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ORIGINAL ARTICLE

Basic Study

Functional analysis of the novel mitochondrial tRNA^{Trp} and tRNA$^{Ser(AGY)}$ variants associated with type 2 diabetes mellitus

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Abstract

BACKGROUND

Mutations in mitochondrial tRNA (*mt-tRNA*) genes that result in mitochondrial dysfunction play important roles in type 2 diabetes mellitus (T2DM). We previously reported a large Chinese pedigree with maternally inherited T2DM that harbors novel *mt-tRNA*^{Trp} *A5514G* and *tRNA*$^{Ser(AGY)}$ *C12237T* variants, however, the effects of these *mt-tRNA* variants on T2DM progression are largely unknown.

AIM

To assess the potential pathogenicity of T2DM-associated *m.A5514G* and *m.C12237T* variants at genetic, molecular, and biochemical levels.

METHODS

Cytoplasmic hybrid (cybrid) cells carrying both *m.A5514G* and *m.C12237T* variants, and healthy control cells without these mitochondrial DNA (mtDNA) variants were generated using trans-mitochondrial technology. Mitochondrial features, including *mt-tRNA* steady-state level, levels of adenosine triphosphate (ATP), mitochondrial membrane potential (MMP), reactive oxygen species (ROS), mtDNA copy number, nicotinamide adenine dinucleotide (NAD^+)/NADH ratio, enzymatic activities of respiratory chain complexes (RCCs), 8-hydroxy-deoxyguanine (8-OhdG), malondialdehyde (MDA), and superoxide dismutase (SOD) were examined in cell lines with and without these *mt-tRNA* variants.

RESULTS

Compared with control cells, the *m.A5514G* variant caused an approximately 35% reduction in the steady-state level of *mt-tRNA*^{Trp} ($P < 0.0001$); however, the

m.C12237T variant did not affect the *mt-tRNA*$^{Ser(AGY)}$ steady-state level (P = 0.5849). Biochemical analysis revealed that cells with both *m.A5514G* and *m.C12237T* variants exhibited more severe mitochondrial dysfunctions and elevated oxidative stress than control cells: ATP, MMP, NAD^+/NADH ratio, enzyme activities of RCCs and SOD levels were markedly decreased in mutant cells (P < 0.05 for all measures). By contrast, the levels of ROS, 8-OhdG and MDA were significantly increased (P < 0.05 for all measures), but mtDNA copy number was not affected by *m.A5514G* and *m.C12237T* variants (P = 0.5942).

CONCLUSION

The *m.A5514G* variant impaired *mt-tRNA*^{Trp} metabolism, which subsequently caused mitochondrial dysfunction. The *m.C12237T* variant did not alter the steady-state level of *mt-tRNA*$^{Ser(AGY)}$, indicating that it may be a modifier of the *m.A5514G* variant. The *m.A5514G* variant may exacerbate the pathogenesis and progression of T2DM in this Chinese pedigree.

Key Words: Type 2 diabetes mellitus; Mitochondrial tRNA genes; Novel variants; Oxidative stress; Mitochondrial dysfunctions

Core Tip: We established cytoplasmic hybrid (cybrid) cells with *m.A5514G* and *m.C12237T* variants, and control cells without these variants. The *m.A5514G* variant decreased *mt-tRNA*^{Trp} stability, whereas the *m.C12237T* variant did not alter the stability of *mt-tRNA*$^{Ser(AGY)}$. More severe mitochondrial dysfunction was observed in mutant cybrids than in control cells, indicating that the *m.A5514G* variant impaired *mt-tRNA*^{Trp} metabolism and mitochondrial functions and increased cellular oxidative stress, which play central roles in type 2 diabetes mellitus (T2DM) progression. By contrast, the *m.C12237T* variant acted as a modifier of the *m.A5514G* variant. Our study provides novel insight into the pathophysiology of maternally transmitted T2DM caused by novel *mt-tRNA* variants.

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INTRODUCTION

Diabetes is a common endocrine disease caused by the presence of chronic hyperglycemia. This complex disorder can be divided into type 1 and type 2. Type 2 diabetes mellitus (T2DM) is a major public health problem that affects approximately 10% of the Chinese adult population[1]. T2DM is characterized by high blood glucose in the context of insulin resistance (IR) and relative insulin deficiency. Furthermore, T2DM-associated complications, such as cardiovascular and peripheral vascular diseases, diabetic retinopathy, foot problems and nephropathy, remain very big challenge for clinicians[2]. Tremendous progression has been made in understanding the pathophysiology of T2DM over the past decades[3-5], with both genetic variations and environmental factors being involved in T2DM pathogenesis[6,7]. Nevertheless, the early prevention, diagnosis, and treatment of T2DM remain far from satisfactory.

Deregulation of mitochondrial oxidative phosphorylation (OXPHOS) is widely accepted as a major cause of T2DM and IR[8], diminished OXPHOS contributes to IR through elevated reactive oxygen species (ROS) production, and disrupted insulin receptor signaling[9]. Mitochondria are present in most eukaryotic cells and their primary role is to provide energy in the form of adenosine triphosphate (ATP) *via* OXPHOS[10]. They have their own DNA, mitochondrial DNA (mtDNA), which is 16569-bp in length and encodes 13 polypeptides, two rRNAs and 22 tRNAs[11]. Although *mt-tRNA* genes account for only approximately 10% mitochondrial genome, more than two third of mitochondrial disease-related variations are localized in this region[12]. An early landmark discovery in T2DM research was the identification of a 10.4-kb large deletion in mtDNA[13] and the m.A3243G mutation in *mt-tRNA*$^{Leu(UUR)}$[14]. A growing number of mtDNA variants has since been reported, for example, *mt-tRNA*^{Glu} A14692G[15], *mt-tRNA*^{Thr} G15897A[16] and *mt-tRNA*^{Gly} T10003C[17] are potential pathogenic variants affecting T2DM predisposition. Exactly how these *mt-tRNA* variants contribute to disease onset, however, remains poorly understood, and, there is an urgent need for additional studies to determine how mitochondrial dysfunction mediates the onset or progression of T2DM.

To understand the molecular basis of mitochondrial diabetes and to provide valuable information for its diagnosis, prevention, and treatment, we previously performed a systematic and extended screen for *mt-tRNA* gene variants in a cohort of 370 Chinese patients with T2DM and 631 healthy controls who were recruited from Hangzhou First People's Hospital and Quzhou People's Hospital in Zhejiang Province of China. We showed that *mt-tRNA*$^{Leu(UUR)}$ A3243G, *ND6* T14502C[18], *ND4* G11696A[19], *ND5* T12338C and *mt-tRNA*^{Ala} T5587C variants[20] are involved in the pathogenesis of maternally inherited T2DM. More recently, we reported a large Chinese pedigree with maternally transmitted T2DM, intriguingly, among 18 matrilineal relatives of this pedigree, six individuals suffered from diabetes. The age at T2DM onset ranged from 40 to 70 years, with an average of 52 years. Mutational analysis of nuclear genes (*GJB2*, *GJB3*, *GJB6*, and

TRMU) indicated no functional variants. Sequence analysis of entire mitochondrial genomes in matrilineal relatives revealed the presence of novel *mt-tRNA*^{Trp} *A5514G* and *mt-tRNA*$^{Ser(AGY)}$ *C12237T* variants[21]. The *m.A5514G* variant disrupted a highly conserved base-pairing (3A-70T) in the Acceptor arm of *mt-tRNA*^{Trp}, whereas the *m.C12237T* variant created a novel Watson-Crick base-pairing (11A-31T) in the Variable region of *mt-tRNA*$^{Ser(AGY)}$ (Figure 1). We therefore hypothesized that change in the *mt-tRNA*^{Trp} and *mt-tRNA*$^{Ser(AGY)}$ secondary structures caused by the *m.A5514G* and *m.C12237T* variants caused aberrant *mt-tRNA* metabolism which led to mitochondrial dysfunction and T2DM progression [22]. However, the pathophysiology of T2DM-related *m.A5514G* and *m.C12237T* variants remains largely undetermined.

