Mutational Analysis of Mitochondrial tRNA Genes in 200 Patients with Type 2 Diabetes Mellitus

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Objective: Previous studies showed that variants in mitochondrial DNA (mtDNA) are associated with type 2 diabetes mellitus (T2DM). However, the relationships between mitochondrial tRNA (mt-tRNA) variants and T2DM remain poorly understood.

Methods: In this study, we performed a mutational screening of 22 mt-tRNA genes in a cohort of 200 Han Chinese subjects with T2DM and 200 control subjects through PCR–Sanger sequencing. The identified mt-tRNA variants were assessed for their pathogenicity via the phylogenetic approach, structural and functional analysis. Furthermore, two Han Chinese pedigrees with maternally inherited diabetes and deafness (MIDD) were reported by clinical and genetic assessments.

Results: A total of 49 genetic variants in mt-tRNA genes were identified; among them, 31 variants (17 pathogenic/likely pathogenic) were absent in controls, located at extremely conserved nucleotides, may have potential structural and functional significance, thereby considered to be T2DM-associated variants. In addition, sequence analysis of entire mitochondrial genomes of the matrilineal relatives from two MIDD pedigrees revealed the occurrence of tRNA$^{Leu(UUR)}$ A3243G and T3290C mutations, as well as sets of polymorphisms belonging to mitochondrial haplogroups F2 and D4. However, the lack of any functional variants in connexin 26 gene (*GJB2*) and tRNA 5-methylaminomethyl-2-thiouridylate (*TRMU*) suggested that nuclear genes may not play active roles in clinical expression of MIDD in these pedigrees.

Conclusion: Our data indicated that mt-tRNA variants were associated with T2DM, screening for mt-tRNA pathogenic mutations was recommended for early detection and prevention of mitochondrial diabetes.

Keywords: type 2 diabetes mellitus, mitochondrial tRNA, variants, Chinese population

Introduction

Diabetes is a very complex disease characterized by the presence of chronic hyperglycemia. Clinically, insulin-dependent type 1 and non-insulin-dependent type 2 are the main types of diabetes. Among them, type 2 diabetes mellitus (T2DM, [MIM125853]) is a common endocrine disorder affecting approximately 10% of adult population.[1] In most cases, T2DM exhibits high blood glucose in the context of insulin resistance (IR) and relative insulin deficiency. To date, the etiology of T2DM is still undetermined. Now it has been recognized that this disorder can be caused by some acquired factors, including inherited genetic factors or the interactions between genetic and environmental factors.[2] In particular, maternally inheritance has been observed in some pedigrees, highlighting the contributions of mitochondrial DNA (mtDNA) mutations to T2DM.[3] Since the

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landmark discovery of the association between mitochondrial diabetes and 10.4-kb deletion in mtDNA,[4] a growing number of T2DM-associated mtDNA mutations have been identified. Most of them are located at mitochondrial tRNA (mt-tRNA) genes. As adapter molecules to convert the genetic codes into amino acid sequences, mt-tRNAs play central roles in mitochondrial protein synthesis, as well as maintenance of respiratory chain functions.[5] By molecular level, almost every mt-tRNA has a highly conserved cloverleaf structure, consisting of Acceptor arm, DHU-Loop, anticodon stem, variable region, and TψC loop, with an average length of 73 nucleotides. Although mt-tRNAs comprise only around 10% of the total coding capacity of mtDNA genes, more than half of mtDNA mutations causing diseases are located in mt-tRNA genes, as indicated in Mitomap database (https://www.mitomap.org/MITOMAP),[6] emphasizing the importance of mt-tRNAs for mitochondrial function.

Recent experimental studies have suggested that tRNA$^{Leu(UUR)}$ A3243G and T3264C, tRNA^{Gly} T10003C, tRNA^{Glu} T14709C, and tRNA^{Thr} G15897A are potential pathogenic mutations affecting T2DM predisposition.[7–11] In particular, the well-known A3243G mutation affects the processing of mitochondrial RNA precursors,[12] and base modification of this tRNA.[13] In cytoplasmic hybrids (cybrids) harboring the A3243G mutation, the level of aminoacylated tRNA$^{Leu(UUR)}$ is reduced by approximately 70–75%,[14] thereby influencing the protein synthesis and mitochondrial respiratory chain function. Furthermore, the T3290C mutation is localized at very conserved nucleotide of TψC loop in tRNA$^{Leu(UUR)}$, and is important for the steady-state level of tRNA$^{Leu(UUR)}$. Thus, these diabetes-related mtDNA mutations or variants, which are often presented in homoplasmy, exhibit a variability of clinical phenotypes.[15] However, the pathophysiology of mt-tRNA mutations/variants in phenotypic manifestation of T2DM remains poorly understood.

In the current study, with the aim of exploring the T2DM-related mt-tRNA mutations/variants, 200 patients who have been diagnosed with T2DM, together with 200 age- and gender-matched controls, underwent the mutational screening of 22 mt-tRNA genes. As a result, a total of 49 genetic variants on 22 mt-tRNA genes are identified in this study. By the pathogenic evaluations including the phylogenetic analysis, potential structural and functional alternations, 17 T2DM-associated tRNA variants in 23 patients are identified. Furthermore, we perform clinical, genetic and molecular evaluations of two maternally inherited diabetes and deafness (MIDD)

families carrying tRNA$^{Leu(UUR)}$ A3243G and T3290C variants. To understand the contributions of mtDNA genetic background and nuclear genes to MIDD, we initiated the mutational screening of the entire mitochondrial genomes, as well as *GJB2, TRMU* from the matrilineal relatives in these pedigrees.

Methods
Study Population
In this case-control study for mutational screening of T2DM-related mt-tRNA variants, a total of 200 genetically unrelated subjects who were diagnosed with T2DM, participating for this study; moreover, 200 control subjects who came from the Healthy Examination Center of our hospital were also recruited. This study was approved by the Ethics Committee of Yantai Affiliated Hospital of Binzhou Medical University, and each participant provided their written informed consent.

The diagnosis of T2DM was based on the criteria proposed by American Diabetes Association:[16] (1) a fasting plasma glucose (FPG) level ≥7.0 mmol/L; (2) a 2h plasma glucose level after 75-g oral glucose tolerance test (OGTT) ≥11.1 mmol/L; (3) the level of Hemoglobin A1c (HbA1c) ≥6.5%.

