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CURRENT OPINION

Deciphering the DNA Methylome of Polycystic Ovary Syndrome

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Abstract
Polycystic ovary syndrome (PCOS) is a hormonal disorder common among women of reproductive age. PCOS is characterized by ovarian dysfunction and metabolic abnormalities with widely varying clinical manifestations brought about by intricate mechanisms of interplay between the genome and the environment. The popularity of epigenome-wide association studies (EWASs) is helping to facilitate the discovery of environment-mediated molecular modification in PCOS from disease etiology to epigenetic marker discovery. Current epigenetic studies have provided convincing observational evidence linking epigenetic regulation with PCOS origin, manifestation, clinical heterogeneity and comorbidity, which could lead to improved management of the disease through efficient intervention and prevention strategies. Several biological pathways have been consistently reported by independent studies, revealing functional regulation due to endocrine abnormalities and metabolic dysfunction in PCOS, while also suggesting an autoimmune component in the condition. The use of high-throughput sequencing technologies for analysing the epigenome integrated with causal inferences is expected to facilitate effective and efficient PCOS management to promote reproductive health.

Key Points

Epigenetic studies help to elucidate the intricate interplay between the genome and the environment in polycystic ovary syndrome (PCOS) development and manifestation.

New approaches in omics techniques and experiment design will enable identification of causal epigenetic biomarkers to serve as targets for efficient intervention and prevention of PCOS.

1 Background

Polycystic ovary syndrome (PCOS) is a complex condition representing a group of symptoms that result from hormonal and metabolic abnormalities, and is the most common endocrine dysfunction in reproductive age women and is often associated with infertility. According to the widely accepted Rotterdam diagnostic criteria, any woman presenting with at least two of the three following characteristics can be diagnosed as having PCOS: clinical and/or biochemical hyperandrogenism, menstrual irregularities (oligo- and/or anovulation) due to ovulatory dysfunction, polycystic ovarian morphology, and exclusion of other aetiologies (congenital adrenal hyperplasias, androgen-secreting tumours, Cushing's syndrome) [1]. The assessment of PCOS is usually straightforward and includes clinical (hirsutism, oligomenorrhoea or amenorrhoea and evaluation of ovarian morphology by ultrasonography), endocrine [high serum concentrations of free testosterone or high circulating concentrations of anti-Mullërian hormone, increased luteinizing hormone (LH)/follicle-stimulating hormone (FSH) ratio] and metabolic (insulin resistance, metabolic cardiovascular syndrome, dyslipidaemia, high rates of premature impaired glucose tolerance, type 2 diabetes and increased cardiovascular risk factors) features. The treatment strategies are mostly symptom-oriented, with therapeutic approaches

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targeting hyperandrogenism and/or the associated metabolic disorders [2]. PCOS is likely a result of interactions between genetic susceptibility and environmental exposures, including toxins, diet and nutrition, inactivity, socioeconomic status, and geography [3], leading to heterogeneous expression of the syndrome. A twin-family study has estimated a heritability of around 60% [4]. A recent large-scale, genome-wide meta-analysis reported a dozen loci associated with the risk of PCOS [5], indicating shared genetic architecture across the heterogeneous expression of the condition. Moreover, animal studies have shown PCOS-like phenotypes including ovarian dysfunction induced by prenatal exposure to excessive androgen [6], suggesting the important roles of environmental interference in PCOS development through foetal programming.

By definition, epigenetics is the study of readily reversible mitotically and/or meiotically heritable changes that do not entail a change in DNA sequence [7]. Epigenetic modifications can be grouped into three main mechanisms: DNA methylation, histone modifications and RNA-based mechanisms. Among them, DNA methylation is one of the best understood, most readily measureable and widely analysed molecular modifications in current epigenome-wide association studies (EWASs). The widespread use of high-throughput technologies such as microarray-based genome-wide DNA methylation analysis (e.g. Illumina BeadChip Arrays) and whole-genome bisulfite sequencing (WGBS) powered by next-generation sequencing (NGS) [8] is revolutionizing our exploration and understanding of the molecular basis of PCOS development and clinical manifestations brought about by an intricate interplay between our genome and environmental exposure. This paper focuses on recent developments in DNA methylome association studies on PCOS by summarizing the major findings and giving author opinions on the biological and clinical implications as well as pointing out limitations for further improvement.

2 Epigenetics in PCOS Pathogenesis

The pathogenesis of PCOS is still not precisely known [9, 10], with different hypotheses [11–13] concerning hormonal imbalance, insulin resistance, genetic inheritance and inflammation. Among the different hypotheses, the most widely accepted is the developmental origin of PCOS [12, 13]. There has been firm evidence from experimental studies of animal models showing that exposure to excess androgen during foetal life of rhesus monkeys [14, 15], sheep [16–18] and rats [19, 20] could lead to pathophysiological changes closely resembling the clinical features in PCOS women. It is ethically challenging to replicate these experiments in humans. However, supportive clinical data have been reported by studies on female foetuses with molecular deficiencies that led to prenatal hyperandrogenaemia [21, 22]. Interestingly, these females manifested PCOS features later in life.

