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ORIGINAL RESEARCH

Mitochondrial Diabetes is Associated with tRNA$^{Leu(UUR)}$ A3243G and *ND6* T14502C Mutations

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Background: Mutations in mitochondrial DNA (mtDNA) are associated with type 2 diabetes mellitus (T2DM). In particular, m.A3243G is the most common T2DM-related mtDNA mutation in many families worldwide. However, the clinical features and pathophysiology of m.A3243G-induced T2DM are largely undefined.

Methods: Two pedigrees with maternally inherited T2DM were underwent clinical, molecular and biochemical assessments. The mtDNA genes were PCR amplified and sequenced. Mitochondrial adenosine triphosphate (ATP) and reactive oxygen species (ROS) were measured in polymononuclear leukocytes derived from three patients with both the m.A3243G and m.T14502C mutations, three patients with only the m.A3243G mutation and three controls without these mutations. Moreover, *GJB2, GJB3* and *GJB6* mutations were screened by PCR-Sanger sequencing.

Results: Members of the two pedigrees manifestated variable clinical phenotypes including diabetes and hearing and vision impairments. The age at onset of T2DM varied from 31 to 66 years, with an average of 41 years. Mutational analysis of mitochondrial genomes indicated the presence of the m.A3243G mutation in both pedigrees. Matrilineal relatives in one of the pedigrees harbored the coexisting of m.A3243G and m.T14502C mutations. Remarkably, the m.T14502C mutation, which causes the substitution of a conserved isoleucine for valine at position 58 in *ND6* mRNA, may affect the mitochondrial respiratory chain functions. Biochemical analysis revealed that cell lines bearing both the m.A3243G and m.T14502C mutations exhibited greater reductions in ATP levels and increased ROS production compared with those carrying only the m.A3243G mutation. However, we did not find any mutations in the *GJB2, GJB3* and *GJB6* genes.

Conclusion: Our study indicated that mitochondrial diabetes is associated with the tRNA$^{Leu(UUR)}$ A3243G and *ND6* T14502C mutations.

Keywords: T2DM, mt-tRNA, *ND6*, m.A3243G, m.T14502C, mutations

Introduction

Mitochondria are present in most eukaryotic cells and are required for a variety of key deeply involved in various important cell functions, especially energy production in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS).[1] Mitochondria carry their own circular DNA, which exists in multiple copies per cell. The inheritance of this DNA is only maternal.[2] Moreover, because of the lack of protection of histones and a poor DNA repair system, mtDNA has a higher mutation rate than nuclear DNA (nDNA).[3] Mitochondrial diseases are a group of disorders characterized by genetic or biochemical abnormalities of the OXPHOS.

Mitochondrial diabetes, defined as a mitochondrial disease with chronic hyperglycemia due to inappropriate secretion of insulin, insulin resistance (IR) or combined defects, remains a big challenge for clinicians.[4] It is a rare monogenic form of diabetes with a frequency of 1% and classified into type l and type 2.[5–7] Accumulating evidences shows that deletions,[8] insertions or point mutations[9] of mtDNA are associated with diabetes mellitus (DM). Human biopsies have shown that mitochondrial dysfunction caused by mtDNA mutations affects insulin-resistant metabolic tissues, including muscle, liver, and fat.[10] It has been proposed that an inefficient metabolism caused by mitochondrial dysfunction may inhibit insulin-stimulated glucose uptake or reduce glucose-stimulated insulin secretion from pancreatic-β cells, thereby leading to the development of T2DM.[11,12]

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In most cases, mitochondrial diabetes is associated with m.A3243G mutation, which was located in mt-tRNA$^{Leu(UUR)}$.[13] The phenotypic expression of this mutation is quite variable, ranging from mild to severe clinical phenotypes. Notably, the m.A3243G mutation is an important cause of mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS),[14] as well as myoclonic epilepsy and ragged-red fiber disease (MERRF).[15] Furthermore, the mtDNA genetic background may affect the phenotypic expression of the m.A3243G mutation.[16,17] However, the molecular pathogenesis is still unclear.

Human mtDNA encodes 13 proteins that are essential for OXPHOS. The only protein encoded on the mtDNA light-strand, mitochondrial NADH-dehydrogenase 6 (*ND6*), plays a critical role in the proper assembly of complex I.[18] Mutation of the *ND6* gene causes severe mitochondrial respiratory dysfunction and several mitochondrial genetic diseases.[19] A recent study by Cao et al[20] demonstrated that hypermethylation of hepatic mitochondrial *ND6* provokes systemic IR that is linked to T2DM, emphasizing the importance of the *ND6* gene in diabetes.

In this study, we reported here the molecular features of two Han Chinese pedigrees, DM1 and DM2 with maternally-transmitted T2DM, which exhibit variable phenotypes including IR and hearing and vision impairments. Sequence analysis of the whole mitochondrial genomes indicated the presence of the m.A3243G mutation in both families, as well as 56 mtDNA variants. The m.T14502C mutation alters isoleucine to valine at amino acid position 58 in *ND6*, and presented in matrilineal relatives of the DM2 pedigree but not in unaffected family members and 270 controls suggesting that this mutation may be a potential modifying factor for T2DM predisposition. To further explore the synergistic role of the m.T14502C mutation in m.A3243G-induced diabetes, we analyzed the mitochondrial functions in polymononuclear leukocytes (PMNs) derived from three patients with both the m.A3243G and m.T14502C mutations, three patients carrying only the m.A3243G mutation, and three controls without these mtDNA mutations. Additionally, mutations in connexins genes have been suggested to be common etiologic factors of hereditary deafness.[21] In particular, the majority of more than 300 reported *GJB2* gene mutations account for about 50% of all cases of autosomal recessive non-syndromic hereditary deafness.[22] While *GJB3* has two exons and 810 nucleotides, encoding a protein of 270 amino acids with a molecular mass of 30.8 kDa.[23] Furthermore, *GJB6* gene mutations also cause hereditary deafness, ranging from moderate to profound.[24] To examine the roles of these genes in deafness expression, we performed PCR-Sanger sequencing to detect mutations in *GJB2, GJB3* and *GJB6*.

