Premature ovarian insufficiency may be associated with the mutations in mitochondrial tRNA genes

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Abstract. Premature ovarian insufficiency (POI) is a common endocrine disorder featured by the triad constituting of amenorrhea for at least four months, to date, the molecular pathogenesis of POI is largely undetermined. Despite several investigations have reported an increase in reactive oxygen species (ROS) content in idiopathic POI, the role of mitochondrial DNA (mtDNA) mutations/variants in the progression of POI has not been widely investigated. The current study aimed to explore the association between mt-tRNA mutations/variants and POI; we first used the PCR-Sanger sequencing to detect the mutations/variants in mt-tRNA genes from 50 POI patients and 30 healthy subjects. In addition, we evaluated the mitochondrial functions by using trans-mitochondrial cybrid cells containing these potential pathogenic mt-tRNA mutations. Consequently, five mutations: tRNA_{Leu(UUR)} C3303T, tRNA_{Met} A4435G, tRNA_{Gln} T4363C, tRNA_{Cys} G5821A and tRNA_{Thr} A15951G were identified. Notably, these mutations occurred at the extremely conserved nucleotides of the corresponding mt-tRNAs and may result the failure in mt-tRNA metabolism and subsequently lead to the impairment in mitochondrial protein synthesis. Furthermore, biochemical and molecular analyses of the cybrid cells containing these mutations showed a significantly lower level of ATP production when compared with the controls, whereas the ROS levels were much higher in POI patients carrying these mt-tRNA mutations, strongly indicated that these mt-tRNA mutations may cause the mitochondrial dysfunction, and play active roles in the progression and pathogenesis of POI. Together, this study shaded additional light on the molecular mechanism of POI that was manifested by mt-tRNA mutations.

Key words: Premature ovarian insufficiency, Mitochondrial tRNA mutations, Mitochondrial dysfunction

Mitochondria are very important organelles in human cells, whose primary role is generating adenosine-triphosphate (ATP) via oxidative phosphorylation (OXPHOS). Human mitochondrial genome contains 13 polypeptides, as well as 2 rRNAs and 22 tRNAs. MtDNA is inherited exclusively maternally and is present in multiple copies in a cell [4]. Notably, because of the lack of histone protection and a poor DNA repair system, mtDNA has a much higher mutation rate than nuclear DNA [5].

On the other hand, mutations/variants in mt-tRNA genes are being increasingly recognized as important causes of clinical disease. Such pathogenic mutations can lead to transcriptional and translational deficiency and result the impairment of mitochondrial respiratory chain functions [6], and are associated with some clinical diseases including the cardiomyopathy, vision loss, diabetes and deafness [7]. Nevertheless, to date, no studies have been performed to evaluate the association between mt-tRNA mutations/variants and POI.

In the current study, we analyzed the frequency of mt-tRNA mutations in 50 POI patients and 30 healthy con-
trols from Hangzhou First People’s Hospital, Zhejiang University. Moreover, to see the contributions of mt-tRNA mutations/variants to mitochondrial functions, we established the cybrid cells harboring these mt-tRNA mutations/variants.

Materials and Methods

Subjects
This study was consisted of 50 POI patients and 30 age-matched healthy females, recruited from Hangzhou First People’s Hospital affiliated to Zhejiang University. The inclusion criteria were as follows: (1) the age less than 40 years old, (2) amenorrhea time more than 6 months, (3) the level of serum FSH more than 40 IU/L, (4) chromosome karyotype 46 XX. While the selection criteria for controls were as follows: (1) with gender- and age-matched with the patients’ group, (2) the normal menstrual cycles, (3) the serum hormone levels were normal. Women who had the histories of chemotherapy or radiotherapy, pelvic surgery, cigarette smoking, or carrying deafness, vision loss, diabetes mellitus (DM) or cardiovascular diseases were excluded from the study. This study had been approved by the institutional review board of the Hangzhou First People’s Hospital affiliated to Zhejiang University, and each participant provided his/her written informed consent.

Laboratory assessment
Blood samples of 80 subjects (50 POI and 30 controls) were collected in the morning between 7:00 and 10:00 AM after an overnight fast. The levels of serum FSH, luteinizing hormone (LH), estradiol (E2) and total testosterone (TT) were analyzed by using electrochemiluminescence immunoassay on the Cobas 601 machine (Roche Diagnostics, Mannheim, Germany).

