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The Mitochondrial tRNA$^{Leu(UUR)}$ A3302G Mutation may be Associated With Insulin Resistance in Woman With Polycystic Ovary Syndrome

Yu Ding, MD[1,2], Guangchao Zhuo, MD[1,2], and Caijuan Zhang, MD[2,3]

Abstract

The aim of this study was to investigate the role of mitochondrial DNA (mtDNA) mutations in polycystic ovary syndrome (PCOS) with insulin resistance (IR), and to explore the possible maternally effects on PCOS. We performed clinical, genetic, and molecular characterization of a Han Chinese family with maternally inherited IR, and we further investigated the possible relationship between mitochondrial genetic background, copy number, and IR. Most strikingly, members from the first and second generation of this family exhibited the type 2 diabetes mellitus (T2DM) with IR, while the member in the third generation of this family manifested the PCOS. Sequence analysis of the complete mitochondrial genome showed the presence of a homoplasmic A3302G in the acceptor arm of transfer RNA$^{Leu(UUR)}$ (tRNA$^{Leu(UUR)}$) gene. This mutation disrupted the highly conserved base pairing (2T-71A) and resulted a failure in mt-tRNA metabolism. Analysis of the mitochondrial copy number showed that the patients with PCOS and IR had lower copy number than the health controls, suggesting that mitochondrial dysfunction may be involved in the pathogenesis of IR. Taken together, the A3302G mutation was a pathogenic mutation associated with IR in this Chinese family.

Keywords

mitochondrial tRNA$^{Leu(UUR)}$, A3302G mutation, PCOS, IR, maternally inherited

Introduction

Polycystic ovary syndrome (PCOS) was one of the most common endocrine disorders of reproductive-age women and was characterized by hirsutism, chronic anovulation, and infertility.[1] It was the most common endocrinopathy in younger women, with an estimated prevalence of 5% to 10% in the general population.[2] Disturbance of carbohydrate metabolism, which was manifested by insulin resistance (IR) or compensatory hyperinsulinemia, was now known to be intrinsic to PCOS and contributed in a major way to its pathogenesis. It was reported that 50% to 70% of patients with PCOS had various degree of IR.[3]

IR was defined as a relative impairment in the ability of insulin to exert its effects on glucose, protein, and lipid metabolism in target tissues. Mitochondria were the main cellular sites devoted to adenosine triphosphate (ATP) production and fatty acid oxidation. As ATP was essential for many cellular processes, mitochondrial function (dysfunction) played an important role in metabolic health and cellular fate. There was considerable evidence suggesting a causal link between mitochondrial dysfunction and IR.[4-6] Moreover, in patients with PCOS, reduced expression of nuclear-encoded genes involved in oxidative phosphorylation (OXPHOS) had been demonstrated in skeletal muscle.[7] This reduction in expression of OXPHOS genes was similar to what had been demonstrated in patients with type 2 diabetes mellitus (T2DM) and participants with a family history of T2DM.[8] The demonstration of impaired skeletal muscle mitochondrial function would provide stronger evidence for the central role of mitochondrial impairment in PCOS.

Most recently, with the purpose of understanding the molecular mechanism underlying mitochondrial DNA (mtDNA) mutations and PCOS, we sequenced the complete mitochondrial genome of 57women with PCOS and 38 controls. We found that mtDNA mutations occurred more frequently in women with PCOS, moreover, mitochondrial OXPHOS complexes and mitochondrial transfer RNA (mt-tRNA) genes were hot spots for pathogenic mutations associated with PCOS.[9] It is interestingly to note that the tRNA^{Gln} T4395C,

[1] Central laboratory, Hangzhou First People's Hospital, Hangzhou, China
[2] Affiliated Hangzhou Hospital, Nanjing Medical University, Hangzhou, China
[3] Department of Gynecology and Obstetrics, Hangzhou First People's Hospital, Hangzhou, China

Corresponding Author:
Yu Ding, Central laboratory, Hangzhou First People's Hospital, Nanjing Medical University, Hangzhou, 310006, China.
Email: dingyu.zj@gmail.com