Materials and Methods

Families and Subjects

We recruited two Han Chinese families with T2DM through Hangzhou First People's Hospital (Figure 1). Additionally, 270 genetically unrelated healthy subjects including 120 males and 150 females, aged from 30 to 51 years, were recruited as controls. The protocol used in this investigation was in accordance with the principles expressed in the 1975 Declaration of Helsinki, which was revised in 2008. The Ethics Committee of Hangzhou First People's Hospital

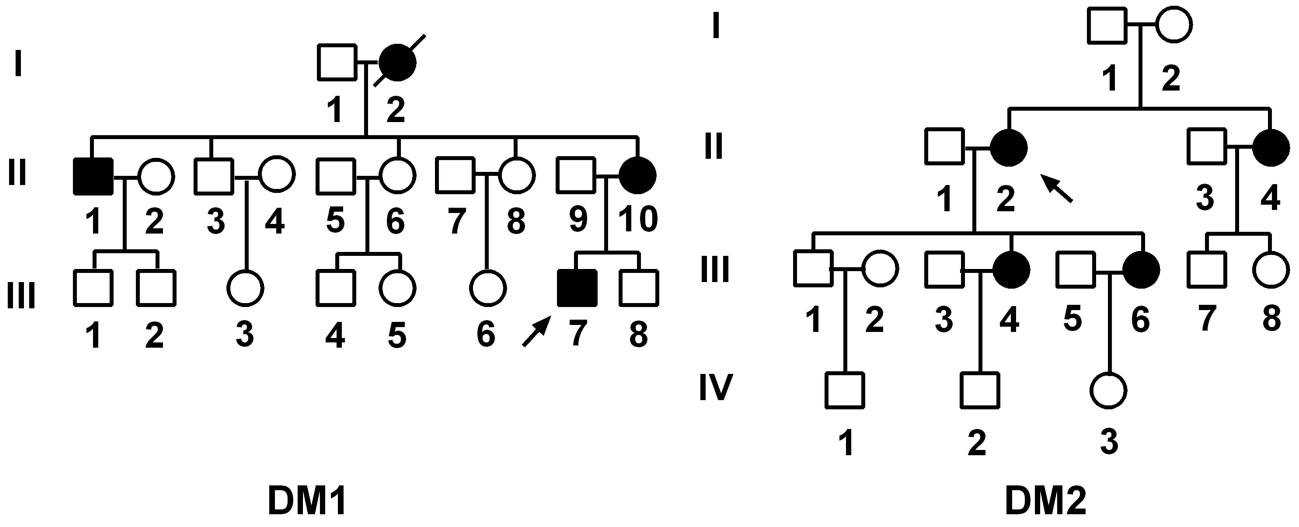

Figure 1 Two Han Chinese pedigrees with maternally transmitted T2DM, arrows indicate the probands.

approved this study (No. 2021-171-01). All participants, including seven matrilineal relatives in these pedigrees (DM1: II-1, II-10 and III-7; DM2: II-2, II-4, III-4 and III-6) and controls, provided informed consent to participate in this study. The written informed consent for participating in this study, as well as informed consent to have their case details published were obtained from all subjects enrolled in this study.

The diagnosis of DM was based on the guidelines of the American Diabetes Association,[25] which was as follows: (1) fasting plasma glucose ≥ 7.0mmol/dL; (2) A 2-h plasma glucose level of 200mg/dL (11.1mmol/L) or higher during a 75-g oral glucose tolerance test (OGTT); and (3) hemoglobin A1c (HbA1c) ≥ 6.5%.

Patients Assessment

Detailed demographics, vital parameters, medical and family histories were recorded for each participant through personal interviews. Subjects of these pedigrees underwent a physical examination and laboratory assessment: body mass index (BMI) was calculated as the body weight (kg) divided by the square of the height (m^2).[26] Obesity was defined using the BMI for Chinese adults: normal = 18.5–24 kg/m^2, overweight = 24–28 kg/m^2 and obese ≥ 28 kg/m^2.