DNA isolation, PCR amplification and sequencing
The genomic DNA of 80 subjects were directly extracted from 5 mL EDTA-coagulated whole blood using a Puregene DNA extraction Kit (Qiagen, Valencia, CA) according to the protocols provided by the manufacturer. The 22 mt-tRNA genes were genetic amplified in all POI patients and controls using 11 primers, as shown in Table 1. The PCR reagents (all obtained from Takara Bio, Inc) and the thermocycling conditions were performed according to a previous investigation [8]. The PCR products of the entire mt-tRNA genes were sequenced by the ABI PRISM™ 3700 machine (Applied Biosystems; Thermo Fisher Scientific, Inc.), then the sequence data was compared with the reverse Cambridge sequence to detect the mt-tRNA mutations/variants (GenBank accessible number: NC_012920) [9].

Mt-tRNA secondary structural analysis
The analysis of mt-tRNA secondary structure was performed according to the study by Florentz et al. [10].

Phylogenetic conservation assessments
Total of 17 vertebrates’ mt-tRNA sequences from NCBI database (https://www.ncbi.nlm.nih.gov/) were chose for the inter-specific analyses. We further determined the values of conservation index (CI) by comparing the human nucleotide mutations/variants with the rest species that listed in this analysis. The CI was then calculated as the percentage of species from the 17 species that have the wild-type nucleotide at that position.

Cell lines
Lymphoblastoid cell lines were immortalized by transformation with the Epstein-Barr virus, as described elsewhere [11]. The mtDNA-free rho- cells cell line (143B rho zero), which was generated from 143B.TK- (143B wild) was cultured in the same conditions as the parents except for the addition of 50 μg/mL uridine. Cell lines derived from six POI patients harboring the tRNA{Leu(UUR)} C3303T, tRNA{Met} A4435G, tRNA{Gln} T4363C, tRNA{Cys} G5821A and tRNA{Thr} A15951G mutations, together with six healthy subjects lacking these pathogenic mtDNA mutations were grown in RPMI1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS). All cybrids cell lines constructed with blood platelet and 143B rho zero cell line.

Mitochondrial ATP analysis
The mitochondrial ATP levels of the mutant POI patients and controls were analyzed by using the Cell Titer-Glo® Luminescent Cell Viability Assay kit (Promega), according to the protocols provided by the manufacturer [12].

ROS analysis
Since the mitochondrion was the major site for ROS generation. The fluorometry was used to determine the ROS level. Approximately 2 × 10⁶ cells were first incubated with the fluorescent probe 2,7-dichlorodihydrofluorescein (DCFH) for about 30 min, subsequently, the cells were analyzed by fluorescence plate reader, as mentioned in a previous study [8].

Statistical analysis
The SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) was used to analysis the statistical significance. For unpaired samples, the Student’s t-test was used to calculate the p values. The p < 0.05 was regarded as having statistical importance.
Results

Clinical information for the POI patients and controls

Since January, 2017 to January, 2018, we recruited 50 POI patients and 30 age-matched healthy subjects from the department of Gynecology and Obstetrics, Hangzhou First People’s Hospital affiliated to Zhejiang University. Table 2 showed that there were significant differences in FSH, LH, E2, TT levels between POI and control groups (p < 0.05 for all).

Screening for the potential pathogenic mutations in mt-tRNA genes

We carried out a mutational analysis of mt-tRNAs in 50 POI patients and 30 age-matched healthy females from Hangzhou First People’s Hospital affiliated to Zhejiang University. After PCR amplification and direct sequencing analysis, we identified five mutations: tRNA^{Leu(UUR)} C3303T, tRNA^{Met} A4435G, tRNA^{Gln} T4363C, tRNA^{Cys} G5821A and tRNA^{Thr} A15951G (Fig. 1). Of these, the C3303T mutation was observed in two POI patients, one patient carried the A4435G mutation, one patient carried T4363C mutation, one patient harbored the G5821A mutation and one patient carried the A15951G mutation (Table 3). However, we failed to detect any of these mutations in the healthy subjects.

