Mitochondrial DNA 4977 bp Deletion in Peripheral Blood is Associated with Polycystic Ovary Syndrome.

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Abstract

Background PCOS is a common worldwide endocrine disorder. We aimed to examine the associations of two mtDNA biomarkers in the peripheral blood, mtDNA CN and mtDNA⁴⁹⁷⁷ DR, with PCOS in a clinical setting.

Methods We performed a study involving 263 women with PCOS and 326 age-matched controls between January 2016 and December 2018. The mtDNA CN and mtDNA⁴⁹⁷⁷ DR were measured via a multiplex probe-based qPCR. The associations of mtDNA CN and mtDNA DR with the risk of PCOS were estimated by logistic regression.

Results In the analysis between mtDNA biomarkers and PCOS, the mtDNA CN ($P = 0.003$) and mtDNA⁴⁹⁷⁷ DR ($P < 0.001$) levels in PCOS patients were significantly higher than those in the controls. After adjusting for BMI, LH/FSH, and T, only higher mtDNA⁴⁹⁷⁷ DR was associated with PCOS (odds ratio 1.053, 95% confidence interval 1.024 to 1.083, $P < 0.001$). The linear dose-response trends of mtDNA⁴⁹⁷⁷ DR were also supported by the quartile analyses.

Conclusions Multivariable models suggest that mtDNA⁴⁹⁷⁷ DR levels are strongly associated with PCOS and represent an independent risk factor for PCOS. Future investigation of the utility of mtDNA as a biomarker for PCOS is warranted.

1. Background

Polycystic ovary syndrome (PCOS) is a common worldwide endocrine disorder with a prevalence ranging from 4–19.9% according to the 2003 Rotterdam criteria [1, 2]. As a heterogeneous disease, PCOS is characterized by polycystic ovarian morphology, hyperandrogenism, and ovulatory dysfunction. Women with PCOS are frequently accompanied by infertility, chronic inflammation, and metabolic dysfunction such as insulin resistance (IR) and obesity. Consequently, PCOS increases the risk of type 2 diabetes, cardiovascular events, pregnancy complications such as gestational diabetes, premature birth, eclampsia, etc. [3–5]. Over the last few years, increased oxidative stress (OS) has been confirmed to be involved in PCOS progression. An earlier report showed that circulating markers of OS levels in PCOS were significantly higher than that of normal women [6]. Additionally, Chen et al. found a higher level of OS in the visceral adipose tissue in PCOS and its strong correlation with IR independent of obesity [7]. Besides, other studies have shown that OS has close interactions with related complications of PCOS, such as IR, low-level inflammation, and hyperandrogenism [8–10].

The mitochondrion is a semiautonomous organelle containing its own circular double-stranded genome and an independent biogenesis system. Mitochondrial DNA encodes 13 essential genes for oxidative phosphorylation (OXPHOS), together with two ribosomal RNAs and 22 transfer RNAs. As the powerhouse of cells, mitochondria regulate the production of cellular ATP and the generation of reactive oxygen species (ROS). Mitochondria have been universally acknowledged to be critically involved in the

regulation of OS. Mitochondrial DNA (mtDNA) copy number (CN) is a relative measure of the mitochondrial content or the mass of mitochondrion. Many studies have indicated that abnormal mtDNA is associated with the pathophysiology of PCOS [11–15]. However, due to the conflicting results of these studies, the exact association of mtDNA content and PCOS remain undetermined. Similar to mtDNA CN, mtDNA 4977-bp (mtDNA⁴⁹⁷⁷) deletion, occurred in two 13 bp direct repeats in mtDNA, is another biomarker that reflects the relative integrity and damage of mtDNA. To our knowledge, it is unknown whether mtDNA⁴⁹⁷⁷ deletion rate (DR) is linked with PCOS. The purpose of this study is to examine the association of two mtDNA biomarkers in the peripheral blood, mtDNA CN and mtDNA⁴⁹⁷⁷ DR, with PCOS in the clinical setting.

