doi: 10.4149/gpb_2018049

Identification of the key pathways and genes related to polycystic ovary syndrome using bioinformatics analysis

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Abstract. Polycystic ovary syndrome (PCOS) is the most common hormonal and metabolic disorder among women of reproductive age, but the mechanisms underlying this unique pathogenesis remain unknown. This study was therefore designed to identify candidate genes involved in the pathogenesis of PCOS, using bioinformatics analysis. The gene expression profiles of GSE34526 from 7 PCOS patients and 3 controls were downloaded from Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were identified using GCBI online tool. Expression levels of candidate genes were verified using quantitative RT-PCR (qRT-PCR) and Western blot. 426 DEGs were identified by GCBI, including 418 up-regulated and 8 down-regulated genes. Function and pathway enrichment analyses showed that these DEGs were significantly enriched in inflammation and immune-related pathways. Additionally, protein–protein interaction (PPI) network and module analyses showed that two modules involved the Toll-like receptor signaling pathway were ranked among the most upregulated modules, and the candidate genes involved in this signaling pathway consisted of *TLR1*, *TLR2*, *TLR8*, and *CD14*. Finally, expression levels of *TLR2*, *TLR8* and *CD14* were significantly increased in samples from PCOS patients. Collectively, the results suggested that the Toll-like receptor signaling pathway might play an important role in the pathogenesis of PCOS.

Key words: Bioinformatics analysis — Polycystic ovary syndrome — Microarray differentially expressed genes (DEGs) — Gene ontology

Introduction

Polycystic ovary syndrome (PCOS) is a common cause of anovulatory infertility in 5–10% reproductive-aged women (Wang and Alvero 2013). It is characterized by hyperandrogenmia, irregular or absent ovulation, and polycystic ovary. Currently, it is believed that both genetic and environmental factors may play important roles in the occurrence and development of PCOS (de Melo et al. 2015). Due to the diversity and complexity of PCOS, its etiologies and mechanisms are largely unknown. Therefore, understanding the molecular mechanism of occurrence and development in PCOS is crucial to develop the more effective diagnostic and therapeutic strategies.

Microarrays technology, as one of the large-scale and efficient techniques to collect biological information, can monitor genome-wide changes in gene expression levels and detect sequence changes of tens of thousands of genes simultaneously (Zhang et al. 2004). At present, microarray technology has been widely employed in studies on many diseases (Duan et al. 2017; Pereira et al. 2017; Zhang et al. 2017). Recently, there are studies using microarrays to identify potentially candidate genes associated with PCOS (Aydos et al. 2016; Lei et al. 2017; Su et al. 2017). However, the involved signaling pathways and related candidate genes in the occurrence and development of PCOS remain to be further defined.

Electronic supplementary material. The online version of this article (doi: 10.4149/gpb_2018049) contains supplementary material, which is available to authorized users.

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In this study, we downloaded the original data (GSE21815) from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). The differentially expressed genes (DEGs) of granulosa cells (GCs) from PCOS patients were screened using GCBI online tool. Subsequently, the function and pathways enrichment analysis for DEGs were analyzed. Additionally, we established protein-protein interaction (PPI) network of the DEGs and picked out major signaling pathways and the related candidate genes. Expression levels of these candidate genes were finally verified by qRT-PCR analysis. Overall, our systematic analysis will gain insights into PCOS pathogenesis at molecular level and help to identify the potential candidate biomarkers for diagnosis, prognosis, and drug targets for PCOS.

Materials and Methods

Microarray data

The gene expression profiles of GSE34526 were downloaded from GEO database (http://www.ncbi.nlm.nih.gov/geo). The GSE34526 was based on Agilent GPL570 platform ([HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array).

Differentially expressed genes (DEGs) analysis

The DEGs was analyzed by web-based tool GCBI (https://www.gcbi. com.cn/gclib/html/index), which is a platform that combines a variety of research findings, genetic informations, sample informations, data algorithms and bioinformatics (Xiao et al. 2017). Cluster analysis was performed to identify DEGs, using p value < 0.05 and fold change |logFC| ≥ 2 as cut-off criteria.

Enrichment analysis of DEGs

In order to analyze the biological processes involved in the pathogenesis of PCOS, gene ontology (GO) enrichment analysis was performed using DAVID (The Database for Annotation, Visualization and Integrated Discovery). $p < 0.05$ was considered significantly different. To further improve interpretation of the biological significance, we constructed pathway relation network for the DEGs and identified the relationship among the pathways using the ClueGO plug-in of Cytoscape software.

PPI network and module analysis

To evaluate the functional interactions between DEGs, we carried out PPI network analysis. First, we mapped the DEGs to STRING version 10.5 (http://string-db.org/), and a combined score with values > 0.4 was set as the cut off criterion. Then, PPI networks were constructed and visualized using the Cytoscape version 3.6.0. The plug-in Molecular Complex Detection (MCODE) app was used to screen the modules of PPI network in Cytoscape. The criteria were set as follows: MCODE scores ≥ 4 and number of nodes > 4. Moreover, the genes of pathway analysis were performed in the top 3 modules by DAVID.

Subjects

78 patients were enrolled into the study from Reproductive Medicine Center, Shanxi women and children's hospital. PCOS patients were diagnosed according to 2003 Rotterdam diagnostic criteria and patients with normal ovulatory function, due to tubal blockage or male factor infertility, were included as controls. The patients, who had undergone IVF/intracytoplasmic sperm injection (ICSI)-embryo transfer for the first time, were subjected to the same ovulation protocol. The demographic and clinical data were recorded accordingly. Human luteinized GCs were obtained during oocyte retrieval and stored at –80°C until further use. The remaining follicular fluid was used for ELISA detection. The study protocol, strictly following the ethical standards of Helsinki Declaration, was approved by the Ethics Committee of Shanxi women and children's hospital, and informed consents were obtained from all participants.

