Key genes and pathways of ovarian granulosa cells in polycystic ovary syndrome identified by bioinformatics analysis

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Original Article

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Abstract

Purpose

Polycystic ovary syndrome (PCOS) is one of the factors leading to infertility. The specific pathogenesis of PCOS is still unclear. The purpose of this study was to determine key changes in gene expression during the formation of PCOS and to provide a theoretical basis for the clinical diagnosis and treatment of PCOS.

Methods

We analyzed differentially expressed genes (DEGs) in the dataset GSE34526 from the bioinformatics array research tool (BART) online analysis tool (bart.salk.edu). Then, through the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) online analysis software for gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) rich path analysis, STRING (https://string-db.org/) online analysis tool for protein-protein interaction (PPI) network, Cytoscape software for Mcode module and HUB gene analysis

Results

A total of 91 DEGs, 7 up-regulated and 84 down-regulated, were found. Seven central HUB genes were identified, including integrin alpha-M (ITGAM), cytochrome BMUR 245 beta chain (CYBB), toll like receptor 1 (TLR1), platelet activating factor receptor (PTAFR), CD163 molecule, caspase 1 (CASP1), and matrix metallopeptidase 9 (MMP 9).

Conclusion

The DEGs, HUB genes and signaling pathways identified in this study help us understand the molecular mechanism of PCOS formation and provide new targets for the diagnosis and treatment of PCOS.

Introduction:

Polycystic ovary syndrome (PCOS) is a common reproductive endocrine disease in women of childbearing age with an incidence of 5.6–16% and is increasing every year. PCOS is considered a systemic multisystem disease, such as hyperandrogenemia, anovulation, irregular menstruation, infertility and metabolic abnormalities, including insulin resistance and hyperlipidemia [1, 2]. PCOS is caused by many causes, including genetic, psychosocial factors, bad living habits and environmental factors (chemicals: pesticides, industrial pollutants; personal care products: perfumes, deodorants, hair dyes, perfume compounds and bisphenol A). The specific etiology and pathogenesis of the disease are not currently clear [3–6]. Ovarian granulosa cells (GCs) have the function of secretion, which plays an
important role in the process of folliculogenesis [7]. Understanding gene expression of PCOS GCs is of great significance for effective diagnosis and treatment. In previous literature, many scholars have analyzed potential differential genes in the GSE34526 gene expression profile, performing gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, PPI network analysis and Mcode module analysis but not Hub gene analysis [8–12].

In this study, we used the bioinformatics array research tool (BART) online analysis tool to analyze the original microarray dataset GSE34526 (healthy samples and PCOS female ovarian GCs for differentially expressed genes (DEGs). GO enrichment and KEGG pathway analysis, PPI network, Mcode module and Hub analysis were used to determine the genes, pathways and molecular mechanisms related to ovarian granulosa cells in women with PCOS to provide a theoretical basis for clinical diagnosis, treatment and prevention of PCOS.

**Materials And Methods:**

**Microarray Data and Identification of DEGs**

GEO (http://www.ncbi.nlm.nih.gov/GEO) [13] is a public functional genome database that contains high-throughput gene expression data, chips and microarrays. A gene expression dataset [GSE34526] is selected through GEO (GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array). The GSE3526 dataset includes 10 samples, including 3 normal female ovarian granulosa cell samples and 7 PCOS female ovarian granulosa cell samples (Human granulosa cells were isolated from ovarian fluid aspiration of normal and PCOS women who received *in vitro* fertilization)

DEGs were downloaded from the BART (bart.salk.edu) [14]. The platform can process raw microarray data from GEO or local into a list of differential genes and related pathways. DEGs is determined by logFC greater than 1 or less than -1 and t-tests with adj. \( P < 0.05 \).

**GO Enrichment and KEGG Pathway Analysis of DEGs**

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) (Version 6.8) online analysis software was used for GO enrichment and KEGG pathway analysis of differential genes. GO analysis included three terms: BP (Biological Process), CC (Cellular Component), MF (Molecular Function). \( P < 0.05 \) had statistical significance in screening important GO terms and KEGG pathway.

