Mechanistic exploration of Cangfu Daotan Decoction for patients with polycystic ovary syndrome by transcriptome sequencing

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Research

Keywords: Cangfu Daotan Decoction, polycystic ovary syndrome, transcriptome sequencing, insulin resistance, immune response

DOI: https://doi.org/10.21203/rs.3.rs-19780/v1

Abstract

Background Polycystic ovary syndrome (PCOS) is a common and complex female endocrine disorder. The pathogenesis of PCOS is not fully elucidated. Cangfu Daotan Decoction is a kind of traditional Chinese medicine prescription widely used for PCOS patients.

Methods In this study, we treated two PCOS patients with Cangfu Daotan Decoction (CDD) and both patients presented favorable responses to the treatment. We performed RNA-seq to explore the underlying molecular mechanism.

Results After data analysis, we found 4 differential expressed genes including S1PR1 , which has been found to be involved in islet β-cell proliferation and cell apoptosis in diabetic mouse models. We also identified 28 differential expressed transcripts including NFATC2 , CD14 and FCGR3A , which are involved in many immune processes.

Conclusions After GO enrichment analysis, we found that pathways including viral gene expression and immune response are involved in the efficacy of CDD treatment. Our study provides new evidence to better understand the pathogenesis of PCOS.

Background

Polycystic ovary syndrome (PCOS) is a complex and chronic endocrine disorder found in 5-10% of premenopausal women [1]. The clinical characteristics of PCOS can be divided into 3 broad segments: reproductive manifestations, metabolic features, and psychological sequelae [2]. The spectrum of clinical features varies among individuals and across the life cycle. Based on 2003 Rotterdam criteria, a woman with the diagnosis of PCOS should meet 2 of the 3 following criteria: (1) oligo-anovulation or chronic anovulation; (2) clinical and/or biochemical signs of hyperandrogenism; (3) polycystic ovaries [3]. PCOS is also associated with several long-term health consequences, including hyperinsulinemia and insulin resistance [4], obesity [5], hyperlipidemia [6, 7], cardiovascular disease [8, 9], endometrial cancer [10, 11], pregnancy complications [12, 13].

It has been demonstrated that multiple risk factors are associated with the etiology of PCOS. Environmental factors have been found to associate with the pathogenesis of PCOS, including smoking, poor diet, and lack of exercise [14, 15]. The different prevalence among ethnicities implies that genetic factors play a role in the development of PCOS [16, 17]. Two GWAS studies identified 11 loci, accounting for 17 SNPs, in Han Chinese women with a strong association risk for PCOS [18, 19]. Twins study indicates that monozygotic has a higher risk to develop PCOS than dizygotic twins [20]. Premature pubarche in adolescents and prenatal exposure to androgens is considered to be involved in later development of PCOS [21, 22]. In addition to the influences throughout life, the *in utero* environment is associated with the increased risk of development of PCOS [23]. Many studies indicate that a defect in insulin action may be the primary cause of PCOS [24, 25].

Symptomic treatment of women with PCOS mainly includes lifestyle intervention and oral contraceptives (OCPs) [26]. Of them, metformin and thiazolidinediones improve hyperandrogenic symptoms through reducing the blood insulin level [27]. Chinese herbal medicine serves as another important treatment for PCOS. In Taiwan, approximately 90% of women with PCOS consulted Chinese medicine practitioners, and more than half of them were treated with herbal medicine [28]. One Chinese herbal medication - Liuwei Dihuang Pills, have been found to improve the PCOS pathogenesis through improving insulin resistance [29]. Another Chinese herbal medication - Cangfu Daotan Decoction (CDD) has been reported to treat PCOS [30], while its underlying mechanism remains largely unknown.

In this study, we treated two Chinese PCOS patients with CDD and evaluate its safety and efficacy. We performed transcriptome sequencing (RNA-Seq) to explore the underlying molecular mechanism involved in the efficacy of CDD.

Methods

Subjects

In this study, two Chinese patients were collected from Zhejiang Provincial Integrated Chinese and Western Medicine Hospital, Hangzhou, China. Insulin resistance was indirectly assessed using the homeostatic index of insulin resistance (HOMA-IR) index according to this formula: HOMA-IR= [Fasting plasma insulin (mU/L) x Fasting plasma glucose (mmol/L)]/22.5 [31]. Common anthropometric parameters were screened before and after the three-month treatment with CDD: BMI, luteinizing hormone (LH), follicle-stimulating hormone (FSH), fasting blood glucose, 2-hour Postprandial blood glucose, fasting insulin, 2-hour postprandial blood insulin, total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were also assessed. Estimation of free serum testosterone (T) was obtained based on SHBG and total T as described previously [32]. This study was conducted in conformity with the Helsinki Declaration II and approved by the Ethics Committee of Hangzhou Redcross Hospital. Written informed consent was given to all subjects before their inclusion.