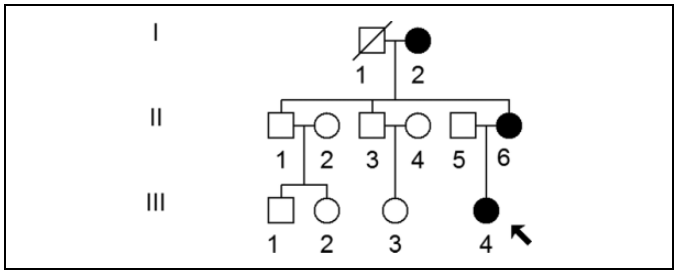


Figure 1. One Han Chinese pedigree with insulin resistance (IR), patients are indicated by filled symbols. Arrowhead denoted the proband.

$tRNA^{Cys}$ G5821A, $tRNA^{Asp}$ A7543G, $tRNA^{Lys}$ A8343G, $tRNA^{Arg}$ T10454C, and $tRNA^{Glu}$ A14693G mutations occurred at the highly conserved nucleotides of the corresponding tRNA genes may cause the structural and functional alternations and consequently result a failure in tRNA metabolism. Impairment in tRNA metabolism will lead to the reduced rate of mitochondrial protein synthesis that was responsible for the mitochondrial dysfunction and contributed to the clinical expression of PCOS. To further understand the molecular mechanism of PCOS, we performed a systematic and extensive mutational screening for the mtDNA mutations of patients with PCOS in Hangzhou from Zhejiang Province. In this study, we reported a Han Chinese family with IR, sequence analysis of the mitochondrial genome showed the presence of A3302G mutation in $tRNA^{Leu(UUR)}$ gene.

Materials and Methods

Participants

As a part of genetic screening program for PCOS, one Han Chinese family, as shown in Figure 1, was ascertained through the Department of Gynecology and Obstetrics in Hangzhou First People's Hospital, China. Moreover, a total of 200 Han Chinese controls were obtained from a panel of unaffected individuals from the same area. Informed consent, blood samples, and clinical evaluation were obtained from all family members. The study protocol was approved by the Ethics Committee of Hangzhou First People's Hospital. Notably, PCOS was diagnosed when the phenotypes of the patients satisfied 2 of the following 3 criteria: oligomenorrhea or amenorrhea, clinical or biochemical hyperandrogenism, and ultrasonographic polycystic ovarian morphology.[1]

Laboratory Assessment

Blood samples were collected in the morning between 7:00 AM and 10:00 AM after an overnight fast. Luteinizing hormone, follicle stimulating hormone, estradiol (E2), total testosterone, and glycosylated hemoglobin (HbA_{1c}) were analyzed, and fasting insulin levels were measured by electrochemiluminescence immunoassays (Roche, Indianapolis). Moreover, the oral glucose tolerance test was performed. Briefly, blood sample was drawn from the antecubital vein at 0 and 2 hours for measurement of plasma glucose concentrations. Patients with a 2-hour plasma glucose concentration <7.8 mmol/L were categorized as having normal glucose tolerance, >7.8 and <11.1 mmol/L as impaired glucose tolerance, and >11.1 mmol/L as diabetes mellitus. While the IR was estimated using the homeostasis model assessment of IR (HOMA-IR) index, the HOMA-IR = (fasting insulin [mIU/L] × fasting glucose [mmol/L])/22.5, HOMA-IR≥2.69 was regarded as IR.

Mutational Screening for the Mitochondrial Genome

Genomic DNA was isolated from whole blood of the individuals for this investigation using Puregene DNA Isolation Kits (Gentra Systems, Minneapolis). The complete mitochondrial genome of the matrilineal relatives (I-2, II-6, III-4) were polymerase chain reaction (PCR) amplified using 24 overlapping primers as described previously.[10] Each fragment was purified and subsequently analyzed by the direct sequencing in an ABI 3700 automatic DNA sequencer using Big Dye Terminator Cycle sequencing reaction Kit. The resultant sequence data were compared with the updated Revised Cambridge Sequence (GenBank Accessible No. NC_ 012920).[11]

Phylogenetic Analysis

A total of 17 vertebrate mtDNA sequences were used in the interspecific analysis. The conservation index (CI) was then calculated by comparing the human nucleotide variants with other 16 vertebrates.[12] The CI was defined as the percentage of species from the list of 16 different vertebrates that had the wild type nucleotide at that position. Notably, the CI $\geq$ 70% was considered as having the functional potential.

