

Identification of a Novel Mitochondrial tRNA Mutation in Chinese Family with Type 2 Diabetes Mellitus

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Background: Mutations in mitochondrial tRNA (mt-tRNA) could be the origin of some type 2 diabetes mellitus (T2DM) cases, but the mechanism remained largely unknown.

Aim: The aim of this study was to assess the impact of a novel mitochondrial tRNA^{Cys}/tRNA^{Tyr} A5826G mutation on the development and progression of T2DM.

Methods: A four-generation Han Chinese family with maternally inherited diabetes underwent clinical, genetic and biochemical analyses. The mitochondrial DNA (mtDNA) mutations of three matrilineal relatives were screened by PCR-Sanger sequencing. Furthermore, to see whether m.A5826G mutations affected mitochondrial functions, the cybrid cell lines were derived from three subjects with m.A5826G mutation and three controls without this mutation. ATP was evaluated by luminescent cell viability assay, mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) were determined by flow cytometry. The student's two-tailed, unpaired *t*-test was used to assess the statistical significance between the control and mutant results.

Results: The age at onset of diabetes in this pedigree varied from 40 to 63 years, with an average of 54 years. Mutational analysis of mitochondrial genomes revealed the presence of a novel m.A5826G mutation. Interestingly, the m.A5826G mutation occurred at the conjunction between tRNA^{Cys} and tRNA^{Tyr}, a very conserved position that was critical for tRNAs processing and functions. Using trans-mitochondrial cybrid cells, we found that mutant cells carrying the m.A5826G showed approximately 36.5% and 22.4% reductions in ATP and MMP, respectively. By contrast, mitochondrial ROS levels increased approximately 33.3%, as compared with the wild type cells.

Conclusion: A novel m.A5826G mutation was identified in a pedigree with T2DM, and this mutation would lead to mitochondrial dysfunction. Thus, the genetic spectrum of mitochondrial diabetes was expanded by including m.A5826G mutation in tRNA^{Cys}/tRNA^{Tyr}, our study provided novel insight into the molecular pathogenesis, early diagnosis, prevention and clinical treatment for mitochondrial diabetes.

Keywords: type 2 diabetes, m.A5826G, tRNA^{Cys}/tRNA^{Tyr}, mitochondrial dysfunction

Introduction

Mitochondria are distinct organelles within eukaryotic cells that are involved in oxidative phosphorylation (OXPHOS). They carry their own genetic material, in humans, mitochondrial DNA (mtDNA) is a circular molecule comprising 16,569-bp that is essential for respiratory chain functions.¹ It encodes 13 OXPHOS-related proteins, as well as 22 tRNAs and 2 rRNAs. MtDNA in humans shows a strictly maternal inheritance as during fertilization of the oocyte the paternal mitochondria are not retained within the fertilized oocyte.² Mitochondrial diseases are a group of genetic disorders characterized by defects in OXPHOS and caused by mutations in nuclear DNA (nDNA) and mtDNA that encode proteins involved in mitochondrial functions.³ Mitochondrial diseases are frequently related to the damage of nerves and muscle conducting to deafness and visual loss. Among them, T2DM is one of the most common types of mitochondrial diseases. It is a complex, heritable and heterogeneous condition that manifests in a range of clinical phenotypes, including

hyperglycemia, diabetic ketoacidosis, retinopathy, nephropathy, foot problem which remain big challenging for clinicians.⁴ However, its pathophysiology is still undetermined.

In recent years, it had been found that mutations in mtDNA may lead to T2DM concomitant with symptoms caused by damage to nerves and muscles.^{5–8} In particular, the well-known m.A3243G mutation in tRNA^{Leu(UUR)} was implicated in the pathogenesis and progression of T2DM in many families worldwide.^{9–11} This mutation affected the structural stability, aminoacylation rate and post-transcriptional modification of tRNA^{Leu(UUR)}.¹² In addition to the m.A3243G mutation, some case-control studies suggested that tRNA^{Trp} A5514G and tRNA^{Ser(AGY)} C12337T,¹³ tRNA^{Glu} A14692G,¹⁴ tRNA^{Ala} T5587C and ND5 T12338C¹⁵ mutations were associated with T2DM. In fact, mt-tRNA pathogenic mutations have structural and functional consequences, such as affecting the tRNA structure, altering 5' or 3' processing of tRNAs, CCA addition and leading to defects in chemical modifications. Thus, these mutations would impair the normal functions of OXPHOS system,¹⁶ thereby exacerbating the mitochondrial dysfunction that is responsible for T2DM. However, the detailed mechanisms remain largely unknown.

To address this problem, most recently, we carried out a mutational screening of mt-tRNA genes from Department of Endocrinology, Ordos Center Hospital, China. Our aim was to describe a Han Chinese family with maternally transmitted T2DM, as well as carrying out a sequence analysis of the complete mitochondrial genomes from matrilineal relatives. As a result, we identified the presence of two potential pathogenic mutations: m.A5826G which resided at a spacer between tRNA^{Cys} and tRNA^{Tyr} gene, together with m.C14668T in ND6 gene. Therefore, our study broadened the mutational spectrum of mitochondrial diabetes. Moreover, we evaluated the impacts of m.A5826G and m.C14668T mutations on mitochondrial functions by creating cybrid cell lines derived from the three patients with these mtDNA mutations, three healthy controls without these mutations. We also examined the levels of ATP, MMP and ROS. Consequently, we found that cells with m.A5826G and m.C14668T mutations exhibited more severe mitochondrial dysfunctions than control cell lines.

