasons. First, these methods are time-consuming and require expertise and expensive equipment. Second, they are unable to handle multiple samples in parallel. Third, mass spectrometry in particular requires a large quantity of RNA for isolating tRNAs, although analyses use only a small amount of tRNA. Thus, there is a need to develop a method that is applicable to the measurement of ms^2 modifications in clinical samples. We found that ms^2 modification attenuates reverse transcription because it sterically hinders Watson–Crick base pairing. Taking advantage of this finding, we developed a quantitative PCR (qPCR)-based method for detecting methylthiolation of tRNA (qPCR-MtR), which overcomes the disadvantages of the current method by allowing measurement of ms^2 modifications in multiple samples in as little as 3 h.

Materials and Methods

PARTICIPANT RECRUITMENT

Blood samples were obtained from nondiabetic volunteers at Kumamoto University. Informed consent was obtained before blood was collected. The Ethics Committee of Kumamoto University approved the experiments regarding analysis of human genetic materials and collecting human blood (approval ID, Genome 159).

RNA ISOLATION

For isolation of total RNA from mouse liver, we used a guanidinium thiocyanate/phenol/chloroform method with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. One particular tRNA, $tRNA^{Lys}$(UUU), was further purified with reciprocal circulating chromatography, as described previously *(4, 11)*. The $tRNA^{Lys}$(UUU) isolated from wild-type (WT) and *Cdkal1* KO mice were subjected to qPCR-MtR (Fig. 1A–C). Threshold cycle (Ct) values calculated with the manufacturer's software are indicated in this figure. To calculate the modification rate, we combined purified $tRNA^{Lys}$(UUU) isolated from WT mice and *Cdkal1* KO mice at the indicated ratios and carried out qPCR-MtR analyses (Fig. 1D). The calculated modification ratio was obtained as described in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue11. The value for the constant E for the efficiency of target amplification was assumed to be 1.

For absolute quantification of ms^2 modification in crude total RNA, we subjected total RNA isolated from WT and *Cdkal1* KO mice to qPCR-MtR (Fig. 2, A and B). To calculate the modification rate, we combined total RNA isolated from each mouse at the indicated ratios and subjected the combined total RNA to qPCR-MtR (Fig. 2C). Calculations are as presented in Fig. 1 in the online Data Supplement.

We isolated total RNA from peripheral blood by mixing 1.5 mL fresh peripheral blood with the erythrocyte-lysis buffer provided in the QIAamp RNA Blood Mini Kit (Qiagen). Total RNA was isolated according to the manufacturer's protocol (Fig. 3, A and B). To examine the appropriateness of the RNA-isolation methods, we recruited another 18 individuals. We added erythrocyte-lysis buffer to 3 mL blood and then aliquoted identical volumes of the mixture into 2 tubes. We isolated total RNA in one of the tubes with the QIAamp RNA Blood Mini Kit (Qiagen) and the total RNA in the other tube with TRIzol reagent. Total RNA was then subjected to qPCR-MtR amplification as described below.

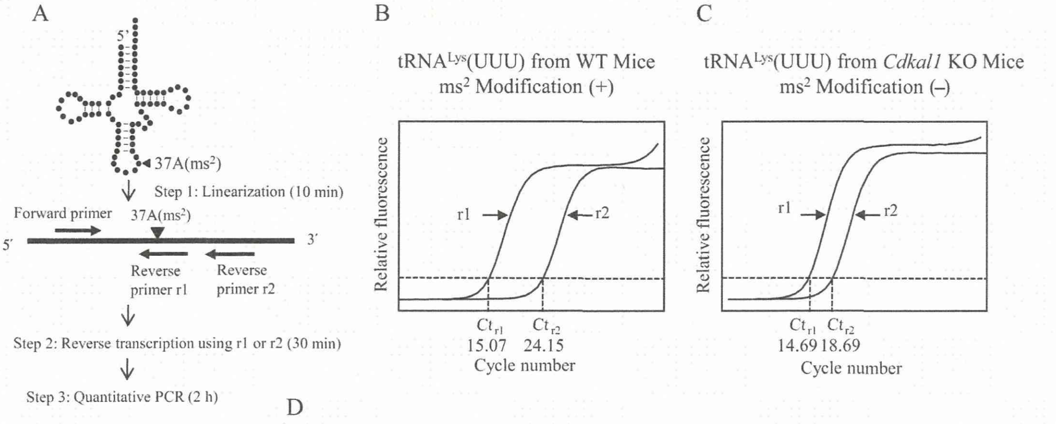
For isolation of total RNA for mass spectrometry analysis, 50-mL peripheral blood samples were collected from each individual. Leukocytes were collected with the erythrocyte-lysis buffer provided in the QIAamp RNA Blood Mini Kit (Qiagen). Total RNA in leukocytes was purified with TRIzol reagent according to the manufacturer's protocol. A particular tRNA, $tRNA^{Lys}$(UUU), was further purified with reciprocal circulating chromatography.