Qualification of the mtDNA Copy Number

The mtDNA copy number was measured using the real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method.[13] The mtDNA content was normalized to a single copy nuclear β-globin gene. Two primers (forward: 5′-GAAGAGCCAAGGACAGGTAC-3′; reverse: 5′-CAACTTCATCCACGTTCACC-3′) complementary to the sequence of β-globin gene were used to amplify a 268 bp product. To amplify the mitochondrial genome, the forward primer 5′-AACATACCCATGGCCAACCT-3′ and the reverse primer 5′-AGCGAAGGGTTGTAGTAGCCC-3,′ which were complementary to the sequence of the mitochondrial ND1 gene, were used to amplify a 153 bp PCR product. We first generated standard curves for both fragments and calculated their respective amplification efficiencies to test if using the $2^{-\Delta\Delta CT}$ method was appropriate. The real-time PCR was then conducted for the calibrator DNA: sample III-4; II-6; I-2, while the III-2 was used as control.

Assigning Pathogenicity to the A3302G Mutation

The pathogenicity classification of the A3302G in $tRNA^{Leu(UUR)}$ gene was assigned using an updating version of a previously

Table 1. Summary of Clinical and Biochemical Data of the Members in this Chinese Family.

Participants	Gender	Age	LH, IU/L	FSH, IU/L	E2, pmol/L	TT, nmol/L	PRL, μg/L	HbA1c, %	Glucose (0 h), mmol/L	Glucose (2 h), mmol/L	Insulin, μU/mL	HOMA-IR
I-2	Female	73	63.25	100.48	<43.31	1.39	8.42	7.7	7.57	14.29	13.6	4.57
II-6	Female	51	40.63	42.84	359.4	2.36	21.89	6.9	7.30	12.29	12.7	4.12
III-2	Female	25	3.89	5.59	169.8	1.78	6.96	5.3	5.1	7.84	11.1	2.51
III-4	Female	28	16.65	7.08	182.8	2.98	13.29	5.5	4.81	6.62	20.8	4.45

Abbreviations: LH, luteinizing hormone; FSH, follicle-stimulating hormone; E2, estradiol; PRL, prolactin; HbA_{1c}, hemoglobin A_{1c}; HOMA-IR, homeostasis model assessment of insulin resistance.

validated scoring system.[14] This pathogenicity scoring system employed a number of weighted criteria covering a range of molecular and genetic data, from which an overall pathogenicity score can be obtained. In particular, a variant was classified as "definitely pathogenic" with a score $\geq$11 points, whereas variant was defined as "possible pathogenic" with a core of 7 to 10 points and a "neutral polymorphism" with a score of $\leq$6 points.

Results

Clinical Features of the Chinese Pedigree With IR

One Chinese family, as shown in Figure 1, was ascertained in Hangzhou First People's Hospital. A comprehensive history and physical examination were performed to identify any clinical abnormalities, genetic factors related to IR in members of this pedigree. Moreover, 200 controls with an average of 30 years were selected from a panel of unaffected women of Han Chinese ancestry in the same region. The proband (III-4) was a 28-year-old woman who came from Hangzhou from Zhejiang Province of China, she went to the Department of Obstetrics and Gynecology in Hangzhou First People's Hospital because of her irregular menstrual periods. According to the PCOS diagnostic criteria, this woman was diagnosed as PCOS-IR. While her mother and grandmother (II-6; I-2) had T2DM. The diagnosis of T2DM was based on the criteria of the American Diabetes Association: fasting plasma glucose level of 7 mmol/dL or higher, oral glucose tolerance level of 1.11 mmol/dL or higher, or HbA_{1c} concentration of 6.5% or more.[15] The clinical and biochemical characterization of this Chinese family was listed in Table 1.