To elucidate the pathogenic mechanism of the novel *mt-tRNA*^{Trp} and *mt-tRNA*$^{Ser(AGY)}$ variants, we generated cybrid cell lines derived from four T2DM patients carrying both *m.A5514G* and *m.C12237T* variants, together with four cybrids derived from control subjects without these mtDNA variants but belonging to the same mtDNA haplogroup. We examined whether the *m.A5514G* and *m.C12237T* variants affected *mt-tRNA* steady-state levels by northern blotting, and evaluated mitochondrial characteristics including levels of mtDNA copy number, malondialdehyde (MDA), superoxide dismutase (SOD), 8-hydroxy-deoxyguanine (8-OhdG), ATP, mitochondrial membrane potential (MMP), ROS, nicotinamide adenine dinucleotide (NAD^+)/NADH ratio and respiratory chain complexes (RCCs) activities in the mutant and control cybrids.

MATERIALS AND METHODS

Subjects

Members of a Han Chinese family with T2DM were enrolled *via* Hangzhou First People's Hospital as a part of genetic screening program for T2DM-associated mtDNA novel variants, as described previously[21]. The protocol used in this investigation was in accordance with the principles expressed in the 1975 Declaration of Helsinki, revised in 2008. The Ethics Committee of Hangzhou First People's Hospital approved this study (No. KY-20240327-0100-01). All participants, including four affected matrilineal relatives with T2DM (III-5, III-10, III-18, and III-22) bearing the *m.A5514G* and *m.C12237T* variants, belonged to mtDNA haplogroup G2a according to East Asian phylogeny[23]. Four genetically unrelated healthy subjects (C1, C2, C3, and C4) lacking these *mt-tRNA* variants and also belonging to human mtDNA haplogroup G2a were selected as controls. Written informed consent for participating in this study and for publication of case details was obtained from all subjects enrolled in the study.

Generation of cybrid cell lines

Cybrid cells can incorporate human mitochondria and perpetuate the mtDNA-encoded components of the incorporated mitochondria, which keeping the nuclear background constant[24]. To generate cybrids, the platelets of four patients with both *m.A5514G* and *m.C12237T* variants (III-5, III-10, III-18, and III-22), together with four controls (C1, C2, C3, and C4) were fused with 143B-ρ^0 206 cells, as described previously[25]. The 143B-ρ0 206 cells were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% FBS (Sigma, Aldrich, St. Louis, MO, United States) and 50 μg/mL uridine. The cybrid transformants were cultured in high-glucose DMEM containing 10% FBS at 37 °C in a humidified CO2 incubator.

To determine successful construction of cybrids, PCR-Sanger sequencing was performed to examine the presence of the *m.A5514G* and *m.C12237T* variants. The primer for amplification of the *mt-tRNA*^{Trp} gene were: Forward, 5′-CTA ACC GGC TTT TTG CCC-3′; reverse: 5′-ACC TAG AAG GTT GCC TGG CT-3′. The primers for amplification of the *mt-tRNA*$^{Ser(AGY)}$ gene were: forward, 5′-TAT CAC TCT CCT ACT TAC AG-3′; reverse: 5′-AGA AGG TTA TAA TTC CTA CG-3′. The PCR products were purified, sequenced and compared with an updated version of the human mitochondrial genome sequence to detect the variants (GenBank Accessible No: NC_012920.1)[26].

Northern blot analysis

Total mitochondrial RNA was obtained from mitochondria isolated from mutant and control cybrid cell lines (approximately 2 × 10^8 cells) using the TOTALLY RNATM kit (Ambion, Thermo Fisher Scientific), as described previously[27]. For northern blotting analysis of *mt-tRNA*, 2 μg of total mitochondrial RNA was electrophoresed through a 10% polyacrylamide/8M urea gel in Tris borate-EDTA buffer. The sequences for digoxigenin (DIG)-labeled probes specific to *mt-tRNA*^{Trp}; *mt-tRNA*$^{Ser(AGY)}$ and 5S rRNA were: 5′-AGA AAT TAA GTA TTG CAA CTT ACT GAG GGC-3′; 5′-GAG AAA GCC ATG TTG TTA GAC ATG GGG GCA-3′ and 5′-GGG TGG TAT GGC GGT AGA C-3′, respectively. Hybridization and quantification of band density were performed as previously described[28].

Analysis of ATP levels

The CellTiter-Glo® luminescent cell viability assay (Promega, Madison, WI, United States) was used to measure ATP levels in mutant and control cybrids according to the manufacturer's instructions[29]. Cells were seeded into white 96-well plates at 1 × 10^5 cells per well and cultured for 20 hours to reach approximately 70% confluence. Cells were then lysed by the addition of 100 μL CellTiter-Glo® working solution to each well and incubation for 2 minutes in the dark. Cell lysate solution (200 μL) was then transferred to an opaque 96-well plate. A 200 μL solution of culture medium and working solution at a ratio of 1:1 was used as a control. ATP concentration was determined using a 96-well fluorescence detector.

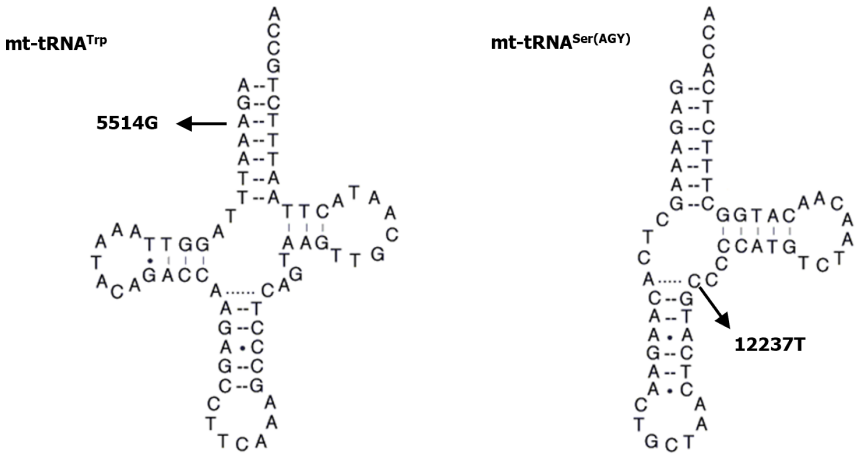

Figure 1 The secondary structures of *mitochondrial tRNA*^{Trp} and *mitochondrial tRNA*$^{Ser(AGY)}$, arrows indicated the positions of the *m.A5514G* and *m.C12237T* variants. mt-tRNA: Mitochondrial tRNA.