**PPI network construction and Module Analysis**

The differential gene protein interaction network (PPI) analysis was performed using STRING (https://string-db.org/) (Version 11.0) online analysis software. Analysis of PPI has a better understanding of the pathogenesis of PCOS, and a minimum required interaction score of 0.400 indicates statistical significance. The TSV file guide Cytoscape (www.Cytoscape.org) (version 3.7.2) of the STRING analysis results was downloaded, which is an open source systems biology analysis software, which can
be used for data visualization. Mcode (version 1.6.1) is a plug-in of Cytoscape software. The functional module of string protein gene network was constructed by clustering, and the network formed by TSV was analyzed again. The selection criteria were as follows: MCODE degree cut-off = 2, node score cut-off = 0.2, Max depth = 100, and k-score = 2. Then, the DEGs in Mcode were analyzed using KEGG and GO software.

**HUB Gene Selection**

The Cytohubba plug-in in Cytoscape software was used for HUB gene selection. The first 10 HUB genes were screened by Radility, MHC, Degree, Stress and Closeness, and an overlapping HUB gene network was constructed.

**Results:**

**Identification of DEGs**

We use BART online analysis software to analyze the DEGs of GSE34526. BART software can automatically download data from GEO and analyze it using the LIMMA bioinformatics software package. The original fluorescence CEL file is used as input, divided into PCOS group and normal group. All samples were isolated from the ovarian fluid of normal and polycystic ovary syndrome patients undergoing in vitro fertilization. There are a total of 54675 genes. The Hclust R function is used to cluster the first 1000 expressed normalized genes (see Figure.1a), by analyzing that logFC is greater than 1 or less than -1 and t-tests with adj. $P < 0.05$. The results showed that there were 91 DEGs, 7 up-regulated genes and 84 down-regulated genes (see Figure.1b).

**GO Term Enrichment and KEGG Pathway Analysis**

The DAVID online tool was used to perform GO enrichment and KEGG pathway analysis on 91 DEGs. As shown in Figure 2, each part of the GO analysis shows the top 10 enrichment analysis results. The biological process (BP) enrichment analysis results show that predicted genes are primarily involved in inflammatory response (GO:0006954), interferon-gamma-mediated signaling pathway (GO:0060333), leukocyte migration (GO:0050900), signal transduction (GO:0007165), adaptive immune response (GO:0002250), immune response (GO:0006955), innate immune response (GO:0045087), B cell receptor signaling pathway (GO:0050853), neutrophil chemotaxis (GO:0030593), and regulation of cell shape (GO:0008360) (Figure. 2a). The results of Cell Component (CC) enrichment analysis showed that the predictive genes were primarily involved in plasma membrane (GO:0005887), integral component of membrane (GO:0016021), extracellular exosome (GO:0070062), endosome membrane (GO:0010008), clathrin-coated endocytic vesicle membrane (GO:0030669), IPAF inflammasome complex (GO:0072557), phagocytic vesicle membrane (GO:0030670), proteinaceous extracellular matrix (GO:0005578), endocytic vesicle membrane (GO:0030666) (Figure. 2b). The results of molecular function (MF) enrichment analysis showed that the predictive genes were primarily involved in protein binding (GO:0005515), transmembrane signaling...
receptor activity (GO:0004888), receptor activity (GO:0004872), and N-formyl peptide receptor activity (GO:0004982), MHC protein binding (GO:0042287), complement receptor activity (GO:0004875), collagen binding (GO:0005518), GTPase activator activity (GO:0005096), RAGE receptor binding (GO:0050786), and IgG binding 2 (GO:0019864) (Figure 2c).

The results of KEGG pathway analysis showed that the predictive genes were primarily involved in Staphylococcus aureus infection (hsa05150), Tuberculosis (hsa05152), Phagosome (hsa04145), Osteoclast differentiation (hsa04380), Leukocyte transendothelial migration (hsa04670) and cRap1 signaling pathway (hsa04015), Leishmaniasis (hsa05140), Fc gamma R-mediated phagocytosis (hsa04666), Inuenza A (hsa05164), Amoebiasis (hsa05146). (Figure 2d)