CALCULATIONS

The mathematical model used to measure the modification rate is described in Fig. 1 in the online Data Supplement. The Ct number for a sample was obtained via qPCR with a cDNA template generated by primer r1 (Ct_{r1}) or by primer r2 (Ct_{r2}). The difference between Ct_{r1} and Ct_{r2} (hereafter dCt_{r2r1}) was calculated by subtracting Ct_{r1} from Ct_{r2}. The dCt_{r2r1} number for individual samples was used to calculate the absolute modification rate directly when the absolute modification rates of 2 samples were known, or to compare the modification rate of one sample relative to that of another.

PRIMER DESIGN

The DNA sequences of $tRNA^{Lys}$(UUU) were retrieved from a tRNA database (tRNAdb, http://trna.bioinf.uni-leipzig.de/) *(12)*. The sequences of the primers used for detecting the ms^2 modification in $tRNA^{Lys}$(UUU) are as follows: forward primer, 5′-GTCGGTAGAGCATCAGACTT-3′; reverse primer r1, 5′-CCTGGACCCTCAGATTAAAA-3′; reverse primer r2, 5′-GAACAGGGACTTGAACCCTG-3′. All primer se-



D

Sample no.	Ratio of samples (WT:KO)	dCt	ddCt	2^{-ddCt}	Calculated ratio	Expected ratio
1	0:100	4	0	1.00	0.00	0.00
2	25:75	4.17	0.17	0.89	0.11	0.25
3	50:50	4.67	0.67	0.63	0.38	0.50
4	75:25	5.79	1.79	0.29	0.73	0.75
5	100:0	9.08	5.08	0.03	1.00	1.00

Fig. 1. Absolute quantification of the ms^2 modification in purified tRNA^{Lys}(UUU) by qPCR-MtR analysis.

A, Flow chart of the qPCR-MtR method for measuring ms^2 modification in tRNA^{Lys}(UUU). B and C, qPCR-MtR of tRNA^{Lys}(UUU) purified from WT and *Cdkal1* KO mice. Shown are representative amplifications of a cDNA template generated with reverse primer r1 or reverse primer r2. Ct values calculated with the manufacturer's software are indicated. D, Purified tRNA^{Lys}(UUU) isolated from WT and *Cdkal1* KO mice were combined at the indicated ratios and subjected to qPCR-MtR. The calculated modification rate was obtained from the ddCt value and compared with the expected modification rate.

quences were screened with Primer 3 software (http://frodo.wi.mit.edu/primer3/) to eliminate primers with self-complementarity and extremely low melting-temperature (T_m) values.