MMP analysis

The JC-1 MMP Assay Kit (Abcam, United States) was used to detect MMP in mutant and wild type cell lines. The JC-1 dye fluoresces in healthy cells with high levels of MMP, and green in cells with low levels of MMP[30]. JC-1 probe (1 μg/mL) was added to approximately 1 × 10^5 cells and incubated for 30 minutes in the dark. Cells were then washed twice with PBS and immediately analyzed by FACSCanto II flow cytometry (BD Biosciences, United States).

Analysis of mitochondrial ROS production

Mutant and control cybrid cells were seeded in 6-well plates (1 × 10^5 cells/well) and loaded with 10 μmol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Beyotime, China) at 37 °C for 20 minutes. Cells were then washed with PBS three times and subsequently evaluated by FACSCanto II flow cytometry (BD Biosciences, United States).

Determination of NAD$^+$/NADH ratio

The NAD$^+$/NADH ratio is a measure of global energy capacity because the activities of rate-limiting enzymes involved in the tricarboxylic acid cycle (TCA), ketone production and glycolysis are regulated by this ratio[31,32]. The NAD$^+$/NADH ratio was determined for 1 × 10^5 cells using the NAD$^+$/NADH Assay Kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's instructions. Standard curves were generated for quantification.

Determination of mtDNA copy number

mtDNA copy number was determined using a quantitative real-time PCR (qRT-PCR) method, as described elsewhere [33]. Genomic DNA was extracted from 3 mL of blood from each participant using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For qRT-PCR analysis, 100 ng genomic DNA was used as template. The primers specific for the mtDNA-ND1 gene were: Forward, 5′-CCTAGC CGT TTA CTC AAT CCT-3′; reverse: 5′-TGA TGG CTA GGG TGA CTTCAT-3′; the primer sequences for nuclear (hemoglobin) gene were: forward, 5′-GCT TCT GAC ACA ACT GTG TTC ACT AGC-3′; reverse: 5′-CAC CAA CTT CAT CCA CGT TCA CC-3′. The qRT-PCR reaction was performed on an ABI 7900 instrument (Thermo Fisher Scientific) using SYBR Green Realtime PCR Master Mix (Bioteke, China). Relative expression levels were normalized by the $2^{-\Delta\Delta CT}$ method[34]. Samples were assayed in triplicate.

Analysis of oxidative stress-related biomarkers

To determine the effects of the *m.A5514G* and *m.C12237T* variants on oxidative stress[35], the concentrations of MDA and SOD in four cybrids with the *m.A5514G* and *m.C12237T* variants (III-5, III-10, III-18, and III-22), and in four control cell lines (C1, C2, C3, and C4) were analyzed using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The serum levels of 8-OhdG were quantified by a competitive enzyme linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Nikken Foods, St. Louis, MO, United States). Samples were assayed in triplicate.

Analysis of enzymatic activities of mitochondrial RCCs

RCCs comprise four enzyme complexes (I-IV), which are embedded in the inner mitochondrial membrane and catalyze the transfer of reducing equivalents from high energy compounds. To analysis the activities of respiratory chain enzymes, an enriched mitochondrial fraction was isolated from mutant and control cybrid cells by centrifugation, as described previously[36]. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, United States). The enzymatic activities of RCCs were assayed spectrophotometrically, and the results were normalized to the activity of citrate synthase, a mitochondrial matrix enzyme[37].

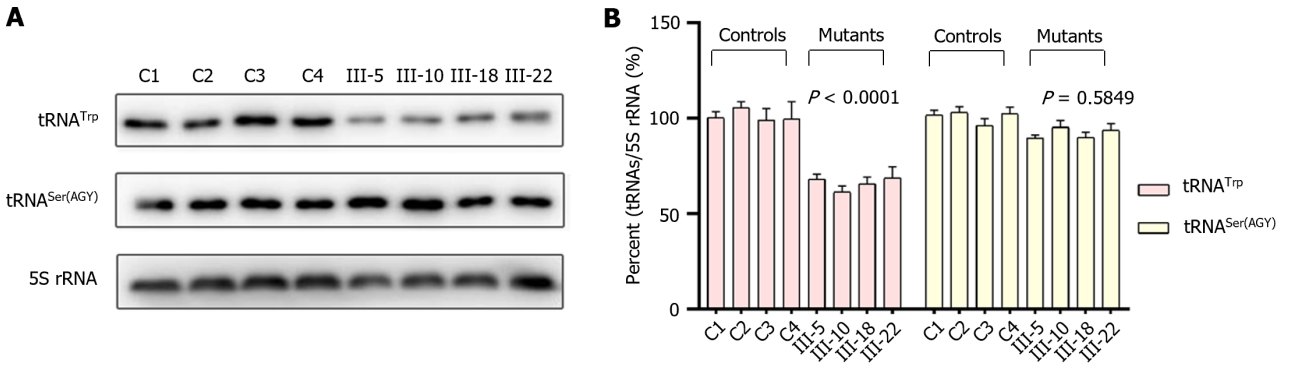

Figure 2 Analysis of steady-state levels of mitochondrial tRNA^{Trp} and mitochondrial tRNA$^{Ser(AGY)}$ by Northern blotting. A: 2 μg mitochondrial tRNA (mt-RNA) were electrophoresed through a denaturing polyacrylamide gel and hybridized with DIG-labeled oligonucleotide probes for *mt-tRNA*^{Trp}, *mt-tRNA*$^{Ser(AGY)}$, and 5S rRNA; B: Qualification of *mt-tRNA* levels.

Redefining the pathogenic roles of the m.A5514G and m.C12237T variants

We used the revised pathogenicity scoring system for *mt-tRNA* variants proposed by Yarham *et al*[38]. This scoring system gives special weight to functional data, which are considered the gold standard methods for assigning pathogenicity. The scoring system was as follows: ≤ 6 points, neutral polymorphism; 7-10 points, possibly pathogenic; 11-13 points (not including evidence from single-fiber, steady-state level, or trans-mitochondrial cybrid studies), probably pathogenic; ≥ 11 points (including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies), definitely pathogenic.

Statistical analysis

Data are presented as the mean ± SD. Statistical analysis of results was performed using GraphPad Prism 9.0 software (GraphPad. Software Inc., La Jolla, CA, United States). Student's *t*-test or the Mann-Whitney test were used to assess the difference between two groups. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Pedigree information and establishment of cybrid cell lines

We previously described a large Han Chinese pedigree with maternally transmitted T2DM that harbors the *mt-tRNA*^{Trp} A5514G and *mt-tRNA*$^{Ser(AGY)}$ C12237T variants[21]. Six individuals (one man and five women) of 18 matrilineal relatives suffered from T2DM. The age at onset varied among these six individuals from 40 to 70 years, with an average of 52 years.