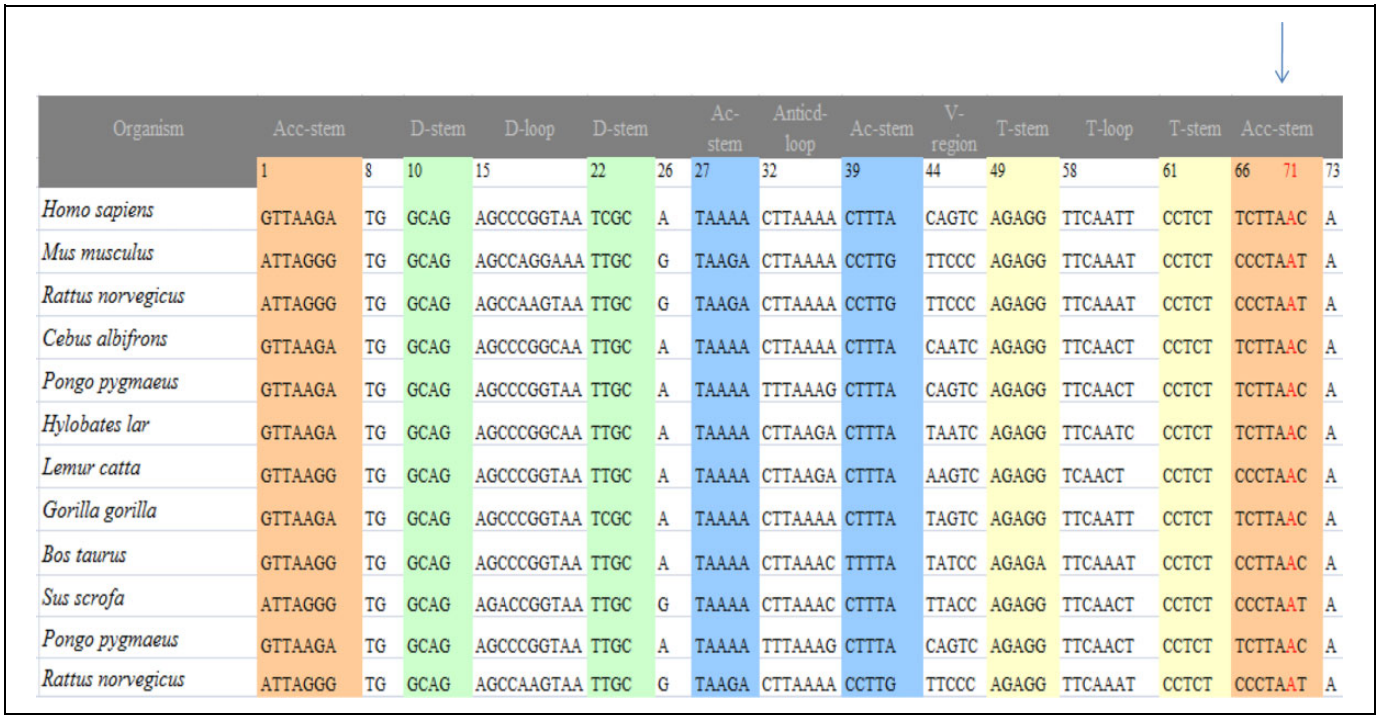
Mitochondrial Genome Analysis

To see whether mitochondrial genetic background contributed to the clinical expression of IR in this family, we sequenced the complete mitochondrial genome of the proband (III-4) and the matrilineal members of this family (I-2; II-6). As shown in Table 2, the comparison of the resultant sequences with the Cambridge consensus sequence identified 29 nucleotide changes in their mitochondrial genomes belonging to human mitochondrial haplogroup C (http://www.phylotree.org/tree/main.htm).[16] Of these, there were 5 variants in D-loop region, 2 variants in 12S ribosomal RNA (rRNA) gene, 1 mutation in tRNA gene, and 19 variants in protein-coding genes. Moreover, there were 7 missense mutations, these mutations included the C8414T (L17F) in the A8 gene, the A8584G (A20T), A8860G (T112A) in the A6 gene, the A10398G (T114A) in the ND3 gene, the G13928C (S531T) in ND5 gene, and C14766T (T7I) and A15326G (T194A) in the CytB gene. These variants in RNAs and polypeptides were further evaluated by phylogenetic analysis of these variants and sequences from other organisms including mouse,[17] bovine,[18] and *Xenopus laevis*.[19] However, none of the variants in the polypeptides were highly evolutionarily conserved except for the A3302G mutation (Figure 2).

