Circular RNA as new serum metabolic biomarkers in patients with Premature Ovarian Insufficiency

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Research Article

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Abstract

Objective

Quantitative Real-time PCR (qPCR) is used to detect the differential expression of Circular RNAs in patients of Premature Ovarian Insufficiency (POI), in order to explore the new biomarkers of POI that can be detected from blood as soon as possible.

Methods

The study collected plasma samples from 30 patients in POI group and 30 normal people group that meet the inclusion criteria, who visited the gynecology clinic of The First Affiliated Hospital of Guangzhou University of Chinese Medicine from July 2019 to December 2020. Then circRNAs in plasma were extracted for qPCR validation.

Results

1. qPCR technology was performed on hsa_circRNA_008901 and hsa_circRNA_403959, and it was found that the levels of both were considerably downregulated in POI group. Clinical evaluation showed that both hsa_circRNA_008901 and hsa_circRNA_403959 have good diagnostic value for POI.

2. According to miRNA Regulatory Element (MRE) analysis, the predicted target miRNAs of hsa_circRNA_008901 are: hsa-miR-548c-3p, hsa-miR-924, hsa-miR-4677-5p, hsa-miR-6786 -3p and hsa-miR-7974; the predicted target miRNAs of hsa_circRNA_403959 are: hsa-miR-1207-5p, hsa-miR-4691-5p, hsa-miR-4763-3p, hsa-miR-6807-5p and hsa -miR-7160-5p.

Conclusion

Compared with the normal group, the expression levels of hsa_circRNA_008901 and hsa_circRNA_403959 in the POI group were downregulated, suggesting that these two circRNAs may be potential biomarkers of POI. Bioinformatics analysis indicated that hsa_circRNA_008901 and hsa_circRNA_403959 may regulate their binding miRNA through the action form of “molecular sponge”, and then regulate the signaling pathway regulated by miRNA, and ultimately affect the disease progression of POI.

What Does This Study Adds To The Clinical Work

The circRNA molecules hsa_circRNA_008901 and hsa_circRNA_403959 in the serum are expected to become the early diagnostic biomarkers of Premature Ovarian Insufficiency. At the same time we also use bioinformatics to reveal the possible mechanism of the above two molecules affecting the Premature Ovarian Insufficiency disease progress.

1. Introduction
Premature ovarian insufficiency (POI) is one of the difficult gynecological diseases, which refers to the symptoms of ovarian hypofunction or even ovarian failure in women under the age of 40, mainly oligomenorrhea or amenorrhea, accompanied by elevated follicle-stimulating hormone (FSH) and decreased estrogen level volatility\(^1\), and may be accompanied by symptoms of perimenopausal syndrome such as hot flashes, sweating, vaginal dryness, decreased libido, and insomnia. POI not only affects the reproductive function of women, causing irregular menstruation, amenorrhea and even infertility, but also increases the risk of cardiovascular disease\(^2\), osteoporosis\(^3\), neurological diseases (dementia and Parkinson's disease), especially their effects on cardiometabolism, increasing the mortality rate of cardiovascular diseases such as coronary heart disease\(^4\), seriously affecting the physical and mental health of patients. The prevalence of POI was previously thought to be 1-1.5%\(^5\), and the incidence of POI has gradually increased in recent years, and a recent study showed that the global prevalence of POI increased to 3.7%\(^6\). The prevalence is higher in low and middle developed countries.