Transcriptome Sequencing

The peripheral blood samples before (T1 for patient I and T3 for patient II) and after (T2 for patient I and T4 for patient II) the CDD treatment were collected from two patients. Total RNA was extracted from blood samples with Tri-reagent LS (Sigma-Aldrich) according to manufacturer’s instructions. RNA concentration and purity were assessed using a NanoDrop ND-1000 (Thermo Fisher Scientific). After total RNA was qualified, the mRNA was enriched by using poly-T oligo-attached magnetic beads. After that, mRNA was randomly fragmented by fragmentation buffer, and the fragmented mRNA was used as a template to synthesize one-strand cDNA with random hexamers, followed by synthesizing double-stranded cDNA. The double-stranded product was purified by AMPure XP beads (Beckman Coulter, Beverly, USA), and the sticky ends were repaired to blunt ends by T4 DNA polymerase and Klenow DNA polymerase. After adenylation of 3' ends of DNA fragments, AMPure XP beads were used for fragment

selection, finally the sequencing library was generated by PCR amplification. After the library was qualified, Illumina Hiseq4000 system was applied for sequencing and the read length was paired-end 2x150 bp (PE150).

Data analysis

High-throughput sequencing data analysis process mainly includes gene expression analysis and alternative splicing analysis. Hisat software was used to assemble and align the sequencing reads to the reference genome (hg39) [33]. The edgeR was used for differential expression analysis and genes with adjusted $P < 0.05$ assigned as differentially expressed [34, 35]. StringTie adopts a network flow algorithm and optional *de novo* assembly to assemble these complex sequencing reads into novel transcripts [36]. Significant differential genes were used for GO (Gene Ontology) enrichment analysis by using R rWikiPathways 1.4.1 package [37]. GO terms with corrected $P < 0.05$ were considered significantly enriched by differentially expressed genes.

Results

Diagnosis and treatment efficacy

Both of the patients exhibited menstrual cycle anomalies, amenorrhoea, oligomenorrhoea or long cycles, clinical and/or biochemical hyperandrogenism and ultrasound appearance of polycystic ovaries. Based on Rotterdam criteria [3], PCOS was diagnosed. After treatment with CDD, we observed an obvious decease of BMI, TG(Triglyceride), TC(total cholesterol) and HOMA-IR in both patients (Table1).

Transcriptome sequencing

We processed the raw data generated by transcriptome sequencing. Clean data was obtained after filtering the unqualified reads by cutadapt. Raw reads filtering as follows: (1) Remove reads containing adaptors; (2) Remove reads containing N > 10% (N represents base that could not be determined); (3) The Q-score (Quality value) of over 50% bases of the read is <= 5. Raw sequencing reads, valid reads, Q20%, Q30% and GC content was also evaluated (Supplementary Table 1). Annotation information was analyzed including chromosomes, genes, transcripts and GO annotation. Hisat was used to align valid reads to reference genome and reads matched to the genome were calculated based on the gene location, including (1) alignment between sequencing data and reference genome; (2) distribution of mapped reads on chromosome (Supplementary Table 2). FPKM (fragments per kilobase of exon model per million mapped reads) was used to estimate the gene expression level. Distribution of gene expression value in each sample was summarized in Supplementary Table 3. As there is difference in the distribution of expressed gene number and gene expression level, FPKM can be divided into different intervals. Gene numbers in different intervals were calculated (Supplementary Table 4).

Identification of differential expressed genes associated with CDD treatment

To determine differential expressed genes (DEGs), RNA-Seq was performed on two PCOS patients with pre- and post-treatment of CDD. According to the inspected RNA-seq data, all the quality control parameters were within the acceptable ranges. For patient I, a total of 29,024 annotated Ensembl genes were detected and included in subsequent analysis, of which 662 genes were upregulated and 429 genes were downregulated in post-treatment of CDD (Extended Table 1). After applying statistical analysis ($P < 0.05$), a FPKM criterion (either one FPKM of pre- (T1) or post-treatment (T2) of CDD >= 1.0), and q-value < 0.05, 130 genes were significantly differentially expressed in patient I with post-treatment of CDD (T2), including 62 upregulated genes and 68 downregulated genes (Extended Table 2). For patient II, a total of 29,034 annotated Ensembl genes were detected and included in subsequent analysis, of which 897 genes were upregulated and 900 genes were downregulated in post-treatment of CDD (Extended Table 3). After applying same statistical analysis and filtering criterion as patient I, 128 genes were significantly differentially expressed in patient I with post-treatment of CDD (T4), including 49 upregulated genes and 79 downregulated genes (Extended Table 4). By comparing the genes identified in both patients, we found 4 differential expressed genes shared by both patients with post-treatment of CDD (Table 2; Extended Table 5). Of them, *S1PR1* is upregulated, and *AL034397.3*, *GNA12* and *MAP1LC3B* are downregulated.