Table 1  Primer sequences for amplification of 22 mt-tRNAs

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer name</th>
<th>Primer Sequence (5'-3')</th>
<th>Tm (°C)*</th>
<th>Product size</th>
</tr>
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<tbody>
<tr>
<td>tRNA^{Phe}</td>
<td>MT-1F</td>
<td>CTCCCTAAAGCAATACACTG</td>
<td>61</td>
<td>802 bp</td>
</tr>
<tr>
<td></td>
<td>MT-1R</td>
<td>TGCTAAATCCACCTTGACC</td>
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<td>tRNA^{Val}</td>
<td>MT-2F</td>
<td>CGATCAACCTCACCACTCT</td>
<td>58</td>
<td>802 bp</td>
</tr>
<tr>
<td></td>
<td>MT-2R</td>
<td>TGGACAACCAGCTATCACCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA^{Leu(UUR)}</td>
<td>MT-4F</td>
<td>AAATCTTACCCCGCTGTTT</td>
<td>60</td>
<td>887 bp</td>
</tr>
<tr>
<td></td>
<td>MT-4R</td>
<td>AGGAATGCCATTGGGATTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA^{Ile}</td>
<td>MT-6F</td>
<td>TGGCTCTTAACTCTCA</td>
<td>60</td>
<td>898 bp</td>
</tr>
<tr>
<td>tRNA^{Met}</td>
<td>MT-6R</td>
<td>AAGGATTATGGCTGCTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tRNA^{Ala}</td>
<td>MT-8F</td>
<td>CTAACCGGCTTTTGCCC</td>
<td>60</td>
<td>814 bp</td>
</tr>
<tr>
<td>tRNA^{Ser}</td>
<td>MT-8R</td>
<td>ACCTAGAAGGTTGCGCTGCT</td>
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<tr>
<td>tRNA^{Glu}</td>
<td>MT-11F</td>
<td>ACGCCTAATTTCCTTACCT</td>
<td>58</td>
<td>987 bp</td>
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<tr>
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<td>CGGAAATTGGACTGTTTTCCT</td>
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<tr>
<td>tRNA^{Val}</td>
<td>MT-12F</td>
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<td>60</td>
<td>900 bp</td>
</tr>
<tr>
<td>tRNA^{Ala}</td>
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<td>TGGCTGTGTGATTAAATG</td>
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<tr>
<td>tRNA^{Thr}</td>
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<td>TCTCCATCTATGAGGCTTCT</td>
<td>60</td>
<td>891 bp</td>
</tr>
<tr>
<td>tRNA^{Cys}</td>
<td>MT-15R</td>
<td>AATAGGCGCTGGCTGGTTTG</td>
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<tr>
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<td>tRNA^{Glu}</td>
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<tr>
<td>tRNA^{Pro}</td>
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<td>60</td>
<td>1,162 bp</td>
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<tr>
<td>tRNA^{Ser}</td>
<td>MT-22R</td>
<td>GAGGTGTTAATAGGGGTATAG</td>
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<td></td>
</tr>
</tbody>
</table>

* Tm: Annealing Temperature

Table 2  Comparison of hormone levels in POI patients and controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>POI patients (n = 50)</th>
<th>Control (n = 30)</th>
<th>p value</th>
</tr>
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<tr>
<td>Age (year)</td>
<td>28.2 ± 4.40</td>
<td>27.8 ± 4.12</td>
<td>0.49</td>
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<tr>
<td>FSH (IU/L)</td>
<td>65.11 ± 11.02</td>
<td>5.77 ± 1.09</td>
<td>0.002</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>41.12 ± 8.55</td>
<td>6.77 ± 3.12</td>
<td>0.001</td>
</tr>
<tr>
<td>E2 (pmol/L)</td>
<td>24.33 ± 10.66</td>
<td>78.66 ± 30.11</td>
<td>0.009</td>
</tr>
<tr>
<td>TT (nmol/L)</td>
<td>0.78 ± 0.31</td>
<td>1.44 ± 0.25</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Evaluation of mt-tRNA mutations