It has been shown that young maternal age and excessive maternal weight gain may increase the prenatal androgen exposure of female foetuses [23]. Barrett et al. [24] reported that prenatal exposure to stressful life events may masculinize some aspects of female reproductive development in humans. A recent methylation profiling of granulosa cells found that altered methylation in *AKR1C3*, *GHRHR*, *MAMLD1*, *RETN* and *TNF* genes indirectly contributed to androgen excess in PCOS patients [25]. Li and Huang [26] proposed the 'epigenetic abnormality' hypothesis for PCOS development, which considers that in utero exposure of the developing female foetus to high maternal androgens may disturb the epigenetic reprogramming of its reproductive tissue and result in development of PCOS-like phenotypes in adulthood. The hypothesis further postulated that the incomplete erasure of the epigenetic abnormality in germ cells after fertilization would confer intergenerational inheritance of PCOS. The epigenetic mechanism underlying the development of PCOS-like phenotypes in prenatally androgenized animals and the intergenerational inheritance of these phenotypes have also been revealed using the microarray technology [15, 20]. Multiple biological pathways were found to be significantly regulated, including the antiproliferative role of TOB in T-cell signalling and transforming growth factor-β signalling [15]. Their results suggest that prenatal androgenization may predispose individuals to PCOS via alteration of the epigenome. Even those who argue against the foetal origin hypothesis have speculated that an epigenetic mechanism is involved in the 'developmental window' of PCOS, spanning well beyond foetal life [27].

3 EWASs and PCOS Comorbidity

Epidemiological studies have shown that PCOS patients are at a higher risk for metabolic and psychiatric comorbidities [28], with the prevalence of metabolic diseases differing across geographic regions and ethnicities. For example, while De Leo et al. [29] found about 60–70% of Italian PCOS patients are obese, a Chinese study [30] reported a prevalence of 18.2% for metabolic syndrome in Chinese PCOS patients. Nevertheless, metabolic disorder is the most common comorbidity with PCOS. It is interesting that, although pathways related to metabolic and psychiatric diseases are also enriched by significant methylation sites, the most frequently reported functional pathways in EWASs are implicated in autoimmune diseases (e.g. type I diabetes mellitus, thyroid disease and asthma pathways), as suggested by EWASs on PCOS in ovarian tissue [31], whole blood [32, 33] and skeletal muscle [34], suggesting a

common mechanism across tissues. Note that the study by Shen et al. [33] was conducted using a genome-wide methylated DNA immunoprecipitation (MeDIP) analysis with a higher coverage of the DNA methylome than the microarray-based analysis used in the other studies. By comparing the nationwide Danish population of PCOS patients with a large control group, Glintborg et al. [35] reported a significantly increased prevalence for multiple diseases in PCOS patients, including autoimmune diseases. In their analysis, about 10% of PCOS patients were also diagnosed with autoimmune diseases. In the literature, a high prevalence of autoimmune disorders was also observed in PCOS patients [36–38]. Although the prevalence of autoimmune diseases is not as high as metabolic disorders in PCOS patients, it is present across populations, with notably high frequencies. Here, it is interesting that the epidemiological observations are supported by biological pathways that were significantly over-represented by the differentially methylated genomic sites identified by EWASs on different tissues of PCOS patients, and using different platforms for DNA methylation analysis. In a published EWAS on PCOS [32], we also found the systemic lupus erythematosus pathway as differentially regulated under the PCOS condition, providing further support for autoimmune involvement in PCOS. To sum up, results from current epigenetic studies on PCOS provide revealing observational evidences that link PCOS with autoimmune diseases [39, 40].