Identification of differential expressed transcripts associated with CDD treatment

For patient I, a total of 114,144 annotated Ensembl genes were detected and included in subsequent analysis, of which 3,623 genes were upregulated and 3,621 genes were downregulated in post-treatment (T2) of CDD compared with pre-treatment (T1) (Extended Table 6). After applying statistical analysis ($P < 0.05$), a FPKM criterion (either one FPKM of T1 or T2 >= 1.0), and q-value < 0.05, 1,362 transcripts were significantly differentially expressed in patient I with post-treatment of CDD (T2) compared with pre-treatment (T1), including 746 upregulated genes and 616 downregulated genes (Extended Table 7). For patient II, a total of 114,143 annotated Ensembl transcripts were detected and included in subsequent analysis, of which 4,150 transcripts were upregulated and 4,154 transcripts were downregulated in post-treatment (T4) of CDD compared with pre-treatment (T3) (Extended Table 8). After applying identical statistical analysis ($P < 0.05$) with patient I, we found that 2,022 transcripts were significantly differentially expressed in patient II with post-treatment (T4) of CDD compared with pre-treatment (T3), including 931 upregulated transcripts and 1091 downregulated transcripts (Extended Table 9). To find out the recurrent differential transcripts, we selected those transcripts with identical “start” and “end” in both patients and fold change > 2.5. We obtained totally 27 differential expressed transcripts shared by two patients with CDD treatment (Table 3; Extended Table 10).

GO enrichment

To further analyze differential gene expression associated with CDD, GO enrichment analysis was performed using the rWikiPathways R package. For patient I, 1,091 differential expressed genes (662 genes were upregulated and 429 genes were downregulated) were fed into pathway analysis with 29,024 annotated Ensembl genes as background genes (Extended Table 1). Top 15 pathways were presented

including viral gene expression and protein synthesis (Figure 1A; Extended Table 11). For patient II, 1,797 differential expressed genes (897 genes were upregulated and 900 genes were downregulated) were put into pathway analysis with 29,024 annotated Ensembl genes as background genes (Extended Table 3). Top 15 pathways were presented including immune response (Figure 1B; Extended Table 12).

Discussion

Several infertility treatments for PCOS have been used in clinic. OCPs contain estrogens and progestins which control the release of luteinizing hormone. However, the use of OCPs has a negative impact on women who plan to conceive [38], and also may increase the risk of cardiovascular complications [39]. Metformin as an insulin-sensitizing agent, has been found to increase the risk of gastrointestinal diseases and lactic acidosis [40, 41]. Other medications like clomiphene citrate may be associated with side effects including hot flashes, breast discomfort and abdominal distention [42]. Recent studies indicate that herbal medicine therapy has improved the symptoms and pathology of PCOS [43]. Preclinical and clinical studies suggest that beneficial effects on PCOS has been accomplished with the treatment of six different herbal medicines individually [44]. However, the quantity of pre-clinical data was limited, and the quality of clinical evidence was variable. In this study, we treated two Chinese PCOS patients with CDD and found that CDD has a favorable efficacy on CDD patients. Our study serves as a good example that CDD treatment may be an option for PCOS. CDD could also be considered as one of complementary and alternative medicine therapies [45].

Insulin resistance (IR) was found to associate with the development and pathogenesis of PCOS [46]. In addition, women with PCOS exhibit defective β-cell function expressed as a reduced disposition index in the absence of glucose intolerance [25]. However, there is an unresolved issue as to whether β-cell dysfunction is a primary defect to IR and a result of progressive β-cell exhaustion. Therefore, serum pro-insulin was recognized as a potential marker of metabolic dysfunction. By RNA-Seq in this study, we found four genes differentially expressed in PCOS patients with CDD treatment including *S1PR1*. S1PR1 has been found to involve in islet β-cell proliferation and cell apoptosis in high-fat diet/streptozotocin diabetic mice [47]. Plus, biased S1PR1 signaling has been reported to involve in immune response [48]. Therefore, targeting S1PR1 signaling may be a potential candidate therapeutic strategy for PCOS therapy.

Recent study by a polygenic integrative analysis suggested that PCOS patients presented dysregulated immunological and metabolic functions [49]. Many regulators of immune responses have been found to implicate in the pathogenesis of PCOS [50]. In this study, we found 28 differential expressed transcripts in patients with CDD treatment including *NFATC2*, *CD14* and *FCGR3A*. NFATc2 can assist BCR-mediated anergic effect in anti-insulin B cells [51]. CD14, working with Toll-like family of immune receptors, plays important roles in immune response to a variety of antigens [52]. *FCGR3A* F158V polymorphism has been found to significantly associate with immune-mediated thrombocytopenia [53]. Furthermore, our GO enrichment analysis found pathways including viral gene expression and immune response are involved in CDD treatment.