Validation of the expression levels of candidate genes by qRT-PCR

Total RNA was extracted from human GCs using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthetized using a SuperScript® III Kit (Thermo Fisher Scientific, Shanghai, China). Samples were amplified according to the following protocol: an initial denaturation of 3 min at 95°C followed by 40 cycles denaturing for 10 s at 95°C, annealing for 1 min at 60°C, and extension for 1 min at 72°C. Primer sequences and product size of genes are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control for quantification. The relative expression levels of target transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method (Dong et al. 2016). Quantification of qRT-PCR results was performed in which the level of a target transcript was normalized against the target transcript level in Ctrl patient #1, which was arbitrarily set at 1.

Determination of inflammatory biomarkers in follicle fluid supernatants

According to the manufacturer's instructions, inflammatory biomarkers (TNF-α, IL-6, and CRP) were measured in follicle fluid supernatants. TNF-α, IL-6, and CRP were determined using an ELISA (R&D Systems, Minneapolis, MN, USA).

Table 1. Primers sequences and product sizes of qRT-PCR analysis in the study

Gene	Primer sequences	Product size (bp)
TLR1	F: TGAACCTCAAGCACTTGGACC R: CCCATAAGTCTCTCCTAAGACCA	188
TLR2	F: TTATCCAGCACACGAATACACAG R: AGGCATCTGGTAGAGTCATCAA	160
TLR8	F: ATGTTCCTTCAGTCGTCAATGC R: TTGCTGCACTCTGCAATAACT	143
CD14	F: GACCTAAAGATAACCGGCACC R: GCAATGCTCAGTACCTTGAGG	161
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	120

F, forward; R, reverse; TLR, toll-like receptor; CD14, cluster of differentiation 14; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Western blot

Western blot was performed as described elsewhere (Zhang et al. 2014). Briefly, total protein samples were isolated and purified using Total Protein Extraction Kit from Merck (Burlington, MA, USA), as *per* the manufacturer's instructions. ~25 mg of protein samples were separated by on SDS/PAGE and transferred to Polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific), followed by incubation with the primary antibodies at 4°C overnight. The primary antibodies employed in the current study were rabbit anti- toll-like receptor 1 (TLR1), anti-TLR2 and anti-TLR8 polyclonal (Thermo Fisher Scientific) and goat anti-CD14 polyclonal (Abcam, Shanghai, China). Final signals were finally detected using an ECL system (Amersham Biosciences) according to the manufacturer's instructions. Densitometric scanning of immunoblots was performed with the aid of Image J software.

Statistical analysis

The statistical analyses were performed with SPSS13.0 software package (SPSS Inc., Chicago, IL, USA). Results were presented as the mean ± S.E.M. Continuous variables between two groups were compared using Student's *t*-test. The p-value < 0.05 was considered significantly different.

Results

Identification of DEGs

The GSE34526 dataset contained 10 granulosa cell samples, including 7 PCOS and 3 controls. The gene expression profiles were analyzed using GCBI and identified the DEGs. Based on the GCBI analysis, p value < 0.05 and $|\log FC| \geq 2$ were used as cut-off criteria. 426 DEGs, which included 418 up-regulated and 8 down-regulated genes, were identified. The details of DEGs expression heat map were shown in Figure 1.

Function and pathways enrichment analysis

All DEGs was uploaded to the DAVID software to identify GO function. GO enrichment analysis results were presented in Table 3. For biological processes, the top 5 GO terms of DEGs, namely inflammatory response, innate immune response, leukocyte migration, interferon-gamma-mediated signaling pathway, and adaptive immune response, were included. The top 5 GO terms of DEGs for molecular function were enriched in receptor activity, signaling pattern recognition receptor activity, low-density lipoprotein particle binding, transmembrane signaling receptor activity, and receptor binding. In addition, GO cell componentanalysis also showed that the top 5 GO terms of DEGs were significantly enriched in plasma membrane, extracellular exosome, integral component of plasma membrane, cell surface, and cytosol.

In order to visualize the gene interactions among DEGs, a pathway relation network for the DEGs was constructed using the ClueGO plug-in of Cytoscape software (Supplementary Fig. S1). The pathway relation network for DEGs consisted of B cell receptor signaling pathway, Natural killer cell mediated cytotoxicity, TNF signaling pathway, cytokine-