Mutational Analysis of Mt-tRNA Genes
Genomic DNA of each subject was extracted from the peripheral blood by using QIAamp Blood Kit (QIAGEN, Hilden, Germany). To detect T2DM-related mt-tRNA variants, polymerase chain reaction (PCR) was used to amplify fragments of all mt-tRNA genes in these subjects with appropriate primers, as described previously.[17] For the subjects carrying the putative variants in mt-tRNA genes, fragments spanning the remaining regions of mtDNA genes were PCR amplified and sequenced to define the mtDNA haplogroups, according to a method described in a previous study.[18] The PCR products were purified and sequenced by ABI 3730 DNA automatic sequencer (Applied Biosystems, Darmstadt, Germany).[19] The sequence data were compared with the revised Cambridge reference sequence (rCRS, GenBank Accessible Number: NC_012920.1) using DNA STAR software package version 5.01 (DNASTAR Inc., Madison, USA) to detect the nucleotides alternations.[20]

Analysis of Conservation Index (CI)
To further assess the pathogenic roles of mt-tRNA variants, the phylogenetic analysis was performed. Briefly, 17

species were used for conservation analysis as described in a previous investigation.[21] These species included *Bos taurus, Cebus albifrons, Colobus guereza, Gorilla gorilla, Homo sapiens, Hylobates lar, Lemur catta, Macaca mulatta, Macaca sylvanus, Mus musculus, Nycticebus coucang, Pan paniscus, Pan troglodytes, Pongo pygmaeus, Pongo abelii, Papio hamadryas*, and *Tarsius bancanus*. The CI was then calculated by comparing the human mtDNA variants with other 16 species. Notably, CI≥75% was regarded as having functional potential.[22]

Structural Analysis

The published secondary structures for the mt-tRNAs were used to define the stem and loop structure.[23,24]

Characterization of Two Han Chinese Pedigrees Harboring Mt-tRNA[Leu(UUR)] Variants

As shown in Figure 1, two Han Chinese families with MIDD were ascertained in Yantai Affiliated Hospital of Binzhou Medical University. To identify personal or family histories of T2DM and other clinical abnormalities, all participants were interviewed and evaluated by physical examinations. For laboratory analysis, each participant's blood sample was collected between 7:00 AM and 10:00 AM after an overnight fast. The level of HbA1c was measured by using high-performance liquid chromatography (HPLC, Bio-Rad, CA, USA). In addition, the OGTT was performed by measuring the 0 and 2h of plasma glucose concentrations after glucose administration.

Furthermore, the age-appropriate audiological examination of hearing loss was performed as described previously,[25] including pure-tone audiometry (PTA),

auditory brainstem response, acoustic immittance measurement and distortion product otoacoustic emission. The PTA was calculated from the sum of the audiometric thresholds at 500, 1000, 2000, 4000 and 8000 Hz. The severity of hearing loss was classified into 5 grades: normal <26 Decibel (dB); mild = 26–40 dB; moderate = 41–70 dB; severe = 71–90 dB and profound >90 dB, as suggested previously.[26] The blood pressure (BP) was measured by an electronic sphygmomanometer and repeated for 3 times. Hypertension was defined according to the guidelines of the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC VI), as a systolic BP≥140 mmHg or the diastolic BP≥90 mmHg.[27]

Screening for the Entire Mitochondrial Genome Variants

The complete mitochondrial genomes of the matrilineal individuals from two MIDD pedigrees (DM-101: II-3, II-6 and III-5; DM-102: II-5, II-8, II-10 and III-7) were PCR amplified by using 24 primers, as previously described.[28] The PCR products were purified and subsequently sequenced by ABI 3730 DNA automatic sequencer (Applied Biosystems, Darmstadt, Germany). The sequence data was then compared with the rCRS (GenBank Accessible Number: NC_012920.1) using DNA STAR software package version 5.01 (DNASTAR Inc., Madison, USA) to detect the nucleotides alternations.[20]

Determining the Mitochondrial Haplogroups

The classification of the mitochondrial haplogroups was based on the phylogenetic tree by Kong et al.[29]
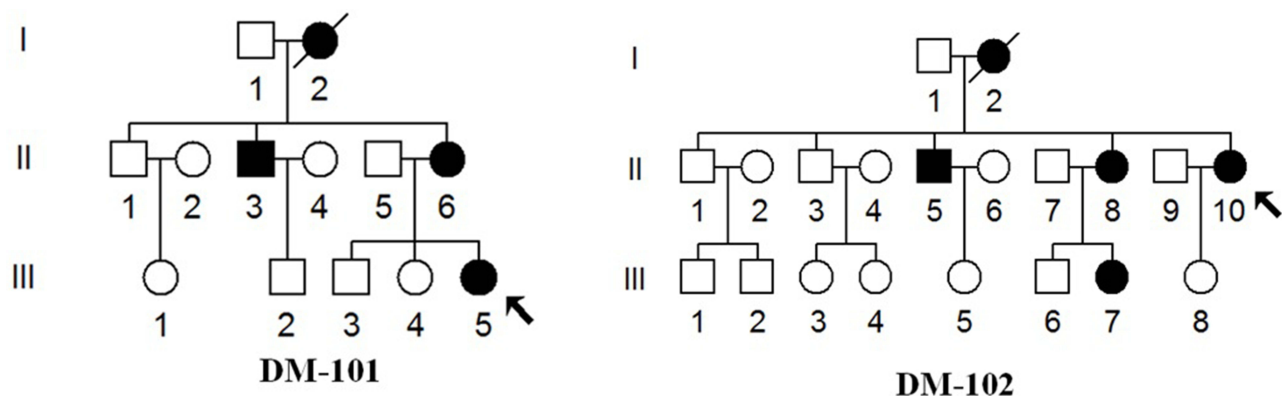
Figure 1 Two Han Chinese pedigrees with MIDD, affected individuals are indicated by filled symbols. Arrows indicate the probands.

Mutational Screening for *GJB2* Gene

To see the contributions of *GJB2* variants to clinical expression of MIDD, we conducted a mutational screening by using PCR amplification of the exons of *GJB2* gene in matrilineal individuals (DM-101: II-3, II-6 and III-5; DM-102: II-5, II-8, II-10 and III-7), the primer sequence for amplification of *GJB2* gene were: forward-5′-TATGACA CTCCCCAGCACAG-3′, and reverse-5′-GGGCAATGCT TAAACTGGC-3′.[30] After PCR amplification and direct Sanger sequence analysis, the data were compared with the wild-type versions of *GJB2* sequence (GenBank Accessible Number: M86849) to identify mutations/ variants.[30]

Analysis of *TRMU* Variants

Previous study indicated that *TRMU* A10S variant may contribute to the phenotypic manifestation of deafness-associated 12S rRNA mutations.[31] To understand the role of *TRMU* in hearing impairment, the *TRMU* A10S variant was screened in the matrilineal relatives (DM-101: II-3, II-6 and III-5; DM-102: II-5, II-8, II-10 and III-7) by using PCR amplification of exon 1 of this gene. The primer sequences for *TRMU* exon 1 were: forward-5′-ACAGCGC AGAAGAAGAGCAGT-3′, and reverse-5′-ACAACGCCA CGACGGACG-3′. After PCR amplification and direct Sanger sequence, the data were compared with the wild-type versions of *TRMU* (GenBank Accessible Number: AF448221) to detect the variants.[31]

Results

Clinical Characterization of 200 Patients with T2DM

A total of 200 T2DM patients (117 females and 83 males) were recruited in this study. The age of these patients ranged from 30 to 68 years, with a median age of 42 years. The age at the onset of T2DM varied from 28 to 66 years, with an average of 40 years. Furthermore, 200 Han Chinese controls from the same region were healthy individuals and did not have any family history of T2DM, deafness, cardiovascular diseases or mitochondrial disorders. The age of these controls varied from 25 to 53 years, with an average of 38 years.