2 Methods

2.1 Participants

This study was conducted in the International Peace Maternal and Child Health Hospital (IPMCH) of Shanghai Jiao Tong University School of Medicine (Shanghai, China). The study population consisted of 263 PCOS cases and 326 age-matched controls. The cases represented approximately 90% of the patients who had sought IVF treatment due to PCOS from January 2016 to December 2018. The PCOS were diagnosed according to the 2003 Rotterdam criteria, which requires the presence of at least two of the following conditions: (1) ultrasonographic polycystic ovarian morphology; (2) clinical or biochemical hyperandrogenism; (3) oligomenorrhea or amenorrhea [16]. Women with other causes of oligomenorrhea or hyperandrogenism, such as congenital adrenal hyperplasia, 21-hydroxylase-deficient non-classic adrenal hyperplasia, hyperprolactinemia, Cushing's syndrome, androgen-secreting tumors, or androgenic/anabolic drug use or abuse, were excluded from this study. During the same period, women who attended the IVF clinics due to male infertility or tubal factor infertility were recruited as controls and matched by age (in 5y bands) with cases. All the control participants had regular menstrual cycles, normal androgen levels, and no hirsutism, diabetes, galactorrhea, or any endocrine or systemic disease that could affect reproductive physiology. Information concerning their demographic characteristics, including age, body mass index (BMI), and biochemical parameters, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E₂), and progesterone (P), was collected.

2.2 DNA extraction

Peripheral blood samples of the participants were collected, processed, aliquoted, and frozen at -80 °C according to standardized procedures. The total DNA was extracted by DNeasy Blood & Tissue Kit (QIAGEN, cat #69504) following the manufacturer's instructions. DNA was quantified, and the DNA integrity was examined.

2.3 Quantification of mtDNA CN and mtDNA⁴⁹⁷⁷ DR

The mtDNA CN and mtDNA⁴⁹⁷⁷ DR were measured by real-time quantitative polymerase chain reaction (qPCR) using a Quantstudio 7 Flex real-time PCR machine. A multiplex qPCR method, which has been validated by several scientific studies, was used to measure the mtDNA CN and mtDNA⁴⁹⁷⁷ DR. Briefly, two segments, one in the major arc (mtMajArc) and the other in the minor arc (mtMinArc) of the mitochondrial genome, were used to assess mtDNA⁴⁹⁷⁷ DR and CN, respectively. Taqman assays of mtMajArc and mtMinArc are detailed in **Supplementary Table S1**. RNase P (ThermoFisher, cat# 4403326) was chosen as the genomic DNA reference. The reaction components (given in final concentrations) were as follows: 250 µM for mtMajArc probes and primers, 250 µM for mtMinArc probes and primers, and 1× concentration for RNase P. Real-time PCR was performed at the same cycling conditions as previously described: 95 °C for 10 min for activation, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 60 °C [17, 18]. Three technical replicates were performed. The mtDNA CN and the mtDNA⁴⁹⁷⁷ DR were calculated by the following formulas: $\text{mtDNA CN} = 2^{\Delta\text{CT}(\text{mtDNA CN})}$, where $\Delta\text{CT}(\text{mtDNA CN}) = \text{CT}_{\text{RNase P}} - \text{CT}_{\text{mtMinArc}}$; $\text{mtDNA}^{4977} \text{ DR}(\%) = 2^{\Delta\text{CT}(\text{mtDNA}^{4977} \text{ DR})} \times 100$, where $\Delta\text{CT}(\text{mtDNA}^{4977} \text{ DR}) = \text{CT}_{\text{mtMinArc}} - \text{CT}_{\text{mtMajArc}}$.

