Mutational Screening for Mitochondrial tRNA Genes in 100 Women with Pre-Eclampsia

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Abstract
Introduction: Impairment of mitochondrial function caused by pathogenic mitochondrial DNA (mtDNA) mutations has been found to be associated with pre-eclampsia (PE). However, the underlying mechanism of PE remains poorly understood. The aim of this study is to evaluate the relationship between mitochondrial tRNA (mt-tRNA) variants and PE.

Methods: The mt-tRNA variants in a cohort of 100 pregnant women with PE and 100 healthy subjects were examined by PCR-Sanger sequencing. Moreover, the phylogenetic conservation analysis, mitochondrial haplogroup analysis, and pathogenicity scoring system were used to assess the potential pathogenicity of these tRNA variants.

Results: We identified five possible pathogenic mt-tRNA variants: tRNA\textsuperscript{Phe} A608G, tRNA\textsuperscript{Ile} A4263G, tRNA\textsuperscript{Ala} T5587C, tRNA\textsuperscript{Leu(CUN)} G12294C, and tRNA\textsuperscript{Pro} G15995A. We noticed that these variants were not detected in control subjects and occurred at the positions which were extremely conserved. Alternations in tRNA structure caused by these variants may lead to the failures in tRNA metabolism, which may subsequently lead to the impairment of mitochondrial translation as well as the respiratory chain functions. Thus, mt-tRNA variants may be involved in the pathogenesis of PE. Conclusion: Taken together, our data indicated that variants in mt-tRNA genes were the important contributors to PE; screening for mt-tRNA variants was recommended for early detection and prevention of PE.

Introduction
Pre-eclampsia (PE) is a life-threatening pregnancy-specific syndrome characterized by gestational hypertension and proteinuria, affecting 2–8% of pregnant women in industrialized countries [1, 2]. In addition, PE can increase the susceptibility to cardiovascular and cerebrovascular diseases, such as hypertension, kidney diseases, coronary heart diseases, stroke, and metabolic syndrome in later life of both mothers and their offspring [3]. To date, the underlying mechanism of this disease remains poorly understood, and the leading hypotheses suggest that systemic inflammation, vascular endothelial dys-

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function, and oxidative stress play an important role in PE progression and pathogenesis [4–6].

Most recently, increasing studies suggested that the aberrations in oxidative phosphorylation (OXPHOS) system due to mutations in mitochondrial DNA (mtDNA) played important roles in the pathogenesis of PE [7, 8]. Moreover, elevated oxidative stress associated with early shallow trophoblastic invasion was evident as early as 8–10 weeks’ gestation and manifests in both the placenta and the maternal circulation [9]. Oxidative stress may be defined as inadequate redox homeostasis, resulting from an intensified generation of reactive oxygen species (ROS). It was speculated that increased ROS generation had been implicated in intrauterine perturbations, like placental insufficiency, and may result in intrauterine growth restriction, observed in small for gestational age newborns [10]. Indeed, placental mitochondria were important components of oxidative stress injury, compared to its nuclear counterpart; mtDNA accumulates damage more extensively when exposed to ROS [11]. In fact, the human mtDNA was a circular, double-stranded, maternally inherited chromosome of 16,569-bp, encoded 37 genes, all involved in OXPHOS pathways. Thirteen encoded enzymatic subunits of the complexes participating in the respiratory chain; the remaining encodes tRNA and rRNA that were involved in protein synthesis [12].

mt-tRNA was a short, noncoding RNA that constitutes approximately 4–10% of all cellular RNAs [13]. In fact, most mt-tRNAs from all domains of life had a highly conserved cloverleaf structure, consisting of an acceptor arm, D-arm, anticodon stem, variable region, and T-loop, with an average length of 73 nucleotides. In addition, the secondary structure of mt-tRNAs contains a significant amount of unstable nucleotide pairs, such as non-Watson-Crick pairs and A-U pairs, and these features mean that mt-tRNAs had lower thermal stability and were more susceptible to mutation than nuclear-encoded tRNAs [14, 15]. mt-tRNA point mutations typically caused a loss of mt-tRNA stability, leading to defective mitochondrial translation and a combined respiratory chain deficiency. Potential mechanisms included aberrant processing of the mRNA transcript by RNases P and ZL, impaired posttranscriptional mt-tRNA modification (e.g., specific base modifications, 3′-end addition of the CCA sequence and mt-tRNA aminoacylation), and compromised interaction of the mt-tRNA with both mitochondrial elongation factor Tu (mt-EF-Tu) and mitochondrial ribosome [16].