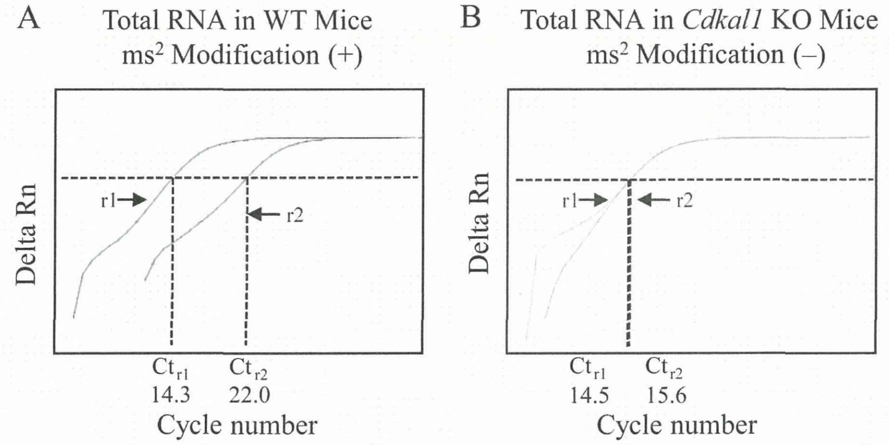
REVERSE TRANSCRIPTION AND qPCR

The total amount of RNA isolated from cells and tissues was adjusted to 100 ng/μL in RNase-free water, unless otherwise indicated. To avoid genomic contamination, we digested 2 μL (200 ng) total RNA with 5 U DNase I (Roche Applied Science) in a 20-μL reaction at 37 °C for 20 min. We then heat-inactivated the DNase I at 75 °C for 10 min. After the DNase treatment, we mixed 2.5 μL digested total RNA with 1 μL 20 μmol/L reverse primer r1 or reverse primer r2, heat-denatured the mixture at 65 °C for 10 min, and cooled it rapidly on ice for at least 5 min. With the mixture still on ice, recombinant reverse transcriptase (Transcriptor; Roche Applied Science) was added to a final concentration of 0.5 U/μL. Reverse transcription was performed in a total reaction volume of 10 μL at 55 °C for 30 min and then heat-inactivated at 85 °C for 5 min. We subjected 2 μL synthesized cDNA to qPCR with the SYBR Premix Ex *Taq* kit (Takara) and the ABI PRISM 7300 Real-Time PCR System (Life Technologies) according to the manufacturers' directions.

For analysis of isolated tRNA^{Lys}(UUU), we used the Transcriptor First Strand cDNA Synthesis Kit, the LightCycler 480 SYBR Green I Master, and the LightCycler 480 real-time PCR system (Roche Applied Science). cDNA was synthesized in a 20-μL volume from 3 fmol of tRNA prepared at defined proportions of tRNA with and without the ms^2 modification. The qPCR was then performed with 0.75 μL synthesized cDNA.

MASS SPECTROMETRIC ANALYSIS OF tRNA

Isolated tRNA^{Lys}(UUU) was digested with RNase A to obtain pyrimidine-ending oligonucleotides, which



C

Sample no.	Ratio of samples (WT:KO)	dCt	ddCt	2^{-ddCt}	Calculated ratio	Expected ratio
1	0:100	1.10	0.00	1.00	0.00	0.00
2	25:75	1.58	0.48	0.72	0.28	0.25
3	50:50	2.23	1.12	0.46	0.55	0.50
4	75:25	4.03	2.93	0.13	0.88	0.75
5	100:0	7.71	6.61	0.01	1.00	1.00

Fig. 2. Absolute quantification of the ms^2 modification in crude samples of total RNA.
A and B, qPCR-MtR of total RNA isolated from WT and *Cdkal1* KO mice. Shown are representative qPCR amplifications with a cDNA template generated by the r1 or r2 primer. C, Samples of total RNA isolated from each mouse were combined at the indicated ratios and subjected to qPCR-MtR. The calculated modification rate was obtained from the ddCt value and compared with the expected modification rate.

were then analyzed with liquid chromatography–mass spectrometry, as described previously *(4)*.

ANIMALS AND CELL CULTURE

Cdkal1 KO mice were generated as described previously *(4)*. The Animal Ethics Committee of Kumamoto University approved all animal procedures (approval ID: B24–134, B24–132).

ORAL GLUCOSE TOLERANCE TEST

Human individuals underwent fasting beginning at 9 PM the night before the test day. They were then administered a 75-g load of glucose (Ajinomoto) between 9 AM and 11 AM on the test day. Peripheral blood samples were obtained while the individuals were fasting and 30 min after glucose loading. Plasma glucose and serum insulin concentrations were measured by SRL Inc. The corrected insulin response at 30 min (CIR_{30}) was calculated as $I_{30}/[G_{30} \times (G_{30} - 70)]$, where I_{30} is the insulin concentration in microunits per milliliter at 30 min and G_{30} is the glucose concentration in milligrams per deciliter at 30 min *(7)*.