2.4 Statistical analysis

The study included all adults with PCOS and age-matched controls who fulfilled the inclusion criteria at a ratio of 1:1.2, and thus no formal calculation of the sample size was done. Data were shown as median (interquartile range, IQR) for non-normal distributions or mean ± standard deviation (SD) for normal distributions. The mtDNA biomarkers were transformed by logarithmic conversion. Comparisons of normally distributed variables between groups were performed using unpaired *t*-tests, whereas comparisons of non-normally distributed ones were conducted using the Mann–Whitney U-test. When more than two groups were compared, the Kruskal-Wallis test, followed by the Bonferroni's posthoc test, was used. The mtDNA CN and mtDNA⁴⁹⁷⁷ DR were analyzed as both continuous variables and quartiles, with the first quartile as the reference group. Binary logistic regression was implemented to assess the associations of mtDNA CN and mtDNA⁴⁹⁷⁷ DR with PCOS. Odds ratios (ORs) and 95% confidence intervals (CIs) were reported for each analysis. All statistical analyses were performed by R statistical software, version 3.6.2. A *P*-value of 0.05 or less (two-sided) was considered significantly different.

3 Results

3.1 Demographic and biochemical characteristics of cases and controls

From June 2015 to June 2019, 263 women with PCOS and 326 controls were included in this study. Demographic characteristics and biochemical parameters were summarized in **Table 1**. Significant differences were found between PCOS cases and controls in BMI, FSH, LH, LH/FSH, and T (*P* < 0.001 for all comparisons), except for E₂ (*P* = 0.07).

Table 1. Clinical characteristics of the women with PCOS and the control subjects.

Variable	Control (n = 326)	PCOS (n = 263)	<i>P</i> -value ^a
Age (y)	29.00 (28.00, 33.00)	29.00 (27.50, 31.00)	0.133
BMI (kg/m ²)	20.80 (19.49, 22.86)	23.80 (21.69, 25.96)	≪0.001
FSH (IU/L)	7.70 (6.40, 8.96)	6.80 (5.70, 7.90)	≪0.001
LH (IU/L)	4.20 (3.10, 5.30)	7.90 (4.90, 12.40)	≪0.001
LH/FSH	0.53 (0.40, 0.71)	1.16 (0.72, 1.75)	≪0.001
E ₂ (pmol/L)	164.00 (119.20, 206.80)	173.00 (125.50, 230.50)	0.07
T (nmol/L)	1.41 ± 0.46	2.24 ± 0.76	≪0.001

Data are given as median (IQR) or mean ± SD.

IQR, interquartile range. SD, standard deviation.

^a *p* values obtained by comparison of variables between PCOS and controls using the Mann–Whitney U-test.

3.2 Increased relative mtDNA CN and mtDNA⁴⁹⁷⁷ DR in PCOS

The mtDNA CN and mtDNA⁴⁹⁷⁷ DR between the two groups were compared using the Mann–Whitney U-test and the Student's *t*-test, respectively. As shown in **Figure 1**, the mean log-transformed mtDNA CN in women with PCOS was statistically higher than that in the controls (2.00 versus 1.95, *P* = 0.002). Additionally, the mtDNA⁴⁹⁷⁷ DR was significantly higher in the PCOS patients compared with controls (1.52 versus 1.48, *P* < 0.001).

Further, all subjects were divided into four groups according to the quartiles of LH/FSH. A significant difference was observed in mtDNA CN among the four groups, as well as in mtDNA⁴⁹⁷⁷ DR (**Supplementary Table S2**). After Bonferroni's correction, a significant difference was confirmed between the lowest quartile and highest quartile (*P* = 0.011) for mtDNA⁴⁹⁷⁷ DR. However, no differences were found between the four groups in terms of mtDNA CN.

3.3 Associations of mtDNA CN and mtDNA⁴⁹⁷⁷ DR with PCOS

The associations of mtDNA CN and mtDNA⁴⁹⁷⁷ DR with PCOS were assessed using logistic regression models (**Table 2**). The mtDNA CN was significantly associated with PCOS. However, after adjustment for BMI, LH/FSH, and T, mtDNA CN as a linear continuous variable was not associated with PCOS (OR = 1.004, 95% CI: 0.999, 1.008). It was consistent with the quartile analyses results, which showed no statistically significant associations between the first quartile and higher quartiles (**Figure 2**, **Supplementary Table S3**).