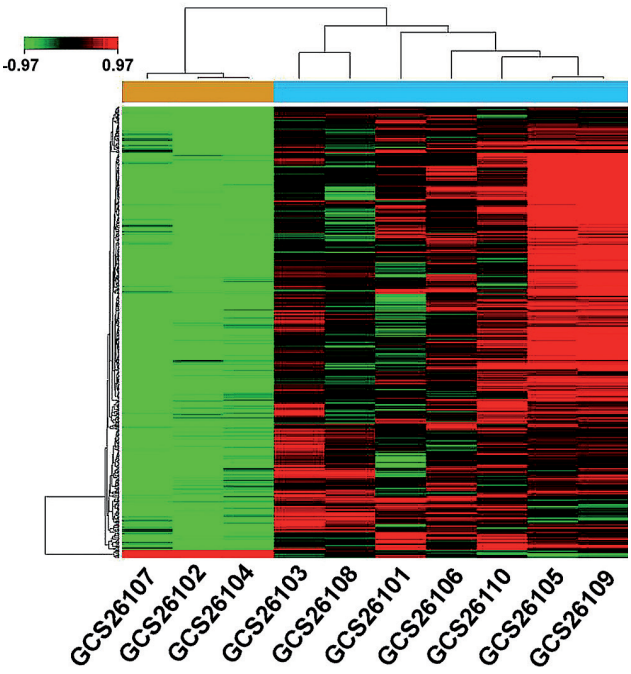
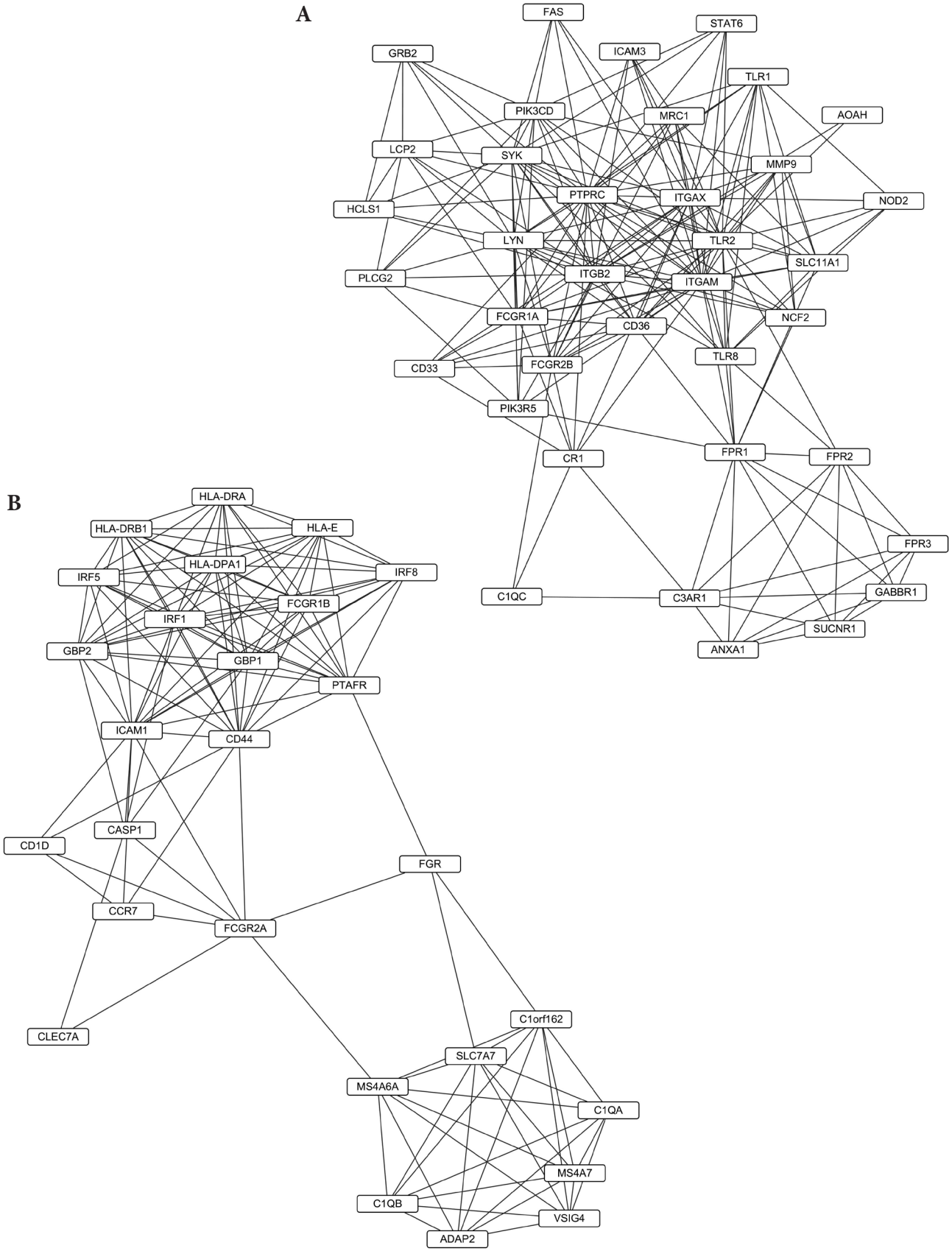


Figure 1. Heat map of the differentially expressed genes of GSE34526. Red: up-regulation; green: down-regulation. (See online version for color figure).


A
FAS
STAT6
GRB2
ICAM3
TLR1
PIK3CD
MRC1
AOAH
LCP2
SYK
MMP9
PTPRC
ITGAX
NOD2
HCLS1
LYN
TLR2
SLC11A1
PLCG2
ITGB2
ITGAM
FCGR1A
CD36
NCF2
TLR8
CD33
FCGR2B
PIK3R5
CR1
FPR1
FPR2
FPR3
C1QC
C3AR1
GABBR1
SUCNR1
ANXA1
B
HLA-DRA
HLA-DRB1
HLA-E
HLA-DPA1
IRF5
IRF8
FCGR1B
IRF1
GBP2
GBP1
PTAFR
ICAM1
CD44
CASP1
CD1D
FGR
CCR7
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CLEC7A
C1orf162
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C1QA
MS4A7
C1QB
VSIG4
ADAP2