To analyze mitochondrial functions, cybrid cell lines were derived from four subjects (III-5, III-10, III-18, and III-22) aged < 50 and from four genetically unrelated healthy subjects (C1, C2, C3, and C4) belonging to the same mtDNA haplogroup for use as controls. The platelets of these subjects were fused with the mtDNA-less human ρo206 cell line and cybrid clones were isolated by growing in selective DMEM, according to a previously described protocol[25]. The cybrid cell lines were analyzed by direct sequencing for the presence of the *m.A5514G* and *m.C12237T* variants and for homoplasmy.

mt-tRNA analysis

To investigate whether the *m.A5514G* and *m.C12237T* variants perturbed *mt-tRNA* metabolism, we subjected mt-RNAs from mutant and control cell lines to northern blotting and hybridized them with DIG-labeled oligodeoxynucleotide probes for *mt-tRNA*^{Trp} and *mt-tRNA*$^{Ser(AGY)}$. As shown in Figure 2A, the steady-state level of *mt-tRNA*^{Trp} in mutant cell lines was significantly decreased, whereas the steady-state level of *mt-tRNA*$^{Ser(AGY)}$ was not affected in either mutant or wild type cells. For comparison, the *m.A5514G* variant caused approximately 35% reduction in the level of *mt-tRNA*^{Trp} (P < 0.0001) after normalization of 5S rRNA, however, the *m.C12237T* variant had little impact on the *mt-tRNA*$^{Ser(AGY)}$ steady-state level (P = 0.5849) (Figure 2B).

ATP levels are decreased in m.A5514G and m.C12237T cybrid cells

We next assessed whether the variants influenced ATP generation by using a luciferin/Luciferase assay. As shown in Figure 3A, the average levels of ATP production in mutant cells decreased approximately 22.1% compared with control cells (P = 0.0016).

The m.A5514G and m.C12237T variants reduce the levels of MMP

The effects of *m.A5514G* and *m.C12237T* variants on MMP was measured using the fluorescence probe, JC-1, in control and mutant cells. The relative ratios of the FL_{590}/FL_{529} geometric means between control and mutant cells were assessed to

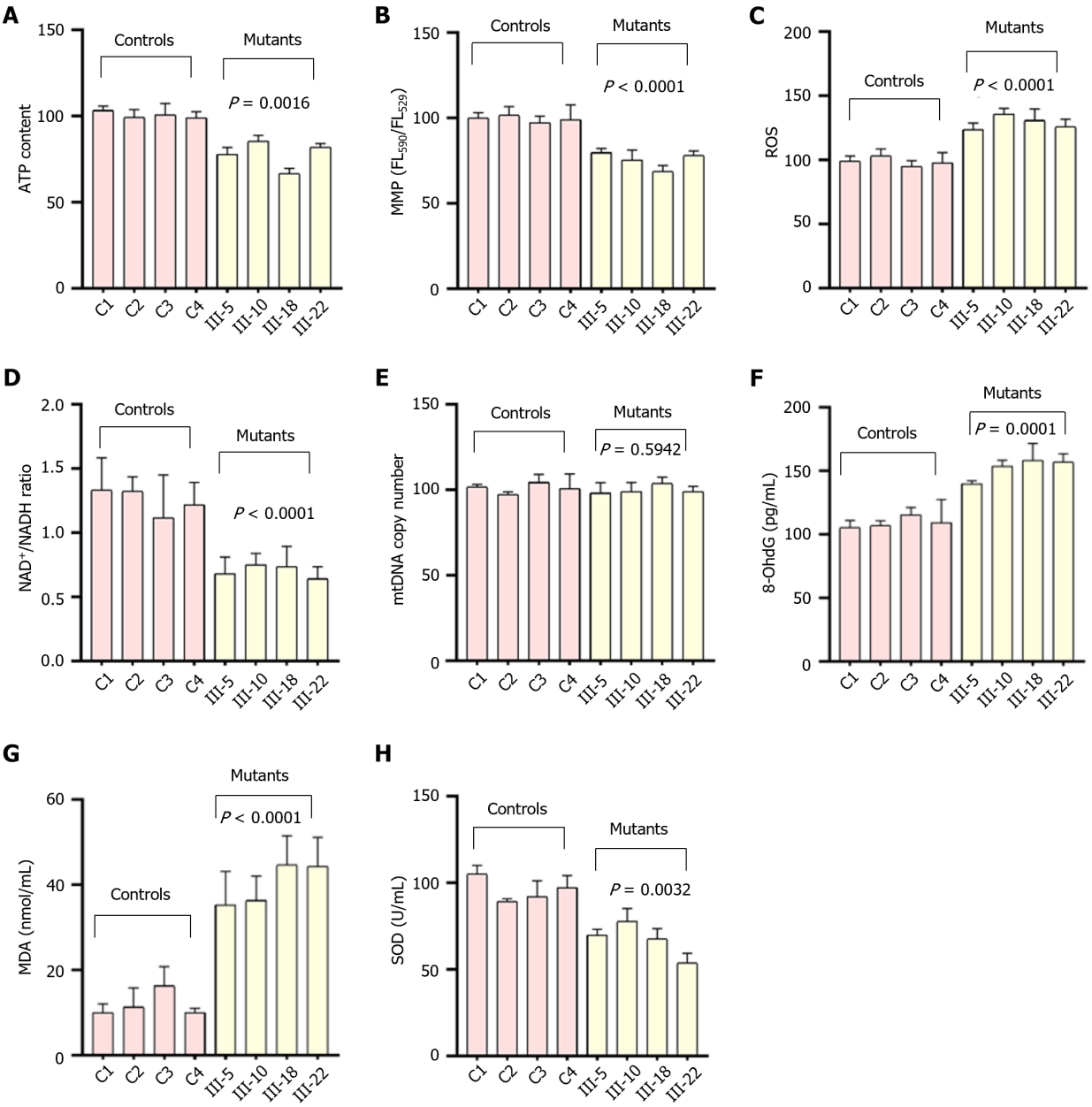

Figure 3 Analysis of mitochondrial functions in mutant and control cybrids. A: Adenosine triphosphate analysis; B: Mitochondrial membrane potential levels; C: Reactive oxygen species analysis; D: Nicotinamide adenine dinucleotide/NADH ratio; E: Mitochondrial DNA copy number; F: 8-hydroxy-deoxyguanine levels; G: Malondialdehyde levels; H: Superoxide dismutase levels. ATP: Adenosine triphosphate; MMP: Mitochondrial membrane potential; ROS: Reactive oxygen species; NAD: Nicotinamide adenine dinucleotide; mtDNA: Mitochondrial DNA; 8-OhdG: 8-hydroxy-deoxyguanine; MDA: Malondialdehyde; SOD: Superoxide dismutase.

depict the MMP level. As shown in Figure 3B, cells carrying *mt-tRNA* variants had 25.6% less MMP compared with control cells ($P < 0.0001$).

ROS production is enhanced in m.A5514G and m.C12237T cybrid cells

We used flow cytometry and DCFH-DA probe loading to assess ROS production in mutant and control cell lines. As shown in Figure 3C, mutant cells bearing the *m.A5514G* and *m.C12237T* variants exhibited increased ROS production, with an average increase of 127.5% relative to control cells ($P < 0.0001$).