Table 2. mtDNA Sequence Variants in this Family With IR.

Gene	Position	Replacement	Conservation (H/B/M/X)[a]
D-loop	73	A to G	
	152	T to C	
	310	T to CTC	
	16189	T to C	
	16519	T to C	
12S rRNA	750	A to G	A/A/G/-
	1438	A to G	A/A/A/G
16S rRNA	1734	C to T	
	2706	A to G	A/G/A/A
$tRNA^{Leu(UUR)}$	3302	A to G	A/A/A/A
ND1	3552	T to A	
	4047	T to C	
CO1	6392	T to C	
	7028	C to T	
CO2	8020	G to A	
A8	8414	C to T (Leu to Phe)	L/F/M/W
A6	8584	A to G (Thr to Ala)	T/S/L/Q
	8860	A to G (Thr to Ala)	T/A/A/T
CO3	9540	T to C	
	9824	T to A	
ND3	10398	A to G (Thr to Ala)	T/T/T/A
	10400	C to T	
ND4	10873	T to C	
	11719	G to A	
ND5	12705	C to T	
	13928	G to C (Ser to Thr)	S/S/V/V
CytB	14766	C to T (Thr to Ile)	T/S/T/S
	15301	G to A	
	15326	A to G (Thr to Ala)	T/M/I/I/

Abbreviations: IR, insulin resistance; mtDNA, mitochondrial DNA; rRNA, ribosomal RNA; H, human; B, bovine; M, mouse; X, *Xenopus laevis*; Leu, leucine; Phe, phenylalanine; Thr, threonine; Ala, alanine; Ser, serine; Ile, isoleucine.
[a]Conservation of amino acid for polypeptides of nucleotides for rRNAs in H, B, M, and X.



Organism	Acc-stem		D-stem	D-loop	D-stem		Ac-stem	Anticd-loop	Ac-stem	V-region	T-stem	T-loop	T-stem	Acc-stem	
	1	8	10	15	22	26	27	32	39	44	49	58	61	66 71	73
Homo sapiens	GTTAAGA	TG	GCAG	AGCCCGGTAA	TCGC	A	TAAAA	CTTAAAA	CTTTA	CAGTC	AGAGG	TTCAATT	CCTCT	TCTTAAC	A
Mus musculus	ATTAGGG	TG	GCAG	AGCCAGGAAA	TTGC	G	TAAGA	CTTAAAA	CCTTG	TTCCC	AGAGG	TTCAAAT	CCTCT	CCCTAAT	A
Rattus norvegicus	ATTAGGG	TG	GCAG	AGCCAAGTAA	TTGC	G	TAAGA	CTTAAAA	CCTTG	TTCCC	AGAGG	TTCAAAT	CCTCT	CCCTAAT	A
Cebus albifrons	GTTAAGA	TG	GCAG	AGCCCGGCAA	TTGC	A	TAAAA	CTTAAAA	CTTTA	CAATC	AGAGG	TTCAACT	CCTCT	TCTTAAC	A
Pongo pygmaeus	GTTAAGA	TG	GCAG	AGCCCGGTAA	TTGC	A	TAAAA	TTTAAAG	CTTTA	CAGTC	AGAGG	TTCAACT	CCTCT	TCTTAAC	A
Hylobates lar	GTTAAGA	TG	GCAG	AGCCCGGCAA	TTGC	A	TAAAA	CTTAAGA	CTTTA	TAATC	AGAGG	TTCAATC	CCTCT	TCTTAAC	A
Lemur catta	GTTAAGG	TG	GCAG	AGCCCGGTAA	TTGC	A	TAAAA	CTTAAGA	CTTTA	AAGTC	AGAGG	TCAACT	CCTCT	CCCTAAC	A
Gorilla gorilla	GTTAAGA	TG	GCAG	AGCCCGGTAA	TCGC	A	TAAAA	CTTAAAA	CTTTA	TAGTC	AGAGG	TTCAATT	CCTCT	TCTTAAC	A
Bos taurus	GTTAAGG	TG	GCAG	AGCCCGGTAA	TTGC	A	TAAAA	CTTAAAC	TTTTA	TATCC	AGAGA	TTCAAAT	CCTCT	CCTTAAC	A
Sus scrofa	ATTAGGG	TG	GCAG	AGACCGGTAA	TTGC	G	TAAAA	CTTAAAC	CTTTA	TTACC	AGAGG	TTCAACT	CCTCT	CCCTAAT	A
Pongo pygmaeus	GTTAAGA	TG	GCAG	AGCCCGGTAA	TTGC	A	TAAAA	TTTAAAG	CTTTA	CAGTC	AGAGG	TTCAACT	CCTCT	TCTTAAC	A
Rattus norvegicus	ATTAGGG	TG	GCAG	AGCCAAGTAA	TTGC	G	TAAGA	CTTAAAA	CCTTG	TTCCC	AGAGG	TTCAAAT	CCTCT	CCCTAAT	A