GENOTYPING OF SNPs IN *CDKAL1*

Genomic DNA was purified from 200 μL peripheral human blood with the QIAamp DNA Blood Mini Kit (Qiagen) and adjusted to 10 ng/μL with distilled water. The

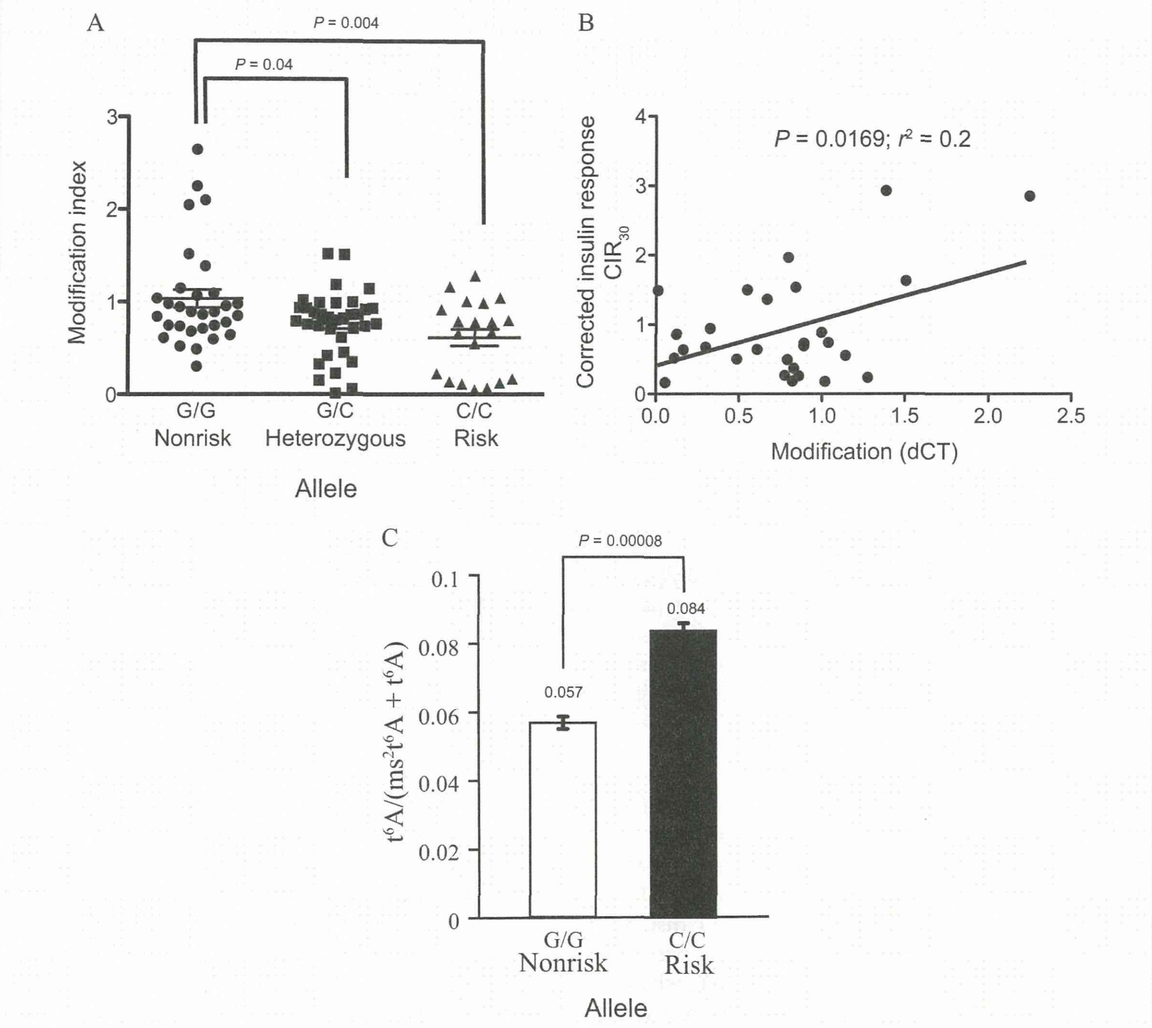


Fig. 3. Decrease in the ms^2-modification level in individuals carrying the CDKAL1 SNP risk allele.
A, Comparison of dCt$_{r2r1}$ values for individuals homozygous for the nonrisk allele (G/G, n = 31) and the risk alleles (C/C, n = 20), and heterozygous for the nonrisk and risk alleles (G/C, n = 35) of the *CDKAL1* SNP. Statistical analyses were one-way ANOVA followed by the Tukey test for multiple comparisons. B, Insulin secretion (CIR$_{30}$) plotted against dCt$_{r2r1}$ for a group with mixed genotypes (C/C, n = 9; G/C, n = 12; C/C = 7). Pearson correlation coefficient (*r*) = 0.44. C, Mass spectrometric analysis of ms^2 modifications in individuals homozygous for the nonrisk allele (G/G) and the risk allele (C/C) for the *CDKAL1* SNP. Statistical analysis by the Student *t*-test.