The NAD⁺/NADH ratio is decreased in m.A5514G and m.C12237T cybrid cells

The NAD⁺/NADH ratio is involved in central carbon metabolism, nucleotide synthesis and lipid metabolism[39]. We measured the NAD⁺/NADH ratio in mutant and control cell lines and demonstrated that the *mt-tRNA* variants significantly reduced the NAD⁺/NADH ratio (Figure 3D).

mtDNA copy number is not affected by m.A5514G and m.C12237T variants

mtDNA copy number is a biomarker for both mitochondrial quality and function[40]. We therefore analyzed the mtDNA

Table 1 Redefining the pathogenic roles of the *m.A5514G* and *m.C12237T* variants

Scoring criteria	*m.A5514G* variant	Score/20	*m.C12237T* variant	Score/20	Classification
More than one independent report	Yes	2	Yes	2	≤ 6 points: Neutral polymorphisms; 7-10 points: Possibly pathogenic; 11-13 points (not including evidence from single fiber, steady-state level, or trans-mitochondrial cybrid studies): Probably pathogenic; ≥ 11 points (including evidence from single fiber, steady-state level or trans-mitochondrial cybrid studies): Definitely pathogenic
Evolutionary conservation of the base pair	No changes	2	No changes	2	
Variant heteroplasmy	No	0	No	0	
Segregation of the mutation with disease	Yes	2	Yes	2	
Biochemical defect in complex I, III, or IV	Yes	2	No	0	
Evidence of mutation segregation with biochemical defect from single fiber studies	No evidence	0	No evidence	0	
Mutant *mt-tRNA* steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Strong evidence	5	No evidence	0	
Maximum score	Definitely pathogenic	13	Neutral polymorphism	6	

content in peripheral blood. As shown in Figure 3E, there were no differences in mtDNA copy number among patients with the *m.A5514G* and *m.C12237T* variants and controls ($P = 0.5942$).

Serum 8-OhdG levels are increased in T2DM individuals

8-OhdG, a critical biomarker of DNA damage, plays an important role in T2DM progression[41]. The concentrations of serum 8-OhdG in T2DM and control subjects were measured by ELISA. As shown in Figure 3F, patients with *mt-tRNA* variants had much higher concentrations of 8-OhdG compared with controls ($P < 0.0001$).

The m.A5514G and m.C12237T variants increase cellular oxidative stress

We next explored whether the *mt-tRNA* variants affected cellular oxidative stress. Individuals carrying the *mt-tRNA* variants showed much higher levels of MDA ($P < 0.0001$; Figure 3G) but significantly decreased levels of SOD ($P = 0.0032$) (Figure 3H) compared with controls.

The m.A5514G and m.C12237T variants inhibit the activities of Complex I and IV in cybrids

Mitochondrial Complex I is the major site for catalyzing oxidation of NADH. We therefore analyzed the enzyme activities of Complex I-IV in cybrids. As shown in Figure 4, the activities of Complex I and IV were dramatically inhibited ($P < 0.0001$, both), while Complex II and III enzyme activities remained unaffected ($P = 0.5019$ and 0.5523, respectively) in the *m.A5514G* and *m.C12237T* variants cybrids.

Pathogenicity of the m.A5514G and m.C12237T variants

Total scores of the *m.A5514G* and *m.C12237T* variants were 13 and 6 points, respectively, (Table 1), indicating that they were "definitely pathogenic" and "neutral polymorphism", respectively.

DISCUSSION

In the present study, we investigated the causal roles of T2DM-related *m.A5514G* and *m.C12237T* variants at genetic, molecular, and biochemical levels. The *m.A5514G* variant was initially described in a neonatal patient with multiple RCC defects[42], and the *m.C12237T* variant was first reported in patients with Leber's Hereditary Optic Neuropathy (LHON) [43]. Subsequently, we showed these variants to be associated with T2DM[21]. At the molecular level, the *m.A5514G* variant occurs at conventional position 3 in the Acceptor arm of *mt-tRNA*^{Trp}, which is highly conserved among species. Importantly, the *m.A5514G* variant abolishes the 3A-70T Watson-Crick base-pairing, which is important in recognizing its cognate tryptophanyl-tRNA synthetase during translation[44]. This causes misreading or misrecognition by RNase P[45]. Interestingly, the *m.T7512C* variant, which occurs at the same position as *mt-tRNA*$^{Ser(UCN)}$, was suggested to be associated with mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) and with myoclonic epilepsy with ragged red fibers (MERRF) overlap syndrome[46]. We therefore speculate that the *m.A5514G* variant, which is similar to the *m.T7512C* variant, may alter the stability of mutant *mt-tRNA*^{Trp}, as indicated by our northern blotting

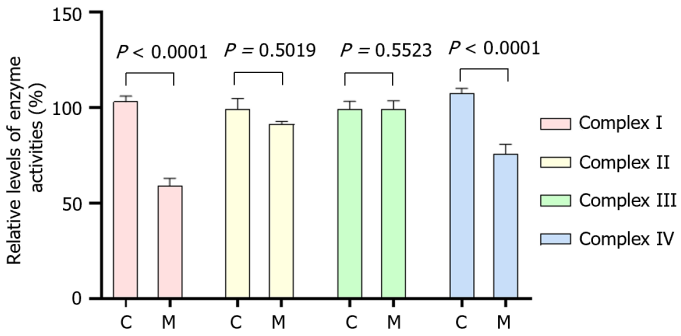

Figure 4 Analysis of enzymatic activities of mitochondrial respiratory chain complexes in four cybrids with mitochondrial tRNA variants and four control cell lines without these variants. C: Control group; M: Mutant group.

results. Indeed, the average steady-state level of *mt-tRNA*^{Trp} was significantly reduced to 35% of control levels, which is below the proposed threshold to produce a clinical phenotype associated with an *mt-tRNA* mutation[47]. These data lead us to believe that the *m.A5514G* variant impairs *mt-tRNA*^{Trp} metabolism.

Genetically, unlike other canonical *mt-tRNA*s, *mt-tRNA*$^{Ser(AGY)}$ is a highly unique tRNA in humans, lacking the entire D-loop structure[22]. In fact, the homoplasmic *m.C12237T* variant is located at position 31 in the Variable region of *mt-tRNA*$^{Ser(AGY)}$, which creates a new 11A-31T Watson-Crick base-pairing. Although RNA Fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) predicted this variant to alter the secondary structure of *mt-tRNA*$^{Ser(AGY)}$[21], functional data indicated no obvious difference in *mt-tRNA*$^{Ser(AGY)}$ steady-state levels between mutant and control cell lines, strongly indicating that the *m.C12237T* variant did not affect *mt-tRNA*$^{Ser(AGY)}$ metabolism. Therefore, the *m.C12237T* variant most probably acts as a modifier of the *m.A5514G* variant in T2DM.

The perturbed tertiary structure caused by the *m.A5514G* variant made the mutant *mt-tRNA*^{Trp} more unstable and led to aberrant *mt-tRNA* metabolism, significantly reducing the activities of Complex I and IV in mutant cells, which was consistent with the clinical phenotypes of the affected patients. As a result, the RCC deficiency caused by the *m.A5514G* and *m.C12237T* variants may result in down-regulated mitochondrial biogenesis and uncoupling of the oxidative pathway for ATP synthesis. Pancreatic beta cells have a high OXPHOS demand and would, therefore, be primarily affected[48]. In the current study, ATP production was reduced by 22.1% in mutant cells as compared with control cells. In addition, defectives OXPHOS complexes can result in impaired MMP production. Importantly, MMP is generated by proton pumps (Complex I, III, and IV), and is an essential in energy storage during OXPHOS[49]. The average MMP level in patients' cells was only 74.4% of that in control cells. As a result, the defective OXPHOS in cells with *mt-tRNA* variants increased the production of ROS, 8-OhdG and MDA. Indeed, MDA is generated in the oxidative degradation of polyunsaturated lipids[50], whereas SOD is an antioxidant enzyme able to break down harmful oxygen molecules within cells and convert them into less toxic products[51]. Therefore, the increased oxidative damage and decreased antioxidant activity may be involved in the occurrence of oxidative stress. This would damage mitochondrial and cellular proteins, lipids, and nucleic acids *via* chemical modifications, including nitrosylation, peroxidation and carbonylation, and, in turn, promote pancreatic beta cell dysfunction and IR[52,53].

