

## Original Article

# Mutations in mitochondrial tRNA genes may be related to insulin resistance in women with polycystic ovary syndrome

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**Abstract:** Polycystic ovary syndrome (PCOS) is a very common endocrine disorder affecting women of reproductive age. Insulin resistance (IR), a central component of this disease, occurs in 30%-40% of women with PCOS. To date, the molecular mechanism underlying PCOS-IR remains largely unknown. Most recently, increasing evidence has shown that mitochondrial dysfunction caused by mitochondrial DNA (mtDNA) mutations plays important roles in the pathogenesis of PCOS-IR. To identify the contribution of mitochondrial tRNA (mt-tRNA) mutations in this disease, we screened 80 women with PCOS-IR and 50 healthy control participants for mt-tRNA mutations. After genetic amplification and direct sequencing, we identified nine mt-tRNA mutations that were potentially associated with PCOS-IR: mt-tRNA<sup>Leu(UUR)</sup> A3302G and C3275A mutations, mt-tRNA<sup>Gln</sup> T4363C and T4395C mutations, mt-tRNA<sup>Ser(UCN)</sup> C7492T mutation, mt-tRNA<sup>Asp</sup> A7543G mutation, mt-tRNA<sup>Lys</sup> A8343G mutation, mt-tRNA<sup>Arg</sup> T10454C mutation and mt-tRNA<sup>Glu</sup> A14693G mutation. These mutations were localized at evolutionarily conserved nucleotides and altered the secondary structure of mt-tRNAs, thus resulting in failure of mt-tRNA metabolism. Moreover, molecular and biochemical analysis revealed that levels of 8-OHdG, malondialdehyde and reactive oxygen species were increased in patients with PCOS-IR carrying these mt-tRNA mutations compared with in healthy control participants, whereas superoxide dismutase levels, mitochondrial copy number, membrane potential and ATP levels were significantly reduced. Taken together, our data indicate that mt-tRNAs are key locations for pathogenic mutations associated with PCOS-IR. Mitochondrial dysfunction caused by mt-tRNA mutations may be involved in the pathogenesis of PCOS-IR. Thus, our findings provide novel insight into the pathophysiology of this disorder.

**Keywords:** Mitochondrial tRNA, mutation, oxidative stress, mitochondrial dysfunction, PCOS-IR

## Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder affecting 5%-10% women of reproductive age [1]. It is characterized by hyperandrogenism, oligomenorrhea or amenorrhea, anovulatory cycles and presence of multiple cysts in the ovaries detectable by ultrasound examination. Insulin resistance (IR), a central component of this disease, occurs in 30%-40% of women with PCOS [2-4]. IR is considered the main pathological factor responsible for the hormonal disturbance associated with the syndrome [5]. However, the molecular mechanism underlying PCOS-IR remains still poorly understood.

Mitochondria play a central role in energy producing in the form of ATP through oxidative phosphorylation (OXPHOS) [6]. Because ATP is essential for many cellular processes, mitochondrial function or dysfunction plays a critical role in metabolic health and cellular fate. Alterations in mitochondrial function, dynamics and biogenesis are often associated with peripheral IR and glucose intolerance [7, 8]. In addition, reduced expression of nuclear-encoded genes involved in OXPHOS has been reported in skeletal muscle of patients with PCOS [9]. Moreover, analysis of oxygen consumption in blood leukocytes has indicated that mitochondrial complex I respiration is reduced in women with PCOS compared with in age- and BMI-

matched control subjects [10]. Because of the significant role of mitochondrial impairment in PCOS, recent clinical and experimental studies have focused on mutations in the mitochondrial genome.

To understand the molecular basis of PCOS, we recently performed a mutational analysis of the mitochondrial genomes of women with PCOS [11]. After PCR amplification and direct sequence analysis, we noted that mtDNA mutations occurred more frequently in patients with PCOS than in the healthy control participants. Specifically, *ND1* T3394C, *ND5* T12811C and A6 G8584A and C8684T mutations in mitochondrial protein-coding genes were found to be associated with PCOS. Interestingly, mt-tRNA<sup>Leu(UUR)</sup> A3302G [12], mt-tRNA<sup>Gln</sup> T4395C, mt-tRNA<sup>Ser(UCN)</sup> C7492T [13], mt-tRNA<sup>Asp</sup> A7543G, mt-tRNA<sup>Lys</sup> A8343G, mt-tRNA<sup>Arg</sup> T10454C and mt-tRNA<sup>Glu</sup> A14693G mutations occurred at evolutionarily conserved nucleotides of the corresponding tRNAs, thus suggesting mt-tRNA dysfunction may be involved in the pathogenesis of PCOS [11]. However, the role of mitochondrial dysfunction in PCOS patients carrying these mt-tRNA mutations remained poorly understood.

In this study, we first performed PCR-Sanger sequencing to detect potential pathogenic mt-tRNA mutations in 80 patients with PCOS-IR and 50 healthy control participants. Additionally, we evaluated the relationships between oxidative stress, mitochondrial dysfunction and PCOS-IR using trans-mitochondrial cybrid cells containing these mt-tRNA mutations.

### Materials and methods

#### *Study population*

Between January 2015 and January 2016, a total of 166 unrelated women with PCOS, aged 18 to 37 years old, were admitted to the Department of Gynecology and Obstetrics of Hangzhou First People's Hospital. After hormone level assessment, 80 women were diagnosed with PCOS-IR and were included in this study. Additionally, 50 age- and BMI-matched healthy control participants were recruited. Informed consent and blood samples were obtained and clinical evaluation was conducted for each participant under a protocol approved by the Ethics Committee of Hangzhou First People's Hospital. Subjects with PCOS were

diagnosed according to the Rotterdam criteria [14], which comprise oligoovulation (cycles lasting longer than 35 d or less than 26 d) [15], elevated free testosterone levels (>0.5 ng/dl, the cutoff level for free testosterone level was the mean  $\pm$  2SD according to normal levels in controls), hirsutism (total Ferriman-Gallwey score >7), and polycystic ovaries (identified by transvaginal ultrasonography and following the Rotterdam criteria, which defines PCOS as the presence of 12 or more small (2~9 mm) follicles in each ovary). Ultrasound scans were performed and scored independently by one of two experienced and blinded reviewers. Control participants had regular menses, normal glucose tolerance, and no family history of diabetes. None of the participants had galactorrhea or any endocrine or systemic disease that could affect her reproductive physiology. No participant reported having used any medication that could interfere with the normal function of the hypothalamic-pituitary-gonadal axis during the previous semester.