As a non-coding RNA molecule, Circular RNA (circRNA) which does not have 5' and 3' ends, is a circular structure with covalent bonding. A majority of circRNAs consist of exon sequences, which are highly conserved in various species, and have expression specificities in tissues and different developmental stages\(^7\). Since circRNAs are insensitive to nucleases, they are more stable than general linear RNAs\(^8\), and some circRNAs are expressed at levels up to 10 times higher than their linear isoforms. Therefore, circRNAs have distinct superiorities in the development and application of novel clinical diagnostic markers. CircRNA can act as a "sponge" for miRNA: the well-known regulatory hypothesis of competing endogenous RNA (ceRNA)\(^9\): the biological function of ceRNA is completed by miRNA response element (MRE). Through various MREs, ceRNAs can competitively bind to miRNAs and regulate the binding of miRNAs and mRNAs. Related studies have found that there are multiple complementary binding sites with miRNA on circRNA\(^10\), which absorb miRNA like a sponge and control the expression and function of target mRNA through sequence complementarity. Indicates that circRNAs can be involved in disease regulation. More and more evidence shows that there is an obvious connection between circRNA and different gynecological diseases\(^11,12\), which have an important impact on the growth and development of follicles and the function of the ovary\(^13\).

In the early stage, we have completed the Arraystar Human circRNA Array V2 analysis of 5 POI patients and 5 normal people(GSE222497). We found that 35 circRNAs were significantly differentially expressed in the POI group, including 12 up-regulated and 23 down-regulated\(^11,14\). The aim of this research was to investigate hsa_circRNA_008901 and hsa_circRNA_403959 as premature ovarian failure biomarker. We explored patterns of hsa_circRNA_008901 and hsa_circRNA_403959 expression in POI patient plasma, and we then evaluated the potential diagnostic utility of an identified POI-related hsa_circRNA_008901 and hsa_circRNA_403959. Overall, these analyses highlighted the promising value of hsa_circRNA_008901 and hsa_circRNA_403959 in the diagnostic assessment of potential POI patients.

2. Materials And Methods
2.1 Patients and ethical statement

The participants in this study were all from July 2019 to October 2020 at the gynecological outpatient clinic of the First Affiliated Hospital of Guangzhou University of Chinese Medicine. There were 30 cases in POI group and 30 cases in normal control group who met the inclusion criteria but did not meet the exclusion criteria. The research was registered in the Chinese Clinical Trial Registry (ChiCTR1800017312) and approved by the Ethics Review Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine. Informed consent was obtained from participants before samples collection.

2.2 Inclusion and exclusion criteria

2.2.1 Inclusion criteria:

POI group: Meet the diagnostic criteria of Western medicine for POI: referring to the diagnostic criteria of Premature Ovarian Insufficiency in the 2017 "Chinese Expert Consensus on Clinical Diagnosis and Treatment of Premature Ovarian Insufficiency"[^15]: female, under 40 years old, oligomenorrhea or menopause for at least 4 months, twice serum FSH > 25IU/L (at least four weeks); Voluntary participation in this researcher.

Health control (HC) group: Female, less than 40 years old, basic regular menstruation (no more than 7 days before and after the menstrual cycle within 12 months), three sex hormones (follicle-stimulating estrogen, luteinizing hormone, estradiol) in the Normal range; Voluntary to participate in this researcher.

2.2.2 Exclusion criteria:

Chromosomal examination found abnormality related to POI; History of ovarian and uterine surgery; History of chemotherapy and radiotherapy; History of ovarian, uterine, breast, hypothalamic, and pituitary tumors; History of autoimmune diseases: such as Hashimoto's thyroiditis, adrenal insufficiency (Addison's disease), toxic diffuse goiter and other thyroid diseases, systemic lupus erythematosus, type I diabetes, rheumatoid arthritis, or positive for antibodies such as adrenal cortex autoimmune antibodies and anti-thyroid antibodies; Metabolic diseases: galactosemia; Pregnant or lactating period in the past 3 months; Have used sex hormone drugs, oral contraceptives, DHEA and other health care drugs in the past 3 months; Smoking history.

2.3 Specimen collection

After obtaining the informed consent of the participants, 5ml of peripheral venous blood was drawn from the participants and collected into an EDTA anticoagulant tube. Gently inverting and mixing, the samples were stored at 4°C in a low temperature. Plasma separation was carried out in the hospital specimen bank within 2 hours (centrifuge was set to 4°C, 3000g for 10min). Then 2-2.5ml of the supernatant was transferred to a cryopreservation tube and transferred to a -80°C refrigerator for storage. All 60 specimens (30 POI group and 30 HC group) were collected and for qPCR detection.