Recent experimental study indicated that tRNA

leu(UUR) A3243G mutation was associated with PE [17]; Furthermore, Ding et al. [18] found that nonsynonymous substitutions in mtDNA may be risk factors for PE in some African-American women. However, the underlying mechanism remained largely undetermined.

In the current study, with the aim of elucidating the association between mt-tRNA variants and PE, we carried out a mutational analysis of mt-tRNA genes in a cohort of 100 women with PE and 100 age-matched control subjects. As a result, we found five potential pathogenic mt-tRNA variants that may be related to PE.

Materials and Methods

Subjects
The study was conducted in accordance with the Declaration of Helsinki and Institutional Ethical Clearance was obtained from Hangzhou Yuhang Woman’s Hospital, and the Ethics Committee of Hangzhou Yuhang Woman’s Hospital approved this study, and written informed consent was obtained from all the subjects prior to this study. From January 2019 to December 2020, a total of 100 women with PE (aged from 25 to 37 years, with an average of 31 years) as well as 100 controls (aged from 27 to 36 years, with an average of 29 years) were enrolled in this study. Controls were women with pregnancies uncomplicated by pregnancy-induced hypertension or proteinuria. Women with chronic or gestational hypertension, preconceptual or gestational diabetes mellitus, multiple gestation, or fetal anomalies were excluded from this study.

The diagnosis of PE was made based on the standard recommendations from the International Society for the Study of Hypertension in Pregnancy (ISSHP). All women who were included as having PE had persistent blood pressure (BP) >140/90 mm Hg or proteinuria (2+ on dipstick or >300 mg/24 h), after 20 weeks of gestation [19, 20].

mt-tRNA Gene Sequencing
Genomic DNA was isolated from peripheral blood of all the subjects using Paxgene Blood DNA Isolation Kits (QIAGEN). The 22 mt-tRNA genes were genetically amplified in all PE patients and controls using 11 primers, as described in a previous study [21]. The PCR reagents (all obtained from Takara Bio, Inc.) and the thermocycling conditions were performed according to a recent investigation [22]. After genetic amplification, the PCR products were purified and analyzed by direct Sanger sequencing. The sequence data were compared with the revised Cambridge Reference Sequences to identify mtDNA variants (GenBank Accession Number: NC_012920.1) [23].

In individuals found to have potential pathogenic variants in tRNA genes, a detailed demographics, anthropometrics, vital parameters, medical history, and pedigree information were recorded. Body mass index was calculated as the body weight (kg) divided by the square of the height (m²). BP was measured by a sphygmomanometer by three times. Furthermore, to examine the contributions of mtDNA genetic background to PE, the fragments that spanned the remainder of the mitochondrial genome were amplified via PCR and were used to establish the mtDNA haplogroups of these individuals.

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**Table 1. Molecular characterizations of PE-associated mt-tRNA variants**

| tRNA species | Nucleotide alterations | Homoplasy, heteroplasy | Location | Numbering in tRNAs | Watson-Crick base-pairing | CI, % | 100 PE patients, n (%) | 100 controls, n (%) | Disease association | References |
|--------------|------------------------|------------------------|----------|-------------------|--------------------------|------|------------------------|-------------------|------------------|------------|
| tRNA\textsuperscript{Phe} | A608G | Homoplasy | Anticodon stem | 31 | A-T↓ | 100 | 1 (1) | 0 | Tubulointerstitial nephritis | [48] |
| tRNA\textsuperscript{Ile} | A4263G | Homoplasy | Acceptor arm | 1 | A-T↓ | 100 | 1 (1) | 0 | Hypertension | [50] |
| tRNA\textsuperscript{Ala} | T5587C | Homoplasy | Acceptor arm | 73 | | 100 | 2 (2) | 0 | LHON | [52] |
| tRNA\textsuperscript{Leu(CUN)} | G12294C | Heteroplasy | Anticodon stem | 29 | G-C↓ | 100 | 1 (1) | 0 | CPEO | [54] |
| tRNA\textsuperscript{Pro} | G15995A | Heteroplasy | Anticodon stem | 29 | G-C↓ | 100 | 1 (1) | 0 | Mitochondrial cytopathy | [55] |

LHON, Leber’s hereditary optic neuropathy; CPEO, chronic progressive external ophthalmoplegia; CI, conservation index. \( ^{\text{a}} \) Classic Watson-Crick base pairing: abolish ↓.

*Phylogenetic Conservation Analysis*

The mtDNA sequences of 15 vertebrates were used for interspecific analysis. The conservation index (CI) was calculated by comparing the human nucleotide variants with the corresponding sequences in the other 14 vertebrates [24]. A CI ≥75% was considered to have functional potential [25].