Materials and Methods

Participants and Clinical Examinations

In this case-control study for genetic screening of T2DM-associated novel mtDNA mutations, a four-generation Han Chinese pedigree with diabetes, as shown in Figure 1, was ascertained via the Department of Endocrinology, Ordos Center Hospital. This pedigree was designed according to the proposed system by the National Society of Genetic Counselors (NSGC), arrow indicated the proband, and affected individuals were indicated by filled symbols.¹⁷ Members of this family were evaluated at length to identify both personal and family medical histories of diabetes, deafness, vision loss and other clinical abnormalities. Moreover, 200 healthy Han Chinese subjects, including 100 males and 100 females, aged from 33 to 50 years, with an average of 41 years were recruited from the Physical Examination Center of our

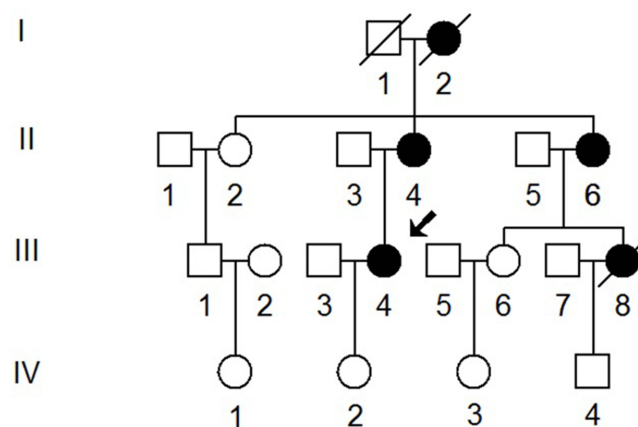


Figure 1 T2DM pedigree information carrying m.A5826G and m.C14668T mutations. Affected individuals (I-2; II-2, II-3, III-2 and III-3) are indicated by filled symbols. Diagonal lines (I-1, I-2 and III-3) are used to show that persons are deceased, arrow shows the proband (III-2).

hospital as controls. The inclusion criteria for these control subjects were as follows: healthy subjects without any diseases or did not have any family history of mitochondrial diseases; subjects who have any history of mitochondrial diseases were excluded. The protocol used in this investigation was in accordance with the principles expressed in 1975 Declaration of Helsinki, which was revised in 2008, and the Ethic Committee of Ordos Center Hospital approved this study (Approval No. 2023–036). The written informed consent for participating in this study as well as informed consent to have their case details published were obtained from all individuals enrolled in this study, and all information was kept in confidential in order to protect their privacy.

The diagnosis of T2DM was based on the standard proposed by the American Diabetes Association,¹⁸ there were four possible ways to diagnose diabetes and each, in the absence of hyperglycemia symptoms, must be confirmed on a different day by any one of the other three: (1) The fasting plasma glucose concentration ≥ 7.0 mmol/L; (2) The 2-h plasma glucose level ≥ 11.1 mmol/L post the oral glucose tolerance test (OGTT). It should be noted that OGTT has poor reproducibility in adolescents, with a concordance rate of $<30\%$ between tests performed a few weeks apart.¹⁹ (3) Random plasma glucose ≥ 11.1 mmol/L in the presence of diabetes symptoms. (4) The concentration of hemoglobin A1c (HbA1c) $>6.5\%$. Notably, the fasting plasma glucose was performed 0.5-h prior to vein puncture and was measured by chemiluminescent immunometric assay (IMMULITE[®], Siemens).

All affected subjects underwent a comprehensive physical examinations and laboratory assessments: the body mass index (BMI) was calculated as the body weight (kg) divided by the square of the height (m^2). BMI ≤ 18.5 kg/m^2 suggested the person was underweight, 18.5 $\text{kg}/\text{m}^2 < \text{BMI} < 23.9$ kg/m^2 indicated normal weight, 24.0 $\text{kg}/\text{m}^2 < \text{BMI} < 27.9$ kg/m^2 indicated overweight, BMI ≥ 28.0 kg/m^2 indicated obesity.²⁰

Blood samples were collected in the morning between 7:00 Am and 10:00 Am after an overnight fast. The HbA1c was measured by high-pressure liquid chromatography (Variant II; Bio-Rad, CA, USA). For biochemical assessment, the fasting plasma glucose level was determined by regular laboratory methods (Beckman Coulter, Tokyo, Japan). In addition, OGTT was carried out by measurement of plasma glucose concentrations at 2-h after 75-g glucose administration, while the fasting plasma insulin was measured by chemiluminescent immunometric assay (IMMULITE[®], Siemens).²¹ The degree of insulin resistance (IR) was estimated using the homeostasis model assessment of IR (HOMA-IR) index, the $\text{HOMA-IR} = (\text{insulin} \times \text{glucose})/22.5$. A $\text{HOMA-IR} \geq 2.69$ was regarded to be IR.²² In addition, the serum creatinine (Cr) and estimated glomerular filtration rate (eGFR) were assessed to evaluate the renal functions. Notably, $\text{eGFR} = 175 \times \text{standardized Scr}^{-1.154} \times \text{age}^{-0.203} \times 1.212$ (if black) $\times 0.742$ (if female), where eGFR was expressed as $\text{mL}/\text{min}/1.73\text{m}^2$ of body surface area and serum Cr was expressed in $\mu\text{mol}/\text{L}$.²³