The exclusion criteria were organic, malignant, hematological, infectious or inflammatory disease and/or a history of ischemic heart disease, stroke, thromboembolism, diabetes mellitus, hyperlipidemia or hypertension.

#### *Laboratory assessment*

Detailed demographics and anthropometrics, vital parameters, medical history, drug administration, history of drug administration and medication and pedigree information were recorded for each participant through personal interviews. Blood samples were collected in the morning between 07:00 and 10:00 after an overnight fast. Luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E<sub>2</sub>), total testosterone (TT) and insulin levels were analyzed by the chemiluminescence techniques (Roche, Indianapolis). Glucose levels were measured using enzymatic techniques and a Dax72 autoanalyzer (Bayer Diagnostic, Tarrytown). Additionally, the homeostasis model assessment (HOMA) index was used to estimate IR, and was calculated as follows: HOMA-IR= (fasting insulin [mIU/L]  $\times$  fasting glucose [mmol/L])/22.5, HOMA-IR $\geq$ 2.69 was taken to indicate IR.

#### *Mutational analysis of mt-tRNA genes*

Genomic DNA was isolated from the whole blood of participants using Puregene DNA

## Mt-tRNA mutations and PCOS-IR

**Table 1.** Primer sequences for amplification of 22 mt-tRNAs

Target gene	Primer name	Primer Sequence (5'-3')	Tm (°C)*	Product size
tRNA <sup>Phe</sup>	MT-1F	CTCCTCAAAGCAATACACTG	61	802 bp
	MT-1R	TGCTAAATCCACCTTCGACC		
tRNA <sup>Val</sup>	MT-2F	CGATCAACCTCACCACCTCT	58	802 bp
	MT-2R	TGGACAACCAGCTATCACCA		
tRNA <sup>Leu(UUR)</sup>	MT-4F	AAATCTTACCCCGCTGTTT	60	887 bp
	MT-4R	AGGAATGCCATTGCGATTAG		
tRNA <sup>Ile</sup>	MT-6F	TGG CTC CTT TAA CCT CTC CA	60	898 bp
tRNA <sup>Gln</sup>				
tRNA <sup>Met</sup>	MT-6R	AAG GAT TAT GGA TGC GGT TG		
tRNA <sup>Ala</sup>	MT-8F	CTAACCGGCTTTTGGCC	60	814 bp
tRNA <sup>Asn</sup>				
tRNA <sup>Cys</sup>	MT-8R	ACCTAGAAGGTTGCCTGGCT		
tRNA <sup>Ser(UCN)</sup>	MT-11F	ACGCCAAAATCCATTCTACT	58	987 bp
tRNA <sup>Asp</sup>	MT-11R	CGGGAATTGCATCTGTTTTT		
tRNA <sup>lys</sup>	MT-12F	ACG AGT ACA CCG ACT ACG GC	60	900 bp
	MT-12R	TGG GTG GTT GGT GTA AAT GA		
tRNA <sup>Gly</sup>	MT-15F	TCTCCATCTATTGATGAGGGTCT	60	891 bp
tRNA <sup>Arg</sup>	MT-15R	AATTAGGCTGTGGGTGGTTG		
tRNA <sup>His</sup>	MT-18F	TATCACTCTCCTACTTACAG	55	866 bp
tRNA <sup>Ser(AGY)</sup>				
tRNA <sup>Leu(CUN)</sup>	MT-18R	AGAAGGTTATAATTCCTACG		
tRNA <sup>Glu</sup>	MT-21F	GCATAATTAACCTTTACTTC	55	938 bp
	MT-21R	AGAATATTGAGGCGCCATTG		
tRNA <sup>Thr</sup>	MT-22F	TGAAACTTCGGCTCACTCCT	60	1162 bp
tRNA <sup>Pro</sup>	MT-22R	GAGTGGTTAATAGGGTGATAG		

\*Tm: Annealing Temperature.

Isolation Kits (Genra Systems, Minneapolis). The fragments spanning the 22 mt-tRNA genes of 80 participants with PCOS-IR and 50 controls were amplified by PCR using light-strand and heavy-strand oligonucleotide primer sets (Table 1). Each fragment was purified and subsequently analyzed by direct sequencing in an ABI automated DNA sequencer using a Big Dye Terminator Cycle sequencing reaction kit. The resultant sequence data were compared with the updated consensus Cambridge sequence (GenBank accession number: NC\_012920) [16].

### Phylogenetic conservation analysis

The mtDNA sequences of 15 vertebrates were used for inter-specific analysis. The conservation index (CI) was calculated by comparing the human nucleotide variants with the corresponding sequences in the other 14 verte-

brates [17]. The CI was defined as the percentage of species from the list of 14 different vertebrates that had the wild-type nucleotide at the same position as in the human sequence. A CI  $\geq 70\%$  was considered to have functional potential.

### Assigning pathogenicity to mt-tRNA mutations

To identify mt-tRNA mutations with potential deleterious, we used the following criteria: (1) present in  $<1\%$  of the healthy controls; (2) CI  $\geq 70\%$ , as proposed by Ruiz-Pesini and Wallace [17]; (3) potential to cause structural and functional alterations; and (4) a score of  $\geq 7$  points under an established pathogenicity scoring system [18], according to which a variant was classified as a "neutral polymorphism" if it scored  $\leq 6$  points, as "possibly pathogenic" if it scored 7-10 points, and as "definitely pathogenic" if it scored  $\geq 11$  points. Patients who carried potential pathogenic mt-

tRNA mutations that met these criteria were selected for further molecular and biochemical analysis.