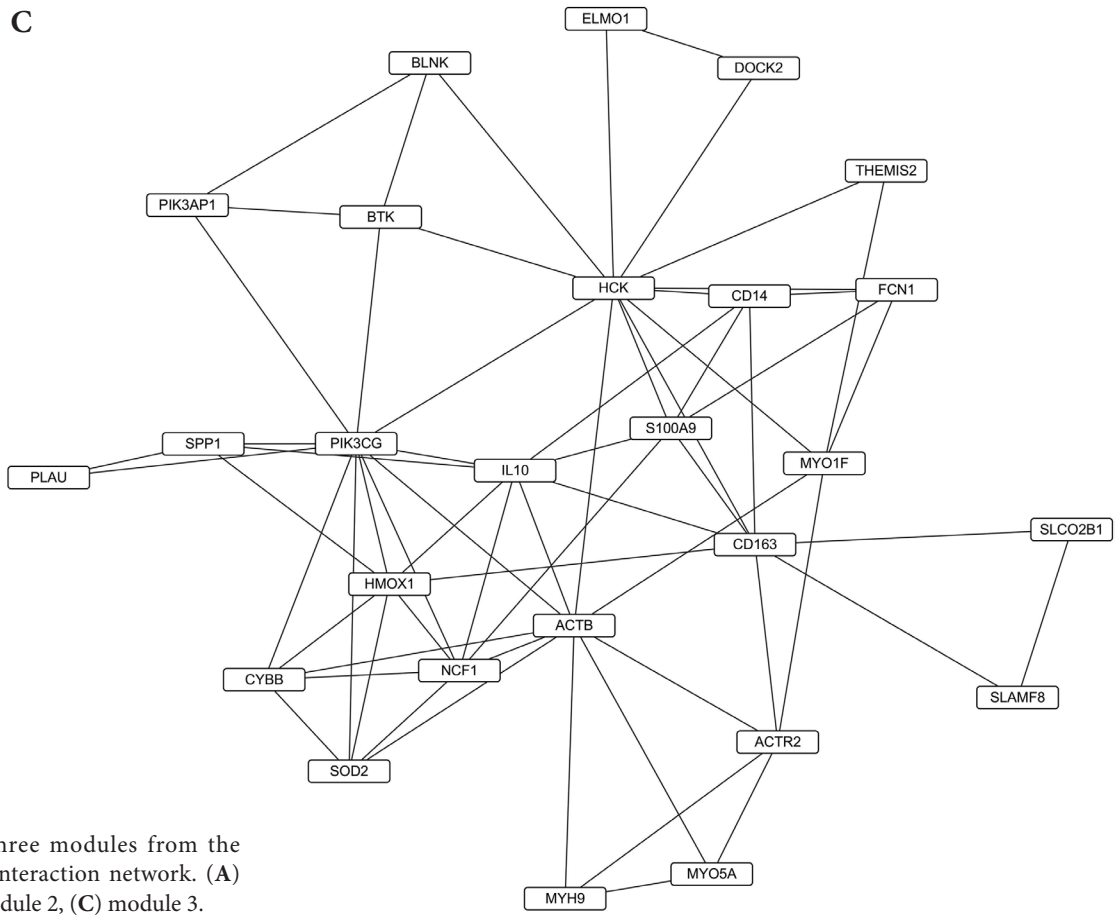

Figure 2. Top three modules from the protein-protein interaction network. (**A**) module 1, (**B**) module 2, (**C**) module 3.

Table 2. KEGG pathway enrichment of the genes involved in the modules 1, 2 and 3

Module 1			
Term	*p* value	FDR	Gene
Fc gamma R-mediated phagocytosis	$1.46 \cdot 10^{-6}$	0.00149	*PTPRC, LYN, FCGR2B, FCGR1A, PIK3CD, PLCG2, PIK3R5, SYK*
B cell receptor signaling pathway	$5.62 \cdot 10^{-6}$	0.00573	*LYN, FCGR2B, GRB2, PIK3CD,PLCG2, PIK3R5, SYK*
Leukocyte transendothelial migration	$7.61 \cdot 10^{-5}$	0.077493	*NCF2, MMP9, PIK3CD, PLCG2, PIK3R5, ITGB2, ITGAM*
Hematopoietic cell lineage	0.001911	1.930742	*CR1, CD36, CD33, FCGR1A, ITGAM*
Toll-like receptor signaling pathway	0.003439	3.450576	*PIK3CD, TLR1, TLR2, PIK3R5, TLR8*
Module 2			
Systemic lupus erythematosus	$4.40 \cdot 10^{-6}$	0.003611	*C1QA, C1QB, HLA-DRB1, HLA-DPA1, FCGR2A, HLA-DRA*
Viral myocarditis	$3.14 \cdot 10^{-5}$	0.025781	*ICAM1, HLA-DRB1, HLA-DPA1, HLA-E, HLA-DRA*
Autoimmune thyroid disease	$3.20 \cdot 10^{-4}$	0.262222	*HLA-DRB1, HLA-DPA1, HLA-E, HLA-DRA*
Cell adhesion molecules (CAMs)	$3.55 \cdot 10^{-4}$	0.29062	*ICAM1, HLA-DRB1, HLA-DPA1, HLA-E, HLA-DRA*
Hematopoietic cell lineage	0.001486	1.212095	*CD44, HLA-DRB1, HLA-DRA, CD1D*
Module 3			
B cell receptor signaling pathway	0.001503	1.46896	*PIK3CG, PIK3AP1, BLNK, BTK*
Chemokine signaling pathway	0.002277	2.217082	*PIK3CG, DOCK2, NCF1, HCK, ELMO1*
Fc gamma R-mediated phagocytosis	0.002965	2.878819	*PIK3CG, DOCK2, NCF1, HCK*
Leukocyte transendothelial migration	0.005471	5.252746	*PIK3CG, ACTB, CYBB, NCF1*
Toll-like receptor signaling pathway	0.039114	32.45961	*PIK3CG, CD14, SPP1*

cytokine receptor interaction, chemokine signaling pathway, etc. These pathways were primarily associated with immune and inflammation.