SNP (rs7754840) in *CDKAL1* was examined with the TaqMan SNP Genotyping Assay Kit (Life Technologies).

Results

MEASURING ms^2 MODIFICATION OF A37 IN PURIFIED tRNA^{Lys}(UUU) BY qPCR-MtR

Posttranscriptional chemical modifications in tRNA are required to stabilize tRNA structure and codon–anticodon interaction *(1, 2)*. Interestingly, previous studies have shown that some of these modifications could attenuate primer extension, possibly owing to steric hindrance *(6, 13–16)*. We investigated the molecular structure of the ms^2 modification of adenine at position 37 (A37) of tRNA^{Lys}(UUU) in the presence of thymine during reverse transcription (Fig. 4A and 4B). In our model, which is based on a previous study of 2-methylthio-N^6-isopentenyladenosine at position 37

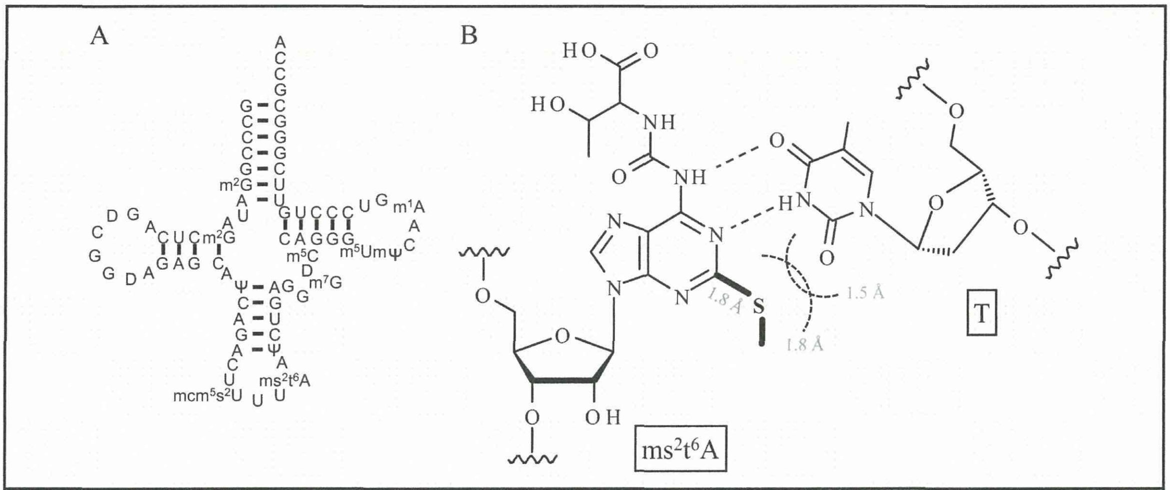


Fig. 4. Structure of the ms^2 modification.

A, Secondary structure of tRNA^{Lys}(UUU). m^{2}G, N^2-methylguanosine; D, dihydrouridine; Ψ, pseudouridine; mcm^{5}s^{2}U, 5-methoxycarbonylmethyl-2-thiouridine; m^{7}G, 7-methylguanosine; m^{5}C, 5-methylcytosine; m^{5}Um, 5,2′-*O*-dimethyluridine; m^{1}A, 1-methyladenosine. B, schematic model of the base pair between ms^{2}t^{6}A and T. The ms^2 modification at position 2 of the adenine base is shown in boldface. Dashed lines indicate hydrogen bonding. Dotted arcs indicate the van der Waals radii of S (0.18 nm) and O (0.15 nm). The original A–U pair geometry and the distance for the C–S bond (0.18 nm) are taken from entry 3I8H in the RCSB Protein Data Bank [see Grosjean et al. *(14)*]; values for van der Waals radii are taken from Bondi *(18)*.