Reduced mtDNA copy number has been theoretically linked to increased oxidative stress *via* increased production of ROS[54]. Our data show no changes in mtDNA content in mutant and control groups. However, the precise mechanisms that led to variation in mtDNA copy number are uncertain and genetic and environmental factors have been hypothesized to interact to determine the number of mitochondria in a cell[55]. Therefore, although the *m.A5514G* and *m.C12237T* variants lead to mitochondrial dysfunctions, other factors such as nuclear background, restored the mtDNA content in the mutant cell lines.

Cellular NAD exists in two forms, oxidized (NAD+) and reduced (NADH). It plays essential roles in cellular redox reactions and is responsible for accepting high energy electrons and carrying them to the electron transport chain for the synthesis of ATP[56]. In diabetic patients, increased NADH levels cause reductive stress, which elevates ROS production and leads to IR and cell death[57]. In our study, mutant cell lines showed a marked decrease in the NAD^+/NADH ratio indicating that the *m.A5514G* and *m.C12237T* variants increases oxidative stress and mitochondrial dysfunction.

CONCLUSION

Our findings indicate that the *m.A5514G* variant reduced the steady-state level of *mt-tRNA*^{Trp}, which affected tRNA metabolism and led to mitochondrial dysfunctions involved in T2DM pathogenesis, whereas the *m.C12237T* variant had little impact on *mt-tRNA*$^{Ser(AGY)}$ stability and was probably a polymorphism with neutral effect on the for expressivity of the *m.A5514G* variant-induced T2DM phenotype. Furthermore, the incomplete penetrance of T2DM and variable clinical phenotypes indicate that *mt-tRNA* variants are not sufficient to produce clinical phenotypes. Therefore, other factors, such as environmental factors, nuclear genes or epigenetic modification, may contribute to T2DM progression. The main limitation of this study was the relatively small sample size, and further studies including more T2DM patients are needed to verify our conclusions.

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FOOTNOTES

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REFERENCES

1 **Xu Y**, Wang L, He J, Bi Y, Li M, Wang T, Wang L, Jiang Y, Dai M, Lu J, Xu M, Li Y, Hu N, Li J, Mi S, Chen CS, Li G, Mu Y, Zhao J, Kong L, Chen J, Lai S, Wang W, Zhao W, Ning G; 2010 China Noncommunicable Disease Surveillance Group. Prevalence and control of diabetes in Chinese adults. *JAMA* 2013; **310**: 948-959 [PMID: 24002281 DOI: 10.1001/jama.2013.168118]

2 **Kahn SE**, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. *Lancet* 2014; **383**: 1068-1083 [PMID: 24315620 DOI: 10.1016/S0140-6736(13)62154-6]

3 **Xourafa G**, Korbmacher M, Roden M. Inter-organ crosstalk during development and progression of type 2 diabetes mellitus. *Nat Rev Endocrinol* 2024; **20**: 27-49 [PMID: 37845351 DOI: 10.1038/s41574-023-00898-1]

4 **Brüning JC**, Fenselau H. Integrative neurocircuits that control metabolism and food intake. *Science* 2023; **381**: eabl7398 [PMID: 37769095 DOI: 10.1126/science.abl7398]

5 **Roberts FL**, Cataldo LR, Fex M. Monoamines' role in islet cell function and type 2 diabetes risk. *Trends Mol Med* 2023; **29**: 1045-1058 [PMID: 37722934 DOI: 10.1016/j.molmed.2023.08.009]

6 **Tonyan ZN**, Nasykhova YA, Danilova MM, Glotov AS. Genetics of macrovascular complications in type 2 diabetes. *World J Diabetes* 2021; **12**: 1200-1219 [PMID: 34512887 DOI: 10.4239/wjd.v12.i8.1200]

7 **Assulyn T**, Khamisy-Farah R, Nseir W, Bashkin A, Farah R. Neutrophil-to-lymphocyte ratio and red blood cell distribution width as predictors of microalbuminuria in type 2 diabetes. *J Clin Lab Anal* 2020; **34**: e23259 [PMID: 32096579 DOI: 10.1002/jcla.23259]

8 **Fang H**, Hu N, Zhao Q, Wang B, Zhou H, Fu Q, Shen L, Chen X, Shen F, Lyu J. mtDNA Haplogroup N9a Increases the Risk of Type 2 Diabetes by Altering Mitochondrial Function and Intracellular Mitochondrial Signals. *Diabetes* 2018; **67**: 1441-1453 [PMID: 29735607 DOI: 10.2337/db17-0974]

9 **Weng SW**, Kuo HM, Chuang JH, Lin TK, Huang HL, Lin HY, Liou CW, Wang PW. Study of insulin resistance in cybrid cells harboring diabetes-susceptible and diabetes-protective mitochondrial haplogroups. *Mitochondrion* 2013; **13**: 888-897 [PMID: 23948373 DOI: 10.1016/j.mito.2013.08.001]

10 **Mohan R**, Jo S, Lockridge A, Ferrington DA, Murray K, Eschenlauer A, Bernal-Mizrachi E, Fujitani Y, Alejandro EU. OGT Regulates Mitochondrial Biogenesis and Function *via* Diabetes Susceptibility Gene Pdx1. *Diabetes* 2021; **70**: 2608-2625 [PMID: 34462257 DOI: 10.2337/db21-0468]

11 **Anderson S**, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature* 1981; **290**: 457-465 [PMID: 7219534 DOI: 10.1038/290457a0]

12 **Florentz C**, Sohm B, Tryoen-Tóth P, Pütz J, Sissler M. Human mitochondrial tRNAs in health and disease. *Cell Mol Life Sci* 2003; **60**: 1356-1375 [PMID: 12943225 DOI: 10.1007/s00018-003-2343-1]

13 **Ballinger SW**, Shoffner JM, Hedaya EV, Trounce I, Polak MA, Koontz DA, Wallace DC. Maternally transmitted diabetes and deafness

associated with a 10.4 kb mitochondrial DNA deletion. *Nat Genet* 1992; **1**: 11-15 [PMID: 1301992 DOI: 10.1038/ng0492-11]

14 **McMillan RP**, Stewart S, Budnick JA, Caswell CC, Hulver MW, Mukherjee K, Srivastava S. Quantitative Variation in m.3243A > G Mutation Produce Discrete Changes in Energy Metabolism. *Sci Rep* 2019; **9**: 5752 [PMID: 30962477 DOI: 10.1038/s41598-019-42262-2]

15 **Wang M**, Liu H, Zheng J, Chen B, Zhou M, Fan W, Wang H, Liang X, Zhou X, Eriani G, Jiang P, Guan MX. A Deafness- and Diabetes-associated tRNA Mutation Causes Deficient Pseudouridinylation at Position 55 in tRNAGlu and Mitochondrial Dysfunction. *J Biol Chem* 2016; **291**: 21029-21041 [PMID: 27519417 DOI: 10.1074/jbc.M116.739482]

16 **Li K**, Wu L, Liu J, Lin W, Qi Q, Zhao T. Maternally Inherited Diabetes Mellitus Associated with a Novel m.15897G>A Mutation in Mitochondrial tRNA(Thr) Gene. *J Diabetes Res* 2020; **2020**: 2057187 [PMID: 32083134 DOI: 10.1155/2020/2057187]