PPI network construction and module analysis

Based on the information in the STRING database, PPI network was constructed using the MCODE plug-in of Cytoscape software. A total of 118 nodes and 421 edges were included in the total of DEGs with significant interaction relation. The top 3 significant modules were selected, and the KEGG pathway enrichment of the genes involved in the modules were analyzed (Figure 2, Table 2). Enrichment analysis revealed that the genes in top 3 modules were mainly associated with Fc gamma R-mediated phagocytosis, B cell receptor signaling pathway, TLR signaling pathway, Cell adhesion molecules (CAMs), Chemokine signaling pathways. These selected pathways were also associated with immune and inflammation. Furthermore, PPI network and module analysis showed that two modules were involved in the TLR signaling pathway in the top 3 modules, and these candidate genes in this signaling pathway consisted of *TLR1*, *TLR2*, *TLR8*, and *CD14*.

Patient's characteristics

A total of 78 patients were enrolled in this study, including 38 PCOS patients and 40 controls. Demographic and clinical characteristic parameters of the subjects were summarized in Table 2. Women in PCOS and control groups had a similar mean age, and duration of infertility except BMI. In basal hormonal levels, there was no significant difference in LH, E2 and P levels. However, the FSH, T and LH/FSH were significantly different between PCOS and Control groups ($p < 0.05$). In addition, concentration of cytokines (IL-6, TNF-a, and CRP) in follicle fluid as inflammatory markers was detected by ELISA (Table 4). The results showed that IL-6, TNF-a, and CRP levels of follicle fluid were significantly increased in PCOS group compared with those in control group.

Validation of candidate Genes

To verify the candidate genes revealed by microarray, four above-mentioned genes (*TLR1, TLR2*, *TLR8*, and *CD14*) were re-examined by qRT-qPCR and Western blot in the 78 samples (38 from PCOS patients and 40 from Controls). As shown in Figure 3A and Supplementary Fig. S2, the mRNA levels of *TLR2*, *TLR8*, and *CD14* were significantly increased in GCs from PCOS when compared to those in GCs from the controls ($p < 0.01$). However, no significant difference in the expression levels of *TLR1* was observed between two experimental groups. These results were further validated at the protein level by Western blot analysis (Figure 3B).

Table 3. Gene ontology function enrichment analysis of the total DEGs associated with PCOS

GO ID	GO name	Gene count	%	*p* value	FDR
	Biological processes				
GO:0006954	inflammatory response	42	12.5	$3.21{\cdot}10^{-20}$	$5.47{\cdot}10^{-17}$
GO:0045087	Innate immune response	38	11.31	$5.07{\cdot}10^{-15}$	$8.70{\cdot}10^{-12}$
GO:0050900	leukocyte migration	21	6.25	$8.34{\cdot}10^{-14}$	$1.42{\cdot}10^{-10}$
GO:0060333	interferon-gamma-mediated signaling pathway	16	4.76	$2.42{\cdot}10^{-12}$	$4.13{\cdot}10^{-9}$
GO:0002250	adaptive immune response	20	5.95	$3.08{\cdot}10^{-11}$	$5.25E{\cdot}10^{-8}$
	Molecular function				
GO:0004872	receptor activity	21	6.25	$3.16{\cdot}10^{-9}$	$4.60{\cdot}10^{-6}$
GO:0008329	signaling pattern recognition receptor activity	5	1.49	$3.64{\cdot}10^{-6}$	0.005302
GO:0030169	low-density lipoprotein particle binding	6	1.79	$5.06{\cdot}10^{-6}$	0.007363
GO:0004888	transmembrane signaling receptor activity	16	4.76	$9.44{\cdot}10^{-6}$	0.013733
GO:0005102	receptor binding	20	5.95	$2.80{\cdot}10^{-5}$	0.040694
	Cellular component				
GO:0005886	plasma membrane	156	46.43	$5.03{\cdot}10^{-24}$	$6.72{\cdot}10^{-21}$
GO:0070062	extracellular exosome	101	30.06	$1.43{\cdot}10^{-12}$	$1.91{\cdot}10^{-9}$
GO:0005887	integral component of plasma membrane	59	17.56	$1.56{\cdot}10^{-9}$	$2.08{\cdot}10^{-6}$
GO:0009986	cell surface	31	9.23	$4.10{\cdot}10^{-8}$	$5.48{\cdot}10^{-5}$
GO:0005829	cytosol	96	28.57	$5.60{\cdot}10^{-7}$	$7.50{\cdot}10^{-4}$

DEGs, differentially expressed genes; PCOS, polycystic ovary syndrome; FDR, false discovery rate; GO, Gene ontology.

Table 4. Demographic and clinical characteristic parameters of the patients

Variables	Controls ($n = 40$)	PCOS ($n = 38$)	p value
Age (year)	29.95 ± 0.751	28.97 ± 0.578	0.306
Infertility duration (year)	4.025 ± 0.455	3.789 ± 0.464	0.718
BMI (kg/m^2)	22.121 ± 0.421	25.915 ± 0.568	0.001
Basal hormone levels			
LH (mIU/ml)	6.242 ± 0.653	7.65 ± 0.618	0.122
FSH (mIU/ml)	9.221 ± 0.558	6.958 ± 0.303	0.001
E2 (pg/ml)	80.55 ± 3.779	77.971 ± 4.132	0.647
P (ng/ml)	0.514 ± 0.052	0.643 ± 0.089	0.217
T (ng/ml)	41.741 ± 2.646	49.986 ± 2.725	0.033
LH/FSH	0.701 ± 0.074	1.144 ± 0.099	0.001
Inflammatory biomarkers			
IL-6 (pg/ml)	45.089 ± 3.547	59.022 ± 4.760	0.022
TNF-α (pg/ml)	30.101 ± 1.652	40.965 ± 3.002	0.002
CRP (mg/l)	2.909 ± 0.279	4.009 ± 0.323	0.012

Data were present by the mean ± SEM. $p < 0.05$ was considered statistically significant. Differences between two groups were analyzed using *Student's t*-test. BMI, body mass index; IL-6, inerleukin-6; TNF-α, tumor necrosis factor- α; CRP, C-reactive protein. PCOS, polycystic ovary syndrome; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; E2, estradiol; T, thyroid; BMI, body mass index.