4 Epigenetics in PCOS Clinical and Biochemical Heterogeneity

As a complex disorder, PCOS is heterogeneous clinically and biochemically, with the commonly associated features neither uniform nor universal [41, 42]. The molecular basis underlying the heterogeneous clinical expression of PCOS has been investigated using high-throughput omics approaches and reported molecular biomarkers for metabolic heterogeneity [33, 43]. Based on genomic DNA methylation profiles measured in our PCOS patients, we conducted epigenetic association analyses on multiple clinical and biochemical features, including metabolic parameters [32]. Highly significant epigenetic associations were observed in PCOS patients for multiple reproductive hormones, including oestradiol, prolactin and progesterone. Although we observed comparable mean levels of the three hormones in PCOS patients and healthy controls ($p > 0.38$), the levels of oestradiol and prolactin displayed larger dispersions (2.5–97.5 quantiles) in PCOS samples (13.8–151.5 pg/ml for oestradiol; 4–55 ng/ml for prolactin) as compared with the controls (27.9–115.9 pg/ml for oestradiol; 5.8–27.2 ng/ml prolactin) [32]. The data suggest higher heterogeneity in the patient groups, which could be regulated by DNA methylation at the identified significant CpG sites. Interestingly, the significant CpGs were identified in association analysis of the hormones in PCOS samples only. Similar epigenetic association analysis on the levels of oestradiol and prolactin performed on the healthy controls failed to detect any significant CpG, indicating that the significant results were driven by the large variation or heterogeneity in the levels of these hormones in PCOS patients. The CpG sites significantly associated with prolactin levels in the patient group were linked to immune reaction and autoimmune diseases (diabetes and thyroid disease), while CpGs associated with oestradiol were involved in biological pathways for synthesis of reproductive hormone and for drug metabolism. Very recently, we performed a genomic region-based association analysis of epigenetic regulation on PCOS heterogeneity and identified seven differentially methylated regions (DMRs) on chromosome 19 (12877188–12876846 bp) and chromosome 6 (MHC region) associated with prolactin level and on chromosomes 11 and 2 associated with metabolic attributes [44]. Functional annotation linked the significant DMRs to functional genes (*HOOK2*, *BDNF*, *HLA-G*, *HLA-H*, *HLA-J*, *RNF39*, etc.) of metabolic disorders and immunity. Interestingly, Jones et al. [45] found genetic variations at *LHCGR* and *INSR* regions differentially methylated/expressed in PCOS patients stratified by obesity, suggesting involvement of cis-methylation quantitative trait loci (meQTL) and cis-expression quantitative loci (eQTL) in regulating PCOS sub-phenotypes. These novel results emphasize the important roles of both genetic and epigenetic regulation behind the observed heterogeneity in the clinical and biochemical expressions of PCOS.

5 Challenges in Epigenetic Biomarker Discovery

Although the literature of epigenetic studies on PCOS has grown considerably, identifying epigenetic biomarkers for PCOS is still challenging due to multiple factors, including limitations in experiment design [46], patient heterogeneity, ethnic differences in the expression of PCOS [47] and the limited availability of tissue biopsies such as skeletal muscle and ovarian tissue for investigating tissue-specific methylation patterns. It is true that consistent functional pathways have been found in studies using different tissues, but no replication of the significant CpG sites across studies has been reported except for some overlapping results at the gene level, e.g. the *OXPHOS* genes in the EWAS by Nilsson et al. [34] and the gene expression analysis by Skov et al. [48], both using skeletal muscle. Instead of focusing on single CpGs, a better strategy would be to perform replications at the gene level to define epigenetic biomarkers for PCOS. Moreover, considering the heterogeneous expression of

PCOS, it is biologically more sensible to look for epigenetic markers for PCOS clinical sub-phenotypes in well categorized/stratified patients, because a specific sub-phenotype is supposed to be under closer epigenetic modification than the composite phenotype of PCOS.

It is necessary to point out that nearly all EWASs on PCOS are observational studies by nature. In epidemiology, observational studies are considered to have less probative force due to the inherent limitation in controlling confounding factors that influence both explanatory (exposure, here, DNA methylation) and clinical or health outcome (here, PCOS) variables, a situation that can result in biased, confusing and even misleading results [49]. As a matter of fact, a significant epigenetic association does not guarantee a causal effect, but just a superficial correlation or coincidence. This means that the identified significant markers from EWASs on PCOS could be meaningless for intervention purposes if they are not causal [47]. In fact, efforts have been made to infer causality from observational studies [50], with effective and valuable approaches proposed such as propensity score matching [51]. Through examining the cross-trait cross-pair correlation between the predictor of one relative and the outcome of the other relative, Hopper and colleagues performed 'inference on causation studies from the examination of familial confounding factors' (ICE FALCON) using regression analysis [52]. The method has been recently applied to EWASs using twins, and reported causal effects of smoking [53] and body mass index [54] on site-specific DNA methylation variations in blood cells. Introducing causal inference in EWASs on PCOS using different tissues should help to verify and identify causal epigenetic biomarkers (tissue specific and non-specific) to serve as targets for efficient prevention, intervention and management.

6 Conclusions

Epigenetic association studies are gaining popularity because they offer an appealing tool for studying PCOS, a complex and heterogeneous condition mediated by individual genetic makeup (nature) and disrupting prenatal and/or postnatal environments (nurture). Current EWASs on PCOS have revealed convincing observational evidence in relating epigenetic modification with PCOS development and manifestation which could contribute to efficient management of the disease and promote reproductive health. The epigenetic study of PCOS will further benefit from NGS-based whole-genome DNA methylation analysis and from emerging approaches for genome-wide profiling of histone modifications and for analysing post-transcriptional regulations. Introducing causal inference in epigenetic association studies is going to enable the identification of causal epigenetic biomarkers, functional genes and biological pathways for revealing the underlying aetiology of PCOS and enabling efficient and effective management of the disorder.

Compliance with Ethical Standards

Conflict of Interest The author (QT) declares that no competing interests exist.

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