We used the following standard to assign the pathogenic mutations: 1. the mutation did not occur in control group; 2. the mutation may affect the secondary or tertiary structures of the mt-tRNAs; 3. the CI > 75%; 4. the mutation may impair the mitochondrial function. For this purpose, we first analyzed the CIs of these mutations, as can be seen from Fig. 2, the C3303T mutation occurred at a position which was very conserved between 17 species (CI = 100%), similarly, the CIs of A4435G, T4363C, G5821A and A15951G mutations were 100% for all. In addition, as displayed in Fig. 1, three pathogenic mutations: C3303T, G5821A and A15951G were located at the acceptor arm of the corresponding tRNAs. While the A4435G and T4363C mutations occurred at the anticodon stem of tRNA\(^\text{Met}\) and tRNA\(^\text{Gln}\), respectively. Moreover, the C3303T, G5821A and A15951G mutations disrupted the highly conserved base-pairings in tRNA\(^\text{Leu(UUR)}\), tRNA\(^\text{Cys}\) and tRNA\(^\text{Thr}\), respectively. By contrast, the A4435G and T4363C mutations created novel base-pairing. We believed that these mutations may cause functional consequences since they altered the
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Secondary or tertiary structures of tRNA\textsuperscript{Leu(UUR)}, tRNA\textsuperscript{Met}, tRNA\textsuperscript{Gln}, tRNA\textsuperscript{Cys} and tRNA\textsuperscript{Thr}.

The construction of cybrid cell lines

The platelets derived from six POI patients carrying the mitochondrial C3303T, A4435G, T4363C, G5821A and A15951G mutations, together with other six control subjects were fused to mtDNA-free ρ\textsuperscript{0} cells which derived from the 143B wild cell lines (Fig. 3). The cybrid clones were isolated and subsequently analyzed for ATP and ROS levels.

Reduced levels in mitochondrial ATP production

To see whether mt-tRNA mutations affected mitochondrial functions, we analyzed the ATP productions in wild and mutant cell lines. As can be seen from Fig. 4A, we found that the levels of mitochondrial ATP production in mutant cell lines carrying the tRNA\textsuperscript{Leu(UUR)} C3303T, tRNA\textsuperscript{Met} A4435G, tRNA\textsuperscript{Gln} T4363C, tRNA\textsuperscript{Cys} G5821A and tRNA\textsuperscript{Thr} A15951G mutations were obviously lower than the control subjects ($p = 0.0029$).

The increase of mitochondrial ROS production

As shown in Fig. 4B, POI patients carried these mt-tRNA mutations exhibited significantly higher levels of ROS when compared with the controls ($p = 0.0035$).

Discussion

The current study investigated the potential association between mt-tRNA mutations and POI. To our knowledge, this was the first study concerning the roles of mt-tRNA mutations in POI. In fact, alternations in mitochondrial function had been found to play an active role in oogenesis and follicle maturation. Since human oocytes contained large amount of mitochondria, recent study indicated that patients with ovarian insufficiency exhibited a much lower level of mtDNA content than women with a healthy ovarian cells [13]. Moreover, increasing evidence suggested that the oxidative stress may act as an important risk factor for reproductive disorders, moreover, ROS overproduction was regarded to be causative in progression of POI [14, 15]. Thus, referring to these studies, we aimed to uncover the mitochondrial genetic alternations by analyzing the entire mt-tRNA genes in women with POI.

Mutational analysis of the 22 mt-tRNA genes from 50 POI patients and 30 controls led us to identify five mt-tRNA mutations (Fig. 1). As early as 1994, Silvestri and colleagues [16] described a C3303T mutation in mt-tRNA\textsuperscript{Leu(UUR)} gene from a pedigree with infantile cardiomyopathy. Patients with this mutation showed a significant reduction in respiratory chain complex I and IV activities [17]. By molecular level, this mutation disrupted the very conserved base-pairing (G1-73C) in the...
acceptor arm of tRNA^Leu(UUR), nucleotide at that position was very important for the tRNA steady-state level and aminoacylation ability, thus, the C3303T mutation may cause the failure in tRNA metabolism. Furthermore, the well-known A4435G mutation was localized at the 3’ prime end to the anticodon stem of tRNA^Met gene (position 37) [18]. A recent study by Zhou et al. indicated that A4435G mutation generated the m1G37 modification of tRNA^Met [19]. Molecular and biochemical analysis of the cybrid cells containing this mutation caused ~30% reduction in the rate of mitochondrial protein synthesis [18]. Moreover, this mutation had been identified to be associated with maternally inherited hypertension in a Chinese pedigree [18].