*Haplogroup Classification*

The entire mtDNA sequences of six subjects with putative pathogenic mt-tRNA variants were assigned to the Asian mitochondrial haplogroups based on the PhyloTree database (http://www.phylotree.org) [26] and East Asian phylogeny [27].

*Pathogenicity Scoring System*

To identify mt-tRNA variants with potential deleterious, we used the following criteria: (1) present in <1% of the healthy controls; (2) CI ≥75%, as proposed by Ruiz-Pesini and Wallace [24]; (3) potential to cause structural and functional alterations; and (4) a score of ≥7 points under an established pathogenicity scoring system [28], according to which a variant was classified as a “neutral polymorphism” if it scored ≤6 points, as “possibly pathogenic” if it scored 7–10 points, and as “definitely pathogenic” if it scored ≥11 points.

**Results**

*Screening for PE-Associated mt-tRNA Variants*

We carried out a genetic screening program for PE-associated mt-tRNA variants. The PCR and direct sequencing analysis revealed five possibly pathogenic variants: tRNA\textsuperscript{Phe} A608G, tRNA\textsuperscript{Ile} A4263G, tRNA\textsuperscript{Ala} T5587C, tRNA\textsuperscript{Leu(CUN)} G12294C, and tRNA\textsuperscript{Pro} G15995A. Furthermore, the A608G variant occurred in 1 out of 100 PE women (1%), the A4263G variant in 1 patient with PE (1%), the T5587C variant was found in 2 patients with PE (2%), the G12294C variant was identified in one woman with PE (1%), and the G15995A variant was found in 1 patient with PE (1%). Further studies revealed that the G12294C and G15995A variants were heteroplasy, whereas other variants were in homoplasmic forms. But we did not find any mt-tRNA variants in controls. The molecular characterization of these mt-tRNA variants is listed in Table 1.

*Assessment of the Pathogenicity*

To classify the potential pathogenic variants, the following criteria were used: first, the variant itself occurred <1% in control subjects; second, the CI ≥75%, as proposed by Ruiz-Pesini and Wallace [24]; and third, the variant may have functional impact on mt-tRNA genes. With this regard, we found that all these mt-tRNA variants were only identified in PE group but absent in controls; moreover, they had high levels of CI (CI = 100% for all). The locations of these variants within tRNA secondary structures are shown in Figure 1, two variants occurred at acceptor arm (tRNA\textsuperscript{Ile} A4263G and tRNA\textsuperscript{Ala} T5587C), and three variants were localized at anticodon stem (tRNA\textsuperscript{Phe} A608G, tRNA\textsuperscript{Leu(CUN)} G12294C, and tRNA\textsuperscript{Pro} G15995A). Furthermore, four variants disrupted the Watson-Crick base-pairings (tRNA\textsuperscript{Phe} A608G, tRNA\textsuperscript{Ile} A4263G, tRNA\textsuperscript{Leu(CUN)} G12294C, and tRNA\textsuperscript{Pro} G15995A).

*Clinical Features of Six Proband Carrying One of the Putative Pathogenic mt-tRNA Variants*

As shown in Table 2, 6 patients with PE, carrying the pathogenic/likely pathogenic tRNA variants, account for...

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6% of the case in our cohort. The age at test in these subjects varied from 28 to 32 years, with an average of 30 years. A comprehensive medical history showed that one subject (PE-026) had a family history of hypertension, while another subject (PE-089) had a family history of diabetes.

**Analysis of the Entire mtDNA Genes in Six Probands Carrying One of the Pathogenic mt-tRNA Variants**

We next sequenced the complete mitochondrial genomes of six probands carrying one of the putative pathogenic mt-tRNA variants in order to examine if there were any other functional mtDNA variants. As shown in Table 2, four functional mtDNA variants were identified by direct sequencing analysis. Of them, the ND1 G3635A variant coexisted with tRNA^Phe^ A608G, the known ND1 T3394C variant coexisted with tRNA^Ala^ T5587C, the ND4 G11696A variant was found in a patient carrying the tRNA^Leu(CUN)^ G12294C variant, and the 12S rRNA 961insC was identified in a subject with tRNA^Pro^ G15995A variant. In fact, the G3635A variant caused a change of highly conserved serine at position 110 with asparagine (S110N) in ND1, the structural component of complex I [29, 30]. Biochemical analysis revealed that G3635A variant reduced the level of ND1 and affected the OXPHOS subunits [31]. Therefore, the