2.4 Total RNA extraction
Following the manufacturer's instructions, after thawing the specimen, TRIzol LS Reagent (Invitrogen life technologies: 10296028) was added for homogenization. After further adding chloroform and centrifuging, the upper colorless aqueous phase contained all RNA. The aqueous phase was removed to a new centrifuge tube with adding isopropanol, and the RNA was precipitated by centrifugation after mixing. After adding 75% ethanol to wash the RNA precipitate, the ethanol solution was removed, and after drying the RNA precipitate, RNase-free water was added to finally obtain the RNA solution. After extracting RNA from tissue samples, the concentration and purity of RNA were determined by NanoDrop 2000 spectrophotometer and denaturing agarose gel electrophoresis.

### 2.5 cDNA synthesis and qPCR

1 µg of total RNA was reverse transcribed into cDNA using the SuperScript™ III Reverse Transcriptase Kit (Invitrogen: 18080-044). Primer 5.0 was used to synthesize primers used in real-time quantitative PCR (Table 1), and real-time PCR reaction systems (Table 2) were configured for all the cDNA samples obtained by reverse transcription. Adding 8ul real-time PCR reaction system mixture + corresponding 2µl cDNA to each well of the 384-PCR plate, placing it on the real-time PCR machine, and using the Vii7 system to perform qPCR. The reaction conditions were set as: 95°C, 10 min; 40 PCR cycles (95°C, 10 sec; 60°C, 60 sec (to collect fluorescence)). With β-actin as the internal reference, $2^{-\Delta\Delta CT}$ was used to represent the relative expression of circRNA relative to the internal reference gene in the validation results.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>List of primers used in real-time quantitative PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td><strong>Bidirectional primer sequences</strong></td>
</tr>
</tbody>
</table>
| β-actin(H) | F:5' GTGGCCGAGGACTTTGATTG3'  
R:5’CTGTAACAACGCATCTCATATT3’ | 60 | 73 |
| hsa_circRNA_403959 | F:5’CCTCAGAAGACAGGAATCGAAT3'  
R:5’GGGGGGGTAGCAGACAAAC 3’ | 60 | 91 |
| hsa_circRNA_008901 | F:5’GGGAAGGAAAAATGTGAGC 3’  
R:5’GGTAGACAATCTTTCTTAACCGAC3’ | 60 | 101 |

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Realtime PCR reaction system configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent name</strong></td>
<td><strong>Dose(µl)</strong></td>
</tr>
<tr>
<td>2X PCR master mix(Arraystar: AS-MR-006-5)</td>
<td>5</td>
</tr>
<tr>
<td>10uM PCR-specific primer F</td>
<td>0.5</td>
</tr>
<tr>
<td>10uM PCR specific primer R</td>
<td>0.5</td>
</tr>
<tr>
<td>Add water to a total volume of</td>
<td>8</td>
</tr>
</tbody>
</table>
2.6 Statistical processing

The relative expression $2^{-\Delta\Delta CT}$ of the two circRNAs was statistically analyzed by SPSS software. The results were tested for normal distribution, t-test was used for data that accorded with normal distribution, and rank-sum test was used for data that accorded with non-normal distribution. When $P < 0.05$, there was a statistically significant difference in the expression of circRNAs between the two groups.

3. Results

3.1 Age comparison between POI group and HC group

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>Age (average)</th>
<th>Standard deviation</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>POI</td>
<td>30</td>
<td>31.83</td>
<td>5.65</td>
<td>0.597</td>
</tr>
<tr>
<td>Health control</td>
<td>30</td>
<td>31.10</td>
<td>5.02</td>
<td></td>
</tr>
</tbody>
</table>

The age of 30 cases of POI group and 30 cases of normal group were tested for homogeneity of variance ($P = 0.952$), and then t-test was performed for age, $P = 0.597$, so there was no significant difference in age between the two groups (Table 3).