Conclusion

In summary, we treated two Chinese PCOS patients with CDD, which obviously improved the symptoms of PCOS. Our transcriptome analysis found CDD that insulin resistance and immune response are involved in the underlying mechanisms of CDD efficacy. Our study provides new evidence to better understand the pathogenesis of PCOS.

Abbreviations

Polycystic ovary syndrome (PCOS)

Cangfu Daotan Decoction (CDD)

homeostatic index of insulin resistance (HOMA-IR)

Genome-Wide Association Studies(GWAS)

luteinizing hormone (LH)

follicle-stimulating hormone (FSH)

low-density lipoprotein cholesterol (LDL-C)

high-density lipoprotein cholesterol (HDL-C)

testosterone (T)

Gene Ontology (GO)

Triglyceride (TG)

total cholesterol (TC)

differential expressed genes (DEGs)

Declarations

Ethics approval and consent to participate:

Ethical approval was received from Zhejiang Chinese Medicine and Western Medicine Integrated Hospital Human Research Ethics Committee. Informed consent was obtained from all individual participants included in the study

Consent for publication:

All participants gave consent for direct quotes from their interviews to be published in this manuscript.

Availability of data and materials:

The supporting materials used in this study are contained within the article.

Competing interests:

No conflict of interest was reported by the authors.

Funding:

This study was funded by Zhejiang Administration of traditional Chinese Medicine.(grant number 2017ZA107).

Authors' contributions :

CC conceived and designed the study with continuous support from CW, XJ and CD. CC collected the data. All authors involved in data analysis and interpretation. CC drafted the manuscript. All authors have read and approved the submission

Acknowledgements:

We sincerely thank all the participants in this study. This study is supported by Project of Zhejiang Administration of traditional Chinese Medicine (2017ZA107).

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Note Regarding Tables

Table 1 mentioned on page 4, and Tables 2 and 3 mentioned on page 5, were omitted by the authors in this version of the paper.

Figures

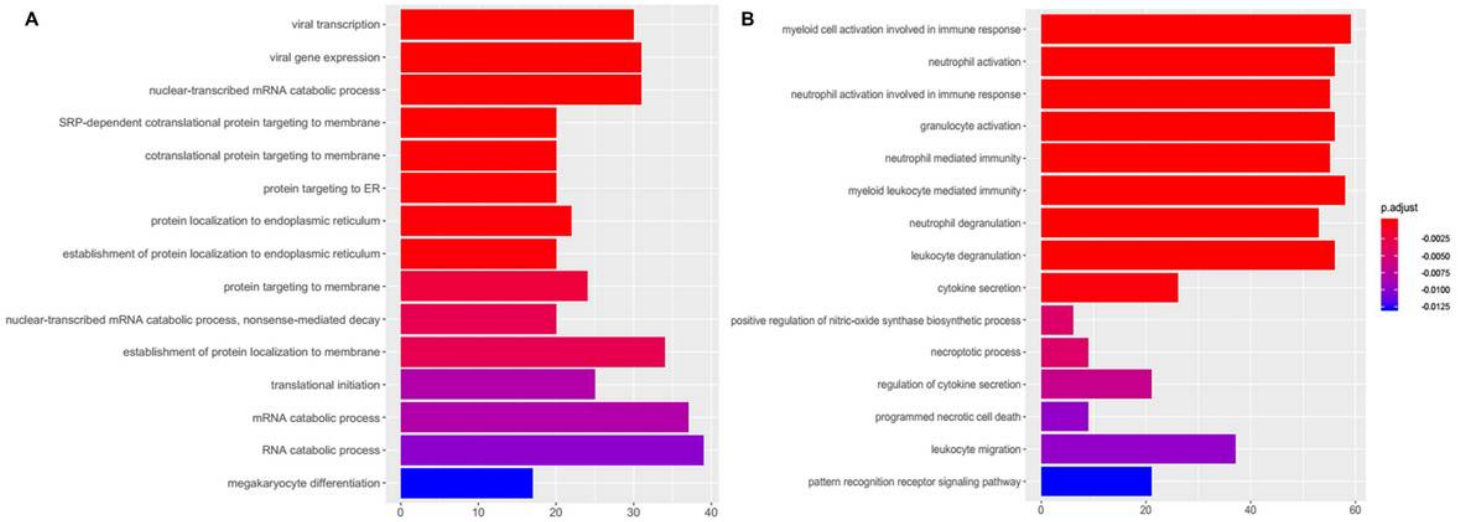

Figure 2

GO enrichment analysis in PCOS patients with CDD treatment. (A) Patient I; (B) Patient II.

Supplementary Files

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