The levels of hearing loss were tested in each affected subject by using pure-tone audiometry (PTA), as described previously.²⁴ The PTA was calculated as the average of the thresholds measured at 0.5, 1.0, 2.0, 4.0, and 8.0 kHz. The severity of hearing impairment was defined as mild (21–40 dB), moderate (41–70 dB), severe (71–90 dB), and profound (>91 dB). Moreover, the visual acuity was tested separately for each eye without refraction at 4-m using Early Treatment Diabetic Retinopathy Tumbling-E study charts (Precision Vision, La Salle, IL, USA), and the degree of visual impairment was classified on the basis of visual acuity as follows: normal (>0.3), mild (0.1–0.3), moderate (0.05–0.1), severe (0.02–0.05), and profound (<0.02).²⁵

Analysis of mtDNA Mutations

The genomic DNA of three matrilineal relatives (II-4, II-6 and III-4), together with 200 controls were extracted by using the Puregene DNA Isolation Kits (Gentra Systems). Subsequently, the affected individuals' DNA fragments spanning the whole mitochondrial genomes were PCR amplified in 24 overlapping fragments using sets of the light and the heavy strand primers as described previously.²⁶ Furthermore, the ABI 3700 DNA instrument was employed to analyze the sequences by comparing with the revised Cambridge sequences (rCRS, GenBank accession number: NC_012920.1).²⁷ The DNA STAR software package version 5.01 (Madison) was used to detect mtDNA mutations or variants.

Phylogenetic Conservation Analysis

A total of 14 species' mtDNA sequences were used in the interspecific analysis. The conservation index (CI) was calculated by comparing the human nucleotide variants with other 13 vertebrates.²⁸ The CI was defined as the percentage

of species from the list of 13 different primate species that have the wild-type nucleotide at that position. Its value $\geq 75\%$ was regarded as having functional importance.²⁹

Generation of Cybrid Cell Lines

Cybrids were obtained by transforming platelets of patients containing the m.A5826G and m.C14668T mutations (II-4, II-6 and III-8), together with three control subjects without these mtDNA mutations (C1, C2 and C3) into mtDNA-less p⁰ human osteosarcoma 143B cells, as described previously.³⁰ Cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) containing 12% FBS (Sigma, Aldrich, St. Louis, MO, USA) at 37°C.

To confirm the successful establishment of cybrid cells, PCR-Sanger sequencing was performed to detect the presence of m.A5826G and m.C14668T mutations in cybrids. The genomic DNA was first isolated from cell lines, and the concentration of DNA was then measured. The primer sequences for detection of m.A5826G mutation were: forward-5'-CTA ACC GGC TTT TTG CCC-3'; reversed-5'-ACC TAG AAG GTT GCC TGG CT-3'; the primer sequence for detection of m.C14668T mutation was: forward-5'-GCA TAA TTA AAC TTT ACT TC-3'; reversed-5'-AGA ATA TTG AGG CGC CAT TG-3'. The PCR primers were supplied by BGI (Shenzhen, China) and the PCR mixture included 200 μ M dNTP, 10 \times buffer, Taq DNA polymerase and 15 mmol/L Mg²⁺ (Takara Biotechnology, Dalian, China). After amplification, the PCR products were purified and sequenced by ABI 3700 DNA instrument, and the data were then compared with rCRS to detect the presence of m.A5826G and m.C14668T mutations (GenBank accession number: NC_012920.1).²⁷

ATP Measurement

The levels of ATP production in six cell lines were determined by CellTiter-Glo[®] Luminescent cell viability assay (Promega, G7572) in accordance with the protocol provided by the manufacturer's instructions.³¹

MMP Analysis

MMP from six cell lines were examined with JC-10 Assay Kit-Flow Cytometry (Abcam) following general manufacturer's recommendations with some modifications, as described previously.³² Cells were first plated onto 96-well cell culture plate overnight in growth medium. JC-10 dye loading solution was added for 30 min at 37°C, 5% CO₂. Alternatively, plated cells were preincubated with 10 μ M of the protonophore uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) for 30 min at 37°C, 5% CO₂ prior to staining with JC-10 dye. The fluorescent intensities for both J-aggregates and monomeric forms of JC-10 were measured at Ex/Em=490/530 and 490/590 nm with flow cytometry.

ROS Analysis

Approximate 2×10^6 cells were grown in 96-well plates, first washed twice with PBS to remove the medium. Next, adding 0.5 mL of measurement buffer containing 2 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Immediately after addition, flow cytometry was used to measure the fluorescence in kinetic mode at 485 nm excitation and 520 nm emission wavelengths.³³

Statistical Analysis

The mitochondrial functional data were expressed as mean \pm standard deviation (SD). The student's two-tailed, unpaired *t*-test was used to assess the statistical significance between the control and mutant results. All analyses were performed using the SPSS software version 22.0. We regarded the $p < 0.05$ as statistically significant.