Screening for Mt-tRNA Variants

By sequencing 22 mt-tRNA genes in T2DM subjects and controls, we were able to identify 49 nucleotide alternations, as shown in Table 1. Among these, 3 variants were identified in tRNA[Phe], 2 variants in tRNA[Val], 2 variants in tRNA[Leu(UUR)], 2 variants in tRNA[Gln], 3 variants in tRNA[Met], 1 variant in tRNA[Trp], 2 variants in tRNA[Ala], 3 variants in tRNA[Cys,] 1 variant in tRNA[Asp], 3 variants in tRNA[Ser(UCN)], 2 variants in tRNA[Lys], 2 variants in tRNA[Gly], 2 variants in tRNA[Leu(CUN)], 2 variants in tRNA[Arg], 3 variants in tRNA[His], 2 variants in tRNA[Ser(AGY)], 1 variant in tRNA[Glu], 9 variants in tRNA[Thr] and 2 variants in tRNA[Pro].

Assessment of the Pathogenicity

We used the following criteria to evaluate the potential pathogenicity of mt-tRNA variants: (1) occurred in <1% in the control subjects; (2) CI≥75%, consistent with the evolutionary conservation at a given locus, as proposed by Ruiz-Pesini and Wallace;[32] (3) the variants were predicted to alter the structure or function of tRNA molecules. As shown in Table 1, among them, the CIs of 22 variants were ≥75%, the CIs of 15 variants were between 50% and 75%, and the CIs of the remaining variants were <50%. Next, we used the secondary structure of mt-tRNAs to localize each variant with either a stem or a loop, in order to test whether the base changes altered the classic Watson-Crick base-pairing. We noticed that there were 13 variants occurring in the Acceptor arm, 12 variants in DHU-Loop, 5 variants in variable region, 12 variants in TψC loop, 1 variant in ACC Terminus. Notably, 12 variants disrupted the Watson-Crick base-pairings, whereas 6 variants created novel Watson-Crick base-pairings. This analysis suggested that 17 mt-tRNA variants, which were well conserved and not detected in control subjects, may be pathogenic/likely pathogenic mutations (Table 1). These variants were as follows: tRNA[Leu(UUR)] A3243G and T3290C, tRNA[Met] A4435G and C4467A, tRNA[Trp] A5514G, tRNA[Ala] T5587C and A5655G, tRNA[Ser(UCN)] C7502T and T7505C, tRNA[Lys] G8313A, tRNA[Gly] T10003C and A10055G, tRNA[Leu(CUN)] A12308G and A12330G, tRNA[Thr] G15897A, A15924G and G15927A. Whereas other 32 variants were classified as "neutral polymorphisms" that were not well conserved or presented >1% in control groups.

As shown in Figure 2, among 17 pathogenic/likely pathogenic variants, 5 of them occurred at Acceptor arm including A4467C in tRNA[Met], A5514G in tRNA[Trp], A5655G in tRNA[Ala], A10055G in tRNA[Gly] and A12330G in tRNA[Leu(CUN)], abolished the classic Watson-Crick base-pairings of corresponding tRNAs. In addition, variants T7505C and C7502T in tRNA[Ser(UCN)], G8313A in tRNA[Lys], T10003C in tRNA[Gly] and G15897A in tRNA[Thr]

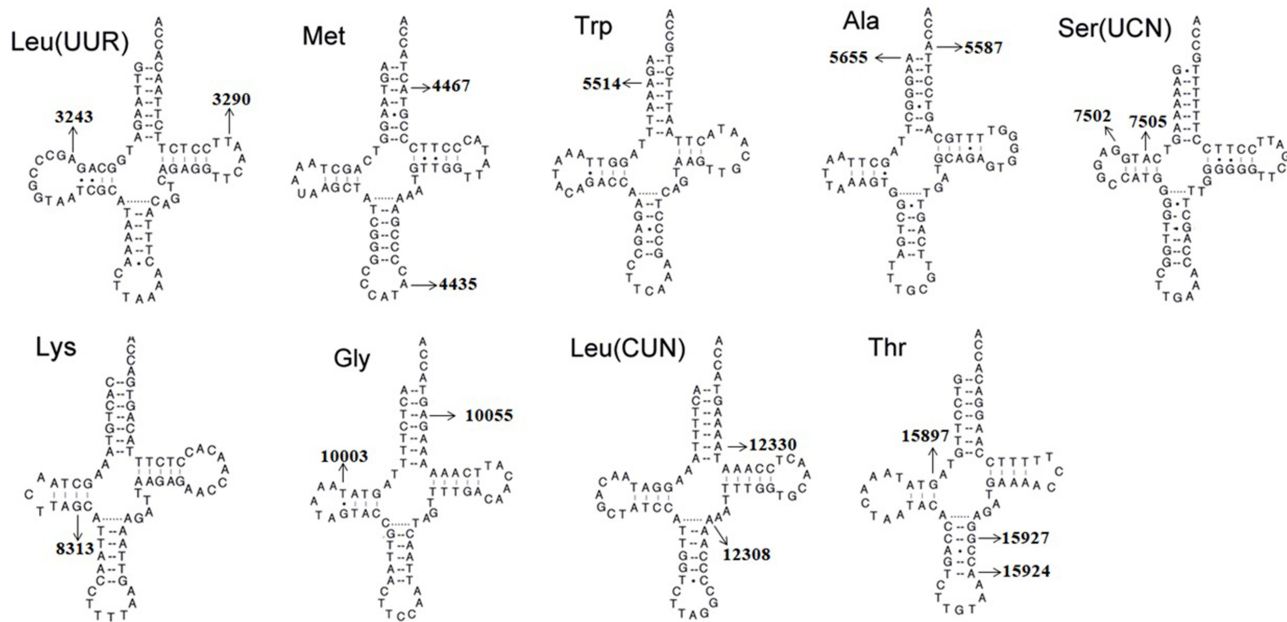**Table 1** Mitochondrial tRNA Variants in 200 Chinese Patients with T2DM