**PPI network construction and Mcode Analysis**

The TSV le was downloaded and analyzed by the STRING online analysis software, imported into Cytoscape, and inserted into the Mcode module, revealing 60 nodes and 193 edges. The nodes represent the DEGs, and edges in the PPI network represent interactions between DEGs. Based on this, two modules were obtained from the PPI network, as shown in Figure 3. Next, GO enrichment and KEGG pathway analysis of the DEGs of the two modules were performed again using the DAVID online tool. The results of GO enrichment and KEGG pathway analysis of DEGs in Mcode1 were as follows: BP enrichment analysis results primarily included innate immune response (GO: 0045087), and inflammatory response (GO: 0006954). CC enrichment analysis results included IPAF inflammasome complex (GO: 0072557). MF enrichment analysis results identified protein binding (GO: 0005515). KEGG pathways primarily identified two, Phagosome (hsa04145) and Tuberculosis (hsa0515) (Table 1). The results of GO enrichment and KEGG pathway analysis of DEGs in Mcode 2 were as follows: BP enrichment analysis results included interferon-gamma-mediated signaling pathway (GO: 0060333) and immune response (GO: 0006955). CC enrichment analysis results included clathrin-coated endocytic vesicle membrane (GO: 003066) and plasma membrane (GO: 0005886). MF enrichment analysis results identified MHC class II receptor activity (GO: 0032395), and KEGG pathway primarily identified Staphylococcus aureus infection (hsa05150) (Table 2).

**HUB Genes Selection**

In this study, we used the cytoHubba plug-in in Cytoscape software to select the HUB gene and screened the first ten genes according to the methods of Radility, MHC, Degree, Stress and Closeness (see Table 3). Finally, seven central genes were identified by overlapping the first 10 genes (see Figure 4). including integrin alpha-M (ITGAM), cytochrome BMUR 245 beta chain (CYBB), toll like receptor 1 (TLR1), platelet activating factor receptor (PTAFR), CD163 molecule, caspase 1 (CASP1), and matrix metallopeptidase 9 (MMP 9) were also selected as hub genes..

**Discussion:**
GCs play an important role in the formation of follicles and the formation of cumulus-oocyte complex around the egg [15]. Recently, many scholars have found that cumulus GC genes can predict oocyte development [16, 17].

Is the pathogenesis of PCOS related to GCs? Many scholars have found that there are changes in oocyte growth and embryonic potential in patients with PCOS. Abnormal GC function is one of the primary causes of follicular dysplasia in PCOS [18, 19]. Victor Blasco and colleagues investigated the decreased expression of TAC3, TACR3 and KISS1 mRNA in mural granulosa and cumulus cells of patients with PCOS, which may be related to abnormal follicular development and ovulation disturbance in PCOS patients [20]. These data show that abnormal gene expression in ovarian GCs is closely related to the pathogenesis of PCOS. However, there are many genes in GCs, and whether there are other additional gene abnormalities and gene interactions leading to the pathogenesis of PCOS need to be further explored.

Many scholars have performed DEGs analysis, PPI network, GO enrichment and KEGG pathway analysis of GSE34526 datasets [8–12]. Different genes were identified using different research methods, providing important clues for the diagnosis and treatment of PCOS. However, no HUB genes were analyzed. In this study, analysis of DEGs in the GSE34526 dataset (3 normal GCs and 7 PCOS female GCs) was performed using the BART online analysis software. This analysis tool has six modules. Users can test differential expression of the original microarray data from GEO or local data using the LIMMA bioinformatics software package [14]. Exclusion criteria were LogFC > 1 or <-1 and t-tests with adj. P < 0.05. A total of 91 DEGs, 7 up-regulated and 84 down-regulated, were found. GO enrichment analysis of DEGs using DAVID software showed that these genes were primarily involved in inflammatory reactions, plasma membrane and protein binding. The abnormalities of inflammatory cytokines and GC cell membrane receptors are reportedly related to the pathogenesis of PCOS [21–23]. In addition, recent studies have reported that SRAGE plays a protective role in the development of PCOS by inhibiting inflammation [24]. KEGG pathway analysis also showed that DEGs was primarily associated with infection and bacteriophage, consistent with Go enrichment analysis.