in bacterial tRNA^{Phe} *(17)*, the calculated distance between the S of the ms^2 group and the O at position 2 of the thymine base was 0.22 nm, whereas the calculated van der Waals radii of S and O were 0.18 nm and 0.15 nm, respectively *(18)*. Therefore, the S–O distance is sufficiently short that steric hindrance prevents Watson–Crick pairing between ms^{2}t^{6}A and T, whereas the N^6-threonylcarbamoyl group does not affect Watson–Crick pairing with T (Fig. 4B). We then speculated that the ms^2 group might affect Watson–Crick pairing with thymine during reverse transcription because of the former's large van der Waals radii. To verify this speculation, we carried out reverse transcription with 2 primers specifically designed to amplify the region including position 37 in tRNA^{Lys}(UUU) (step 2, Fig. 1A). Reverse primer r1 was designed to anneal with the specific region encompassing A37, whereas reverse primer r2 was designed to anneal with a specific region downstream (3′) of A37. The cDNA generated with either primer r1 or r2 was quantified by qPCR (step 3, Fig. 1A). If the ms^2 modification attenuates reverse transcription of fully modified tRNA^{Lys}(UUU), we would expect the amount of cDNA generated by primer r2 to be less than that generated by primer r1. In hypomodified tRNA^{Lys}(UUU), on the other hand, we would expect the amount of cDNA generated by primer r2 to be close to that generated by primer r1, in proportion to the reduction in ms^2 modification.

tRNA^{Lys}(UUU) with and tRNA^{Lys}(UUU) without the ms^2 modification were purified from liver of a WT mouse or a *Cdkal1* null mouse *(4)*, respectively, and subjected to qPCR-MtR. As expected, use of a fully modified WT tRNA^{Lys}(UUU) as template markedly increased Ct_{r2} compared with CT_{r1} (Fig. 1B). In contrast, use of unmodified tRNA^{Lys}(UUU) as template reduced Ct_{r2} to a level close to Ct_{r1} (Fig. 1C). These results suggest that ms^2 modification at A37 indeed attenuated reverse transcription when primer r2 was used, whereas reverse transcription was unaffected when primer r1 was used. Thus, Ct_{r2} reflects the extent of ms^2 modification, whereas Ct_{r1} reflects the total number of tRNA molecules in a tRNA sample.

ABSOLUTE QUANTIFICATION OF ms^2 MODIFICATION BY qPCR-MtR

By combining the conventional ddCt method and an original mathematical model, we demonstrated that the difference between Ct_{r1} and Ct_{r2} (i.e., $dCt_{r2r1} = Ct_{r2} - Ct_{r1}$) in a given sample can be used as an index that represents the modification rate (see Fig. 1 in the online Data Supplement). In short, the smaller the dCt_{r2r1} value, the less the ms^2 modification of the tRNA. In addition, the model enabled us to calculate the absolute modification rate of an unknown sample from 2 reference samples with known absolute modification rates.

To verify our calculation, we prepared tRNA^{Lys}(UUU) with a known ms^2-modification rate by combining tRNA^{Lys}(UUU) molecules from WT and *Cdkal1* KO mice at defined ratios and then performed qPCR-MtR (Fig. 1D). The dCt value decreased in proportion to the reduction in the ms^2-modification rate. Furthermore, the calculated ms^2-modification rate for the WT and modified tRNA mixtures (samples 2–4, Fig. 1D) accurately predicted the expected ms^2-modification rate when fully ms^2-modified tRNA (sample 5 in Fig. 1D, modification rate = 1; *c* in equation 14 in Fig. 1 in the online Data Supplement) and unmodified tRNA (sample 1 in Fig. 1D, modification rate = 0; *a* in equation 14 in Fig. 1 in the online Data Supplement] were used as references.