Gene	Position	Replacement	CI (%)[a]	Homoplasmy/ Heteroplasmy	Numbering in tRNA	Watson-Crick Base-Pairing[b]	Location in tRNA	No. of 200 Patients (%)	No. of 200 Controls (%)	Previously Reported[c]
Pathogenic/likely pathogenic mutations										
tRNA^Leu(UUR)	3243	A to G	100	Heteroplasmy	14		DHU-loop	3 (1.5)	0	Yes
	3290	T to C	83	Homoplasmy	59		TψC loop	1(0.5)	0	Yes
tRNA^Met	4435	A to G	100	Homoplasmy	37		Anticodon stem	1(0.5)	0	Yes
	4467	C to A	100	Homoplasmy	71	G-C↓	Acceptor arm	1(0.5)	0	Yes
tRNA^Trp	5514	A to G	92	Homoplasmy	3	A-U↓	Acceptor arm	1(0.5)	0	Yes
tRNA^Ala	5587	T to C	86	Heteroplasmy	73		ACC Terminus	2(1.0)	0	Yes
	5655	A to G	86	Homoplasmy	1	A-U↓	Acceptor arm	1(0.5)	0	Yes
tRNA^Ser(UCN)	7502	C to T	77	Homoplasmy	14		DHU-loop	1(0.5)	0	Yes
	7505	T to C	90	Homoplasmy	11	A-U↓	DHU-loop	1(0.5)	0	Yes
tRNA^Lys	8313	G to A	100	Heteroplasmy	19	C-G↓	DHU-loop	1(0.5)	0	Yes
tRNA^Gly	10,003	T to C	98	Homoplasmy	13	G-C↑	DHU-loop	2(1.0)	0	Yes
	10,055	A to G	96	Homoplasmy	70	A-U↓	Acceptor arm	1(0.5)	0	Yes
tRNA^Leu(CUN)	12,308	A to G	100	Homoplasmy	43	A-U↑	Variable region	1(0.5)	0	Yes
	12,330	A to G	100	Homoplasmy	68	A-U↓	Acceptor arm	1(0.5)	0	Yes
tRNA^Thr	15,897	G to A	98	Homoplasmy	10	G-C↓	DHU-loop	1(0.5)	0	Yes
	15,924	A to G	86	Homoplasmy	39	A-U↓	Anticodon stem	2(1.0)	0	Yes
	15,927	G to A	75	Homoplasmy	42	G-C↓	Anticodon stem	2(1.0)	0	Yes
Other variants										
tRNA^Phe	606	A to G	65	Homoplasmy	30	A- U↓	Anticodon stem	1(0.5)	1(0.5)	Yes
	628	C to T	52	Homoplasmy	52		TψC loop	2(1.0)	1(0.5)	Yes
	633	A to G	25	Homoplasmy	57		TψC loop	1(0.5)	0	Yes
tRNA^Val	1607	T to C	73	Homoplasmy	6	C-G↑	Acceptor arm	2(1.0)	1(0.5)	Yes

(*Continued*)

Table I (Continued).

Gene	Position	Replacement	CI (%)[a]	Homoplasmy/ Heteroplasmy	Numbering in tRNA	Watson-Crick Base-Pairing[b]	Location in tRNA	No. of 200 Patients (%)	No. of 200 Controls (%)	Previously Reported[c]
	1664	G to A	31	Homoplasmy	67	A-U↑	Acceptor arm	1(0.5)	0	Yes
tRNA^Gln	4363	T to C	75	Homoplasmy	38		Acceptor arm	2(1.0)	2(1.0)	Yes
	4395	T to C	82	Homoplasmy	6	C-G↑	Acceptor arm	1(0.5)	2(1.0)	Yes
tRNA^Met	4454	T to A	59	Homoplasmy	53		TψC loop	3(1.5)	2(1.0)	Yes
tRNA^Ala	5601	C to T	63	Homoplasmy	59		TψC loop	2(1.0)	4(2.0)	Yes
	5603	G to A	70	Homoplasmy	61		TψC loop	1(0.5)	3(1.5)	Yes
tRNA^Cys	5788	A to G	44	Homoplasmy	39		Variable region	1(0.5)	5(2.5)	Yes
	5811	T to C	67	Homoplasmy	16		DHU-loop	1(0.5)	2(1.0)	Yes
	5821	C to T	86	Homoplasmy	6	C-G↓	Acceptor arm	2(1)	0	Yes
tRNA^Asp	6571	T to C	66	Homoplasmy	44		TψC loop	1(0.5)	1(0.5)	Yes
tRNA^Ser(UCN)	7498	C to T	33	Homoplasmy	17		DHU-loop	1(0.5)	2(1.0)	Yes
tRNA^Lys	8343	A to G	46	Homoplasmy	54		TψC loop	2(1.0)	4(2.0)	Yes
tRNA^Arg	10,410	T to C	11	Homoplasmy	6		Acceptor arm	1(0.5)	3(1.5)	Yes
	10,454	T to C	69	Homoplasmy	54		TψC loop	2(1)	4(2.0)	Yes
tRNA^His	12,153	C to T	54	Homoplasmy	16		DHU-loop	1(0.5)	3(1.5)	Yes
	12,172	A to G	55	Homoplasmy	38		Anticodon stem	2(1)	4(2.0)	Yes
	12,189	T to C	36	Homoplasmy	56		TψC loop	1(0.5)	1(0.5)	Yes
tRNA^Ser(AGY)	12,234	A to G	67	Homoplasmy	28	A-U↑	Anticodon stem	1(0.5)	0	Yes
	12,237	C to T	63	Homoplasmy	31		Variable region	1(0.5)	2(1.0)	Yes
tRNA^Glu	14,693	A to G	96	Homoplasmy	54		TψC loop	2(1)	4(2.0)	Yes

tRNA^Thr	15,889	T to C	36	2	Homoplasmy	Acceptor arm	1(0.5)	4(2.0)	Yes
	15,900	T to C	78	13	Homoplasmy	DHU-loop	1(0.5)	3(1.5)	Yes
	15,904	C to T	67	17	Homoplasmy	DHU-loop	1(0.5)	3(1.5)	Yes
	15,907	A to G	65	20	Homoplasmy	DHU-loop	1(0.5)	4(2.0)	Yes
	15,930	A to G	21	43	Homoplasmy	Variable region	1(0.5)	1(0.5)	Yes
	15,941	T to C	48	54	Homoplasmy	TψC loop	1(0.5)	3(1.5)	Yes
tRNA^Pro	16,000	G to T	11	26	Homoplasmy	Variable region	1(0.5)	2(1.0)	Yes
	16,017	A to G	35	7	Homoplasmy	Acceptor arm	1(0.5)	3(1.5)	Yes

Notes: [a]CI: conservation index. [b]Classic Watson-Crick base pairing: created (↑) or abolished (↓). [c]Please see Mitomap database (www.mitomap.org).

resided at DHU-loop, affected the conserved Watson-Crick base-pairings. Furthermore, variants A4435G in tRNA^Met, A15924G and G15927A in tRNA^Thr occurred at anticodon stem may affect the steady-state level of mt-tRNAs. While variant A3290G in tRNA^Leu(UUR) occurred at TψC loop, variant T5587C in tRNA^Ala occurred at ACC Terminus, variant A12330G in tRNA^Leu(CUN) located at variable region may also affect the structure and function of mt-tRNAs.

