Differential expression of plasma-derived exosomal miRNAs in polycystic ovary syndrome as a circulating biomarker

YANFEI LIU, XINYAN SHI, BING XU, ZHEN WANG, YU CHEN and MIAO DENG

Clinical Laboratory, Hangzhou Women's Hospital, Hangzhou, Zheijiang 310008, P.R. China

Received April 22, 2023; Accepted September 8, 2023

DOI: 10.3892/br.2023.1674

Abstract. Identifying biomarkers with high sensitivity and stability is helpful for the timely and accurate diagnosis, and effective management of polycystic ovary syndrome (PCOS), a long-term, progressive endocrine disorder. Circulating microRNAs (miRNAs/miRs) are being increasingly recognized as promising biomarkers given the stability and enrichment of miRNAs in exosomes. The high sensitivity of the reverse transcription-quantitative PCR (RT-qPCR) has enabled accurate quantification of miRNAs and small fragments, present in a low abundance, in the circulation. In the present study, the potential of miRNAs in the diagnosis of PCOS was evaluated. Exosomal miRNAs were extracted and screened, and three miRNAs (miR-4488, miR-151a-5p, and miR-223-3p) were found to be differentially expressed between the PCOS group and age-matched controls by sequencing analysis. RT-qPCR was performed on a clinically confirmed PCOS cohort (n=107) and a non-PCOS control cohort (n=101) from South China to validate the PCOS-related RNA sequencing results. miR-151a-5p and miR-4488 expression levels were significantly upregulated, and miR-223-3p expression was downregulated in the PCOS cohort compared with the control cohort ($P<0.05$). The areas under the receiver operating characteristic curve were 0.889, 0.871, and 0.664 for miR-4488, miR-151a-5p, and miR-223-3p, respectively. Combining anti-Müllerian hormone levels with the three miRNAs resulted in an AUC of 0.967, and higher sensitivity and specificity. These results suggest that miRNAs may prove useful in the early diagnosis and effective management of PCOS, and that these three miRNAs may be involved in the pathogenesis of PCOS. In addition, bioinformatics analysis showed that these three exosomal miRNAs were involved in key signaling pathways related to cancer.

Correspondence to: Dr Xinyan Shi, Clinical Laboratory, Hangzhou Women's Hospital, 369 Kunpeng Road, Hangzhou, Zhejiang 310008, P.R. China
E-mail: 240471882@qq.com

Key words: polycystic ovary syndrome, microRNA, bioinformatic analysis, diagnosis

Introduction

Polycystic ovary syndrome (PCOS) is commonly associated with anovulation, hyperandrogenism (clinical and/or biochemical), and polycystic ovaries (excluding other androgenic, pituitary, or adrenal causes). It is estimated that 5-10% of all women of reproductive age suffer from PCOS (1,2). In addition to its association with multifactorial endocrine disorders, PCOS is also associated with an increased lifetime risk of obesity and infertility, which are present in ~74% of PCOS patients (3). Therefore, timely diagnosis and treatment of PCOS are not only important for reproductive health, but also for monitoring and intervention of complications. These factors are also prerequisites for gaining a clearer understanding of the pathogenesis of PCOS. Serum biochemical markers, such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), and androgen concentration, have been long used as biochemical indices for the diagnosis of PCOS. However, serum biochemical marker levels are affected by different physiological cycles diminishing their utility for diagnosing PCOS.

The role of exosomal microRNAs (miRNAs/miRs) in the occurrence, progression, and outcomes of tumors has been reported (4-6), while the study of miRNAs related to PCOS in exosomes is in the early stages (7,8). The etiology of PCOS cases is highly heterogeneous with genetically complex and individual differences that are not only related to the endocrine system but are also greatly influenced by the environment and gene expression factors, such as miRNAs (9).

miRNAs are short non-coding RNAs that regulate gene expression at the post-transcriptional level (10,11). miRNAs have been confirmed to be encapsulated in microvesicles (12-16), and exist in most body fluids such as saliva, serum, plasma, urine, milk, and follicular fluid (17). Studies comparing the differences in miRNA expression between follicular fluid exosomes and plasma in women of reproductive age have shown that, among the 37 miRNAs with upregulated expression in follicular fluid, 32 were expressed in exosomes and may be involved in several key signaling pathways of follicular development and egg cell maturation, such as the WNT, mitogen-activated protein kinase (MAPK), ErbB, and transforming growth factor (TGF)-β1 signaling pathways (18). miRNAs in serum are rich, stable, and easy to detect, and may thus serve as non-invasive diagnostic markers of PCOS.