Discussion

In the current study, we used published microarray data and bioinformatics analysis method to explore the DEGs in ovarian granulosa cells of PCOS, and our systematic analysis will help to understand the complicated pathogenesis of PCOS at the molecular level.

The GO term analysis showed that the top 5 GO terms of DEGs were mainly engaged in inflammatory response, innate immune response, leukocyte migration, interferon-gamma-mediated signaling pathways, and adaptive immune response in biological processes. In order to visualize of gene interactions among DEGs, KEGG pathway enrichment analysis was performed using ClueGO plug-in of Cytoscape software. The results showed that these significant pathways with the DEGs, including B cell receptor signaling pathway, Natural killer cell mediated cytotoxicity, TNF signaling pathway, cytokine-cytokine receptor interaction, chemokine signaling pathway, etc. Hence, we speculated that inflammation may play an essential role in the induction of PCOS.

PCOS is towel known to be correlated to low-grade chronic inflammation (El Khoudary et al. 2011), and the alternation of ovarian environment is an fundamental pathophysiological characteristic of PCOS, while GCs as the dominant cell community, regulate the development of follicles and oocytes (Zuo et al. 2017). In 2001, Kelly et al. firstly reported that women with PCOS have significantly higher CRP levels compared to those in healthy women with normal menstrual rhythm and normal androgens (Kelly et al. 2001). In recent years, several studies have shown

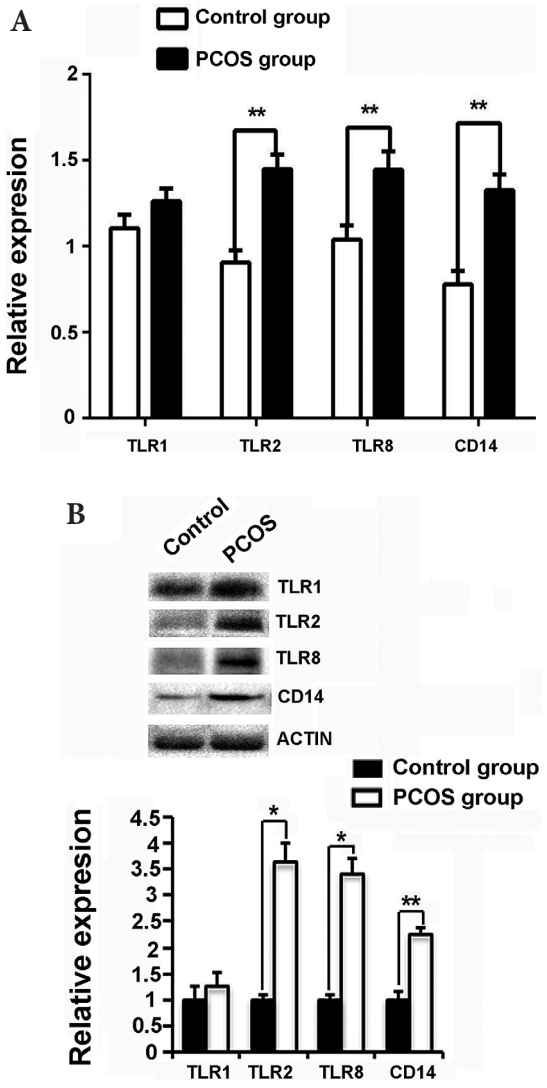

Figure 3. A. qRT-PCR analysis of the expression of *TLR1*, *TLR2*, *TLR8*, and *CD14* gene in human ovarian luteinized granulosa cells (GCs) from PCOS and Controls. * $p < 0.05$, ** $p < 0.01$. **B.** Western blot analysis of the expression levels of TLR1, TLR2, TLR8, and CD14 in human ovarian luteinized GCs from PCOS and Controls. Densitometric scanning of immunoblots was performed in which the level of a target protein was normalized against the protein level in Patient #1 from control group, which was arbitrarily set at 1 (lower panel). Each bar represents the mean ± S.E.M. of results from three experiments using different batches of GCs. Each experiment had replicate cultures. * $p < 0.05$, ** $p < 0.01$.

that the levels of inflammatory factors (TNF, CRP, Il-6) in peripheral blood and follicle fluid from PCOS patients are significantly increased (Atabekoglu et al. 2011; Escobar-Morreale et al. 2011). These data suggest that low degree of chronic inflammation may be related to the pathogenesis of PCOS. In our study, the expressions of TNF-a, IL-6 and CRP were all upregulated in the follicle fluid of PCOS patients. At present, chronic low inflammation has not been applied in the clinical diagnosis and treatment of PCOS patients. It is necessary to further study the potential mechanisms of low-degree inflammation in PCOS pathophysiological process.