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**Fig. 1.** Cloverleaf structures of mt-tRNA with standard nucleotide numbering. Arrows indicate the PE-related mt-tRNA variants.
G3635A variant may alter both the structure and function of complex I, thereby causing mitochondrial dysfunction. However, the homoplasmic T3394C variant occurred at a highly conserved tyrosine at position 30 of ND1 was believed to increase the penetrance and expressivity of Leber’s hereditary optic neuropathy-associated ND4 G11778A variant [32]. Previous study indicated that the T3394C variant altered the structure and function of complex I, diminished the ATP and mitochondrial membrane potential, and increased the production of ROS [33]. Interestingly, the G-to-A transition at position 11696 (G11696A) in the ND4 gene, resulting in the substitution of an isoleucine for valine at amino acid position 312, was regarded to enhance the clinical expression of deafness-associated A1555G and Leber’s hereditary optic neuropathy-associated G11778A variant in Chinese families [34, 35]. Thus, it can be speculated that the G11696A may act as a synergistic role in clinical expression of PE-related tRNA Leu(CUN) G12294C variant, while the 961insC had been found to play a role in enhancing the penetrance of the deafness-associated A1555G variant in a large Chinese family [36].

In addition, the complete mitochondrial genomes of six subjects carrying one of putative pathogenic mt-tRNA variants had been assigned to East Asian mitochondrial haplogroups based on the PhyloTree database (http://www.phylotree.org/) [26] and East Asian phylogeny [27]. According to their distinct sets of polymorphisms, the mtDNA of these subjects belonged to East Asian mitochondrial haplogroup D4b1, N9a3, M9a, D5a, D4j, and B5b1, respectively.

### Assessment of the Pathogenicity

According to the revised pathogenicity scoring system [28], we found that the total scores of tRNA Phe A608G, tRNA Ile A4263G, tRNA Ala T5587C, tRNA Leu(CUN) G12294C, and tRNA Pro G15995A variants were 11, 13, 15, 15, and 10 points, respectively, and belonged to “definitely pathogenic” and “possibly pathogenic” (Table 3).

## Discussion

In the present study, we analyzed the frequencies of mt-tRNA variants in a cohort of 100 women with PE and 100 control subjects by using PCR and Sanger sequencing. In fact, PE was a heterogeneous and multisystem disorder characterized by the new onset of hypertension associated with various organ damages and/or fetal growth restriction [37]. The etiology of PE remained elusive, but three lines of evidence suggest a role for aberrations in OXPHOS due to mutations in mitochondrial genes. First, both increased generation of ROS and decreased levels of the antioxidants that normally scavenge ROS had been observed both in placental tissue and in the maternal circulation during PE [38, 39]. Second, mitochondria were the major sites of ROS production and removal, and mutations in mitochondrial genes resulted in increased ROS production [40]. Lastly, an increased prevalence of PE in families and individuals with myopathies due to mitochondrial mutations had been reported according to recent studies [41, 42].
The mitochondrial genome was a circular molecule containing 13 protein-encoding genes that contributed proteins to complexes I, III, IV, and V of the OXPHOS system, as well as 2 rRNAs and 22 tRNAs [43]. Point mutations in the genes encoding mt-tRNAs had been increasingly recognized as important causes of diseases; such variants can result in transcriptional and translational defects and consequently mitochondrial respiratory chain dysfunction [44–46]. Therefore, we hypothesized that mt-tRNA variants may play key roles in clinical expression of PE.

With this regard, the variants in 22 mt-tRNA genes were screened by Sanger sequencing. Compared with the revised Cambridge Reference Sequences, we identified five possible pathogenic variants: tRNA Phe A608G, tRNA Ile A4263G, tRNA Ala T5587C, tRNA Leu(CUN) G12294C, and tRNA Pro G15995A variants. Among these, the A608G variant occurred at position 31 in the anticodon stem of tRNA Phe, which was extremely conserved from various species [47]. Importantly, the A608G variant disrupted the conserved base-pairing (31A-39T), which caused the shorting of the anticodon stem and the lengthening of the anticodon loop. Previous study suggested that the A608G was a pathogenic variant for tubulointerstitial nephritis [48]. However, the A-to-G substitution at position 4263 changed the stop codon TAA of the ND1 mRNA to an equivalent TAG stop codon and, at the same time, caused an A-to-G transition at the 5′ end of the tRNA Ile gene [49]. Functional analysis of cybrid cells containing the A4263G variant revealed that it caused ∼30% reduction in the efficiency of the 5′ end processing of tRNA Ile precursor; in addition, approximately 46% reduction in the steady-state level of tRNA Ile was observed in the mutant cell line harboring this variant [50].