### Functional characterization of mt-tRNA mutations

*Determining plasma 8-OHdG, malondialdehyde (MDA) and superoxide dismutase (SOD) level in subjects carrying pathogenic mutations:* The blood samples were placed into sterile ethylenediaminetetraacetic acid test tubes and centrifuged at 3000 rpm for 20 min at 4°C to separate the plasma. The plasma was stored at -70°C until further analysis. The concentration of 8-OHdG, a critical biomarker of oxidative stress and DNA damage in blood, was measured by ELISA in accordance with the manufacturer's instructions (Nikken Foods, Fukuroi, Japan). The MDA level was determined by the thiobarbituric acid reaction, as described else-

## Mt-tRNA mutations and PCOS-IR

**Table 2.** Clinical and metabolic features of PCOS-IR patients and control subjects

Characteristics	PCOS-IR (n=80)	Control (n=50)	P
Age (y)	27.54±4.74	28.14±4.47	0.47
FSH (IU/L)	5.85±2.08	6.59±3.07	0.10
LH (IU/L)	11.37±7.47	6.9±8.7	<0.05
LH/FSH	1.97±1.08	1.00±0.71	<0.05
PRL (µg/L)	19.94±65.85	15.29±14.15	0.62
E <sub>2</sub> (pmol/L)	245.45±183.31	278.55±193.23	0.33
PRGE (nmol/L)	2.89±1.65	2.17±0.87	0.35
TT (nmol/L)	3.02±0.6	1.48±0.45	<0.001
Insulin (0 h) (µU/mL)	16.79±8.4	6.57±1.45	<0.001
Glucose (0 h) (mmol/L)	5.4±1.53	5.23±0.53	0.45
HOMA-IR	4±2.29	1.53±0.36	<0.001

where [19]. Moreover, the activity of SOD was analyzed using the method described by Winterbourn [20], based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium by superoxide.

### Qualification of mtDNA copy number

The mtDNA content was determined in duplicate runs using a multiplex quantitative PCR (QPCR; Applied Biosystems, Foster City) targeting the nuclear human  $\beta$ -globins gene and the mitochondrial *ND1* gene [21]. The specific primers and probes used to amplify the nuclear  $\beta$ -globins and mitochondrial *ND1* genes were as follows: nuclear  $\beta$ -globin gene; forward, 5'-CTGGGCATGTGGAGACAGAGAAGACT; reverse, 5'-AGGCCATCACTAAAGGCACCGAGC, probe 5'-FAM-CCCTTAGGCTGCTGGTGGTCTACCCTT-TAMRA. Mitochondrial *ND1* gene; forward, 5'-GACGCCATAAACTCTTCACCAA, reverse, 5'-AGGTTGAGGTTGACCAGGGG, probe 5'-FAM-CCATCACCTCTACATCACCGCCC-TAMRA. Reactions included 1x Absolute QPCR Mix containing SYBR Green and ROX (AB-1163, Life Technologies) in the presence of 100 nM mtDNA primers, 360 nM nDNA primers and 100 nM probes. The 45-cycle PCR was carried out at a 62°C annealing temperature and probe fluorescence was monitored using ROX, HEX, CY5 and FAM filters on an Mx3000P or Mx3005P qPCR systems (Agilent Technologies, Waldbronn).

### Cell cultures

Lymphoblastoid cell lines were immortalized by transformation with the Epstein-Barr virus, as described elsewhere [22]. Cell lines derived

from the patients carrying candidate pathogenic mt-tRNA mutations and from 10 age- and BMI-matched control subjects were grown in RPMI1640 medium (Invitrogen, CA), supplemented with 10% fetal bovine serum.

### ATP measurement

ATP levels were determined using a CellTiter-Glo<sup>®</sup> Luminescent Cell Viability Assay kit (Promega, Madison) according to the manufacturer's instructions. Cells were grown in 6-well plates to approximately 80% confluence ( $2 \times 10^4$  cells/well). Briefly, the assay buffer and substrate were equilibrated to room temperature, and the buffer was transferred to 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 and the contents were mixed for 2 min on an orbital shaker to induce cell lysis. After 10 min incubation in room temperature, the luminescence was read on a microplate reader (Synergy H1, Bio-Tek).

### Mitochondrial ROS assessment

Production of ROS was assayed by fluorometry using a Fluoroskan (Thermo LabSystems, Thermo Scientific, Rockford) after incubation of cells for 30 min with the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate ( $5 \times 10^{-6}$  mol/liter), as described elsewhere [23].

### Measurement of mitochondrial membrane potential (MMP)

MMP was assessed with a JC-1 Assay Kit-Microplate (Abcam, Cambridge) following the manufacturer's general recommendations. Cells were incubated for 30 min with the fluorescent probe tetramethylrhodamine methyl ester ( $1 \times 10^{-7}$  mol/liter), and fluorescence was detected using a Fluoroskan (Thermo LabSystems).

### Statistical analysis

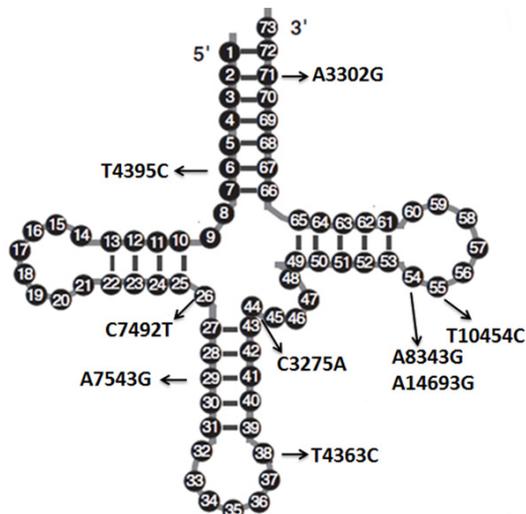
Data are presented as mean  $\pm$  standard deviation of three independent experiments.