Results

Clinical Features

We ascertained a maternally transmitted T2DM via the Department of Endocrinology, Ordos Center Hospital, China (Figure 1). Detailed information was obtained from each subject of this pedigree, as well as the family history of

diabetes, deafness, myopia or renal diseases. The proband (III-4) was a 42-year-old woman who came from Ordos City of Inner Mongolia Autonomous Region. The fasting glucose level, as well as the HbA1c and OGTT results, strongly indicated that she was a diabetic carrier. She also exhibited IR and obesity ($\text{HOMA-IR} = 7.019$, $\text{BMI} = 28 \text{ kg/m}^2$). After the genetic counseling, we noticed that she began to develop T2DM when she was 40, she was treated with metformin ($0.5 \mu\text{kg}$). In addition, family history suggested that the subjects (I-2 and III-8) died several years ago due to diabetes and its complications. Meanwhile, the matrilineal relatives (II-4 and II-6) were also diabetic carriers. Notably, both of them (II-4 and II-6) had hearing and vision loss, as well as declining in kidney functions. The detailed clinical features of these affected individuals were listed in Table 1.

MtDNA Mutations Analysis

Owing to the maternally transmission found in this four-generation Han Chinese pedigree, which suggested that mutations in mtDNA genes were the molecular basis for T2DM and its complications, a PCR-Sanger sequencing was performed in matrilineal relatives (II-4, II-6 and III-4), and as a result, a total of 32 variants were identified (Table 2), which belonged to East Asian mitochondrial haplogroup D4.³⁴ There were eight variants in D-loop, two variants in 12S rRNA and two variants in 16S rRNA, one mutation in tRNA^{Cys} and tRNA^{Tyr} (m.A5826G), and other variants occurred at OXPHOS-associated genes. Moreover, six missense variants were identified, including *A6* A8701G (Ala to Thr) and A8860G (Thr to Ala), *ND3* A10398G (Thr to Ala), *ND6* C14668T (Tyr to Asn), *CytB* C14766T (Thr to Ile) and A15326G (Thr to Ala). We further used phylogenetic conservation analysis including mouse,³⁵ bovine³⁶ and *Xenopus laevis*³⁷ to evaluate the potential pathogenicity. We found that, except for the m.A5826G and m.C14668T mutations (Figures 2 and 3), other mutations were not well conserved and may not be involved in the pathogenesis of T2DM. Furthermore, the m.A5826G and m.C14668T mutations were not detected in 200 healthy subjects, highlighting the contributions of these mutations to T2DM.

As shown in Figure 4, the novel m.A5826G mutation occurred at a spacer immediately to the 3' end of tRNA^{Cys} and 5' end of tRNA^{Tyr} in the light chain of mitochondrial genome.³⁸ A position was important for tRNA steady state level and function. Furthermore, the m.C14668T mutation led to an alternation of evolutionary conserved Tyr to Asn that may cause an aberrant in *ND6* metabolism. A recent case-control study revealed that m.C14668T mutation may be involved in schizophrenia, bipolar disorder, and major depressive disorder, highlighting the importance of this mutation to mitochondrial dysfunction.³⁹

Reduced in ATP Production

Mitochondrial ATP production is the main energy source for intracellular metabolic pathways.⁴⁰ To see whether m.A5826G and m.C14668T mutations affected mitochondrial function, we measured ATP levels in three subjects with these mutations and three controls, as shown in Figure 5A, the mutant cell lines (relative level: 63.5 ± 8.2) exhibited approximately 36.5% reductions in ATP production as compared with controls (relative level: 100 ± 11.3) ($p < 0.0001$).

Decreased in MMP

The MMP generated by proton pumps (Complexes I, III and IV) is an essential component in the process of energy storage during OXPHOS.⁴¹ As can be seen in Figure 5B, mutant cells with m.A5826G and m.C14668T (relative fluorescence level: 78.6 ± 6.2) showed a markedly decreased in MMP level as compared with controls (relative fluorescence level: 100 ± 9.3) ($p < 0.0001$).

ROS Increased

Mitochondria are the main source of ROS in the cell and, particularly, the enzymes in the electron transport chain are the major contributors to this phenomenon.⁴² As shown in Figure 5C, we noticed that mutant cells with m.A5826G and m.C14668T (relative level: 133.3 ± 10.7) exhibited much higher levels of ROS when comparing with the controls (relative level: 100 ± 4.3) ($p = 0.0018$).