The homoplasmic T5587C variant occurred at position 73 in the acceptor arm of tRNA Ala, which was extremely conserved from different species [51]. In vitro processing experiments showed that the T5587C variant impaired the 3′-end processing of tRNA Ala precursors by RNase Z and inhibited the addition of CCA by tRNA nucleotidyltransferase. Furthermore, Northern blot analysis revealed that the T5587C variant perturbed tRNA Ala aminoaclonylation, as evidenced by decreased efficiency of aminoaclonylation [52]. Thus, the T5587C variant may cause the failure in tRNA metabolism and lead to mitochondrial dysfunction. Moreover, the heteroplasmic G12294A variant disrupted a Watson-Crick base-pairing that was highly conserved in the anticodon stem of the tRNA Leu(CUN) [53], and biochemical analysis revealed that the G12294A significantly reduced activities of complexes I, III, and IV.

| Score | Classification |
|-------|----------------|
| ≤6 points: neutral polymorphisms; 7–10 points: possibly pathogenic; 11–13 points (not including evidence from single fiber, steady-state level, or transmitochondrial cybrid studies): probably pathogenic; ≥11 points (including evidence from single fiber, steady-state level, or transmitochondrial cybrid studies): definitely pathogenic |

Table 3. The predicted pathogenicity of PE-associated mt-tRNA variants

- **A608G variant**
  - Score: 2
  - More than one independent report: Yes
  - Evolutionary conservation of the base pair: No change
  - Segregation of the mutation with disease: No
  - Biochemical defect in complex I, III, or IV: No
  - Mutant mt-tRNA steady-state level or evidence of pathogenicity in transmitochondrial cybrid studies: No evidence
  - Maximum score: 10
  - Classification: Possibly pathogenic

- **A4263G variant**
  - Score: 2
  - More than one independent report: Yes
  - Evolutionary conservation of the base pair: No change
  - Segregation of the mutation with disease: No
  - Biochemical defect in complex I, III, or IV: No
  - Mutant mt-tRNA steady-state level or evidence of pathogenicity in transmitochondrial cybrid studies: No evidence
  - Maximum score: 10
  - Classification: Possibly pathogenic
Exercise physiological studies in the patient with this variant demonstrated a significantly reduced maximal oxygen uptake as well as threefold elevated lactate/pyruvate ratios strongly indicated that G12294A was pathogenic [54]. Furthermore, the G-to-A substitution at position 15995 occurred at anticodon stem of tRNA\textsubscript{Pro}, which was predicted to abolish the highly conserved Watson-Crick base-pairing. Previous investigation suggested that the G15995A variant was associated with mitochondrial myopathy [55]. Therefore, these variants may have a structural and functional impact on the corresponding tRNAs and may lead to the failure in tRNA metabolism.

We believed that the possible molecular mechanisms may be as follows: these variants may influence the three-dimensional structure of tRNAs, induce an abnormal tRNA conformation, or impair their functions such as the recognition by CCA-adding enzyme, steady-state level, aminoacylation efficiency, and binding to EF-Tu or post-transcriptional modifications [56, 57]. As a result, these biochemical processes will result the failure in tRNA metabolism and in turn reduce mitochondrial protein synthesis [58]. Subsequently, defects in mitochondrial protein synthesis will impair mitochondrial function, decrease in ATP, enhance the ROS production, and increase the oxidative stress that is responsible for PE [59, 60].

In conclusion, our study provided the direct evidence that mt-tRNA variants were associated with PE; in particular, the tRNA\textsubscript{Phe} A608G, tRNA\textsubscript{Ile} A4263G, tRNA\textsubscript{Ala} T5587C, tRNA\textsubscript{Leu(CUN)} G12294C, and tRNA\textsubscript{Pro} G15995A should be added as risk factors for this disorder. The main limitations of this study were the relatively small sample size; further studies including more PE patients were needed to verify this conclusion.

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**Statement of Ethics**

This work is approved by the Ethics Committee of Hangzhou Yuhang Woman’s Hospital (approval number: LLSC-KYKT-2022-0020-A). Written informed consent was obtained from the patient for participation in this study.

**Conflict of Interest Statement**

The authors declare no competing interests.

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**Author Contributions**

Baohua Zhou and Xuelian Chu designed the study, Caijuan Zhang and Xiufeng Liang collected the PE samples and control subjects, Baohua Zhou performed molecular and genetic analysis, and Xuelian Chu analyzed the data. All authors read and approved the final draft.

**Data Availability Statement**

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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