## Mt-tRNA mutations and PCOS-IR

**Table 3.** Molecular characterization of mt-tRNA mutations identified by PCR-Sanger sequencing

Genes	Mutation	Number of nucleotide in tRNAs	Location	Homoplasmy/Heteroplasmy	CI (%)	80 patients (%)	50 controls (%)	Disease association
tRNA <sup>Leu(UUR)</sup>	A3302G	71	Acceptor arm	Homoplasmy	100	1 (1.25)	0	MELAS
	C3275A	44	Extra loop	Homoplasmy	86.6	2 (2.5)	0	LHON
tRNA <sup>Gln</sup>	T4363C	38	Anticodon stem	Homoplasmy	73.3	4 (5)	0	Hypertension, Development delay
	T4395C	6	Acceptor arm	Homoplasmy	100	1 (1.25)	0	Hypertension
tRNA <sup>Gys</sup>	G5821A	6	Acceptor arm	Homoplasmy	66.6	3 (3.75)	1 (2)	Deafness
tRNA <sup>Ser(UCN)</sup>	C7492T	26	Anticodon stem	Homoplasmy	100	1 (1.25)	0	PCOS-IR
tRNA <sup>Asp</sup>	A7543G	29	Anticodon stem	Homoplasmy	73.3	1 (1.25)	0	MEPR
tRNA <sup>Lys</sup>	A8343G	54	T-loop	Homoplasmy	86.6	2 (2.5)	0	PD risk factor
tRNA <sup>Arg</sup>	T10454C	55	T-loop	Homoplasmy	93.3	2 (2.5)	0	Deafness
tRNA <sup>Glu</sup>	A14693G	54	T-loop	Homoplasmy	100	1 (1.25)	0	Deafness, Hypertension
tRNA <sup>Met</sup>	T4454C	58	T-loop	Homoplasmy	60	0	2 (4)	Neutral polymorphism
tRNA <sup>Thr</sup>	T15900C	13	D-arm	Homoplasmy	46.6	0	1 (2)	Neutral polymorphism

Abbreviations: MELAS: Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; LHON: Leber's hereditary optic neuropathy; MEPR: Myoclonic epilepsy and psychomotor regression; PD: Parkinson's disease.



**Figure 1.** Cloverleaf structure of mt-tRNA with standard nucleotide numbering, arrows indicated the mutations that were identified to be associated with PCOS-IR.

Statistical significance was evaluated by one-way ANOVA or an independent Student's *t* test using SPSS18.0 software (IBM, Armonk).  $P < 0.05$  was considered statistically significant.

## Results

### *Clinical and biochemical characterization of women with PCOS-IR*

The clinical and biochemical characteristics of participants in this study are listed in **Table 2**. The 216 participants enrolled in this study included 80 patients diagnosed with PCOS-IR and 50 healthy controls. There were no significant difference in age or FSH, PRL,  $E_2$ , PRGE or fasting glucose levels between patients with PCOS-IR and healthy control participants. In contrast, women with PCOS-IR had significantly higher LH, LH/FSH, TT and fasting insulin levels and HOMA-IR with respect to the control group ( $P < 0.05$  for all).

### *Mutational analysis of the 22 mt-tRNA genes*

DNA fragments spanning the 22 mt-tRNA genes were amplified by PCR from genomic DNA of each individual. After PCR amplification, the fragments were purified and subsequently analyzed by DNA sequencing. Comparison of the resultant sequence with the Cambridge consensus sequence identified 12 nucleotide changes in 10 mt-tRNA genes, as shown in

**Table 3** and **Figure S1**. These mutations were as follows: mt-tRNA<sup>Leu(UUR)</sup> A3302G and C3275A, mt-tRNA<sup>Gln</sup> T4363C and T4395C, mt-tRNA<sup>Cys</sup> G5821A, mt-tRNA<sup>Ser(UCN)</sup> C7492T, mt-tRNA<sup>Asp</sup> A7543G, mt-tRNA<sup>Lys</sup> A8343G, mt-tRNA<sup>Arg</sup> T10454C, mt-tRNA<sup>Glu</sup> A14693G, mt-tRNA<sup>Met</sup> T4454C and mt-tRNA<sup>Thr</sup> T15900C. All of these nucleotide alternations were verified by sequence analysis of both strands and appeared to be homoplasmy. Among the 50 healthy subjects, one carried the mt-tRNA<sup>Cys</sup> G5821A mutation, two carried the tRNA<sup>Met</sup> T4454C mutation and one carried the mt-tRNA<sup>Thr</sup> T15900C mutation. None of other 76 subjects harbored any mt-tRNA mutations.

### *Evaluation of PCOS-IR related mt-tRNA mutations*

We evaluated candidate pathogenic mt-tRNA mutations using the four criteria described in the Materials and methods. We first used the secondary structure of tRNAs to localize each mutation to either a stem or a loop and analyzed whether the base changes within stems altered the Watson-Crick base pairing. As shown in **Figure 1**, three mutations (A3302G, T4395C and A7543G) disrupted putative base-pairing. Additionally, we performed a phylogenetic conservation analysis by comparing the human mt-tRNA nucleotide alternations with the relevant sequences in 14 other vertebrates. For this analysis, we used the tRNA<sup>Gln</sup> T4395C and T4363C mutations as examples. The T4395C (position 6) and T4363C (position 38) mutations were localized at highly conserved nucleotides in the corresponding tRNA, with CIs of 100% and 73.3%, respectively (**Figure 2**). The CIs among the mutations ranged from 46.6% (tRNA<sup>Thr</sup> T15900C mutation) to 100% (tRNA<sup>Leu(UUR)</sup> A3302G, tRNA<sup>Gln</sup> T4395C, tRNA<sup>Ser(UCN)</sup> C7492T and tRNA<sup>Glu</sup> A14693G mutations) (**Table 3**). Notably, there were nine mutations with  $CI \geq 70\%$ . Moreover, we identified two mutations (tRNA<sup>Met</sup> T4454C and tRNA<sup>Thr</sup> T15900C) that occurred only in healthy participants but were absent in women with PCOS-IR, which suggests that they were neutral polymorphisms.

### *Pathogenicity scoring system for mt-tRNA mutations*

To further evaluate the deleterious role of mt-tRNA mutations, we used an updated version of the pathogenicity scoring system originally pro-