Whole Mitochondrial Genome Analysis of the 23 Subjects Carrying Putative Pathogenic Mt-tRNA Variants

As shown in Table 2, 23 patients with T2DM carried the pathogenic/likely pathogenic mt-tRNA variants, accounting for 11.5% of the cases in our cohort. The age at onset of T2DM in these subjects ranged from 30 to 71 years. Moreover, a comprehensive medical history showed that 2 of 17 probands carrying putative pathogenic mt-tRNA variants had an obvious family history of T2DM, in particular, the family members of DM-101 and DM-102 carrying tRNA^Leu(UUR) A3243G and T3290C variants suffered both hearing loss and diabetes. There were variable clinical phenotypes of diabetes among these probands, with different levels of HbA1c, as well as FPG. In addition, analysis of entire mitochondrial genomes in 23 probands revealed 3 possible functional mtDNA variants: NADH dehydrogenase 1 (*ND1*) T3394C and T3398C, *ND5* T12338C, which co-existed with tRNA^Leu(UUR) T3290C, tRNA^Trp A5514G and tRNA^Gly T10003C, respectively. Interestingly, the T3394C (Tyr to His) and T3398C (Met to Thr) variants occurred at very conserved nucleotides of *ND1* polypeptide.[33,34] While the *ND5* T12338C (Met to Thr) variant resulted in replacement of the first amino acid, translation-initiating methionine with a threonine, which may alter the respiratory function, as well as the processing of RNA precursors.[35]

The entire mtDNA sequences of 23 probands carrying these putative mt-tRNA variants were performed and then assigned to East Asian mitochondrial haplogroups based on the Phylotree database (http://www.phylotree.org/).[36] As shown in Table 2, according to their distinct sets of polymorphisms, the mtDNA of 23 probands belonged to East Asian haplogroups F2, D4, G2b, N1a, N9a, G2a1, F1, D5b1b, C4c, A4, M11b, M11, D5a, U, D4b1, D4b2b, B5b and B5b1, respectively.[29]

Figure 2 Summary of 17 pathogenic/likely pathogenic mt-tRNA variants at the cloverleaf structures of canonical tRNAs. Arrows indicate the positions of the variants in the mt-tRNA.

Clinical and Biochemical Characterization of Two MIDD Pedigrees Carrying tRNA^Leu(UUR) Variants

As shown in Figure 1, two Han Chinese families (DM-101 and DM-102) with MIDD were ascertained in Yantai Affiliated Hospital of Binzhou Medical University. A comprehensive history and physical examinations were performed to identify any clinical abnormalities, genetic factors related to diabetes in members of these two families. In DM-101 pedigree, the proband (III-5) was a 32-year-old woman who came from Yantai city of Shandong province. She began to suffer from T2DM when she was 30 (Table 3), she also developed bilateral hearing loss (55 dB at right ear and 70 dB at left ear). Moreover, the family history suggested that other matrilineal relatives (II-3 and II-6) suffered from T2DM at different ages at onset. In particular, subject II-3 had profound hearing loss (90 dB at right ear and 95 dB at left ear) and hypertension (145/80 mmHg). Subject II-6 had moderate hearing impairment (55 dB at right ear and 35 dB at left ear) and hypertension (150/100 mmHg), whereas other members of this family were normal.

In DM-102 pedigree, the proband (II-10) was a 68-year-old woman who also lived in Yantai city of Shandong province. She suffered from T2DM when she was 55. Comprehensive physical examinations indicated that she

had very high BP (140/95 mmHg) for 3 years, she also exhibited mild hearing impairment (38 dB at right ear and 40 dB at left ear). Genetic counseling revealed that matrilineal relatives (II-5, II-8, III-7 and III-3) were also T2DM carriers. Besides, other members in DM-102 were normal subjects. The clinical and biochemical data of these subjects are listed in Table 3.

Analysis of mtDNA Variants

The maternally inherited pattern of these two pedigrees indicated that mitochondrial dysfunctions may be involved in the pathogenesis of MIDD. In order to investigate the contributions of mtDNA variants to MIDD, the complete mitochondrial genomes of matrilineal relatives from these families (DM-101: II-3, II-6 and III-5; DM-102: II-5, II-8, II-10 and III-7) were PCR amplified and sequenced by ABI 3730 automated DNA instrument, subsequently the data was compared with the rCRS and the mtDNA variants were screened. As can be seen in Table 4, matrilineal relatives of these pedigrees harbored 76 mtDNA variants, of these, 21 variants occurred at D-loop, 3 variants in 12S rRNA, 3 variants in 16S rRNA, 2 variants in tRNA^Leu(UUR), as well as the common COII/tRNA^Lys intergenic 9-bp deletion occurred at position between 8271 and 8279. Other variants were localized at oxidative phosphorylation (OXPHOS)-related genes. In addition, 12 missense variants were

Table 2 Mitochondrial Genetic Background and Clinical Features of 23 Probands Carrying One of the Pathogenic/Likely Pathogenic T2DM-Associated tRNA Variants

Genes	Variants	Probands	Gender	Age at Test (Year)	Age at Onset (Year)	HbA1c (%)	Glucose (0h)	Family History	Other Functional mtDNA Variants	Haplogroup
tRNA$^{Leu(UUR)}$	A3243G	DM-101	M	32	30	7.1	9.1	Yes	/	F2
		DM-210	F	44	38	6.5	10.8	No	/	F2
		DM-225	F	51	42	7.0	11.1	No	/	F2
	T3290C	DM-102	M	68	55	6.9	5.6	Yes	*ND1* T3394C	D4
tRNA^{Met}	A4435G	DM-132	M	70	65	7.1	9.1	No	/	G2b
	C4467A	DM-126	M	52	41	7.2	4.8	No	/	N1a
tRNA^{Trp}	A5514G	DM-108	F	46	39	7.9	8.7	No	*ND1* T3398C	N9a
tRNA^{Ala}	T5587C	DM-213	F	66	61	8.3	5.2	No	/	G2a1
		DM-259	M	59	52	8.0	5.6	No	/	F1
	A5655G	DM-277	M	41	38	7.7	7.0	No	/	D5b1b
tRNA$^{Ser(UCN)}$	C7502T	DM-239	M	39	33	7.4	4.9	No	/	C4c
	T7505C	DM-184	F	57	53	7.0	13.0	No	/	F1
tRNA^{Lys}	G8313A	DM-199	F	65	50	6.6	11.6	No	/	A4
tRNA^{Gly}	T10003C	DM-217	F	71	70	6.8	8.0	No	*ND5* T12338C	M11b
		DM-290	M	66	61	6.9	7.9	No	/	M11
	A10055G	DM-255	M	49	41	7.2	5.5	No	/	D5a
tRNA$^{Leu(CUN)}$	A12308G	DM-230	M	46	39	7.9	5.8	No	/	U
	A12330G	DM-222	M	55	51	8.0	6.0	No	/	F2
tRNA^{Thr}	G15897A	DM-266	M	57	53	7.4	5.1	No	/	D4b1
	A15924G	DM-277	F	79	71	7.3	6.8	No	/	M11
		DM-281	F	60	58	7.1	6.6	No	/	D4b2b
	G15927A	DM-155	M	59	52	6.8	6.8	No	/	B5b
		DM-260	F	61	60	6.6	7.0	No	/	B5b1