17 **Liu H**, Li R, Li W, Wang M, Ji J, Zheng J, Mao Z, Mo JQ, Jiang P, Lu J, Guan MX. Maternally inherited diabetes is associated with a homoplasmic T10003C mutation in the mitochondrial tRNA(Gly) gene. *Mitochondrion* 2015; **21**: 49-57 [PMID: 25615420 DOI: 10.1016/j.mito.2015.01.004]

18 **Ding Y**, Zhang S, Guo Q, Zheng H. Mitochondrial Diabetes is Associated with tRNA(Leu(UUR)) A3243G and ND6 T14502C Mutations. *Diabetes Metab Syndr Obes* 2022; **15**: 1687-1701 [PMID: 35685248 DOI: 10.2147/DMSO.S363978]

19 **Ding Y**, Zhang S, Guo Q, Leng J. Mitochondrial Diabetes Is Associated with the ND4 G11696A Mutation. *Biomolecules* 2023; **13** [PMID: 37371486 DOI: 10.3390/biom13060907]

20 **Jiang Z**, Cai X, Kong J, Zhang R, Ding Y. Maternally transmitted diabetes mellitus may be associated with mitochondrial ND5 T12338C and tRNA(Ala) T5587C variants. *Ir J Med Sci* 2022; **191**: 2625-2633 [PMID: 34993838 DOI: 10.1007/s11845-021-02911-w]

21 **Yang L**, Guo Q, Leng J, Wang K, Ding Y. Late onset of type 2 diabetes is associated with mitochondrial tRNA(Trp) A5514G and tRNA(Ser(AGY)) C12237T mutations. *J Clin Lab Anal* 2022; **36**: e24102 [PMID: 34811812 DOI: 10.1002/jcla.24102]

22 **Sharma S**, Fazal FM. Localization of RNAs to the mitochondria-mechanisms and functions. *RNA* 2024; **30**: 597-608 [PMID: 38448244 DOI: 10.1261/rna.079999.124]

23 **Kong QP**, Bandelt HJ, Sun C, Yao YG, Salas A, Achilli A, Wang CY, Zhong L, Zhu CL, Wu SF, Torroni A, Zhang YP. Updating the East Asian mtDNA phylogeny: a prerequisite for the identification of pathogenic mutations. *Hum Mol Genet* 2006; **15**: 2076-2086 [PMID: 16714301 DOI: 10.1093/hmg/ddl130]

24 **Wilkins HM**, Carl SM, Swerdlow RH. Cytoplasmic hybrid (cybrid) cell lines as a practical model for mitochondriopathies. *Redox Biol* 2014; **2**: 619-631 [PMID: 25460729 DOI: 10.1016/j.redox.2014.03.006]

25 **Li D**, Sun Y, Zhuang Q, Song Y, Wu B, Jia Z, Pan H, Zhou H, Hu S, Zhang B, Qiu Y, Dai Y, Chen S, Xu X, Zhu X, Lin A, Huang W, Liu Z, Yan Q. Mitochondrial dysfunction caused by m.2336T>C mutation with hypertrophic cardiomyopathy in cybrid cell lines. *Mitochondrion* 2019; **46**: 313-320 [PMID: 30196098 DOI: 10.1016/j.mito.2018.08.005]

26 **Andrews RM**, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. *Nat Genet* 1999; **23**: 147 [PMID: 10508508 DOI: 10.1038/13779]

27 **Zhou M**, Wang M, Xue L, Lin Z, He Q, Shi W, Chen Y, Jin X, Li H, Jiang P, Guan MX. A hypertension-associated mitochondrial DNA mutation alters the tertiary interaction and function of tRNA(Leu(UUR)). *J Biol Chem* 2017; **292**: 13934-13946 [PMID: 28679533 DOI: 10.1074/jbc.M117.787028]

28 **Fan W**, Zheng J, Kong W, Cui L, Aishanjiang M, Yi Q, Wang M, Cang X, Tang X, Chen Y, Mo JQ, Sondheimer N, Ge W, Guan MX. Contribution of a mitochondrial tyrosyl-tRNA synthetase mutation to the phenotypic expression of the deafness-associated tRNA(Ser(UCN)) 7511A>G mutation. *J Biol Chem* 2019; **294**: 19292-19305 [PMID: 31685661 DOI: 10.1074/jbc.RA119.010598]

29 **Lou X**, Zhou Y, Liu Z, Xie Y, Zhang L, Zhao S, Gong S, Zhuo X, Wang J, Dai L, Ren X, Tong X, Jiang L, Fang H, Fang F, Lyu J. De novo frameshift variant in MT-ND1 causes a mitochondrial complex I deficiency associated with MELAS syndrome. *Gene* 2023; **860**: 147229 [PMID: 36717040 DOI: 10.1016/j.gene.2023.147229]

30 **Sivandzade F**, Bhalerao A, Cucullo L. Analysis of the Mitochondrial Membrane Potential Using the Cationic JC-1 Dye as a Sensitive Fluorescent Probe. *Bio Protoc* 2019; **9** [PMID: 30687773 DOI: 10.21769/BioProtoc.3128]

31 **Butterfield DA**, Halliwell B. Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. *Nat Rev Neurosci* 2019; **20**: 148-160 [PMID: 30737462 DOI: 10.1038/s41583-019-0132-6]

32 **Xin L**, Ipek Ö, Beaumont M, Shevlyakova M, Christinat N, Masoodi M, Greenberg N, Gruetter R, Cuenoud B. Nutritional Ketosis Increases NAD(+)/NADH Ratio in Healthy Human Brain: An in Vivo Study by (31)P-MRS. *Front Nutr* 2018; **5**: 62 [PMID: 30050907 DOI: 10.3389/fnut.2018.00062]

33 **Elyamany A**, Ghazala R, Fayed O, Hamed Y, El-Shendidi A. Mitochondrial DNA copy number in Hepatitis C virus-related chronic liver disease: impact of direct-acting antiviral therapy. *Sci Rep* 2023; **13**: 18330 [PMID: 37884543 DOI: 10.1038/s41598-023-44665-8]

34 **Fragouli E**, Spath K, Alfarawati S, Kaper F, Craig A, Michel CE, Kokocinski F, Cohen J, Munne S, Wells D. Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. *PLoS Genet* 2015; **11**: e1005241 [PMID: 26039092 DOI: 10.1371/journal.pgen.1005241]

35 **Demirci-Çekiç S**, Özkan G, Avan AN, Uzunboy S, Çapanoğlu E, Apak R. Biomarkers of Oxidative Stress and Antioxidant Defense. *J Pharm Biomed Anal* 2022; **209**: 114477 [PMID: 34920302 DOI: 10.1016/j.jpba.2021.114477]

36 **Misceo D**, Strømme P, Bitarafan F, Chawla MS, Sheng Y, Bach de Courtade SM, Eide L, Frengen E. Biallelic NDUFA4 Deletion Causes Mitochondrial Complex IV Deficiency in a Patient with Leigh Syndrome. *Genes (Basel)* 2024; **15** [PMID: 38674434 DOI: 10.3390/genes15040500]