## Mt-tRNA mutations and PCOS-IR

Organism	4395				4363												
	Acc-stem	D-stem	D-loop	D-stem	Acc-stem	Anticd-loop	Acc-stem	V-region	T-stem	T-loop	T-stem	Acc-stem					
	1 6 8 10			22	26 27	32 38	39	44	49	58	61	66	73				
<i>Cebus albifrons</i>	TAGAGTA	TG GTGT	AATAGGTA	GCAC	G	GAGGA	TTTTGAG	TTCTT	AGGA	ATAGG	TTGAGT	CCTAT	AATTCTA	G			
<i>Didelphis virginiana</i>	TAGAAATA	TG GTGT	AAAGGAA	ACAC	A	TGGA	TTTTGAG	TTCTA	TAAT	GTAGG	TTCAATT	CCTAT	TGTTCTA	G			
<i>Mus musculus</i>	TAGGATA	AG GTGT	TTAGGTA	GCAC	G	GAGAA	TTTTGAA	TTCTT	AAGT	GTAGG	TTCAATT	CCTAT	TGTOCTA	G			
<i>Gorilla gorilla</i>	TAGGATG	GG GTGT	GACAGGTG	GCAC	G	GAGAA	TTTTGGA	TTCTC	AGGG	ATGGG	TTCAATT	CTCAT	AGTOCTA	G			
<i>Rattus norvegicus</i>	TAGGATA	GG GTGT	ATTGGTG	GCAC	G	GAGAA	TTTTGAA	TTCTT	AGGT	GTAGG	TTCAATT	CCTAT	TGTOCTA	G			
<i>Homo sapiens</i>	TAGGATG	GG GTGT	GATAGGTG	GCAC	G	GAGAA	TTTTGGA	TTCTC	AGGG	ATGGG	TTGATT	CTCAT	AGTOCTA	G			
<i>Pan troglodytes</i>	TAGGATG	GG GTGT	GATAGGTG	GCAC	G	GAGAA	TTTTGGA	TTCTC	AGGG	ATGAG	TTGATT	CTTAT	AGTOCTA	G			
<i>Papio hamadryas</i>	TAGGATG	GA GTGT	GAGGGGTA	GCAC	G	GAGAA	GTTTGA	TTCTC	AGGA	GCAGG	TTGAGT	CCTGT	AATCTA	G			
<i>Tarsius bancanus</i>	TAGAAATA	TT GTGT	AATTGGGTA	GCAC	G	AAGAA	TTTTGGA	TTCTT	AAGT	ATAGG	TTCAATT	CCTGT	AATTCTA	G			
<i>Lemur catta</i>	TAGAAATA	GG GTGT	GTTTAGGTA	GCAC	G	AAGAA	CTTTGAA	TCCTT	AAGA	GCAGG	TTGATC	CTGT	GATTCTA	G			
<i>Macaca sylvanus</i>	TAGGATG	GG GTGT	ATGAGGTA	GCAC	G	GAGAA	GTTTGGG	TTCTC	AGGG	GTAGG	TTGATA	CCTAC	AGTOCTA	G			
<i>Pongo pygmaeus</i>	TAGGATG	GG GTGT	GATGGGTG	GCAC	G	GAGAA	TTTTGGA	TTCTC	AGGG	GTGGG	TTCAATT	CCCAT	AGTOCTA	G			
<i>Vombatus ursinus</i>	TAGAAATG	TG GTGT	AATGGAA	ACAC	A	GAGGA	TTTTGAG	TTCTC	TGAT	ATGGG	TTGAGT	CCTAT	TGTTCTA	G			
<i>Chrysochloris asiatica</i>	TAGAAATA	TG GTGT	ATATGGTA	GCAC	G	AAGAT	TTTTGAA	TTCTT	AGGT	TTAGG	TTGAGT	CCTAA	AATTCTA	G			
<i>Tachyglossus aculeatus</i>	TAGAGTA	TG GTGT	AATTGAGA	GCAC	G	GAGGT	TTTTGAA	GCCTT	AGAT	GTAGG	TTTAAGT	CCTAT	TATTCTA	G			

**Figure 2.** Alignment of mt-tRNA<sup>Gln</sup> genes from different species. The arrows indicated the position 6 and 38, corresponding to the T4395C and T4363C mutations.

posed by Yarham et al. [20]. This pathogenicity scoring system employs a number of weighted criteria covering a range of molecular and genetic data, from which an overall pathogenicity score can be obtained. We again used the T4395C and T4363C mutations as examples. The T4395C and T4363C mutations scored 13 and 11 points, respectively, which suggest that both should be regarded as “definitely pathogenic” (Table 4). The A3302G, C3275T, G5821A, C7492T, A7543G, A8343G, T10454C, A14693G, T4454C and T15900C mutations scored 18, 11, 6, 8, 7, 8, 8, 10, 4 and 4 points, respectively. With the exception of the G5821A, T4454C and T15900C variants, the mutations identified were therefore classified as pathogenic mutations according to this scoring system.

### Increased oxidative stress in women with PCOS-IR

To verify the hypothesis that oxidative stress level was altered in the PCOS-IR group, MDA and intracellular 8-OHdG levels were tested. Levels of 8-OHdG and MDA were significantly higher in the PCOS-IR group than in healthy controls (Figure 3;  $P < 0.05$  for all). Conversely, SOD levels were lower in the PCOS-IR group than in the control participants ( $P < 0.05$ ).

### MtDNA copy number analysis

The mtDNA copy number is a relative measure of the cellular number of mitochondria. Damage to mtDNA is considered to be an alternate mea-

sure of mitochondrial dysfunction because it leads to reduced cellular metabolic activity [24]. As shown in Figure 4, the mitochondrial copy number was significantly lower in the PCOS-IR group carrying the mt-tRNA mutations than in the control group ( $P < 0.05$ ).

### Analysis of mitochondrial ATP, ROS and MMP

To examine whether mutations in mt-tRNA genes impaired mitochondrial function, cells derived from the individuals carrying the mtDNA mutations identified above and from 10 healthy age- and BMI-matched participants were used for analyzing mitochondrial functions including mitochondrial ATP synthesis, ROS production and MMP. As shown in Figure 4, mitochondrial ATP and MMP were significantly lower in cells derived from patients with PCOS-IR than in those derived from control subjects, whereas increased ROS levels were observed in cells from the PCOS-IR group ( $P < 0.05$  for all). This suggests that impaired mitochondrial function may be associated with PCOS-IR.