Blood samples were collected in the morning between 07:00 and 10:00 after an overnight fast. The level of HbA1c was detected by high-pressure liquid chromatography (Variant II; Bio-Rad, CA, USA). Moreover, serum glucose levels were measured using enzymatic techniques and a Dax72 auto-analyzer (Bayer Diagnostic, New York, USA), while plasma insulin (0h) was analyzed by chemiluminescent immunometric assay (Immulite 2000 System; Siemens Health Diagnostics, USA). The IR was estimated using the homeostasis model assessment of IR (HOMA-IR) index, the HOMA-IR = (fasting insulin [μU/mL] × fasting glucose [mmol/L])/22.5. Notably, a HOMA-IR ≥ 2.69 was regarded as IR.[27]

Audiometric evaluations and otological examinations included otoscopy, pure tone audiometry (PTA), acoustic immittance measurement, auditory brainstem responses, and distortion product otoacoustic emissions (DPOAE) were evaluated. 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 (>90 dB).[28]

Visual acuity was tested separately for each eye without refraction at 4m using Early Treatment Diabetic Retinopathy Tumbling-E study charts (Precision Vision, La Salle, IL, USA) in a well-lit, indoor area.[29] The degrees of visual impairment were 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).[30]

mtDNA Analysis

Genomic DNA was isolated from the peripheral blood using a DNA Isolation Kit (QIAGEN, Germany). The entire mitochondrial genomes of matrilineal relatives from the two pedigrees (DM1: II-1, II-10 and III-7; DM2: II-2, II-4, III-4 and III-6) were PCR amplified using 24 primers as described previously.[31] The PCR products were purified and subsequently sequenced by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequence data were then compared with the revised Cambridge sequence (rCRS, GenBank accession number: NC_012920.1) to detect the mutations or variants.[32] Furthermore, the allele frequencies of the m.A3243G and m.T14502C mutations in 270 controls were examined by PCR-Sanger sequencing, using the protocol as described previously.[31]

Classification of mtDNA Haplogroups

Using the nomenclature of mitochondrial haplogroups,[33] we assigned the entire mtDNA sequences of the probands (DM1: III-7; DM2: II-2) to a certain mitochondrial haplogroup.

Phylogenetic Conservation Analysis

We compared human mtDNA nucleotide variation with 16 species to determine the conservation index (CI).[34] A CI ≥ 75% was regarded as having functional potential.[35]

Cell Lines and Culture Conditions

The PMNs from three patients (DM2: II-2, II-4 and III-4) with both the m.A3243G and m.T14502C mutations, three subjects (DM1: II-1, II-10 and III-7) with only the m.A3243G mutation, and three controls (DM1: III-1, III-2 and III-3) without these mutations were first isolated from blood samples and then incubated for 45 min with dextran (3%). The supernatant was centrifuged at 250×g for 25 min over Ficoll-Hypaque. Lysis buffer was added to the pellet and centrifuged at room temperature (100g, 5 min), following the protocol as described previously.[36] All cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), and incubated at 37°C in an atmosphere of 5% CO_2.

Analysis of ATP Production

The levels of ATP in the three subjects with both the m.A3243G and m.T14502C mutations (DM2: II-2, II-4 and III-4), three patients with only the m.A3243G mutation (DM1: II-1, II-10 and III-7) and three healthy individuals (DM1: III-1, III-2 and III-3) without these mutations were determined by CellTiter-Glo® Luminescent cell viability assay (Promega, G7572) in accordance with the protocol provided by the manufacturer's protocol.[37] Briefly, the assay buffer and substrate were equilibrated to room temperature, and the buffer was then transferred and gently mixed with the substrate to obtain a homogeneous solution. After a 30 min equilibration of the cell plate to room temperature, 100 μL of the assay reagent was added into each well with 2×10^5 cells and the contents were mixed for 2 min on an orbital shaker to induce cell lysis. After 10 min incubation at room temperature, the luminescence was read on a microplate reader.

ROS Analysis

To analyze the ROS level, a total of 2×10^6 cells were first incubated with the fluorescent probe 2,7-dichlorodihydro-fluorescein for 30 min, after which the cells were analyzed using a fluorescence plate reader, as described previously.[38]

Mutational Analysis of the *GJB2, GJB3*, and *GJB6* Genes

Mutations in *GJB2, GJB3*, and *GJB6* are the important causes of hearing loss.[39] To examine the contributions of these genes to deafness expression, we conducted a mutational screening of *GJB2, GJB3*, and *GJB6* in matrilineal relatives of these pedigrees. The primer sequences for the *GJB2* gene were: forward-5'-TATGACACTCCCCAGCACAG-3' and reverse-5'-GGGCAATGCTTAAACTGGC-3'. While the primers for genetic amplification of the *GJB3* were: forward-5'-GTCACCTATTCATTCATACGATGG-3' and reverse-5'-TCACTCAGCCCCTGTAGGAC-3'. The primer sequences for amplification of the *GJB6* were: forward-5'-CCTTAAAATAAAGTTGGCTTCAG-3', reverse-5'-GGAACTTTCAGGT TGGTATTG-3'. After PCR amplification and direct Sanger sequencing, the data were compared with the wild-type sequences of *GJB2, GJB3*, and *GJB6* (GenBank accessible numbers: M86849, AF052692 and NG_008323, respectively) to detect variants.

Statistical Analysis

Student's *t*-test was used to assess the statistical significance between unpaired samples. All analyses were performed using SPSS software version 20.0. We regarded the $p < 0.05$ as statistically significant.

Results

Clinical Presentations

We recruited two Chinese families (DM1 and DM2) with maternally-transmitted T2DM from Hangzhou First People's Hospital (Figure 1). A comprehensive history was obtained and physical examinations were performed to identify any clinical abnormalities, and genetic factors related to diabetes in the members of these two families. In the DM1 family, the proband (III-7) was a 38-year-old woman who came from Hangzhou city of Zhejiang Province; she suffered from T2DM when she was 36. She also exhibited IR and obesity (HOMA-IR=6.57, BMI=27.0 kg/m^2). Moreover, the family history suggested that other matrilineal relatives (II-1 and II-10) were T2DM carriers. In particular, the subject II-10 had moderate hearing loss (55 dB at right ear and 30 dB at left ear), mild vision loss (0.2 in the right eye and 0.1 in the left

eye), and IR. These matrilineal relatives showed no other clinical abnormalities, such as coronary heart disease or cancer (Table 1).