The present study aimed to identify novel miRNAs that are differentially expressed in PCOS patients by screening exosomal miRNAs in a PCOS cohort. The identified differentially expressed miRNAs may have potential value as diagnostic biomarkers for sensitive and accurate diagnosis of PCOS.

Materials and methods

Participants and selection criteria. Between October 2016 and June 2020, 122 patients with PCOS (aged 18-35 years) were recruited from Hangzhou Women's Hospital (Hangzhou, China). Written informed consent was provided by the patients or their legal guardians according to the Declaration of Helsinki (19). All experimental protocols were approved by the Medical Ethics Committee of the hospital and were performed in accordance with the relevant guidelines and regulations. All women were diagnosed according to the Rotterdam criteria and did not have Cushing's syndrome, late-onset congenital adrenal hyperplasia, thyroid dysfunction, hyperprolactinemia, or androgen-secreting tumors. Other exclusion criteria included diabetes, hypertension, chronic renal disease, smoking, and the use of alcohol or medications. A group of 112 age-matched first-trimester individuals (aged 18-36 years) with no previous history of reproductive system diseases or appendicitis served as the control. The control group had normal and regularly cycling menstrual periods, and their ovaries appeared normal on ultrasonography. The exclusion criteria for the control women in the study were the use of drugs, including oral contraceptives or other hormonal drugs, intrauterine device placement, and smoking within the past 3 months (20).

We selected the common gynecological diseases, menopausal syndrome (MPS) and abnormal uterine bleeding (AUB), as control disease groups to improve the experimental study. The 10 women in the MPS group were diagnosed according to the International Clinical Practice Guideline of Chinese Medicine Climacteric Syndrome (21). The 10 women in the AUB group were diagnosed according to the Guidelines on the Diagnosis and Treatment of Abnormal Uterine Bleeding (22).

In the first stage of the study, five pairs of serum samples from five patients with PCOS and five healthy individuals were combined into two group of samples as the screening group. Exosomes and total RNA were extracted, and differentially expressed miRNAs were screened by RNA sequencing (RNA-seq) using a commercial service (Guangzhou RiboBio Co., Ltd.). Following preliminary screening, two miRNAs with significantly upregulated expression (miR-151a-5p and miR-223-3p) and one miRNA with significantly decreased expression (miR-4488a) were identified and validated. Reverse transcription-quantitative PCR (RT-qPCR) was performed to validate the expression of these three miRNAs for the next stage.

Serum collection, blood glycolipid assay, hormone assessment, and exosomal purification. After fasting for 8-12 h, 5 ml blood from controls and patients with PCOS was drawn in the morning. Whole blood was separated by centrifugation at 1,000 x g for 10 min at room temperature, and the isolated serum was centrifuged at 10,000 x g for another 10 min at room temperature to completely remove cells and debris. Prepared serum samples were stored at -80˚C until required.

The serum anti-Müllerian hormone (AMH) concentration was determined using a Roche Cobas e411 automated electrochemiluminescence immunoassay analyzer (Roche GmbH). Serum fasting plasma insulin, testosterone, dehydroepiandrosterone sulphate (DHEA-S), and sex hormone-binding globulin levels were measured using an automated chemiluminescence immunoassay performed on a UniCel DxI 800 analyzer (Beckman Coulter, Inc.). Serum glucose and lipid levels were measured using a Beckman Coulter AU5800 automatic biochemical analyzer (Beckman Coulter, Inc.). All tests were performed in strict accordance with the manufacturer's recommended protocols and reagent instructions. The free androgen index (FAI) was calculated using the equation: FAI=(total testosterone nmol/lx100)/(sex hormone-binding globulin nmol/l) (23). Insulin resistance was calculated using the HOMA method [insulin resistance=(insulin x glucose)/22.5] (24).