### Discussion

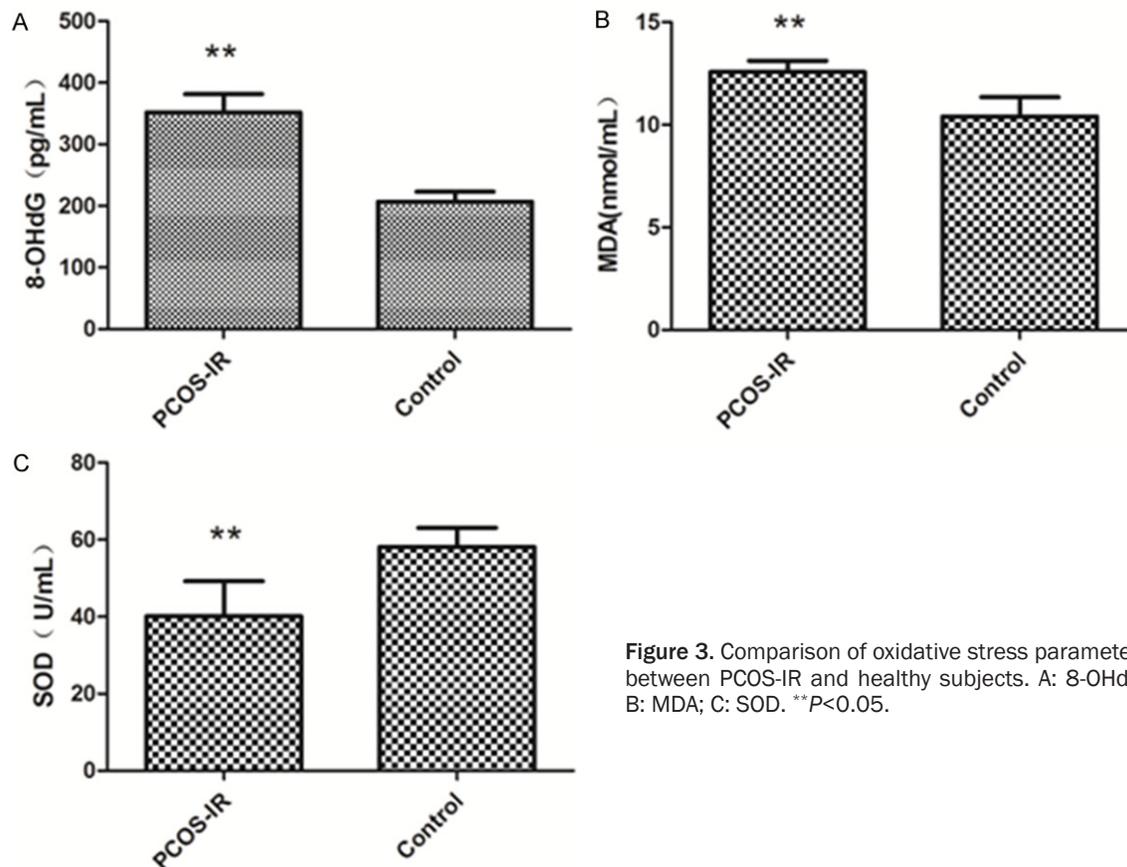
In the present study, we investigated the possible roles of mt-tRNA mutations in women with PCOS-IR. PCOS is a heterogeneous syndrome associated with a wide range of endocrine and metabolic abnormalities, including hyperinsulinemia, hyperglycemia and dyslipidemia, which are regarded as hallmark components of metabolic syndrome. IR plays an important role in the pathogenesis of PCOS and increases the

## Mt-tRNA mutations and PCOS-IR

**Table 4.** The pathogenicity scoring system for T4395C and T4363C mutations

Scoring criteria	T4395C mutation	Score	T4363C mutation	Score	Classification
More than one independent report	Yes	2	Yes	2	
Evolutionary conservation of the base pair	No changes	2	2 changes	0	
Variant heteroplasmy	No	0	No	0	≤6 points: neutral polymorphisms;
Segregation of the mutation with disease	Yes	2	Yes	2	7~10 points: possibly pathogenic;
Histochemical evidence of mitochondrial disease	Strong evidence	2	Strong evidence	2	≥11 points (including trans-mitochondrial cybrid studies): definitely pathogenic.
Biochemical defect in complex I, III or IV	No	0	No	0	
Evidence of mutation segregation with biochemical defect from single-fiber studies	No	0	No	0	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Strong evidence	5	Strong evidence	5	
<b>Maximum score</b>		<b>13</b>		<b>11</b>	<b>Definitely pathogenic</b>

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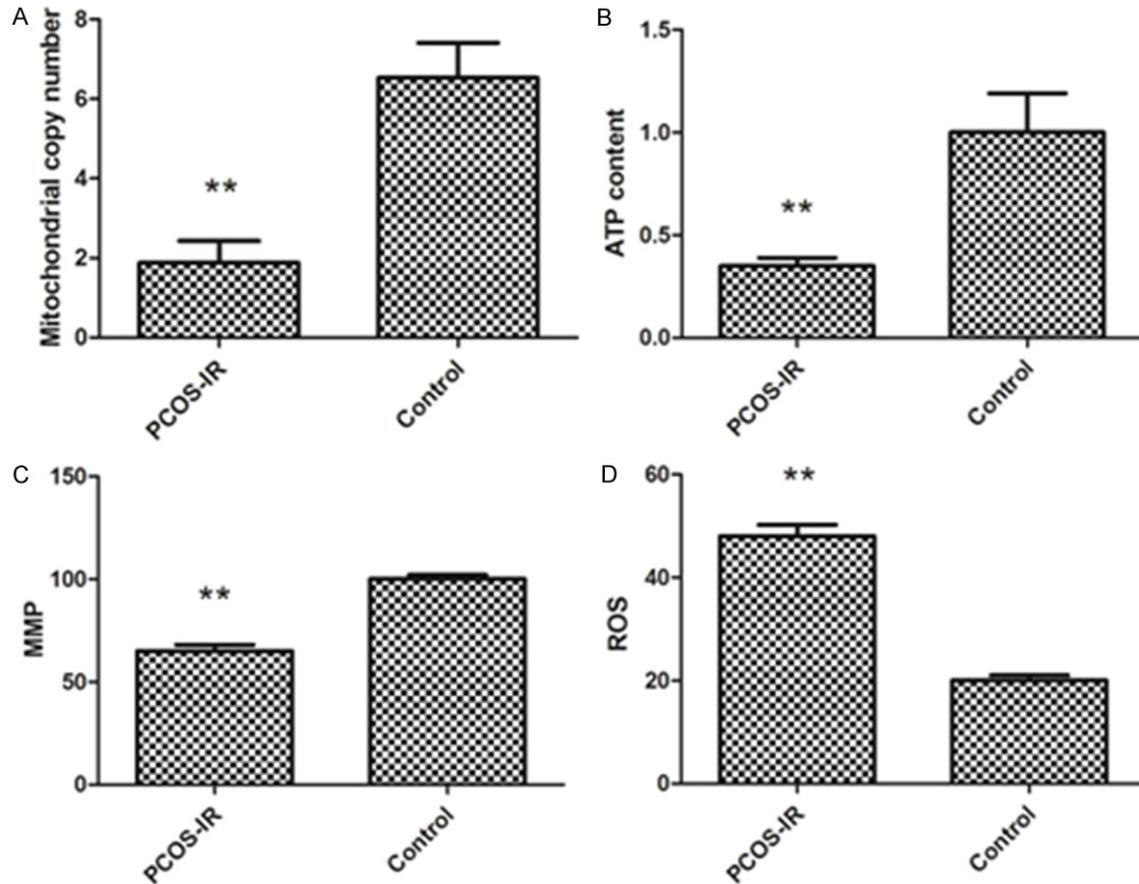
**Figure 3.** Comparison of oxidative stress parameters between PCOS-IR and healthy subjects. A: 8-OHdG; B: MDA; C: SOD. \*\* $P < 0.05$ .