In the DM2 family, the proband (II-2) was a 68-year-old woman who went to Hangzhou First People's Hospital for regular treatment of DM. As shown in Table 1, laboratory analysis suggested that she suffered from T2DM, profound hearing loss (90 dB at right ear and 100 dB at left ear), and vision impairment (0.1 for both eyes). Genetic counseling revealed that the matrilineal relatives (II-4, III-4 and III-6) were also diabetes carriers. The clinical and biochemical features of the two families are listed in Table 1.

Analysis of mtDNA Mutations

The maternal transmission observed in these families suggested that mitochondrial dysfunction may be the molecular basis of their T2DM. Compared with the rCRS,[32] PCR-Sanger sequencing identified 57 mutations in mitochondrial genomes from matrilineal relatives (Table 2), which belonged to the mitochondrial haplogroups M7c and M10a, respectively.[33] Among these, there were 13 variants in the D-loop, four variants in 12S rRNA, two variants in 16S rRNA, and one mutation in $tRNA^{Leu(UUR)}$, together with a 9-bp common deletion (CCCCCTCTA). The other mutations were mainly localized in OXPHOS-related genes. In particular, nine missense mutations were identified, namely *ND2* G4491A (Val to Ile), *A8* C8414T (Leu to Phe), *A6* G8584A (Ala to Thr), A8701G (Thr to Ala) and A8860G (Thr to Ala), *ND6* T14256C (Ile to Val) and T14502C (Ile to Val), and *Cytb* C14766T (Thr to Ile) and A15326G (Thr to Ala).

To identify putative deleterious mtDNA mutations, these variants were further evaluated using the following criteria: (1) missense mutations; (2) CI $\geq$ 75%, proposed by Ruiz-Pesini and Wallace,[40] especially the nucleotide sequences compared with mouse,[41] bovine[42] and *Xenopus laevis* sequences.[43] (3) absent in 270 control subjects and (4) potential structural and functional alterations. We found that, except for the m.A3243G and m.T14502C mutations (Figure 2), the others were not well conserved and may not play active roles in T2DM progression. Furthermore, the m.A3243G and m.T14502C mutations were not detected in 270 control subjects, emphasizing their pathogenic roles in T2DM.

Intriguingly, as shown in Table 1, patients carrying both the m.A3243G and m.T14502C mutations exhibited more severe clinical phenotypes (IR, high myopia and profound hearing loss) than the patients with only the m.A3243G mutation, suggesting that the m.T14502C mutation may increase the clinical expression of m.A3243G-induced diabetes.

Reductions in Mitochondrial ATP Production

As shown in Figure 3, the levels of ATP production in the mutant cells bearing only m.A3243G, and both the m.A3243G and m.T14502C mutations were 78.2% and 60.6% of the average values of control cells, respectively ($p = 0.0037$ and $p = 0.0002$, respectively). Thus, it seemed that patients with both mtDNA mutations exhibited much lower levels of ATP production than patients with only one mtDNA mutation.

Increased of ROS Production

The levels of ROS generation in the mutant cells carrying only the m.A3243G mutation and both the m.A3243G and m.T14502C mutations were 129.6% and 156.2% of the mean values measured in the control cell lines, respectively ($p = 0.0011$ and $p < 0.0001$, respectively). Thus, patients with both mtDNA mutations had much higher ROS levels than patients with only m.A3243G mutation (Figure 4).

Analysis of Nuclear Gene Mutations

To examine the contributions of nuclear genes to the expression of deafness, we performed mutational screening of the exons of the *GJB2, GJB3*, and *GJB6* genes in the matrilineal relatives of the two families. However, no mutations were found.

Discussion

Herein, we reported the clinical, genetic, molecular, and biochemical characterizations of two families with maternally-transmitted T2DM. The age-at-onset of DM in the affected matrilineal relatives of the two families varied from 36 to 62

Table 1 Clinical and Molecular Characterizations of Some Members in Two Chinese Pedigrees with T2DM