Table 1 Clinical and Biochemical Characterizations of Matrilineal Relatives in This Pedigree with DM

Subject	Gender	BMI (kg/m ²)	Age at test (Year)	Age at onset (Year)	HbA1c (%)	Glucose (0h) (mmol/L)	Insulin (0h) (μU/mL)	HOMA- IR	Glucose (2h) (mmol/L)	PTA (dB) Right/Left ear	Level of hearing loss	Visual acuity Right/ Left eye	Level of vision loss	Ketoacidosis	Serum Cr (μmol/L)	eGFR (mL/min)	Functional mtDNA mutations
II-4	Female	21.2	80	63	7.9	7.23	6.6	2.12	14.2	105/110	Profound	0.05/0.05	Severe	No	122	51	m.A5826G and m.C14668T
II-6	Female	24.6	76	60	8.2	7.8	12.6	4.368	15.1	95/80	Profound	0.1/0.2	Moderate	No	155	60	m.A5826G and m.C14668T
III-4	Female	28.0	42	40	6.8	10.6	14.9	7.019	16.3	20/15	Normal	0.5/0.6	Normal	Yes	86	77	m.A5826G and m.C14668T
III-3	Male	22.8	44	/	5.5	5.2	5.1	1.178	7.1	23/18	Normal	0.8/0.6	Normal	No	79	90	/

Abbreviations: BMI, body mass index; HbA1c, Hemoglobin A1c; HOMA-IR, Homeostasis model assessment-insulin resistance; dB, decibels; Cr, creatinine; eGFR, estimated glomerular filtration rate; mtDNA, mitochondrial DNA.

Table 2 mtDNA Variants in This Pedigree with T2DM

Gene	Position	Replacement	Conservation (H/B/M/X) ^a	rCRS ^b	Previously Reported ^c
D-loop	73	A to G		A	Yes
	146	T to C		T	Yes
	204	T to C		T	Yes
	249	Del A		A	Yes
	310	T to CTC		T	Yes
	16,189	T to C		T	Yes
	16,223	C to T		C	Yes
	16,519	C to T		C	Yes
12S rRNA	750	A to G	A/A/A/G	A	Yes
	1438	A to G	A/A/A/G	A	Yes
16S rRNA	2706	A to G	A/G/A/A	A	Yes
	3107	Del N		N	Yes
ND1	3483	G to A		G	Yes
	3970	C to T		C	Yes
ND2	4769	A to G		A	Yes
	5201	T to C		T	Yes
tRNA ^{Cys} /tRNA ^{Tyr}	5826	A to G	A/A/A/A	A	No
CO1	5978	A to G		A	Yes
	7028	C to T		C	Yes
A6	8701	A to G (Ala to Thr)	A/V/V/T	A	Yes
	8860	A to G (Thr to Ala)	T/A/A/T	A	Yes
ND3	10,310	G to A		G	Yes
	10,398	A to G (Thr to Ala)	T/T/T/A	A	Yes
	10,400	C to T		C	Yes
ND4	10,873	T to C		T	Yes
	11,719	G to A		G	Yes
ND5	12,705	C to T		C	Yes
	13,811	T to C		T	Yes
ND6	14,668	C to T (Tyr to Asn)	Y/Y/Y/Y	C	Yes
CytB	14,766	C to T (Thr to Ile)	T/S/T/S	C	Yes
	15,301	G to A		G	Yes
	15,326	A to G (Thr to Ala)	T/M/I/I	A	Yes

Notes: ^aConservation assessment in human (H), bovine (B), mouse (M) and *Xenopus laevis* (X). ^brCRS: revised Cambridge Reference Sequences.

^cPlease see Mitomap (www.mitomap.org/MITOMAP) and mtDB (<http://www.mtDB.igp.uu.se/>) databases.

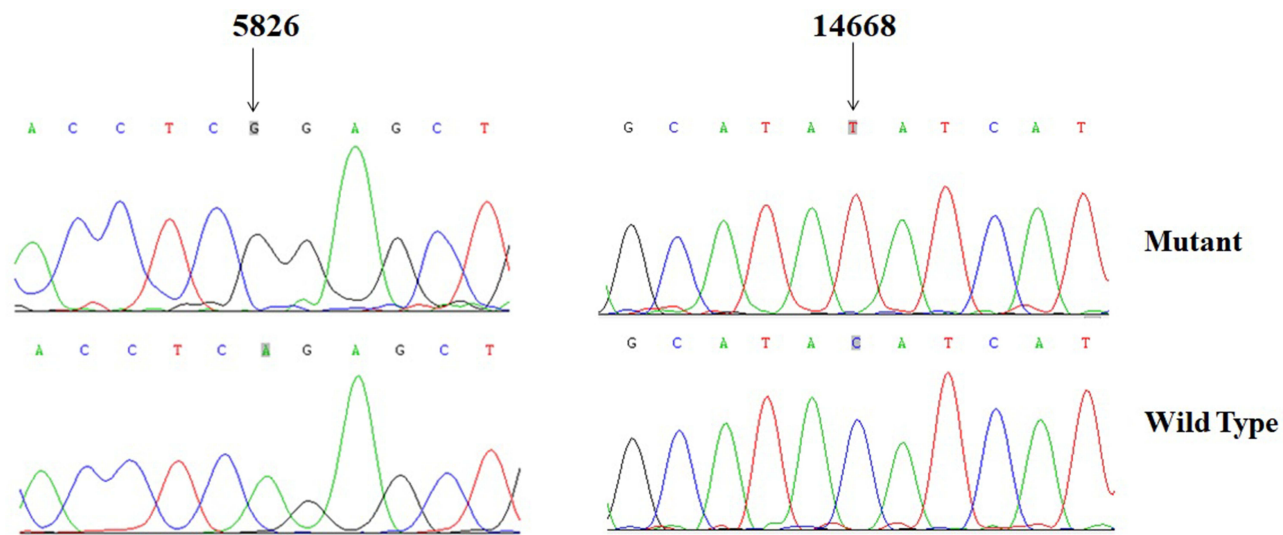


Figure 2 Sequence analysis of m.A5826G mutation in tRNA^{Cys}/tRNA^{Tyr} genes and m.C14668T mutation in ND6 gene.