Table 2. Odds ratios and 95% CIs for PCOS with mtDNA biomarkers and metabolic parameters.

	Odds ratio (95% CI)	<i>P</i> -value	Adjusted† OR (95% CI)	<i>P</i> -value
mtDNA CN	1.005 (1.002, 1.008)	0.003	1.004 (0.999, 1.008)	0.091
mtDNA ⁴⁹⁷⁷ DR	1.047 (1.027, 1.069)	< 0.001	1.053 (1.024, 1.083)	< 0.001

mtDNA, mitochondrial DNA; mtDNA⁴⁹⁷⁷, mitochondrial DNA 4977-bp; CN, copy number; DR, deletion rate; OR, odds ratio; CI, confidence interval.

†Adjusted for body mass index, luteinizing hormone/follicle-stimulating hormone, and testosterone.

Similarly, mtDNA⁴⁹⁷⁷ DR was significantly associated with PCOS ($P < 0.001$). However, unlike mtDNA CN, the linear dose-response trends were still apparent in the adjusted model. As can be observed in **Table 2**, the adjusted OR (95% CI) for PCOS by mtDNA⁴⁹⁷⁷ DR was 1.053 (1.024, 1.083). This relationship was also supported by the quartile analysis results (**Figure 2, Supplementary Table S3**). Compared to the first quartile, the higher quartiles of mtDNA⁴⁹⁷⁷ DR were associated higher risk of PCOS, especially in the highest quartile (OR = 3.867, 95% CI: 1.847, 8.096). These results imply that the association between mtDNA⁴⁹⁷⁷ DR and PCOS is independent of metabolic parameters.

4 Discussion

In this study, we observed elevated mtDNA CN and mtDNA⁴⁹⁷⁷ DR in the peripheral blood of PCOS patients compared with control subjects. However, after adjustments for confounding factors, such as BMI, LH/FSH, and T, only a high level of mtDNA⁴⁹⁷⁷ DR revealed an independent association with PCOS.

Several studies on the mtDNA content in PCOS patients have been conducted. Lee et al. provided the first evidence of decreased peripheral mtDNA CN in PCOS patients [14]. Recently, another two studies indicated the same tendency, showing that mtDNA CN in the peripheral blood of PCOS patients was significantly lower than that in healthy controls matched by age and BMI [11, 12]. However, this study revealed an elevated mtDNA CN in the peripheral blood of PCOS patients. Conflicting results have also been reported in other studies. Rabol et al. demonstrated that the mitochondrial content in skeletal muscle did not differ between the controls and the PCOS group [19]. Min et al. detected decreased mitochondrial respiration ability but increased mtDNA CN in PCOS patient-derived induced pluripotent stem cells (iPSCs) [20]. As a relative measure of the mass of mitochondria and an indicative marker of mitochondrial biogenesis, mtDNA CN is regulated mainly by nuclear-encoded genes, including the mitochondrial transcription factor A (TFAM), the peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), and the nuclear respiratory factors (NRFs). In Min's study, consistent with increased mtDNA CN, the expression levels of PGC-1 α , TFAM, and NRF1 were greater in PCOS patient-derived iPSCs compared with control-derived iPSCs. This inconsistency may be attributed to participants' preference or

the different sample size of the studied population. Furthermore, different stages of disease manifestation might have influenced the mtDNA CN in the peripheral blood. While the exact association between mtDNA content and PCOS remains unclear, it seems that changes in mtDNA content may be causative or indicative of disease. Considering that the association between mtDNA CN and PCOS is not independent of metabolic factors, we speculated that the increased mtDNA CN is a compensatory mechanism for the mitochondrial dysfunction caused by mtDNA damage or mutation. Further research needs to be undertaken to explore the possible biologic mechanisms of abnormal mtDNA CN in PCOS.