Subjects	Gender	BMI (kg/m^2)	Age at Onset (Year)	Age at Test (Year)	Fasting Glucose (mmol/L)	Fasting Insulin (μU/mL)	HOMA-IR	HbA1c (%)	Ketoacidosis	Visual Acuity Right/Left Eye	Level of Vision Loss	PTA (dB) Right/Left Ear	Level of Hearing Loss	Clinical Presentations	Functional mtDNA Mutations
DM1: II-1	Female	25.5	51	55	16.3	13.2	9.56	7.6	No	0.5/0.4	Normal	24/24	Normal	T2DM; IR	m.A3243G
DM1: II-10	Male	26.4	58	61	15.0	11.1	7.4	6.9	No	0.2/0.1	Mild	55/30	Moderate	T2DM; IR; myopia; hearing loss	m.A3243G
DM1: III-7	Female	27.0	36	38	8.80	16.8	6.57	7.4	Yes	0.4/0.3	Normal	20/25	Normal	T2DM; IR	m.A3243G
DM2: II-2	Male	23.5	60	68	8.14	6.9	2.49	7.2	No	0.1/0.1	Moderate	90/100	Profound	T2DM; myopia; hearing loss	m.A3243G and m.T14502C
DM2: II-4	Male	27.8	62	70	7.95	17.9	6.32	7.0	No	0.05/0.05	Severe	90/90	Profound	T2DM; IR; myopia; hearing loss	m.A3243G and m.T14502C
DM2: III-4	Male	28.5	39	39	7.21	11.0	3.52	6.8	Yes	0.2/0.2	Mild	60/70	Severe	T2DM; IR; myopia; hearing loss	m.A3243G and m.T14502C
DM2: III-6	Male	26.0	40	41	7.33	10.5	3.42	6.6	Yes	0.1/0.2	Moderate	85/80	Severe	T2DM; IR; myopia; hearing loss	m.A3243G and m.T14502C
DM1: III-1	Female	21.3	/	30	5.5	6.6	1.61	5.0	No	0.5/0.5	Normal	20/16	Normal	Normal	None
DM1: III-2	Female	22.8	/	28	5.2	5.4	1.25	5.3	No	0.6/0.8	Normal	17/21	Normal	Normal	None
DM1: III-3	Male	23.5	/	29	5.0	7.0	1.55	5.7	No	0.6/0.6	Normal	15/15	Normal	Normal	None

Abbreviations: BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; PTA, pure-tone audiometry; dB, decibel; T2DM, type 2 diabetes mellitus; IR, insulin resistance.

Table 2 mtDNA Variants in Two Pedigrees with T2DM

Gene	Position	Sequence Variant	Amino Acid Change	Conservation (H/B/M/X)[a]	DM1	DM2	rCRS[b]	Previously Reported[c]
D-loop	73	A to G			G	G	A	Yes
	146	T to C			C		T	Yes
	195	T to C			C	C	T	Yes
	310	T to CTC			CTC		T	Yes
	524	delC				delC	C	Yes
	16,051	A to G			G	G	A	Yes
	16,111	C to T				T	C	Yes
	16,182	A to C			C		A	Yes
	16,189	T to C			C	C	T	Yes
	16,274	G to A			A	A	G	Yes
	16,311	T to C			C		T	Yes
	16,519	T to C			C	C	T	Yes
	16,569	T to C				C	T	Yes
12S rRNA	709	G to A		G/G/A/-	A		G	Yes
	750	A to G		A/A/A/-	G	G	A	Yes
	1041	T to C		A/T/T/T		C	T	Yes
	1438	A to G		A/A/A/G	G	G	A	Yes
16S rRNA	2706	A to G		A/G/A/A	G	G	A	Yes
	3107	delN			delN	delN	N	Yes
$tRNA^{Leu(UUR)}$	3243	A to G		A/A/A/A	G	G	A	Yes
ND1	3483	G to A				A	G	Yes
	3970	C to T				T	C	Yes
	4071	C to T			T		C	Yes
	4161	C to T				T	C	Yes
ND2	4491	G to A	Val to Ile	V/I/I/V	A	A	G	Yes
	4769	A to G			G	G	A	Yes
	4883	C to T				T	C	Yes
	4895	A to G			G		A	Yes
CO1	6392	T to C				C	T	Yes
	7028	C to T			T	T	C	Yes
CO2	7785	T to C			C		T	Yes
	8020	G to A			A		G	Yes

(*Continued*)

Table 2 (Continued).

Gene	Position	Sequence Variant	Amino Acid Change	Conservation (H/B/M/X)[a]	DM1	DM2	rCRS[b]	Previously Reported[c]
NC7	8271–79	del 9-bp				9-bp del	9-bp	Yes
A8	8414	C to T	Leu to Phe	L/F/M/W	T		C	Yes
A6	8584	G to A	Ala to Thr	A/V/V/I	A	A	G	Yes
	8701	A to G	Thr to Ala	T/S/L/Q	G	G	A	Yes
	8856	G to A				A	G	Yes
	8860	A to G	Thr to Ala	T/A/A/T	G		A	Yes
CO3	9540	T to C			C		T	Yes
	10,136	A to G				G	A	Yes
ND4	10,873	T to C				C	T	Yes
	11,719	G to A			A	A	G	Yes
	12,091	T to C				C	T	Yes
ND5	12,361	A to G				G	A	Yes
	12,705	C to T			T	T	C	Yes
	13,401	T to C			C		T	Yes
	13,563	A to G				G	A	Yes
ND6	14,256	T to C	Ile to Val	I/M/I/V	C		T	Yes
	14,502	T to C	Ile to Val	I/I/I/I		C	T	Yes
Cytb	14,766	C to T	Thr to Ile	T/S/T/S	T	T	C	Yes
	14,783	T to C			C	C	T	Yes
	15,043	G to A			A		G	Yes
	15,301	G to A			A	A	G	Yes
	15,326	A to G	Thr to Ala	T/M/I/I	G	G	A	Yes
	15,346	G to A			A		G	Yes
	15,784	T to C			C		T	Yes
	15,850	T to C				C	T	Yes

Notes: [a]Conservation of amino acid for polypeptides or nucleotide for RNAs in human (H), bovine (B), mouse (M), and *Xenopus laevis* (X). [b]rCRS: reversed Cambridge Reference Sequence. [c]See online mitochondrial genome databases http://www.mitomap.org.