Abbreviations: T2DM, type 2 diabetes mellitus; mtDNA, mitochondrial DNA; M, male; F, female.

identified, including *ND1* T3394C (Tyr to His), *ND2* T5442C (Phe to Leu), *CO2* G7598A (Ala to Thr), *ATP6* (A6) A8701G (Thr to Ala) and A8860G (Thr to Ala), *ND3* A10398G (Thr to Ala), *ND4L* T10609C (Met to Thr) and A10750G (Asn to Ser), *ND5* G12406A (Val to Ile) and G13928C (Ser to Thr), *CytB* C14766T (Thr to Ile) and A15326G (Thr to Ala). To further assess their pathogenicity, phylogenetic approach was carried out to see the evolutionary conservation of each variant, especially in mouse,[37] bovine[38] and *Xenopus laevis*.[39] We found that except for the A3243G and T3290C in tRNA$^{Leu(UUR)}$, the T3394C in *ND1*, other variants showed lower levels of CIs (Figure 3 and 4). Moreover, the A3243G and T3290C variants were not detected in 200 controls, suggesting that they may be involved in the pathogenesis of MIDD.

Mutational Analysis of *GJB2* Gene

Mutations in *GJB2* were the important causes for hearing loss,[40] to examine the contributions of *GJB2* to deafness expression, we conducted a mutational screening for *GJB2* gene in matrilineal relatives of these MIDD pedigrees

Table 3 Summary of Clinical and Biochemical Data for Several Members in These Two Families with Maternally Inherited Diabetes and Deafness

Subjects	Gender	Age at Test (Years)	Age at Onset (Years)	HbA1c (%)	Glucose (oh) (mmol/L)	Glucose (2h) (mmol/L)	BP (mmHg)	PTA (dB) Right/Left Ear	Level of Hearing Loss
DM-101 (II-3)	Male	55	50	6.6	7.7	14.2	145/80	90/95	Profound
DM-101 (II-6)	Female	58	45	7.0	8.0	12.6	150/100	55/35	Moderate
DM-101 (III-5)	Female	32	30	7.1	9.1	14.8	130/75	55/70	Severe
DM-102 (II-5)	Male	65	50	6.8	5.1	7.8	145/95	20/20	Normal
DM-102 (II-8)	Female	60	49	7.3	8.2	14.6	135/80	23/17	Normal
DM-102 (II-10)	Female	68	55	6.9	5.6	8.0	140/95	38/40	Mild
DM-102 (III-7)	Female	40	38	6.5	5.2	7.1	130/85	52/45	Moderate
DM-102 (III-3)	Female	36	/	5.6	4.8	6.9	125/75	20/15	Normal

Abbreviations: HbA1c, glycosylated hemoglobin; BP, blood pressure; PTA, pure-tone audiometry; dB, decibel.

(DM-101: II-3, II-6 and III-5; DM-102: II-5, II-8, II-10 and III-7). However, we failed to detect any variants in *GJB2* gene, suggesting that *GJB2* may not play an active role in the phenotypic manifestation of MIDD in these families.

Mutational Screening of *TRMU* Gene

Previous study showed that variants in *TRMU* may modulate the clinical manifestation of deafness-associated mitochondrial A1555G or C1494T mutation.[41] To examine whether *TRMU* played an important role in MIDD expression, we screened the *TRMU* A10S variant in matrilineal relatives of these families. But we did not detect the presence of A10S variant suggested that *TRMU* may not be involved in the pathogenesis of MIDD in these families.

Discussion

In the present case-control study, we analyzed the frequencies of mt-tRNA variants in 200 patients with T2DM and 200 controls. Through PCR and direct sequence analysis, a total of 49 genetic variants in mt-tRNA genes were identified. By focusing only on variants that were evolutionary conserved, presented <1% in controls and predicted to induce functional or structural changes in mt-tRNA molecules, we were able to identify 17 pathogenic/likely pathogenic mt-tRNA variants in 23 probands with T2DM. Among them, the heteroplasmic A3243G in tRNA[Leu(UUR)] was one of the most common T2DM-associated pathogenic mutations.[42] This mutation also led to mitochondrial encephalomyopathy, lactic acidosis and stroke-like symptoms (MELAS).[43] Molecular analysis revealed that this mutation reduced the steady-state level, aminoacylation, as well as

codon recognition of tRNA[Leu(UUR)].[44] As a result, the A3243G mutation caused the pre-termination of transcription and expression impeding of normal rRNA, thus compromising mitochondrial protein synthesis, ATP synthesis and organic metabolism.[45] While the homoplasmic T3290C variant occurred at position 59 in the TψC loop of tRNA[Leu(UUR)], which had been regarded as a risk factor for hypertension.[46] In addition, the A4435G variant was localized at immediately 3′ end to the anticodon, corresponding to the conventional position 37 of tRNA[Met].[47] The adenine (A37) at this position of tRNA[Met] was extremely conserved from bacteria to human mitochondria.[48] Furthermore, the A4435G variant introduced an m^{1}G37 modification of tRNA[Met], altered its structure and function. Functional analysis of cybrid cells harboring this variant revealed a markedly diminished ATP levels and mitochondrial membrane potential (MMP), and increased reactive oxygen species (ROS) production.[48] While the C4467A variant occurred at 3′ end of tRNA[Met], which was the processing site for the tRNA[Met] 3′ end precursors of the light strand and was predicted to alter A-U base pairing (A1-U72) at the aminoacyl acceptor stem of tRNA[Met].[49] The C4467A variant may impair the cleavage of polycistronic mtRNA transcripts into mature mt-tRNA species at the tRNAase Z cleavage site.[50] Thus, the point variant located at the 3′ end was important for both mt-tRNA synthesis and function.[51] Moreover, the homoplasmic A5514G variant disrupted an A-U base-pair within the acceptor stem of tRNA[Trp], had been reported to be associated with mitochondria encephalomyopathy.[52] Interestingly, two variants (A5655G and T5587C) were identified in tRNA[Ala] gene, in fact, A5655G variant was

Table 4 mtDNA Sequence Variants in Two Chinese Families with Maternally Inherited Diabetes and Deafness