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No	Organism	Acc-stem		D-stem	D-loop	D-stem		Ac-stem	Ac-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem	
		1	8	10	14	22	26	27	32	40	44	49	54	61	66	73
1	<i>Bos taurus</i>	AGCCCTG	TG	GTGA	ATTT	ACAC	G	TTGAA	TTGCAAA	TTCAG	AGAA	GCAGC	TTCAAT	TCTGC	CGGGGCT	T
2	<i>Chaunax abei</i>	AGCCCCG	AG	GTGT	CA	ACAT	A	TTAGA	TTGCAAA	TCTAA	AGAG	GTAGG	CTAACG	CCTGC	CGGGGCT	T
3	<i>Kogia breviceps</i>	AACATTA	TA	GCAT	AATTGGAA	TTGC	G	TTAGT	TTGCAAA	ATTAT	GAGT	ATAGG	TTCGACT	CCTGT	TAATGTT	T
4	<i>Gorilla gorilla</i>	AGTCCG	AG	GTGA	ATT	TCAT	A	TTGAA	TTGCAAA	TTCGA	AGAA	GCAGC	TTCAAA	CCTGC	CGGGGCT	T
5	<i>Homo sapiens</i>	AGTCCG	AG	GTGA	TTT	TCAT	A	TTGAA	TTGCAAA	TTCGA	AGAA	GCAGC	TTCAAA	CCTGC	CGGGGCT	T
6	<i>Hylobates lar</i>	AGTCCG	AA	GTGG	TTT	TCAC	G	TTGAA	TTGCAAA	TTCAA	AGGA	GCAGC	TTCAAT	CCTGC	CGGGGCT	T
7	<i>Lama glama</i>	AGTCCTG	TG	GTGA	TTA	ACAC	A	TTGAA	TTGCAAG	TTCAA	AGAA	GCAGC	TTCAAAC	TCTGC	CGGGGCT	T
8	<i>Macaca mulatta</i>	AGCCCCG	AG	GTGA	TTT	TCAT	G	TTGAA	TTGCAAG	TTCAA	AGGA	GCAGT	CTTAGAGTT	TCTGC	CGGGGCT	T
9	<i>Mugil cephalus</i>	AGCTTAG	GA	GTGT	TA	CCAT	A	TGAGA	TTGCAAA	TCTCG	AGAA	GCAGA	CTAAC	TCTGC	CTAGGCT	T
10	<i>Pan paniscus</i>	AGCTCTG	AG	GTGA	TTT	TCAT	A	TTGAA	TTGCAAA	TTCAA	AGAA	GCAGC	TTCAAA	CCTGC	CGGGGCT	T
11	<i>Pongo abelii</i>	AGCCCCG	AG	GTGA	TTG	TCAT	G	TTGAA	TTGCAAA	TTCGA	AGGA	GCAGC	TTTAAGG	CCTGC	CGGGGCT	T
12	<i>Rattus rattus</i>	AGTCTTA	AA	GTGT	TTA	TCAT	G	TCGAA	TTGCAAA	TTCGA	AGGT	GTAGA	GAACCCC	TCTAC	TAAGGCT	T
13	<i>Sus scrofa</i>	AGTTCG	TG	GTGA	ATA	TCAT	G	TTGAA	TTGCAAA	TTCAA	AGAA	GCAGC	TTCAAT	TCTGC	CGGGGCT	T
14	<i>Zeus faber</i>	AGTCCG	AG	GTGT	CA	TCAT	G	CTAGA	TTGCAAA	TCTAG	AGTA	GCAGG	CAAATCC	CCTGC	CGGATCT	T

Figure 3 Evolutionary conservation analysis: sequence alignment of tRNA^{Cys} gene from 14 vertebrates, arrow indicates the position 1, corresponding to the m.A5826G mutation, suggesting that the m.A5826G mutation is very conserved between different species.

Discussion

The followings were the major findings of the current study: (1) m.A5826G mutation occurred at the conjunction between tRNA^{Cys} and tRNA^{Tyr}, which was critical for tRNA steady-state level and function; (2) m.C14668T mutation was located at a well-conserved position that may affect mitochondrial ND6 metabolism; (3) Cell lines carrying m. A5826G and m.C14668T mutations exhibited much more severe of mitochondrial damage, ATP and MMP dropped significantly, whereas ROS increased; (4) The novel m.A5826G and m.C14468T mutations were involved in the pathogenesis of T2DM; (5) The incomplete penetrance of T2DM suggested that m.A5826G and m.C14468T mutations were not sufficient to produce clinical phenotypes; hence, other risk factors such as environmental factors, epigenetic modification may contribute to T2DM expression.