years, with an average of 49 years. Using genetic and molecular approaches, we identified two mtDNA mutations: $tRNA^{Leu(UUR)}$ A3243G and *ND6* T14502C, as potential risk factors for T2DM. Notably, the DM2 pedigree, which harbored both the m.A3243G and m.T14502C mutations, had a higher penetrance of T2DM (40%) than the DM1 pedigree that only carried the m.A3243G mutation (27.2%). Interestingly, patients in the DM2 pedigree exhibited more complex clinical phenotypes: four individuals developed both hearing and vision loss as compared with the DM1 pedigree in which only one individual had hearing and vision impairments; and three patients exhibited IR as compared

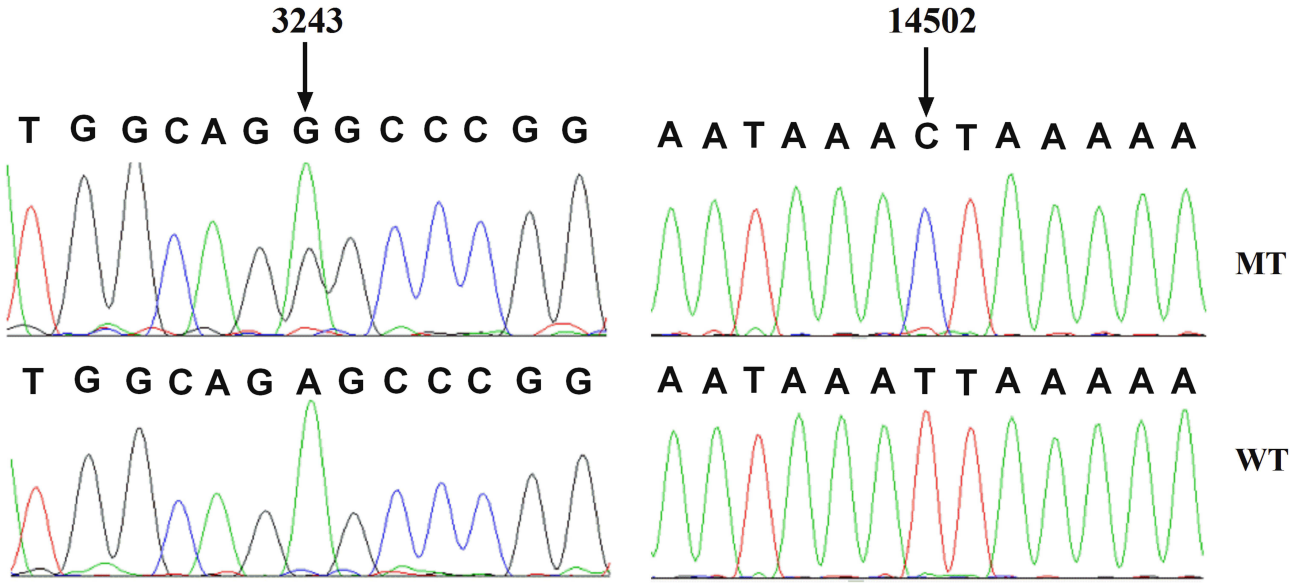

Figure 2 Identification of $tRNA^{Leu(UUR)}$ A3243G and *ND6* T14502C mutations by direct sequencing.
Abbreviations: WT, wild type; MT, mutant.

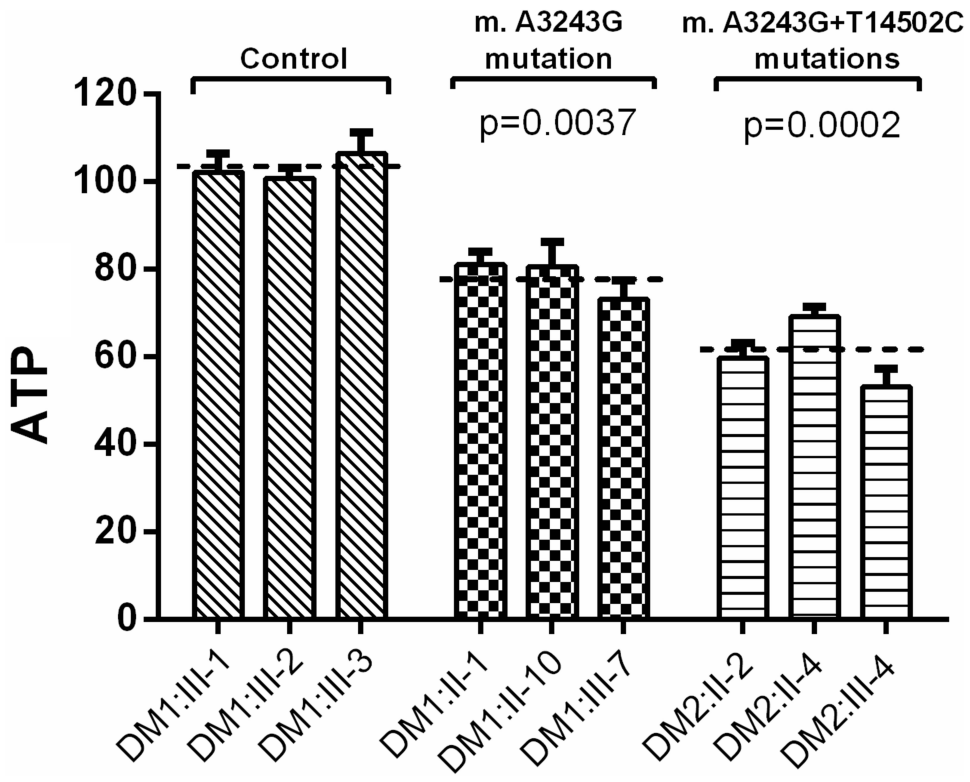

Figure 3 Analysis of ATP levels in three patients with the m.A3243G and m.T14502C mutations, three patients with the only m.A3243G mutation and three controls without these mtDNA mutations.