37 **Yoshida Y**, Tamura Y, Kouzaki K, Nakazato K. Dietary apple polyphenols enhance mitochondrial turnover and respiratory chain enzymes. *Exp Physiol* 2023; **108**: 1295-1307 [PMID: 37658608 DOI: 10.1113/EP091154]

38 **Yarham JW**, Al-Dosary M, Blakely EL, Alston CL, Taylor RW, Elson JL, McFarland R. A comparative analysis approach to determining the pathogenicity of mitochondrial tRNA mutations. *Hum Mutat* 2011; **32**: 1319-1325 [PMID: 21882289 DOI: 10.1002/humu.21575]

39 **Luengo A**, Li Z, Gui DY, Sullivan LB, Zagorulya M, Do BT, Ferreira R, Naamati A, Ali A, Lewis CA, Thomas CJ, Spranger S, Matheson NJ, Vander Heiden MG. Increased demand for NAD(+) relative to ATP drives aerobic glycolysis. *Mol Cell* 2021; **81**: 691-707.e6 [PMID: 33382985 DOI: 10.1016/j.molcel.2020.12.012]

40 **Fukunaga H**, Ikeda A. Mitochondrial DNA copy number variation across three generations: a possible biomarker for assessing perinatal outcomes. *Hum Genomics* 2023; **17**: 113 [PMID: 38098033 DOI: 10.1186/s40246-023-00567-4]

41 **Al-Rawaf HA**, Gabr SA, Iqbal A, Alghadir AH. High-Intensity Interval Training Improves Glycemic Control, Cellular Apoptosis, and Oxidative Stress of Type 2 Diabetic Patients. *Medicina (Kaunas)* 2023; **59** [PMID: 37512131 DOI: 10.3390/medicina59071320]

42 **del Mar O'Callaghan M**, Emperador S, López-Gallardo E, Jou C, Buján N, Montero R, Garcia-Cazorla A, Gonzaga D, Ferrer I, Briones P, Ruiz-Pesini E, Pineda M, Artuch R, Montoya J. New mitochondrial DNA mutations in tRNA associated with three severe encephalopamyopathic phenotypes: neonatal, infantile, and childhood onset. *Neurogenetics* 2012; **13**: 245-250 [PMID: 22638997 DOI: 10.1007/s10048-012-0322-0]

43 **Ji Y**, Zhang J, Liang M, Meng F, Zhang M, Mo JQ, Wang M, Guan MX. Mitochondrial tRNA variants in 811 Chinese probands with Leber's hereditary optic neuropathy. *Mitochondrion* 2022; **65**: 56-66 [PMID: 35623556 DOI: 10.1016/j.mito.2022.05.003]

44 **Williams TL**, Yin YW, Carter CW Jr. Selective Inhibition of Bacterial Tryptophanyl-tRNA Synthetases by Indolmycin Is Mechanism-based. *J Biol Chem* 2016; **291**: 255-265 [PMID: 26555258 DOI: 10.1074/jbc.M115.690321]

45 **Karasik A**, Fierke CA, Koutmos M. Interplay between substrate recognition, 5' end tRNA processing and methylation activity of human mitochondrial RNase P. *RNA* 2019; **25**: 1646-1660 [PMID: 31455609 DOI: 10.1261/rna.069310.118]

46 **Nakamura M**, Nakano S, Goto Y, Ozawa M, Nagahama Y, Fukuyama H, Akiguchi I, Kaji R, Kimura J. A novel point mutation in the mitochondrial tRNA(Ser(UCN)) gene detected in a family with MERRF/MELAS overlap syndrome. *Biochem Biophys Res Commun* 1995; **214**: 86-93 [PMID: 7669057 DOI: 10.1006/bbrc.1995.2260]

47 **Lehmann D**, Schubert K, Joshi PR, Hardy SA, Tuppen HA, Baty K, Blakely EL, Bamberg C, Zierz S, Deschauer M, Taylor RW. Pathogenic mitochondrial mt-tRNA(Ala) variants are uniquely associated with isolated myopathy. *Eur J Hum Genet* 2015; **23**: 1735-1738 [PMID: 25873012 DOI: 10.1038/ejhg.2015.73]

48 **Brun T**, Maechler P. Beta-cell mitochondrial carriers and the diabetogenic stress response. *Biochim Biophys Acta* 2016; **1863**: 2540-2549 [PMID: 26979549 DOI: 10.1016/j.bbamcr.2016.03.012]

49 **Zorova LD**, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, Babenko VA, Zorov SD, Balakireva AV, Juhaszova M, Sollott SJ, Zorov DB. Mitochondrial membrane potential. *Anal Biochem* 2018; **552**: 50-59 [PMID: 28711444 DOI: 10.1016/j.ab.2017.07.009]

50 **Wu C**, Hu X, Jiang Y, Tang J, Ge H, Deng S, Li X, Feng J. Involvement of ERK and Oxidative Stress in Airway Exposure to Cadmium Chloride Aggravates Airway Inflammation in Ovalbumin-Induced Asthmatic Mice. *Toxics* 2024; **12** [PMID: 38668459 DOI: 10.3390/toxics12040235]

51 **Yao B**, Xin ZK, Wang D. The effect of curcumin on on intravitreal proinflammatory cytokines, oxidative stress markers, and vascular endothelial growth factor in an experimental model of diabetic retinopathy. *J Physiol Pharmacol* 2023; **74** [PMID: 38345446 DOI: 10.26402/jpp.2023.6.07]

52 **Picca A**, Calvani R, Coelho-Junior HJ, Landi F, Bernabei R, Marzetti E. Mitochondrial Dysfunction, Oxidative Stress, and Neuroinflammation: Intertwined Roads to Neurodegeneration. *Antioxidants (Basel)* 2020; **9** [PMID: 32707949 DOI: 10.3390/antiox9080647]

53 **Novoselova EG**, Lunin SM, Khrenov MO, Glushkova OV, Novoselova TV, Parfenyuk SB. Pancreas B-Cells in Type 1 and Type 2 Diabetes: Cell Death, Oxidative Stress and Immune Regulation. Recently Appearing Changes in Diabetes Consequences. *Cell Physiol Biochem* 2024; **58**: 144-155 [PMID: 38639210 DOI: 10.33594/000000690]

54 **Nguyen HT**, Do SQ, Kobayashi H, Wakai T, Funahashi H. Negative correlations of mitochondrial DNA copy number in commercial frozen bull spermatozoa with the motility parameters after thawing. *Theriogenology* 2023; **210**: 154-161 [PMID: 37506625 DOI: 10.1016/j.theriogenology.2023.07.027]

55 **Castellani CA**, Longchamps RJ, Sun J, Guallar E, Arking DE. Thinking outside the nucleus: Mitochondrial DNA copy number in health and disease. *Mitochondrion* 2020; **53**: 214-223 [PMID: 32544465 DOI: 10.1016/j.mito.2020.06.004]

56 **Xiao W**, Wang RS, Handy DE, Loscalzo J. NAD(H) and NADP(H) Redox Couples and Cellular Energy Metabolism. *Antioxid Redox Signal* 2018; **28**: 251-272 [PMID: 28648096 DOI: 10.1089/ars.2017.7216]

57 **Wu J**, Jin Z, Zheng H, Yan LJ. Sources and implications of NADH/NAD(+) redox imbalance in diabetes and its complications. *Diabetes Metab Syndr Obes* 2016; **9**: 145-153 [PMID: 27274295 DOI: 10.2147/DMSO.S106087]

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