APPLICATION OF qPCR-MtR TO SAMPLES OF CRUDE TOTAL RNA

Purifying tRNA^{Lys}(UUU) is a time-consuming process requiring milligrams of total RNA. Because clinical samples are usually too small to yield this much total RNA, adapting qPCR-MtR for a small amount of crude RNA would be ideal for clinical samples. We subjected 200 ng total RNA isolated from 2 WT mice and 2 *Cdkal1* KO mice to qPCR-MtR analysis. In agreement with the results obtained with purified tRNA^{Lys}(UUU), we obtained a marked reduction in the Ct_{r2} value for the samples of total RNA isolated from *Cdkal1* KO mice, compared with WT mice (Fig. 2, A and B). Furthermore, the calculated ms^2-modification rate obtained from the dCt_{r2r1} value accurately predicted the expected ms^2-modification rate (Fig. 2C). In addition, we were able to calculate the absolute ms^2-modification rate even when we used only 2 ng total RNA for qPCR-MtR (see Table 1 in the online Data Supplement). All procedures were finished in <3 h.

DECREASED ms^2-MODIFICATION LEVEL OF tRNA^{Lys}(UUU) IN INDIVIDUALS CARRYING RISK *CDKAL1* SNPs

Because qPCR-MtR successfully detected ms^2 modification in tRNA^{Lys}(UUU), we applied this method via a column procedure and with 100 ng total RNA isolated from peripheral blood samples of nondiabetic individuals (n = 86). In addition, we determined relative modification levels by using the dCt_{r2r1} value, because a reference for the absolute ms^2-modification rate in human tRNA^{Lys}(UUU) was not available. We compared the dCt_{r2r1} values obtained for total RNA isolated from 20 individuals homozygous for the T2D-associated risk allele (C/C) of the *CDKAL1* SNP (rs7754840) and 35 individuals heterozygous for the risk allele (G/C) with the dCt_{r2r1} values obtained for 31 individuals homozygous for the nonrisk allele (G/G). dCt_{r2r1} values were significantly lower in individuals carrying the risk allele (G/C and C/C) (Fig. 3A), suggesting that ms^2 modification was suppressed in individuals carrying the T2D-associated risk allele for the *CDKAL1* SNP. The accuracy of the qPCR-MtR result was confirmed by mass spectrometric analysis. tRNA^{Lys}(UUU) was purified from 50 μg of total RNA isolated from 50 mL of blood from 2 individuals of same group carrying risk (C/C) or nonrisk (G/G) alleles of *CDKAL1* SNPs and analyzed with mass spectrometry. The amount of ms^2-unmodified tRNA^{Lys}(UUU) was increased 1.47-fold (Fig. 3C) in individuals carrying the risk allele, compared with individuals carrying the nonrisk allele (see Fig. 2 in the online Data Supplement for a representative mass chromatogram). This result was compatible with the qPCR results obtained with the corresponding total-RNA samples ($dCt_{nonrisk} - dCt_{risk} = 1$).

tRNA is lost during the purification of total RNA when a column method is used, and that loss may interfere with measurement of the correct ms^2-modification level. Therefore, we purified peripheral blood RNA from additional 18 nondiabetic individuals, both by a column method and by a guanidinium thiocyanate/phenol/chloroform method with the same samples; we then compared the dCt_{r2r1} values (see Fig. 3 in the online Data Supplement). We obtained a smaller dCt_{r2r1} value with the column method than with the guanidinium thiocyanate/phenol/chloroform method. These results suggest a partial loss of tRNA during column purification; however, the dCt_{r2r1} values obtained with the 2 methods were significantly correlated (see Fig 3A in the online Data Supplement). We also used electrophoresis to compare the qPCR products obtained with each isolation method. The intensities and sizes of the bands obtained with the 2 methods were identical (see Fig. 3 B in the online Data Supplement).

The risk allele of *CDKAL1* has been associated with impaired insulin secretion *(7)*. To investigate whether the ms^2-modification level of tRNA^{Lys}(UUU) was also associated with insulin secretion, we carried out oral glucose tolerance tests with 28 nondiabetic individuals. Insulin secretion (CIR_{30}) was significantly correlated with dCt_{r2r1} (Fig. 3B), suggesting that the decreased ms^2-modification level is associated with the impairment of insulin secretion.