Gene	Position	Alternation	Conservation (H/B/M/X)[a]	rCRS[b]	DM-101	DM-102	Previously Reported[c]
D-loop	73	A to G		A	G	G	Yes
	143	G to A		G	A		Yes
	146	T to C		T		C	Yes
	150	C to T		C	T		Yes
	195	T to C		T		C	Yes
	207	G to A		G	A		Yes
	263	A to G		A		G	Yes
	310	T to TC/CTC		T	TC	CTC	Yes
	374	A to G		A	G		Yes
	489	T to C		T	C	C	Yes
	514	DelC		C	DelC		Yes
	515	DelA		A		DelA	Yes
	523	Del A		A	Del A		Yes
	16,051	A to G		A	G	G	Yes
	16,093	T to C		T	C		Yes
	16,129	G to A		G	A	A	Yes
	16,175	A to T		A		T	Yes
	16,189	T to C		T	C	C	Yes
	16,223	C to T		C	T	T	Yes
	16,311	T to C		T	C	C	Yes
	16,526	G to A		G	A		Yes
12S rRNA	750	A to G	A/G/G/-	A	G	G	Yes
	1041	A to G	A/T/T/T	A		G	Yes
	1438	A to G	A/A/A/G	A	G	G	Yes
16S rRNA	2706	A to G	A/G/A/A	A	G	G	Yes
	3010	G to A	G/G/A/A	G		A	Yes
	3107	Del N		N	Del N	Del N	Yes
tRNA[Leu(UUR)]	3243	A to G	A/A/A/A	A	G		Yes
	3290	T to C	T/T/T/T	T		C	Yes
ND1	3394	T to C (Tyr to His)	Y/Y/Y/Y	T		C	Yes
	3483	G to A		G	A		Yes
	3970	C to T		C		T	Yes
	4071	C to T		C	T		Yes
ND2	4769	A to G		A	G	G	Yes
	4850	C to T		C	T		Yes
	5442	T to C (Phe to Leu)	F/F/M/L	T		C	Yes
CO1	6392	T to C		T	C	C	Yes
	6455	C to T		C		T	Yes
	6599	A to G		A		G	Yes
	6962	G to A		G		A	Yes
	7028	C to T		C		T	Yes
	7250	A to G		A	G	G	Yes
	7337	G to A		G	A	A	Yes
CO2	7598	G to A (Ala to Thr)	A/M/F/S	G		A	Yes
	7805	G to A		G		A	Yes
NC7	8281–8289	Del 9-bp		CCCCCTCTA		Del 9-bp	Yes

(*Continued*)

Table 4 (Continued).

Gene	Position	Alternation	Conservation (H/B/M/X)[a]	rCRS[b]	DM-101	DM-102	Previously Reported[c]
A8	8440	A to G		A	G		Yes
A6	8701	A to G (Thr to Ala)	T/S/L/Q	A		G	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	G	G	Yes
CO3	9540	T to C		T	C	C	Yes
	9824	T to C		T	C		Yes
	9896	A to G		A		G	Yes
ND3	10,310	G to A		G		A	Yes
	10,398	A to G (Thr to Ala)	T/T/T/A	A	G		Yes
	10,400	C to T		C	T		Yes
ND4L	10609	T to C (Met to Thr)	M/T/T/T	T	C		Yes
	10,750	A to G (Asn to Ser)	N/N/N/N	A		G	Yes
ND4	10,683	G to A		G	A		Yes
	10,873	T to C		T		C	Yes
	11,719	G to A		G		A	Yes
	11,926	A to C		A	C		Yes
ND5	12,360	A to G		A	G		Yes
	12,406	G to A (Val to Ile)	V/F/S/F	G		A	Yes
	12,705	C to G		C	G	G	Yes
	12,882	C to T		C	T	T	Yes
	13,152	A to G		A		G	Yes
	13,708	G to A		G	A		Yes
	13,759	G to A		G		A	Yes
	13,928	G to C (Ser to Thr)	S/T/S/T	G		C	Yes
ND6	14,311	T to C		T		C	Yes
CytB	14766	C to T (Thr to Ile)	T/S/I/S	C	T	T	Yes
	14,783	T to C		T	C		Yes
	15,040	C to T		C	T		Yes
	15,043	G to A		G	A		Yes
	15,301	G to A		G	A	A	Yes
	15,326	A to G (Thr to Ala)	T/M/I/I	A	G	G	Yes

Notes: [a]Conservation of amino acids in polypeptides or nucleotides in RNA in Human (H), cows (B), mice (M) and *Xenopus laevis* (X). [b]rCRS: revised Cambridge reference sequence. [c]Please visit Mitomap database (www.mitomap.org) or mtDB (www.genpat.uu.se/mtDB).

located at processing site for the tRNA 5′ end precursors, catalyzed by RNase P.[53] Furthermore, A5655G variant changed the highly conserved base pairing (A1-U72) at the Acceptor arm of tRNA[Ala]. Functional analysis revealed that this variant caused an improperly aminoacylated tRNA[Ala] and slower electrophoretic mobility of mutated tRNA.[54] While the homoplasmic T5587C variant occurred at position 73 near the end of tRNA[Ala], which was very important for tRNA identity.[55] Notably, the T to C transition at that position was extremely conserved from various species, recent experimental studies indicated that the T5587C variant may be associated with Leber's Hereditary Optic Neuropathy (LHON) and essential hypertension (EH).[56,57] Furthermore, the C7502T and T7505C variants were identified in DHU-loop of tRNA[Ser(UCN)] gene, the C7502T variant occurred at A14-U8 interaction site, which is important for cognate aminoacyl tRNA synthetase recognition.[58] While the T7505C variant was located at a highly conserved base-pairing (A10-U20) of tRNA[Ser(UCN)]. The abolishment of A10-U20 Watson-Crick base-pairing likely altered the tRNA[Ser(UCN)] metabolism. Functional significant of this variant was supported by
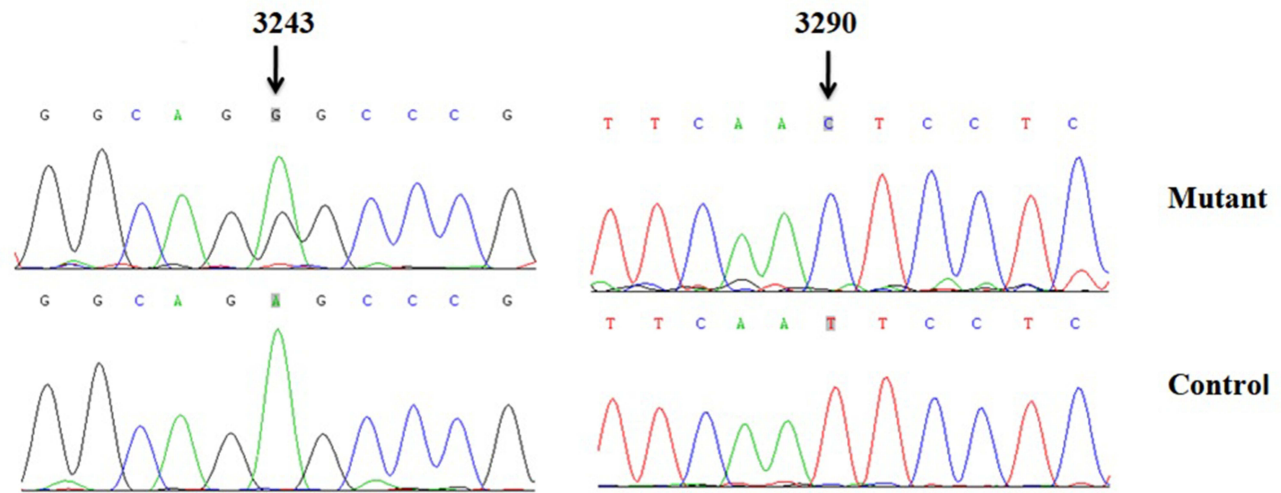
Figure 3 Identification of tRNA^{Leu(UUR)} A3243G and T3290C variants by direct sequence.