Figure 2. Sequence alignment for the $tRNA^{Leu(UUR)}$ from different species. Arrow indicates the position 71, corresponding to the A3302G mutation.

Table 3. The Pathogenicity Scoring System for A3302G Mutation.

Scoring Criteria	A3302G Mutation	Score	Classification
More than one independent report	Yes	2	≤6 points: neutral polymorphisms; 7-10 points: possibly pathogenic; 11-13 points (not including evidence from single fiber, steady-state level): probably pathogenic; ≥11 points (including trans-mitochondrial cybrid studies): definitely pathogenic.
Evolutionary conservation of the base pair	No changes	2	
Variant heteroplasmy	No	0	
Segregation of the mutation with disease	Yes	2	
Histochemical evidence of mitochondrial disease	Strong evidence	2	
Biochemical defect in complex I, III, or IV	Yes	2	
Evidence of mutation segregation with biochemical defect from single-fiber studies	Yes	3	
Mutant mt-tRNA steady-state level or evidence of pathogenicity in trans-mitochondrial cybrid studies	Yes	5	
Maximum score		18	Definitely pathogenic

Mitochondrial Copy Number Analysis

As shown in Figure 3, the relative mtDNA copy number was significantly lower in patient with DM (I-2), probably due to her great age, because during aging, mitochondrial reactive oxygen species (ROS) increased and led to the decreasing of mitochondrial copy number.[20] We also noticed that the II-6 and III-4 had lower copy number, suggested that a possible association between IR and copy number.

Pathogenicity Scoring System for the A3302G Mutation

According to the revised pathogenicity scoring system, the A3302G mutation was classified as "definitely pathogenic" with a total score of 18 points (Table 3).

Discussion

The IR underpinned the tight association between the T2DM and obesity pandemics, in addition, IR was found in 50% to 70% of the women with PCOS, was a major risk factor in the development of metabolic syndrome. It had thus been suggested that primary and genetic abnormalities in mitochondrial function may lead to accumulation of toxic lipid species in muscle and elsewhere, impairing insulin action on glucose metabolism.[21,22] In addition, it was generally believed that IR and reduction in insulin production were the major characterization of the T2DM pathogenesis,[23,24] IR continuously increased or decreased the insulin secretory compensation response, and the deterioration into impaired glucose tolerance occurred. Increased glucose, free fatty acid, and insulin levels

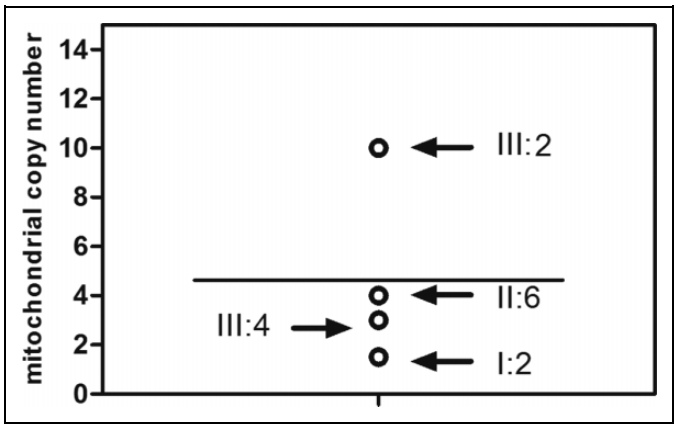


Figure 3. Qualification of mtDNA copy number in subjects (I:2, II:6 and III:4) with the A3302G mutation, III:2, the granddaughter of I:2, was used as control.