The PPI network of DEGs was analyzed by STRING, and the TSV file was downloaded and imported into the Cytoscape software, allowing identification of the two modules by the Mcode plug-in. GO enrichment and KEGG pathway analysis of DEGs in the module were performed using the DAVID online tool. Our research shows that Module 1 significantly participates in innate immune response, inflammatory response, Phagosome and IPAF inflammasome complex, while Module 2 significantly participates in interferon-gamma-mediated signaling pathway, clathrin-coated endocytic vesicle membrane, MHC class II receptor activity and Staphylococcus aureus infection. Previous research has confirmed that serum levels of interferon-γ in patients with PCOS are lower than in healthy women. Interferon-γ may be a new biomarker for the diagnosis and treatment of PCOS [22]. Androgens can induce apoptosis of GCs, a process related to macrophages. Therefore, infection and immunity play an important role in the occurrence and development of PCOS [25].

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In addition, we also analyzed the PPI network of DEGs and used five sequencing methods to identify seven HUB genes: ITGAM, CYBB, tTLR1, PTAFR, CD163, CASP1, and MMP9. ITGAM was the most prominent HUB gene, has been reported to be associated with the pathogenesis of PCOS [26, 27], but no scholars have studied its expression in ovarian GCs or the specific pathogenesis of PCOS. The relationship between CYBB, CASP1 and polycystic ovary syndrome has not been studied. At present, some studies have reported that saturated fat intake promotes the increase of circulating endotoxin levels and TLR-4 gene expression in obese women of childbearing age, especially in the presence of PCOS [28]. However, the role of TLR1 in the pathogenesis of PCOS is still unclear. PTAFR, a member of the G protein coupled receptor family, was detected in the luminal epithelial cells of embryonic diapause and is strongly expressed in all stages of resuscitation [29]. The relationship between PTAFR and PCOS has not been reported. CD163 is a marker of macrophages. Asa Lindholm and other scholars have found that expression of CD163 in peripheral blood is decreased in overweight women with PCOS [30]. Nine members of the family of matrix metalloproteinases, the main proteases involved in extracellular matrix remodeling, were identified. It was found that levels of MMP2 and 9 were higher in the circulation, follicular fluid and granulosa cells of patients with PCOS, while levels of TIMP1 were constant or low. Increased activity of MMPs may disrupt the process of tissue remodeling, as well as the availability of growth factors and gap junctional communication, leading to the development of abnormal ovarian phenotypes in women with PCOS [31].

Based on our analysis, we speculated on the potential mechanism of seven HUB genes in the formation of PCOS. However, our study is limited by a lack of analysis on the dataset related to the peripheral blood of women with PCOS. In our next study, we will further analyze whether genes related to the peripheral blood of women with PCOS are consistent with the expression of GCs. In addition, we will perform molecular biology experiments to confirm the potential mechanism of Hub genes in the formation of PCOS to provide a theoretical basis for clinical diagnosis and treatment of PCOS.

**Conclusion:**

In conclusion, 91 DEGs, 2 network modules and 7 HUB genes were identified. The seven Hub genes, ITGAM, CYBB, TLR1, PTAFR, CD163, CASP1 and MMP9, and their associated signaling pathways identified in this study are worthy of further study. Molecular biological experiments are needed to confirm the role of these genes in the formation of PCOS to provide a basis for clinical diagnosis and treatment.

**Abbreviations**

PCOS
Polycystic ovary syndrome;
DEGs
differentially expressed genes;
BART
bioinformatics array research tool; DAVID Database for Annotation, Visualization and Integrated Discovery; GO gene ontology; KEGG Kyoto Encyclopedia of Genes and Genomes; PPI protein-protein interaction; ITGAM integrin alpha-M; CYBB cytochrome BMUR 245 beta chain; TLR1 toll like receptor 1; PTAFR platelet activating factor receptor; CASP1 caspase 1; MMP 9 matrix metallopeptidase 9; GCs granulosa cells; BP Biological Process; CC Cellular Component; MF Molecular Function

Declarations

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Compliance with ethical standards

Conflict of Interest

The authors declare that there are no conflicts of interest.
Human and Animal Rights

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

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Tables
Due to technical limitations, table 1, 2, 3 is only available as a download in the Supplemental Files section.