Figure 4 Sequence alignment of tRNA^{Leu(UUR)} from various species, arrows indicate the positions of 14 and 59, corresponding to the A3243G and T3290C variants.

approximately 65% reductions in the level of tRNA^{Ser(UCN)} observed in the lymphoblastoid cell lines carrying the T7505C variant, as compared with the wild-type cell lines.[59] In addition, the heteroplasmic G8313A variant had been found in patients with various clinical phenotypes including encephaloneuropathy, short stature, myopathy, peripheral neuropathy, and osteoporosis.[60] Functional analysis of cybrid cells containing this variant showed a marked decreased in tRNA^{Lys} steady-state level and aminoacylation ability, suggesting that these molecular abnormalities may underlie the pathogenesis of the G8313A variant.[61] Moreover, the T10003C and A10055G variants were identified in tRNA^{Gly} gene, in fact, the T10003C variant created a novel Watson-Crick base-pairing (C13-G19), whereas the A10055G variant disrupted the

conserved Watson-Crick base-pairing (U3-A70), therefore, the alteration of mt-tRNA structure caused by A10055G variant may affect mt-tRNA function, and subsequently led to a failure in mt-tRNA metabolism.[62] Moreover, the A12308G variant created a novel Watson-Crick base-pairing (A25-U43) in the variable region of tRNA^{Leu(CUN)}, by contrast, the A12330G variant abolished the conserved base-pairing (U6-A68) in the Acceptor arm of tRNA^{Leu(CUN)}. In fact, the A12308G variant had been implicated to be associated with pigmentary retinal degeneration, short stature, dysphasia-dysarthria and cardiac conduction defects,[63,64] and increased the risk of developing stroke.[65] While the homoplasmic A12330G variant was found in patients with maternally inherited EH.[66] Furthermore, 3 variants were found in tRNA^{Thr} gene:

G15897A, A15924G and G15927A, among these, the G15897A variant occurred at highly conserved nucleotide of tRNA^{Thr}, which was important for the stability and identity of mt-tRNA. This variant was found to decrease the steady-state level, as well as aminoacylation ability of tRNA^{Thr}, and subsequently led to the defects in energetic processes.[11] Notably, the A15924G and G15927A variants were located at anticodon stem of tRNA^{Thr}, which were extremely conserved from different species.[67] Previous studies showed that the A15924G variant was associated with fatal infantile respiratory enzyme deficiency,[68] while the G15927A was regarded as pathogenic mutation associated with coronary heart disease (CHD)[69] and implicated to modulate the clinical expression of deafness-associated 12S rRNA A1555G mutation.[70] The G15927A mutation caused significantly decreased efficiency in aminoacylation and steady-state levels of tRNA^{Thr}.[71] Thus, the failure in tRNA metabolism led to the mitochondrial dysfunction that was responsible for T2DM.

Among these patients carrying putative pathogenic mt-tRNA variants, only 2 of them had an obvious family history of T2DM (Figure 1). In DM-101 pedigree, among 6 matrilineal relatives, 3 individuals suffered from both diabetes and deafness, while in DM-102 pedigree, there were 8 matrilineal relatives, notably, 4 of them suffered from T2DM (2 subjects with diabetes only, 2 subjects with both diabetes and hearing impairment). Interestingly, matrilineal relatives in these two families had earlier age onset of diabetes, indicating that mtDNA variants may be acted as risk factors for molecular diagnosis and detection of this disease.

Sequence analysis of the entire mitochondrial genomes of the matrilineal relatives from two families revealed the presence of tRNA$^{Leu(UUR)}$ A3243G and T3290C variants, together with sets of genetic polymorphisms belonging to East Asian haplogroups F2 and D4, respectively.[29] In fact, the heteroplasmic A3243G mutation accounted for approximately 0.5–3% of all cases of diabetes in general population.[72,73] Patients who carried this mutation demonstrated an impaired OXPHOS,[74] and decreased OXPHOS in glucose-stimulated insulin secretion from β-cells.[75] On the other hand, the homoplasmic T3290C variant occurred at position 59 in the TψC loop of tRNA$^{Leu(UUR)}$, which was very conserved from different species (Figure 4). Thus, it was hypothesized that T3290C variant likely altered the tRNA$^{Leu(UUR)}$ metabolism, in fact, previous study suggested that this variant was found to be associated with EH.[46] Moreover, a variable severity of T2DM, as well as

audiometric configuration of hearing impairment were observed among the matrilineal relatives in these two families indicated the involvement of other modified factors such as nuclear genes, mitochondrial haplogroups, epigenetic or environmental factors. However, the absence of *TRMU* A10S and *GJB2* variants indicated that these nuclear modified genes may not play active roles in MIDD in these families. Furthermore, sequence analysis of the entire mtDNA genes of matrilineal relatives in these families suggested the presence of 74 variants, in addition to the A3243G and T3290C variants, which belonged to mitochondrial haplogroups F2 and D4, respectively.[29] Of these, the *ND1* T3394C variant displayed very highly evolutionary conservation, and was not detected in 200 Chinese control subjects. Notably, this variant disrupted the specific electrostatic interactions between Y30 of *ND1* with the sidechain of E4 and backbone carbonyl group of M1 of NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1 (NDUFA1) of Complex I, thereby altering the structure and function of OXPHOS.[76] Importantly, the T3394C variant altered the stability of *ND1* and Complex I assembly, decreased the activities of Complex I and increased the production of ROS in cybrid cells.[77] Therefore, the mitochondrial dysfunctions caused by the tRNA$^{Leu(UUR)}$ T3290C variant may be worsened by the *ND1* T3394C variant in DM-102 pedigree.

Conclusions

In summary, our study indicated that mt-tRNA variants may be associated with T2DM in Han Chinese population, in particular, tRNA$^{Leu(UUR)}$ A3243G and T3290C, tRNA^{Met} A4435G and C4467A, tRNA^{Trp} A5514G, tRNA^{Ala} T5587C and A5655G, tRNA$^{Ser(UCN)}$ C7502T and T7505C, tRNA^{Lys} G8313A, tRNA^{Gly} T10003C and A10055G, tRNA$^{Leu(CUN)}$ A12308G and A12330G, tRNA^{Thr} G15897A, A15924G and G15927A altered the structure and function of their tRNAs, thereby causing mitochondrial dysfunctions and long-standing increase of ROS in pancreatic β-cells. These variants may be the inherited risk factors for T2DM. Future studies with larger sample size comprising different ethnicities should be performed to confirm our conclusion.

Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of the Yantai Affiliated Hospital of Binzhou

Medical University (No. 20210212001). This study was conducted in accordance with the declaration of Helsinki. Written informed consent was obtained from all participants.

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Disclosure
The authors declare that they have no conflicts of interest for this work.

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