The mtDNA⁴⁹⁷⁷ deletion, involving huge destruction of almost one-third of the length of mtDNA, is the most common large deletion in the mitochondrial genome [21]. The deleted region contains 5 tRNA genes and 7 genes encoding 4 complex I subunits, 1 complex IV subunit, and 2 complex V subunits. The mtDNA⁴⁹⁷⁷ deletion has been considered as a pathogenic mutation in human as the loss of the tRNA genes and coding regions could cause a failure of ATP production and mitochondrial dysfunctions. Increased OS has been reported to trigger the accumulation of deletions in mtDNA [21, 22]. Consistent with evidence linking OS with mitochondrial destruction [7, 23], our study found that mtDNA⁴⁹⁷⁷ DR was a risk factor for PCOS independent of metabolic parameters. It is plausible to hypothesize that the high level of OS in PCOS might be a potential trigger of mtDNA⁴⁹⁷⁷ deletion. When the deletions accumulate to a threshold, it would cause mitochondrial dysfunctions and be involved in the pathogenesis of PCOS. Moreover, it is important to note the significantly higher mtDNA⁴⁹⁷⁷ DR in women with the highest LH/FSH. Increased LH/FSH ratio has been widely accepted as a specific endocrine profile in many PCOS patients. Such results have confirmed that high mtDNA⁴⁹⁷⁷ DR in peripheral blood is associated with PCOS.

To our knowledge, this study is the first to evaluate the associations between mtDNA⁴⁹⁷⁷ DR and PCOS. The quantification of mtDNA⁴⁹⁷⁷ DR and CN were assessed simultaneously using an efficient and effective multiplex real-time PCR assay, which makes our results reliable [17, 18]. Additionally, the sample size of our study was larger than those of previous similar studies assessing the association between mtDNA CN and PCOS. However, it should be noted that this study is a single-center study based on cross-sectional data. Multicenter studies are warranted to validate these findings in the future.

5 Conclusions

In summary, the present study demonstrates the contribution of mtDNA⁴⁹⁷⁷ DR to PCOS independent of metabolic parameters for the first time. Further studies are warranted to elucidate the molecular mechanisms underlying these associations.

6 Abbreviations

BMI Body mass index

CN Copy number

DR Deletion rate

E2 Estradiol

FSH Follicle-stimulating hormone

IR Insulin resistance

iPSCs Induced pluripotent stem cells

LH Luteinizing hormone

mtDNA Mitochondrial DNA

NFRs Nuclear respiratory factors

OS Oxidative stress

OXPHOS Oxidative phosphorylation

P Progesterone

PCOS Polycystic ovary syndrome

PGC-1 α Peroxisome proliferator-activated receptor - γ coactivator-1 α

ROS Reactive oxygen species

TFAM Transcription factor A

7 Declarations

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Authors' contributions

All the authors contributed to the study conception and design. The recruitment of participants and data collection were performed by XL and LJ. The measurement of mtDNA biomarkers and data analysis were performed by WS, BH, FG, and MY. The draft of the manuscript was written by MY. The design of the study and writing review and editing were performed by CX and SL.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of IPMCH (no. GKLW2018-12). The research was carried out according to the relevant guidelines and ethical regulations in research involving human participants. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.

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Figures

A **B**

Figure 1

Mitochondrial DNA (mtDNA) copy number (CN) and mtDNA 4977-bp (mtDNA4977) deletion rate (DR) in women with PCOS and controls. A. Log-transformed mtDNA CN in women with PCOS and controls ($P = 0.002$). B. Log-transformed mtDNA4977 DR in women with PCOS and controls ($P < 0.001$).

A

B

Figure 2

Odds ratios and 95% CIs for PCOS by quartiles of mitochondrial DNA copy number (A) and 4977-bp deletion rate (B), adjusted for body mass index, luteinizing hormone/follicle-stimulating hormone, and testosterone.

Supplementary Files

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