To further understand the possible mechanisms underlying the low-degree inflammation in PCOS, we constructed the PPI network with DEGs. Module analysis of the PPI network revealed that the development of PCOS was associated with Fc gamma R-mediated phagocytosis, B cell receptor signaling pathway, TLR signaling pathway, Cell adhesion molecules, Chemokine signaling pathway. Moreover, TLR signaling pathway was involved in two of top three modules, so we speculate that TLR signaling pathway may play an important role in PCOS. TLR is one of pathogen pattern recognition receptors (Aderem and Ulevitch 2000; Akira and Sato 2003). It has been reported that TLRs are important in adipose tissue inflammation of chronic disease (Lucas and Maes 2013). Meanwhile, it is suggested that TLRs are associated with tissue damage and inflammation (Kawai and Akira 2010). Moreover, Liu et al. indicated that TLRs localized in mammalian granulosa cells, cumulus cells and theca cells, and TLRs expression are related with cumulus-oocyte complex expansion and fertilization (Liu et al. 2008). In addition, the activation of TLR signaling pathway leads to the stimulation of chemokine and cytokine expression including IL-6 and IL-8 (Zarember and Godowski 2002). The cytokines modulated local and systemic inflammatory and immune responses (Lotteau et al. 1990). The study found that TLR activation resulted in excessive expression of COX-2, which was associated with the inflammatory response (Sirois et al. 2004; Williams and DuBois 1996).Conversely, IL-10 as an anti-inflammatory cytokine controlled inflammation response *via* inhibiting TLR signaling pathways (Williams et al. 2004). In addition, our results showed that TLR signaling pathway was related to TLR1, TLR2, TLR8, CD14. But, qRT-PCR results showed that *TLR2*, *TLR8* and *CD14* expression increased in PCOS ovary granulosa cells in the validation cohort. While, Western blot showed that the expression level of TLR8 had significantly difference between PCOS and control group.

TLR2 recognizes LPS ligands and mediates signal transductions. Accumulated evidences indicate that TLR2-mediated inflammation condition may favor sustained cytokine production. On the other hand, it may further favor androgen excess in women (Ojeda-Ojeda et al. 2016). Shimada, et al. proved that the expression of IL-6 can be regulated by TLR2 and TLR4 (Shimada et al. 2006). In 2008, Shimada rediscovered that TLR2/4-stimulated COCs secreted chemokines, which could induce sperm capacitation and enhance fertilization (Shimada et al. 2008). In an *in vitro* fertilization assay, Jiang et al. (2005) proved that cumulus cells activated by TLR2/4 enhanced fertilization by releasing cytokines and chemokines.

TLR8 is an inert receptor, but recent studies have shown that it can identify pathogens RNA and induce immune inflammation (Cervantes et al. 2013; Guiducci et al. 2013). Taghavi S. A. investigated the expression of TLRs in follicular cells of infertile PCOS women, and found the expression levels of TLR1-6, TLR8 and TLR9 were higher in PCOS (Taghavi et al. 2013). Forsbach observed that the activation of TLR-8 led to the secretion of inflammatory cytokines such as IFN-γ, TNF-α and IL-12 (Ospelt and Gay 2010). Moreover, the accumulating evidence indicates that CPG-52364 as TLR antagonist, could block several inflammatory autoimmune diseases induced by activation of TLR7, TLR8 and TLR9 in clinical trials (Lai et al. 2017). It meant that TLR8 was associated with systemic inflammation, which was involved in pathophysiology of PCOS.

Clusters of differentiation 14 (CD14), as a pattern recognition receptor, is expressed on the surfaces of monocytes and macrophages, and contributes to TLR-induced cell activation (Aderem and Ulevitch 2000; Antal-Szalmas 2000). In 2012, Lei et al. indicated that inhibition of CD14 by RNA interference could inhibit TNF-α secretion and NO production in RAW264.7 cells induced by LPS (Lei et al. 2012). Moreover, using *in vitro* test, Thorgersen et al. (2009) found CD14 antibody binding sites on the surface, prevented their combination with LBP and made LPSCD14 TLR4-MyD2 receptor complexes formation blocked, which reduce the secretion of TNF-α and IL-1β. They all suggested that the regulation of CD14 can inhibit the inflammatory response to a certain extent. Above all, the results indicate that these candidate genes (*TLR2, TLR8* and *CD14*) and their related TLR signaling pathway may play key roles in PCOS.

Additionally, there were eight DEGs including *HAS2*, *THSD7A*, *LPHN3*, *DLX2*, *COCH*, *SPOCK3*, *CNTN4* and *ACOT4*, whose expression levels were found to be significantly down-regulated in GCs from PCOS patients when compared to those in GCs from Ctrl patients (data not shown). Some of these downregulated DEGs may also play potential roles during the pathogenesis of PCOS. For example, *HAS2* and *CNTN4* are both distinctly expressed in GCs (Liu et al. 2016; Wigglesworth et al. 2015). Overexpression of *HAS2* promotes resistance to apoptosis in GCs (Liu et al. 2016). More importantly, *HAS2* and *CNTN4* are both regulated fundamentally by insulin signaling pathway (Grado-Ahuir et al. 2009; Kuroda et al. 2001). Given the close association between insulin-signaling pathway and gonadotrophin hormone action in PCOS (Aydos et al. 2016;

Szczuko et al. 2016), we therefore propose that HAS2 and CNTN4 are both functionally involved in the pathogenesis of PCOS. This intriguing hypothesis is currently under investigation in our lab.

In conclusion, our data collectively provide a comprehensive bioinformatics analysis of DEGs in PCOS. Our results confirm that the inflammation and immune play important roles in the occurrence and development of PCOS. Meanwhile, TLR signaling pathway mediating inflammation and immune might be involved in the pathogenesis of PCOS, and *TLR2*, *TLR8*, *CD14* may be core target genes. Therefore, the hub genes and pathways may be potential therapeutic targets of PCOS treatment.