In recent years, mitochondrial genome mutations had been the target of study in the pathogenesis and progression of different chronic metabolic diseases.^{43,44} Mitochondria can store between two and ten copies of mtDNA. The lack of histones in mtDNA led to a mutation rate that was six to 17 times higher than that of nDNA. Furthermore, it had been reported that mitochondrial metabolism was involved in the processes that controlled insulin release from pancreatic β-cells,⁴⁵ in addition, mitochondrial dysfunction was intricately linked to T2DM through several pathways, which included

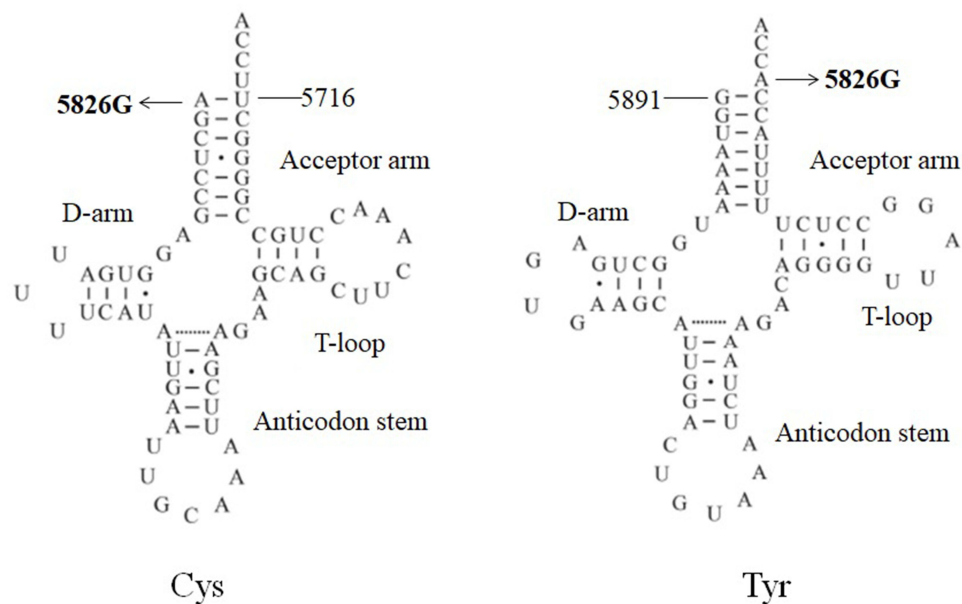


Figure 4 Secondary structure of tRNA^{Cys} and tRNA^{Tyr}, arrows indicate the location of m.A5826G mutation, the secondary structure of mt-tRNA genes are derived from tRNA dB database (<http://mtrna.bioinf.uni-leipzig.de/mtDataOutput/>).

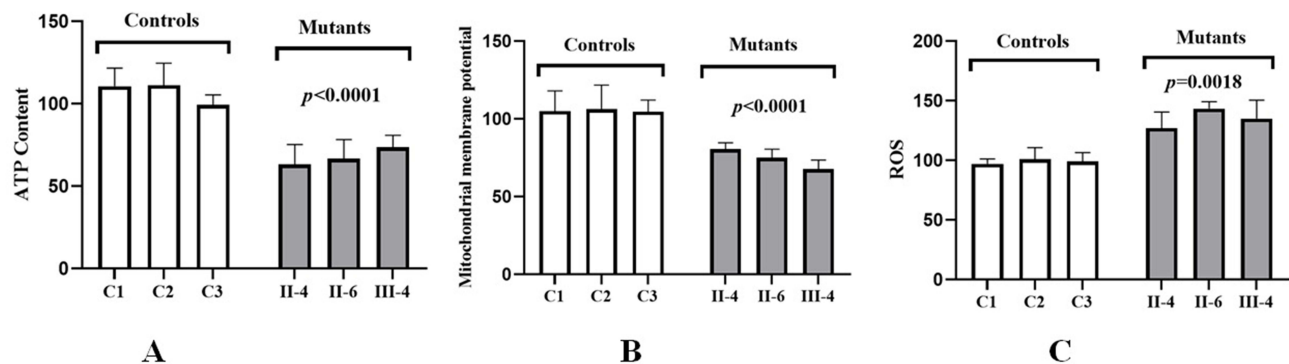


Figure 5 Analyses of mitochondrial functions in cybrids. (A) ATP analysis; (B) Analysis of MMP; (C) ROS measurement.

diminished energy production, oxidative stress, the altered metabolism of fatty acids, inflammation, and aberrations in cell signaling.⁴⁶

To see the contributions of mtDNA mutations to T2DM and its complications, a four-generation Han Chinese family with maternally transmitted T2DM was assessed by clinical and biochemical analyses. Clinically, the age at onset of T2DM varied from 40 to 63 years, with an average of 54 years. Despite the fact that several affected individuals developed a wide range of clinical phenotypes including hearing and vision impairment, as well as declined renal functions, the pattern of T2DM transmission was consistent with maternally inheritance, suggesting that mtDNA mutations were the molecular basis for T2DM in this pedigree.