3.2 The results of qPCR validation of differentially expressed circRNAs

The expressions of hsa_circRNA_008901 and hsa_circRNA_403959 in POI group and normal group were verified by qPCR. The results are as follows: Figs. 1 to 2. qPCR confirmed that was hsa_circRNA_008901 and hsa_circRNA_403959 are low expression in POI patients than in HC patients ($P < 0.01$). The area under the curve (AUC) of hsa_circRNA_008901 is 0.8445 (Fig. 3). The hsa_circRNA_403959 expression was also significantly downregulation in POI group with area under the curve (AUC) = 0.8041 (Fig. 4).

3.3 circRNA-miRNA network construction

Based on TargetScan and miRanda, the circRNA-miRNA interaction was further forecasted. Five miRNA targets for circRNAs may have a "sponge effect" were obtained (Table 4). According to MRE analysis, the predicted targeted miRNAs of hsa_circRNA_008901 were hsa-miR-548c-3p, hsa-miR-924, hsa-miR-4677-5p, hsa-miR-6786-3p and hsa-miR-7974. The predicted targeted miRNAs by hsa_circRNA_403959 are: hsa-miR-1207-5p, hsa-miR-4691-5p, hsa-miR-4763-3p, hsa-miR-6807-5p and hsa-miR-7160-5p. Figure 5 and Fig. 6 show the positions of putative binding sites of these miRNAs in these two circRNAs.
Table 4

Predictions of circRNA-miRNA for differential expression of POI

<table>
<thead>
<tr>
<th>circRNA</th>
<th>MRE1</th>
<th>MRE2</th>
<th>MRE3</th>
<th>MRE4</th>
<th>MRE5</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa_circRNA_403959</td>
<td>hsa-miR-1207-5p</td>
<td>hsa-miR-7160-5p</td>
<td>hsa-miR-6807-5p</td>
<td>hsa-miR-4763-3p</td>
<td>hsa-miR-4691-5p</td>
</tr>
<tr>
<td>hsa_circRNA_008901</td>
<td>hsa-miR-7974</td>
<td>hsa-miR-4677-5p</td>
<td>hsa-miR-548c-3p</td>
<td>hsa-miR-924</td>
<td>hsa-miR-6786-3p</td>
</tr>
</tbody>
</table>

4. Discussion

In this study, qPCR technology was used to reverse transcribe circRNAs into cDNA and amplify them to analyze their initial numbers. The relative expression levels of patients in POI group and patients in normal group were compared. It was verified that the expression levels of hsa_circRNA_008901 and hsa_circRNA_403959 were significantly down-regulated in POI patients, indicating that they may be related to the mechanisms of POI. At the same time, the AUC curves of hsa_circRNA_008901 is 0.8445 and the AUC curves of hsa_circRNA_403959 is 0.8041, suggesting these two circRNAs have the ability to become biomarkers for early diagnosis of POI.

Current studies have found that circRNAs mainly play a role in regulating biological processes by acting as a “sponge” of miRNAs\[^{10}\], and circRNAs are competitively combined with miRNAs through various MREs. miRNAs are known as “small regulators with great potential”. miRNAs can bind to any complementary sequence on mRNA, thereby inhibiting gene transcription or promoting mRNA degradation to inhibit gene-expression, thereby influencing a variety of cellular processes, like cell proliferation, differentiation and apoptosis\[^{16}\]. Therefore, the following relationship can be formed between circRNA, miRNA, mRNA and POI (Fig. 7).