will lead to ROS overproduction, increased oxidative stress, and activate stress transduction factor pathways. This can cause insulin activity inhibition and secretion to accelerate the onset of T2DM.[25]

We previously identified several mt-tRNA mutations that were associated with PCOS-IR, these mt-tRNA mutations/variants occurred at the positions which were highly conserved from different species.[9] Recently, we reported a homoplasmic mutation C7492T in mt-tRNA$^{Ser(UCN)}$ gene, combined with the ND5 T12338C mutation, contributed to IR in women with PCOS (Ding et al., Unpublished data). The well-known ND5 T12338C mutation, caused the replacement of the first amino acid methionine to threonine, may decrease the ND5 mRNA level as well as the processing of RNA precursors,[26] moreover, the C7492T mutation occurred at the highly conserved nucleotide in the anticodon stem of tRNA$^{Ser(UCN)}$ gene and was important for the tRNA steady-state level as well as the aminoacylation ability. Thus, mitochondrial dysfunction may be caused by the co-occurrence of the T12338C and C7492T mutations. In this study, we reported the clinical, genetic, and molecular characterization of a Han Chinese family with IR. Most strikingly, the first generation in this family (I-2) carried the T2DM with IR, while the patient (II-6) in the second generation manifested the IR without T2DM, the proband (III-4) carried PCOS-IR, such IR family was infrequent and rare. Regarding the inherited model, we sequenced the complete mitochondrial genome of members in this family. As a result, a homoplasmic A3302G mutation in mt-tRNA$^{Leu(UUR)}$ was identified. This mutation was located 2 nucleotides away from the 5′ terminal tRNA$^{Leu(UUR)}$ nucleotide at position 3304, which was highly conserved from different species (CI = 100%; Figure 2). It was anticipated that this mutation could affect the addition of the CCA triple to the 3′ terminus by the tRNA nucleotidyltransferase. In addition, the A3302G mutation was described in patients with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS), cardiomyopathy, and myopathy with respiratory insufficiency.[27,28,29,30] Intriguingly, patients in this family did not carry these reported syndromes. This mutation caused the severe respiratory chain complex I deficiency and lowered complex IV activity. Moreover, a significant reduction in the steady-state level of tRNA$^{Leu(UUR)}$ was observed in cell carrying this mutation. Studies concerning the functional role of A3302G mutation showed that it led to abnormal processing of the mt-16S rRNA-tRNA$^{Leu(UUR)}$-ND1 precursor RNA.[30,31] Therefore, the A3302G mutation should be regarded as a pathogenic mutation associated with PCOS-IR.

The mtDNA copy number was a relative measure of the cellular number or mass of mitochondria. Increased mitochondrial ROS generation and defects had been implicated in the pathogenesis of IR.[25,32] Recent experimental studies suggested that alterations in mtDNA played a fundamental role in the increase in ROS, maintenance of mtDNA copy number was essential for the preservation of mitochondrial function and cell growth.[33] In this study, we found that the mtDNA contents in patients with IR were significantly lower than the controls, this observation was consistent with the previous study.[34] Moreover, we noticed that the mtDNA content of I-2 reached the lowest rank may be due to her great age; because during aging, mtDNA volume, integrity, and functionality decrease due to accumulation of mutations and oxidative damage induced by ROS.[35] In addition, the mtDNA copy number negatively correlated with insulin levels and HOMA-IR in this family, these findings suggested that mtDNA mutations may be involved in the pathogenesis of PCOS.

In conclusion, the genetic and molecular evidence of the present study indicated that the mt-tRNA$^{Leu(UUR)}$ A3302G mutation was associated with IR in women with PCOS. The A3302G mutation not only caused the reduction in mitochondrial copy number but also reduced the steady-state level of tRNA.[30,31] Thus, the A3302G mutation should be added to the list of inherited risk factor for future molecular diagnosis of PCOS-IR. Therefore, our findings provided the novel insight into the molecular mechanism, management of maternally inherited IR.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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