Nevertheless, the potential caveats and other alternative explanations would be very insightful for future research, such as the limited control numbers in the database, and future directions of single-RNA profiling.

Acknowledgments. We are grateful to the researchers who have deposited their data in the public database.

Conflict of interest. The authors declare that there are no conflicts of interest.

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Received: October 22, 2018
Final version accepted: December 21, 2018

doi: 10.4149/gpb_2018049

Supplementary Material

Identification of the key pathways and genes related to polycystic ovary syndrome using bioinformatics analysis

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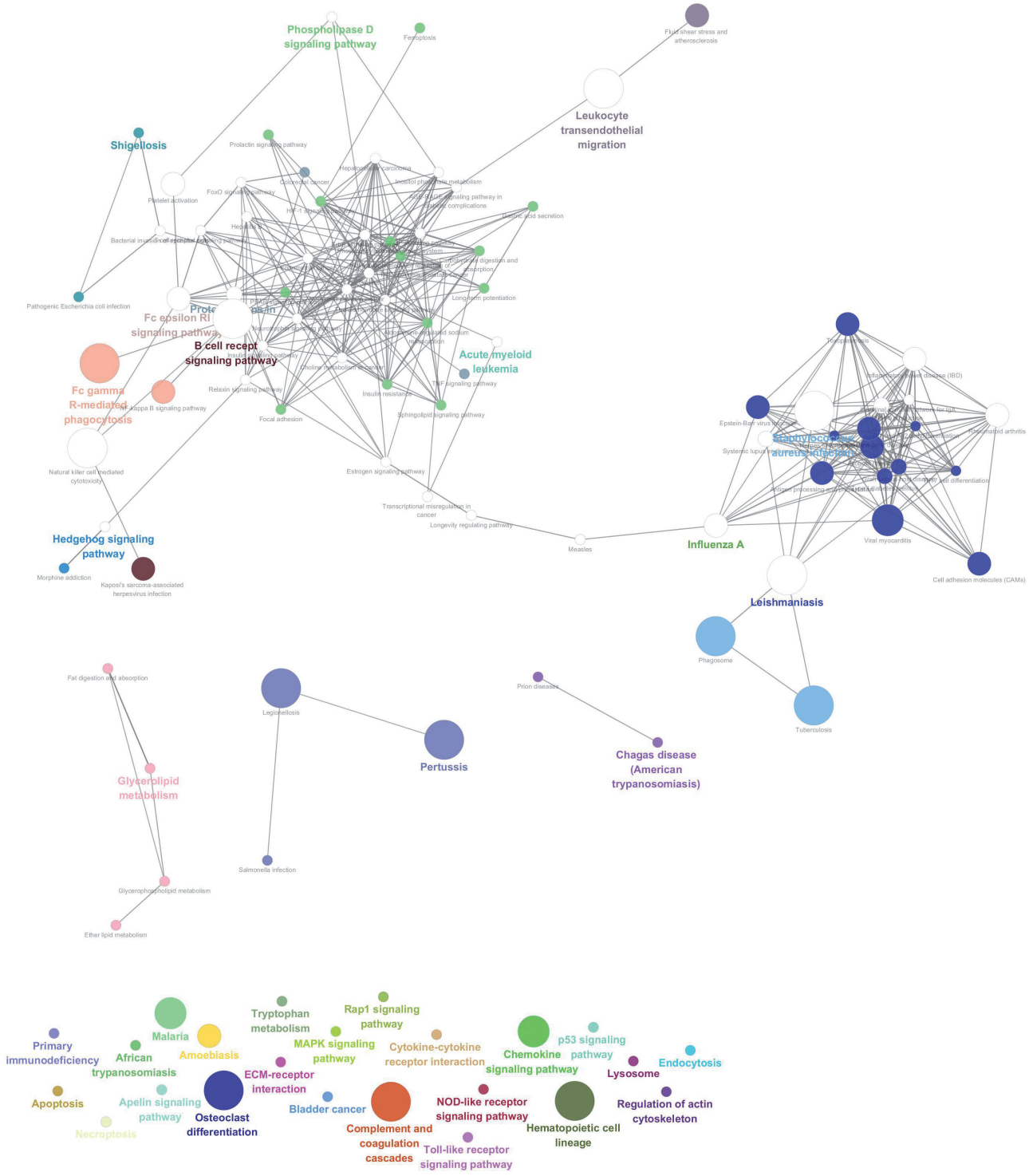

Figure S1. Pathway relation network for the total differentially expressed genes (DEGs). Each GO term is represented by a circle, and each group is represented by a different color. The larger the size of the circle is, the more the number of genes is contributing to the GO term.

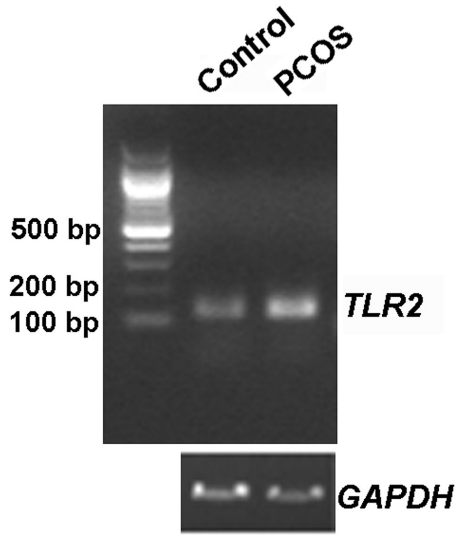

Figure S2. Representative RT-PCR analysis of TLR2 expression in human ovarian luteinized GCs from PCOS and Controls. Parallel amplification of *GAPDH* mRNA served as internal control.