In order to further explore the potential regulatory mechanism of circRNA to POI, we used bioinformatics tools to predict the potential targets of the above two circRNAs. Using MRE prediction, it was found that the target miRNAs predicted by hsa_circRNA_008901 were: hsa-miR-548c-3p, hsa-miR-924, hsa-miR-4677-5p, hsa-miR-6786-3p and hsa-miR-7974; the predicted target miRNAs of hsa_circRNA_403959 were: hsa-miR-1207-5p, hsa-miR-4691-5p, hsa-miR-4763-3p, hsa-miR-6807-5p and hsa-miR-7160-5p. Among them, hsa-miR-548c-3p\[^{17}\] can regulate the expression of genes such as PTEN, AKT, STAT, P13K, CREB and FNIP, which is related to tumor diseases and other metabolic diseases. In the studies that have been found, hsa-miR-924\[^{18, 19}\] is mainly related to liver cancer and lung cancer, and its overexpression inhibits cell proliferation, migration and invasion. Hsa-miR-7974\[^{20}\] has a significant correlation with lung cancer. Hsa-miR-1207-5p can inhibit the expression of AKT and STAT3\[^{21}\] to regulate the function of macrophages, and it is related to inflammatory response, and some studies have shown that it may involved in glucose and lipid metabolism\[^{22}\], especially associated with glycated hemoglobin. The target gene of hsa-miR-4691-5p\[^{23}\] is RBM24, which can regulate its expression. The related target genes of hsa-miR-4763-3p\[^{24}\] and hsa-miR-6807-5p\[^{25}\] are mainly related to inflammatory response. The main target genes predicted by hsa-miR-4677-5p are: STX6, LMAN2, ADSS, STYK1, EIF2S1, etc. It has been confirmed...
that IL-6 up-regulates FSH-induced LHR expression in granulosa cells through the JAK/STAT signal transduction pathway[26]. The main target genes predicted by hsa-miR-7974 are: CABP4, MAP7D1, NUFIP2, SDC4, FKBP15, etc. SDC4 may also promote the apoptosis of intact granulosa cells surrounding apoptotic granulosa cells in atretic follicles[27]. With "Premature Ovarian Insufficiency" and "Premature Ovarian Failure" as key words, retrieve GeneCards and OMIM database, the identified POI-related targets included PTEN, AKT, STAT, EIF2S1 and NUFIP2 in the above genes. It is speculated that hsa_circRNA_008901 or hsa_circRNA_403959 is involved in the expression of these genes by regulating the related predicted targeted miRNAs, causing the occurrence and development of POI. At the same time, KEGG analysis of the above related targeted genes was performed using KOBAS/DAVID database, and it was found that they were mainly enriched in the PI3K-Akt signaling pathway.

The PI3K/Akt signaling pathway (Fig. 8) can promote cell growth and cycle proliferation, and is one of the most significant signaling pathways to inhibit apoptosis[28]. PI3K activated by specific receptors can produce a series of products, some of which can activate AKT, make it phosphorylated, and then activate a series of downstream factors that can regulate cell cycle and apoptosis. PI3K activated by specific receptors can produce a series of products, some of which can activate AKT, make it phosphorylated, and then activate a series of downstream factors that can regulate cell cycle and apoptosis[29]. Gene of phosphate and tension homology deleted on chromosome ten (PTEN) is an important factor in the PI3K/Akt signaling pathway[30], which has a negative regulatory role. Inhibition of PTEN can activate the PI3K/Akt signaling pathway to recruit follicles and facilitate the activation of primordial follicles. At the same time, it affects the oocyte DNA damage repair mechanism and regulates follicular atresia. Therefore, when the PI3K/Akt signaling pathway is abnormal, it will lead to the incomplete growth and development of follicles, resulting in the decline of ovarian function, and even the occurrence of diseases such as POI and POF[31].

Researches have shown that inflammatory aging has significant effects on the pathogenesis of POI[32]. Inflammatory aging refers to the long-term chronic and progressively elevated pro-inflammatory state of the body during the natural aging process. Elevated levels of pro-inflammatory cytokines like tumor necrosis factor (TNF) and interleukin-6 (IL-6) lead to the existence of a hyperpro-inflammatory state, which contributes to inflammatory aging[33]. Activation of PI3K/Akt signaling can inhibit the transduction of nuclear factor kappa-B (NF-κB) signaling which can promote the release of inflammatory factors TNF-α, IL-1 and IL-6, resulting in the presence of a highly pro-inflammatory state[34].