In addition, the T to C transition at position 4363 occurred at 3’ prime end to the anticodon of tRNA^Gln (conventional position 38), which was evolutionarily conserved from different species. Moreover, nucleotide at position 38 was often chemically modified; therefore, defects in tRNA modification caused by the T4363C mutation may affect the efficiency of codon and anticodon interaction. Thus, we proposed that this mutation may have the functional impact on the steady state level of tRNA^Gln [20]. Most recently, several genetic studies found that the T4363C mutation was a risk factor for essential hypertension or myopia [20, 21]. Therefore, the T4363C should be classified as a potential pathogenic mutation associated with POI. Similarly, the G5821A mutation destabilized highly conservative base-pairing (G6-C67) in tRNA^Cys. It was suggested that the G5821A mutation may decrease the stability levels of functional tRNA, and consequently result in failure of tRNA metabolism [22]. Moreover, this mutation had been found to modulate the phenotypic manifestation mitochondrial A1555G mutation-induced hearing loss [23].

While the homoplasmic A15951G mutation was localized at highly conserved nucleotide (conventional position 71) of tRNA^Thr [24], where the position was critical for the recognition of aminoacyl-tRNA synthetase [10]. Previous study showed that the A15951G mutation may have a synergistic effect on Leber’s hereditary optic neuropathy (LHON)-associated ND4 G11778A mutation, increase the LHON penetrance and expressivity [25]. Actually, this mutation caused approximately 35% reduction in the steady-state level of the corresponding tRNA, most probably resulted from an impairment of aminoacylation ability of tRNA^Thr or deficiency in precursor tRNA processing by RNaseP [25]. Interestingly, the A15951G mutation had been implicated to play a putative role in Parkinson’s disease [26]. Therefore, we believed that these five mutations may lead to the failure in mt-tRNAs metabolism in the form of decreasing the tRNA steady state level or aminoacylation ability, and finally caused mitochondrial dysfunction that was involved in the pathogenesis of POI.

To see the contributions of C3303T, A4435G, T4363C, G5821A and A15951G to mitochondrial dysfunction, we generated the cybrid cell lines containing these mutations. As shown in Fig. 4, we found that patients carrying these mutations have a much lower level of ATP when compared with the control, by contrast, the ROS was higher in POI group than control (p = 0.0029 and 0.0035, respectively). In fact, shortage in ATP generation in POI patients was most probably a result of the decreased in the proton electrochemical potential gradient of impaired mitochondria [27]. On the other hand, the increased level of ROS would cause the oxidative stress in human mitochondria, thus, damaging mtDNA and cellular proteins, lipids [28]. Therefore, these mt-tRNA mutations were involved in the pathogenesis of POI.

According to these clinical and biochemical data, we propose that the possibly molecular mechanism underlying mt-tRNA mutations in the progression of POI may be as follows: first of all, these mutations influence the secondary or tertiary structures of the corresponding tRNAs and result in the failure in tRNA metabolism [29-31]. Consequently, it will lead to the defects in mito-

Fig. 4 Analysis of mitochondrial function. A. Mitochondrial ATP analysis; B. Mitochondrial ROS analysis.
The impairment in mitochondrial respiratory chain will reduce the ATP and increase the ROS production. Particularly, lower level of ATP could activate mammalian target of rapamycin (mTOR) and the pool of primordial follicles was activated prematurely due to elevated mammalian target of rapamycin complex 1 (mTORC1) activity in oocytes [32-35], which would accelerate the ovarian follicle development and rate of follicle loss. Moreover, increased ROS would affect the quality of gametes, lead to the cell death and apoptosis [36, 37], thus, contributed to the POI.

In summary, we found a high incidence of mt-tRNA mutations in patients with POI, the C3303T, A4435G, T4363C, G5821A and A15951G should be regarded as pathogenic mutations associated with POI. However, the homoplasmic form, mild mitochondrial dysfunction suggested that mt-tRNA mutation itself was insufficient to produce the clinical phenotype, therefore, other risk factors, such as nuclear genes, mtDNA haplogroup/ haplotype, epigenetic modifications may contribute to the POI progression. Taken together, our study provided novel insight into the molecular pathogenesis of POI that was manifested by mitochondrial dysfunction. The main limitation of the current study was the relative small population, further studies with a larger sample size need to be performed.

Acknowledgments

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Competing Financial Interests Statement

None.

References

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