Discussion

This study has demonstrated that the method we have developed (qPCR-MtR) can detect and quantify chemical modifications in tRNA^{Lys}(UUU), both for purified RNA and crude total RNA samples. This method is based on the unique biochemical feature of ms^2 modification. Previous biochemical and structural studies of synthetic poly(ms^2-A) and poly(U) have demonstrated that Watson–Crick poly(ms^2-A)–poly(U) pairing is not possible because of the large van der Waals radii of the ms^2 group at the C2 position of adenine *(19, 20)*. Given the structural similarity of uracil and thymine, our results suggest that the inhibitory effect of the ms^2 group at A37 of $tRNA^{Lys}$(UUU) is also due to its large van der Waals radii, which prevent Watson–Crick pairing with thymine during reverse transcription. Another possibility is that the ms^2 group causes almost complete loss of reverse transcription, rather than inhibiting it; hence, a trace amount of hypomodification remaining in fully ms^2-modified samples would give a Ct_{r2} value apparently higher than Ct_{r1}. Indeed, signals corresponding to the *m/z* of the ms^2-hypomodified molecule were detected at intensities of about 3% in the mass spectrometric analyses of the isolated $tRNA^{Lys}$(UUU) prepared from the WT mice (data not shown); however, this putative heterogeneity would not affect the calculation in Fig. 2C (by using 0.97 as the modification rate instead of 1). Because achieving complete purification of a single species from a heterogeneous population is almost impossible, the differences between Ct_{r2} and Ct_{r1} may be due to the combined effects of strong inhibition of reverse transcription and the intrinsic heterogeneity of the samples.

Genetic variants of the human *CDKAL1* gene have been associated with impaired insulin secretion and the development of T2D across ethnic groups *(7)*. Because the disease's development in individuals carrying the *CDKAL1* risk allele could be influenced by environmental intervention, the *CDKAL1* genotype alone is less predictive of T2D *(21)*. CDKAL1 enzyme activity is regulated by both genetic variation and environmental conditions. Therefore, T2D risk would be assessed more accurately if genotype information were combined with ms^2-modification measurement, which directly reflects CDKAL1 activity. qPCR-MtR provides an efficient means of detecting and quantifying ms^2 modification of $tRNA^{Lys}$(UUU) in clinical samples. Our method successfully detected ms^2 modification in total RNA isolated from peripheral blood and showed that the ms^2-modification rate was indeed decreased in individuals carrying risk-related genetic variants in the *CDKAL1* gene. Furthermore, the ms^2-modification level was significantly correlated with insulin secretion. These results not only implicate the importance of ms^2 modification of $tRNA^{Lys}$(UUU) in T2D but also demonstrate that qPCR-MtR is fully applicable to the clinical setting. Further investigations into combining the ms^2-modification level with genetic information and other clinical indices may lead to improvements in the accuracy of assessing T2D risk.

CDKAL1 has been implicated in diseases other than T2D, such as obesity and Crohn disease *(22–24)*. Further investigations with qPCR-MtR may shed light on the pathologic function of ms^2 modifications in these diseases. In addition, the lack of chemical modification, such as taurine modification in the anticodon loop of mitochondrial tRNAs, has been reported for mitochondrial diseases *(5, 6)*. Because the onset of such mitochondrial diseases depends on the number of mitochondrial DNA copies with a mutated tRNA gene, quantifying the modification level in the corresponding tRNAs would help in diagnosis and treatment. The qPCR-MtR concept could easily be adapted for measuring the modification level in these mitochondrial tRNAs if these modifications also inhibit reverse transcription.

In this study, we compared the ms^2-modification level in blood samples from which total RNA was isolated, both with a column method and a guanidinium thiocyanate/phenol/chloroform method. The dCt_{r2r1} value obtained by a column method was smaller than that obtained with the guanidinium thiocyanate/phenol/chloroform method, suggesting a partial loss of tRNA during column purification. Nevertheless, the dCt_{r2r1} values obtained with the 2 methods were significantly correlated, and the intensities and sizes of the bands of the qPCR products obtained with the 2 methods were identical. These results suggest that a guanidinium thiocyanate/phenol/chloroform method should be applied for absolute quantification of ms^2 modifications; however, the column method can still be used for relative quantification. With respect to possible diagnostic use, the guanidinium thiocyanate/phenol/chloroform method has the advantages of convenience and producing no organic waste.

To our knowledge, qPCR-MtR is the most convenient, fastest, and least expensive way to reliably measure ms^2 modifications of $tRNA^{Lys}$(UUU) in crude samples of total RNA. This method could be adapted not only to the clinical assessment of T2D risk but also to biomedical research into the functions of ms^2 modifications in other diseases.

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