risk of type 2 diabetes mellitus [25]. Landmark studies relating IR to mitochondrial dysfunction reported reduced  $\text{NADH}_2\text{-O}_2$  oxidoreductase activity and structural mitochondrial aberrations [26], and deficiency of subsarcolemmal mitochondrial function [27], in patients with type 2 diabetes mellitus, as compared with healthy controls, suggesting potential pathogenic roles of mitochondrial dysfunction in IR.

The human mitochondrial genome encodes 13 polypeptides involved in OXPHOS, 2 rRNAs and 22 tRNAs required for mitochondrial protein synthesis [18]. Among these tRNAs, the genes encoding  $\text{tRNA}^{\text{Glu}}$ ,  $\text{tRNA}^{\text{Ala}}$ ,  $\text{tRNA}^{\text{Asn}}$ ,  $\text{tRNA}^{\text{Cys}}$ ,  $\text{tRNA}^{\text{Tyr}}$ ,  $\text{tRNA}^{\text{Ser(UCN)}}$ ,  $\text{tRNA}^{\text{Gln}}$  and  $\text{tRNA}^{\text{Pro}}$  resided in the cytosine-rich light strand; the genes encoding the remaining tRNAs,  $\text{tRNA}^{\text{Phe}}$ ,  $\text{tRNA}^{\text{Val}}$ ,  $\text{tRNA}^{\text{Leu(UUR)}}$ ,  $\text{tRNA}^{\text{Leu(CUN)}}$ ,  $\text{tRNA}^{\text{Ile}}$ ,  $\text{tRNA}^{\text{Met}}$ ,  $\text{tRNA}^{\text{Ser(AGY)}}$ ,  $\text{tRNA}^{\text{Trp}}$ ,  $\text{tRNA}^{\text{Asp}}$ ,  $\text{tRNA}^{\text{Lys}}$ ,  $\text{tRNA}^{\text{Gly}}$ ,  $\text{tRNA}^{\text{Arg}}$ ,  $\text{tRNA}^{\text{His}}$  and  $\text{tRNA}^{\text{Thr}}$  are located in the guanine-rich heavy strand. Point mutations in the genes encoding mt-tRNAs are increasingly being recognized as important causes of diseases; such mutations can result in transcrip-

tional and translational defects and consequently mitochondrial respiratory chain dysfunction [28], and have been shown to be associated with clinical features such as hypertension [29], deafness [30] and PCOS [11-13]. In the current study, we used PCR-Sanger sequencing to identify nine potential pathogenic mt-tRNA mutations. Notably, these tRNA mutations occurred at highly conserved nucleotides. Among the mutations identified, the  $\text{tRNA}^{\text{Leu(UUR)}} \text{A3302G}$  (position 71) and  $\text{tRNA}^{\text{Gln}} \text{T4395C}$  (position 6) mutations were localized in the acceptor arm of mt-tRNA and disrupted highly conserved base-pairings. Moreover, the A3302G mutation has been reported to be associated with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke syndrome), cardiomyopathy, and myopathy with respiratory insufficiency [31-33], the T4395C mutation has been demonstrated to be associated with maternally inherited hypertension [34]. The T4363C (position 38), C7492T (position 26) and A7543G (position 29) mutations occurred in the anticodon stem of the corresponding tRNAs, and were highly conserved in

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**Figure 4.** Mitochondrial function analysis. A: Mitochondrial copy number; B: ATP; C: MMP; D: ROS. \*\* $P < 0.05$ .

14 other vertebrates. Nucleotides at these positions were often modified, and contributed to the high fidelity of codon and anticodon interaction [35], thus, it can be speculated that the T4395C, C7492T and A7543G mutations may reduce the steady state level of tRNAs. The A8343G mutation in the tRNA<sup>Lys</sup> gene is considered a risk factor underlying respiratory chain deficiency [36]. Additionally, the A14693G and T10454C mutations, which were located in the T-loop of tRNA<sup>Glu</sup> and tRNA<sup>Arg</sup>, respectively, have been reported to increase the penetrance and expressivity of the deafness-associated mitochondrial 12S rRNA A1555G mutation [37]. Furthermore, the C3275A mutation, located in the extra loop of the tRNA<sup>Leu(UUR)</sup> was reported to be associated with Leber's Hereditary Optic Neuropathy [38]. Mutations in these tRNAs reduced tRNA stability and affected the tertiary structure of the mt-tRNAs.

To investigate the effects of mt-tRNA mutations, we examined oxidative stress and mitochondrial function using trans-mitochondrial

cybrid cells containing the primary mt-tRNA mutations identified in the patients. Because pathogenic mtDNA mutations have been suggested to be an indicator of oxidative stress, we analyzed ROS generation and levels of oxidative damage (indicated by 8-OHdG and MDA levels) in blood samples at the DNA level. As expected, oxidative stress and damage levels in PCOS-IR were higher than those in control individuals. Moreover, in response to oxidative stress, the antioxidant system was activated to mitigate the deleterious effects [39]. SOD is a superoxide radical-scavenging mitochondrial enzyme and an important component of the cellular antioxidant system. A significantly lower level of SOD was observed in the PCOS-IR group, which indicates that disturbance of the equilibrium via increased of oxidative stress and decreased antioxidant capacity may be involved in the pathogenesis of PCOS-IR.