with the DM1 pedigree, in which two patients showed IR. These observations strongly suggest that the m.T14502C mutation may increase the expression of diabetes in families carrying the m.A3243G mutation, as in the case of m. A4435G mutation in m.G11778A-induced Leber hereditary optic neuropathy (LHON).[44]

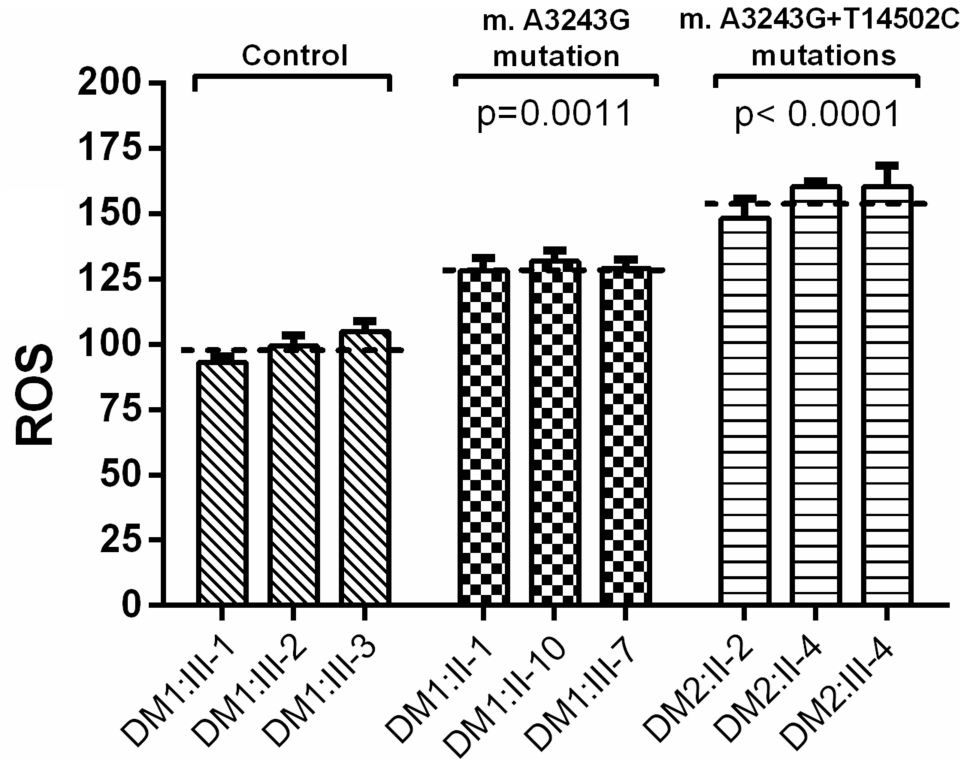

Figure 4 Analysis of ROS production in three patients with the m.A3243G and m.T14502C mutations, three patients with the only m.A3243G mutation and three controls without these mtDNA mutations.

The A-to-G transition at position 3243 of the mtDNA is reported the most prevalent mutation for mitochondrial diabetes worldwide, with a prevalence varying from 0.1% to 10%.[4] In a Tunisian diabetic population, this mutation was only reported in 1.07%,[45] which was similar to the values reported in Japanese[46] and French diabetic populations.[47] In an Asian Indian population, the prevalence of this mutation was 7.8%.[48] However, Khalaf Alharbi et al found that the m.A3243G mutation played no role in Saudi women diagnosed with gestational diabetes, probably because of the small sample size.[49] Interestingly, a recent meta-analysis revealed that patients with the m.A3243G mutation and pigmentary retinopathy maintained highly-functional visual acuity until around the fifth decade of life, after which significant visual decline ensued.[50] Importantly, cybrid cell lines containing the m.A3243G mutation above a certain heteroplasmy threshold led to a reduction in oxygen consumption and OXPHOS.[51] This mutation also affected the processing of longer mitochondrial RNA precursors and the post-transcriptional modification of $tRNA^{Leu(UUR)}$.[52–54] In cybrid cells containing the m.A3243G mutation, an approximately 75% reduction in the steady-state level of $tRNA^{Leu(UUR)}$ was observed, as well as in the aminoacylation ability,[55,56] thereby causing a reduced rate of mitochondrial protein synthesis and respiration defects.[57]

Because the phenotypes associated with the m.A3243G mutation are highly variable and heterogeneous, modifications to other factors, such as nuclear genes, mitochondrial genetic background and environmental factors may contribute to the clinical expression of m.A3243G-induced DM.[58–60] For this purpose, we examined mutations in nuclear genes (*GJB2, GJB3* and *GJB6*), but we did not identify any functional variants, suggesting that nuclear genes may not play important roles in T2DM expression.