Glucose is an important substrate for energy production and is used to support the metabolic and physiological functions of the ovary. Comparing the metabolic characteristics of POI patients and normal women of the same age[35], it was found that the glucose metabolism in POI patients was abnormal. In addition, women with POI are at higher risk of type 2 diabetes compared with women of normal age (45–55 years) at menopause[36]. The above suggests that POI may be related to glucose metabolism. The PI3K/Akt and MAPK signaling pathways are critical for the regulation of glucose metabolism[37]. Different downstream factors of the PI3K/Akt signaling pathway have different regulatory mechanisms for
glucose metabolism\cite{38}. For example, fork head box O1 (Fox O1), which is phosphorylated by Akt, can relieve the regulation of gluconeogenesis-related gene transcription, and inhibit hepatic gluconeogenesis to reduce blood glucose concentration. It is an important target molecule in the insulin signaling pathway.

Glycogen synthase kinase-3 (GSK-3) which is another important downstream factor in the PI3K/Akt signaling pathway, participates in the regulation process of glucose metabolism from different ways such as gluconeogenesis in liver, glycogen synthesis and glucose transport. Therefore, when the PI3K/Akt signaling pathway is abnormal, it can lead to abnormal glucose metabolism, which may lead to the occurrence of POI.

In summary, hsa_circRNA_008901 and hsa_circRNA_403959 may regulate the PI3K/Akt signaling pathway, or by affecting the ovary itself, or by affecting inflammatory aging, or by affecting glucose metabolism, leading to the occurrence and development of POI.

5. Conclusion

Compared with the HC group, the expression levels of hsa_circRNA_008901 and hsa_circRNA_403959 in the POI group were significant downregulated, which may have an important impact on the occurrence and development of POI. Further clinical evaluation showed that both hsa_circRNA_008901 and hsa_circRNA_403959 have good diagnostic value for POI. These results may provide new insights for achieving the goal of early diagnosis of POI and have important value for making treatment decisions.

Bioinformatics analysis revealed that hsa_circRNA_008901 and hsa_circRNA_403959 may act as miRNA sponges, regulating the corresponding mRNAs of miRNAs to inhibit the expression of target genes, then affecting the growth and development of follicles, the differentiation and proliferation of ovarian granulosa cells, and the maturation of oocytes, or affecting inflammatory aging, or affecting glucose metabolism through signaling pathways such as PI3K-AKT. It may cause the occurrence and development of POI. It provides a possible theory for the pathogenesis of POI, which needs further clinical or animal experiments to verify.

Declarations

Consent for publication

The authors confirmed that we have obtained written consent from the patient to publish the manuscript.

Availability of supporting data

The detailed procedures of methods, 8 figures and 4 table are attached.

Competing interests

The authors declare no conflict of interest.
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Authors' contributions
Conception and design: Yu Lin, Ying Zhao;
Acquisition of data: Zhuoya Wang, Caiting Zhong, Yuyang Ou, Yihui Feng;
Analysis and interpretation of data: Yuqi Zheng;
Writing, review, and/or revision of the manuscript: Yu Lin, Ying Zhao

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Conflicts of Interest
The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Figures
Figure 1

Comparison of circRNA_008901

P-value = 5.45E-06
Figure 2

Comparison of circRNA_403959
Figure 3

ROC of hsa_circRNA_008901

AUC=0.8445
P<0.0001
Figure 4

ROC of hsa_circRNA_403959
Figure 5

Binding sites of hsa-miR-548c-3p, hsa-miR-924, hsa-miR-4677-5p, hsa-miR-6786-3p and hsa-miR-7974 in the 3'UTR of hsa_circRNA_008901
### Figure 6

Binding sites of hsa-miR-1207-5p, hsa-miR-4691-5p, hsa-miR-4763-3p, hsa-miR-6807-5p and hsa-miR-7160-5p in the 3′UTR of hsa_circRNA_403959
Figure 7

Relationship between circRNA, miRNA, mRNA and POI

Figure 8

PI3K/Akt signaling pathway diagram