We further analyzed mitochondrial dysfunction by examining mitochondrial copy number, ATP and ROS levels and MMP. Increased mitochon-

drial ROS generation and defects have been implicated in the pathogenesis of IR [40]. Moreover, ROS plays an important role in hyperglycemia-mediated microvascular complications and endothelial dysfunction in IR-related conditions [41, 42]. Recent experimental studies have suggested that mtDNA alternations play a fundamental role in the increase in ROS [43] and that maintenance of mtDNA copy number is essential for the preservation of mitochondrial function and cell growth [44]. In this study, an average reduction of approximately 55% was observed in mitochondrial ATP production in cybrid cells carrying the tRNA mutations identified in patients with PCOS, compared with those carrying the mt-DNA of healthy controls. This was likely a consequence of a decrease in the proton electrochemical potential gradient in mutant mitochondria [45]. MMP reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and OXPHOS [46]. Our results showed approximately a ~35% reduction in MMP in cells carrying the tRNA mutations, which may have been due to substantially decreased efficiency of respiratory chain-mediated proton extrusion from the matrix. The reduction in both mitochondrial copy number and MMP would evaluate the production of ROS in mutant cells carrying these tRNA mutations.

Based on these observations, we propose that the possible molecular mechanisms underlying the role of mt-tRNA mutations in the pathogenesis of PCOS-IR may be as follows: first, the mutation itself disrupts the secondary structure of mt-tRNA, thus causing a failure in mt-tRNA metabolism, such as CCA addition, post-transcriptional modification or aminoacylation [47]. Failures in mt-tRNA metabolism caused by these mutations would impair mitochondrial protein synthesis and respiration. Second, abnormal mitochondrial respiration causes oxidative stress and uncoupling of oxidative pathways for ATP synthesis [48], which causes the pancreatic  $\beta$ -cell dysfunction and apoptosis, leading to decreased insulin secretion [49, 50]. As a result, insulin fails to suppress hepatic glucose production or stimulate glucose uptake by peripheral tissues; this would lead to the IR that is responsible for PCOS.

In conclusion, our study showed that mutations in the mitochondrial genome, especially in mt-

tRNA genes, were important causes of PCOS-IR. Moreover, pathogenic mt-tRNA mutations would lead to mitochondrial dysfunction involved in the pathogenesis of PCOS-IR. The main limitation of this study was the relatively small population. Further studies including a large sample size need to be performed.

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### Disclosure of conflict of interest

None.

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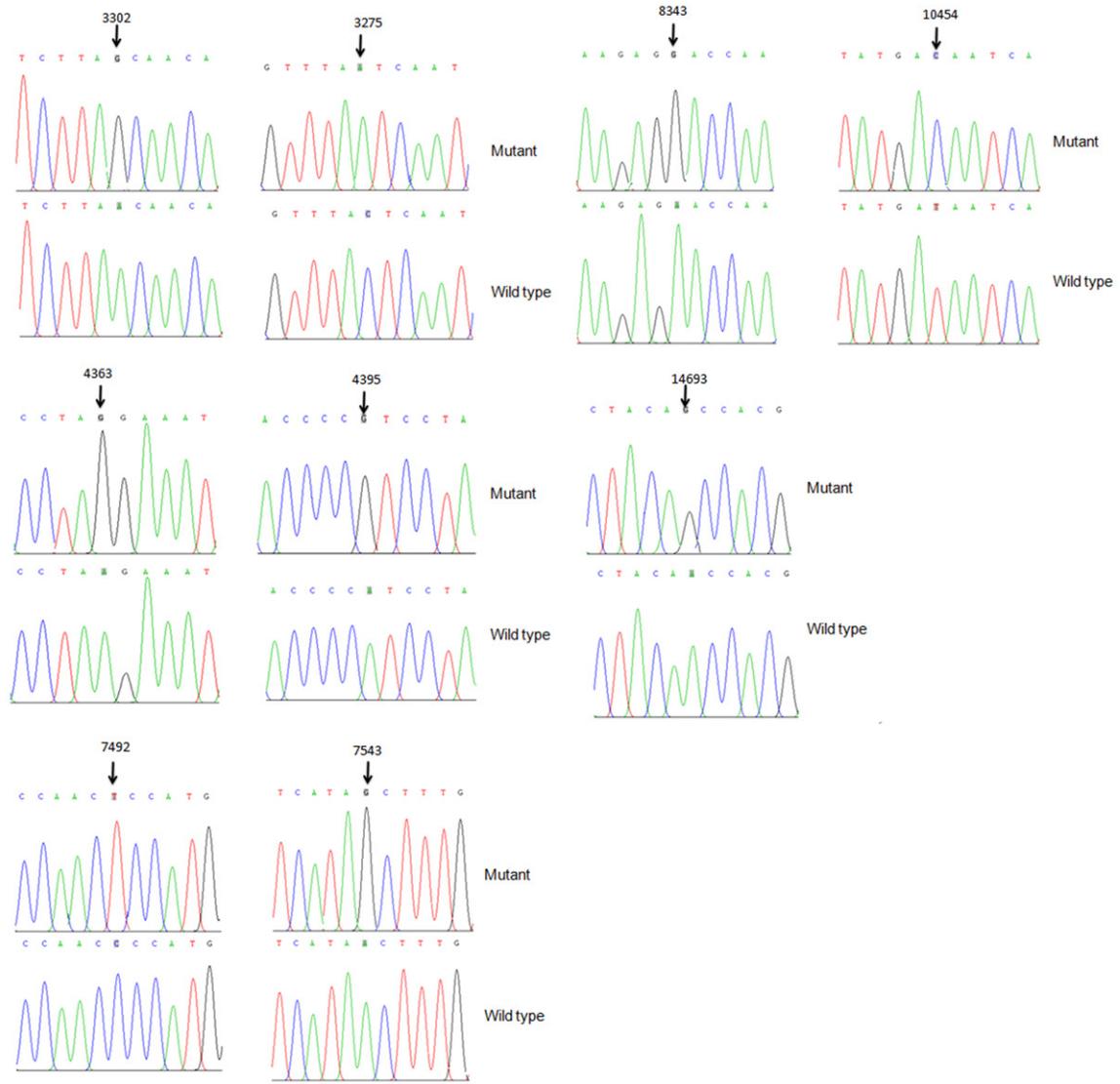
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**Figure S1.** Identification of nine PCOS-IR associated mt-tRNA mutations using PCR-Sanger sequencing.