To uncover the contribution of the mtDNA genetic background to diabetes, we summarized 19 diabetic pedigrees with the m.A3243G mutation (Table 3). We found that the penetrances of DM in these families varied from 18.4% to 55.5%; moreover, matrilineal relatives bearing functional mtDNA deletions (10.4-kb or 1.0-kb) or mutation (m. T15402C) may increase the expressivity of the T2DM-associated m.A3243G mutation.[61–77]

Indeed, the m.T14502C mutation occurred in homoplasmy only in the maternal lineage of the DM2 pedigree, and the isoleucine at amino acid position 58 is extremely conserved in the *ND6* polypeptide between different organisms.[78] This mutation is also associated with LHON[78–81] and other clinical abnormalities (Table 4).[82] In a recent study, functional analysis

Table 3 Summary of Clinical and Molecular Data for 19 DM Pedigrees Carrying $tRNA^{Leu(UUR)}$ A3243G Mutation

Family Number	Country	Number of Matrilineal Relatives	Number of Affected Relatives	Penetrance of DM (%)	Other Functional mtDNA Mutations	References
1	China	11	3	27.2	None	This study
2	China	10	4	40	*ND6* T14502C mutation	This study
3	Italy	9	3	33.3	10.4-kb deletion	[61]
4	Tunisia	9	5	55.5	1.0-kb deletion	[62]
5	Taiwan	5	1	20	None	[63]
6	Taiwan	18	6	33.3	None	[64]
7	China	9	3	33.3	None	[65]
8	USA	11	3	27.2	None	[66]
9	USA	13	4	30.7	None	[67]
10	Japan	10	3	30	None	[68]
11	Japan	5	2	40	None	[69]
12	Japan	11	2	18.2	None	[70]
13	Sweden	23	6	26.1	None	[71]
14	Sweden	22	6	27.2	None	[72]
15	Netherlands	25	11	44	None	[73]
16	Portugal	11	3	27.2	None	[74]
17	Switzerland	16	6	37.5	None	[75]
18	Norway	38	7	18.4	None	[76]
19	Germany	7	2	28.6	None	[77]

Abbreviations: DM, diabetes mellitus; mtDNA, mitochondrial DNA.

Table 4 Overview of Clinical Presentation of Mitochondrial *ND6* T14502C Mutation

Number	Age	Gender	Clinical Features	Family History	References
1	17	Female	LHON	Yes	[78]
2	4	Male	LHON	Yes	[78]
3	40	Female	LHON	Yes	[78]
4	30	Male	LHON	Yes	[79]
5	38	Female	LHON	Yes	[80]
6	24	Male	LHON	Yes	[80]
7	26	Female	LHON	Yes	[80]
8	19	Male	LHON	Yes	[80]
9	32	Female	LHON	Yes	[81]
10	43	Male	Hypertrophic Cardiomyopathy	No	[82]

Abbreviation: LHON, Leber's hereditary optic neuropathy.

of cybrid cells bearing the m.T14502C mutation showed mild effects on mitochondrial functions when compared with the cells containing both the m.T14502C and m.G11778A mutations. Additionally, the m.T14502C mutation altered the assembly of complex I, thereby aggravating the respiratory phenotypes associated with m.G11778A mutation.[83] Similarly, cell lines bearing both the m.A3243G and m.T14502C mutations exhibited more severe mitochondrial dysfunctions than those in the cells carrying only the m.A3243G mutation (Figures 3 and 4), although the observed decreases of approximately 40% in ATP production in cells bearing both the m.A3243G and m.T14502C mutations were below a proposed threshold level to develop a clinical phenotype.[84] Conversely, cells harboring both the m.A3243G and m.T14502C mutations exhibited greater levels of ROS than either cells carrying only the m.A3243G mutation or controls. Therefore, a lower level of ATP and a higher level of ROS production may lead to the impairment of mitochondrial functions, which could be involved in the pathogenesis and progression of T2DM in the DM2 pedigree. Thus, our study highlights the critical role of the m.T14502C mutation in the pathogenesis of T2DM, manifestated by its synergy with the m.A3243G mutation.

The strength of this study was that we analyzed the synergistic roles between the m.T14502C and m.A3243G mutations, and we found that the m.T14502C mutation may modulate the clinical expression of the diabetes-associated m.A3243G mutation. The main limitation of the current study was the relatively small sample size; thus, further studies including more DM samples are needed to verify this conclusion.

Conclusions

In this study, we showed that mitochondrial diabetes was associated with the $tRNA^{Leu(UUR)}$ A3243G and *ND6* T14502C mutations. Moreover, the m.T14502C mutation may increase the penetrance of m.A3243G-induced T2DM. In the future, functional studies of patients with putative T2DM-associated mtDNA pathogenic mutations should be undertaken.

Abbreviations

mtDNA, mitochondrial DNA; T2DM, type 2 diabetes mellitus; PMNs, polymononuclear leukocytes; mt-tRNA, mitochondrial tRNA; ATP, adenosine triphosphate; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; nDNA, nuclear DNA; IR, insulin resistance; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fiber disease; ND6, NADH-dehydrogenase 6; OGTT, oral glucose tolerance test; HbA1c, hemoglobin A1c; BMI, body mass index; BP, blood pressure; HOMA-IR, homeostasis model assessment of IR; PTA, pure tone audiometry; DPOAE, distortion product otoacoustic emissions; rCRS, revised Cambridge sequence; CI, conservation index; FBS, fetal bovine serum; LHON, Leber's hereditary optic neuropathy.

Data Sharing Statement

The datasets used and analysed during the current study are available from corresponding author (Yu Ding, E-mail: dingyu_zj@126.com) on reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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