Sequence analysis of the entire mitochondrial genome from matrilineal relatives revealed a set of polymorphisms, in particular, the m.A5826G and m.C14668T were the two identified as likely involved in the pathogenesis. In fact, the novel homoplasmic m.A5826G mutation was localized at the junction between the tRNA^{Cys} and tRNA^{Tyr} at the light strand.³⁸ Moreover, the m.A5826G mutation disrupted the evolutionary conserved A1-U73 Watson–Crick base-pairing. Interestingly, the homoplasmic m.A4401G mutation also occurred at the junction between tRNA^{Met} and tRNA^{Gln} decreased the 5' end processing efficiency of tRNA^{Gln} and tRNA^{Met} precursors; furthermore, the m.A4401G reduced the steady-state level of tRNA^{Gln} and tRNA^{Met} and was regarded to be a pathogenic mutation for essential

hypertension.⁴⁷ Thus, we proposed that the m.A5826G mutation, which was similar to the m.A4401G mutation, may also affect the tRNA^{Cys} and tRNA^{Tyr} 3' end precursors and lead to the failure in tRNAs metabolism.

Human mtDNA encoded 13 proteins that were essential for OXPHOS. The only protein encoded in the mtDNA light-strand, *ND6* played a critical role in the proper assembly Complex I.⁴⁸ Mutation in the *ND6* gene caused severe mitochondrial respiratory dysfunction and several mitochondrial genetic diseases.⁴⁹ Due to the central role of Complex I in the pathological ROS production, the m.C14668T mutation was believed to be involved in the pathogenesis of T2DM in this pedigree. In fact, the homoplasmic m.C14668T mutation caused the well-conserved Tyr to Asn, was first described to be associated with schizophrenia, bipolar disorder and major depressive disorder,³⁹ and the conserved Tyr to Asn transition perturbed the structural and function of *ND6*, thereby affecting Complex I deficiency, as in the case of T2DM associated *ND6* T14502C mutation.¹¹

Trans-mitochondrial cybrids were created by fusing cells devoid of mtDNA (ρ^0 cells) with cytoplasts from different individuals, so the resultant cybrids had uniform nuclear background but different mtDNA.⁵⁰ With the development and use of the cybrid model, questions related to the importance of the mtDNA variants and mitochondrial–nuclear interactions can be addressed.⁵¹ In fact, cybrid cells were already used to model human mitochondrial defects in the pathogenesis of T2DM. T2DM cybrid cells recapitulated diabetes' pathological features, including excessive oxidative stress, cytochrome oxidase defects, decreased MMP, impaired energy metabolism, and alterations in mitochondrial dynamics.⁵² However, the greatest limiting factor to generation cybrid cell lines may turn out to be mtDNA heteroplasmy. The traditional Sanger-based sequencing approach was not designed to detect low abundance heteroplasmic mutations; if the mutational load was less than 20–30%, the ability to detect the mtDNA change was unreliable.⁵³

In this study, we created cybrid cells containing homoplasmic m.A5826G and m.C14668T mutations, as well as control subjects without these mtDNA mutations. Approximately 36.5% and 31.8% in ATP and MMP reductions were observed in mutant cells with these mutations, which were below a proposed threshold level to develop a clinical phenotype.⁵⁴ Because a certain mt-tRNA mutation can be complemented at the translational level by wild-type tRNA genes to ensure a normal rate of mitochondrial protein synthesis. Thus, genetic and functional complementation of mitochondrial defects can occur at the following levels: transcription, translation, enzyme assembly and activity, mitochondrial respiration and ATP synthesis, cell activity and organ function, and all of these different compensatory mechanisms can combine to result in the observed phenotypic threshold effect.⁵⁵

Indeed, ATP was required for DNA replication, biosynthesis, protein assembly, and biochemical transport. Defects in ATP synthesis revealed an impairment of OXPHOS system.^{56,57} Furthermore, MMP was critical for maintaining the physiological function of the respiratory chain to generate ATP.⁵⁸ Thus, loss of MMP would lead to mitochondria-mediated apoptosis.⁵⁹ As a result, a decrease in ATP and MMP enhanced ROS production that was responsible for the accumulation of ROS-associated damages in DNA, proteins and lipids. Furthermore, mitochondria ROS were closely involved in cellular activity, such as cell differentiation, multiplication and death. This might affect the life and function of pancreatic β -cells.⁶⁰ When cells misplaced, a large amount of ROS contributed to apoptosis condition of pancreatic β -cells, resulting in limited regeneration ability. Thus, in the presence of increased β -cell loss, either by apoptosis or dedifferentiation, a scenario would arise in which β -cells were lost with insufficient replacement, resulting in inadequate insulin secretion to maintain proper glucose homeostasis,^{61,62} and contributing to T2DM progression.⁶³

Conclusion

In conclusion, our study suggests that there is a type of mitochondrial T2DM disease related to tRNA^{Cys}/tRNA^{Tyr} A5826G and *ND6* C14668T mutations. The m.A5826G and m.C14668T should be considered risk polymorphisms for T2DM mitochondrial disease. Furthermore, the incomplete penetrance of T2DM suggested that m.A5826G and m.C14668T mutations were not sufficient to produce clinical phenotypes; hence, other risk factors such as environmental factors or epigenetic modification may contribute to T2DM expression. However, our study only enrolled one pedigree, and further investigation including more patients was needed to verify the conclusions.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are not publicly available due to the original design and validation of the project but are available from the corresponding author on reasonable request.

Ethics Statement

This study was approved by the Ethics Committee of Ordos Center Hospital, and all participants provided their informed consent to participate in this study.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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

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