Exosomal RNA extraction and the RT-qPCR. Plasma exosomes were separated according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.) with minor modifications. Briefly, 500 μl serum was added to a new tube, and 100 μl total exosome isolation reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added. The mixture was then vortexed until a homogeneous solution was obtained. The sample was incubated at 4˚C for 30 min, followed by centrifugation at 10,000 x g for 10 min at room temperature. The supernatant was aspirated and discarded. The exosomes were present in the pellet that was re-suspended in 200 μl PBS. Due to the failure of exosome extraction in some samples, the samples for subsequent RT-qPCR were reduced to 107 samples in the PCOS group and 101 samples in the control group.

miRNAs were extracted and purified using a commercial kit (BioTeke Corporation) and 50 μl miRNA was acquired. cDNA synthesis and RT-qPCR of miR151a, miR223a, and miR4488a were performed as recommended by the manufacturer. Briefly, miRNAs were reverse transcribed into DNA using the RT™ All-in-One Master Mix (Herogen Biotech). The bulge-loop miRNA™ RT-qPCR Primers Sets (one RT primer and a pair of qPCR primers for each set) specific for miR-151a-5p, miR-223-3p, and miR-4488 were designed and synthesized by Guangzhou RiboBio Co., Ltd. The primer sequences are proprietary and are not disclosed.

Synthetic U6 was added routinely to a final concentration of 1,024 pmol/ml in all samples to control for variations during RNA extraction and/or purification due to the absence of homologous sequences in humans (25,26). Furthermore, all study participants were recruited during the same period, and the specimens were stored under the same conditions and processed in equal volumes at each experimental step to control for potential bias.

The RT-PCR solution contained 2.0 μl 5 RT™ Mix, 6.5 μl mRNA template, 0.5 μl each primer, and 1.0 μl nuclease-free water in a total volume of 10 μl (Herogen Biotech). qPCR was performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 25˚C for 10 min, 42˚C for 50 min; followed by 45 cycles of 95˚C for

Table I. Hormonal and metabolic variables in the controls and patients with PCOS.

Variable	PCOS, n=122[c]	Control, n=112[c]	P-value
Age, years	26.0 (23.0-28.25)	28.0 (23.0-30.0)	0.102
AMH, ng/ml	9.26 (7.55-12.99)	2.72 (1.90-4.45)	<0.001[b]
Testosterone, nmol/l	2.43 (1.92-2.84)	2.41 (1.80-3.15)	0.971
DHEA-S, μmol/l	8.99 (6.44-11.51)	5.09 (3.66-6.82)	<0.001[b]
SHBG, nmol/l	38.10 (23.70-65.83)	91.50 (66.10-141.50)	<0.001[b]
FAI	6.21 (2.80-11.57)	2.32 (1.57-3.44)	<0.001[b]
TG, mmol/l	4.85 (4.37-5.45)	3.89 (3.48-4.31)	<0.001[b]
TC, mmol/l	0.98 (0.69-1.51)	0.73 (0.56-0.94)	<0.001[b]
HDLC, mmol/l	1.65±0.40	1.57±0.32	0.058
LDLC, mmol/l	2.75 (2.31-3.21)	2.01 (1.74-2.34)	<0.001[b]
FBG, mmol/l	4.76 (4.48-5.22)	4.64 (4.41-4.88)	0.019[a]
FINS, μIU/ml	6.80 (4.68-11.78)	5.30 (3.70-6.60)	<0.001[b]
HOMA-IR	1.47 (0.97-2.69)	1.05 (0.78-1.36)	<0.001[b]
LH/FSH, IU/l	2.24 (1.21-3.94)	-	-

[a]P<0.05 and [b]P≤0.001. [c]Data are presented as the median (range) or the mean ± SD. PCOS, polycystic ovary syndrome; AMH, anti-Müllerian hormone; DHEA-S, dehydroepiandrosterone sulphate; SHBG, sex hormone-binding globulin; FAI, free androgen index; TG, triglycerides; TC, total cholesterol; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; FBG, fasting blood glucose; FINS, fasting serum insulin; HOMA-IR, homeostatic model assessment-insulin resistance; LH/FSH, luteinizing hormone/follicle-stimulating hormone.

15 sec, and 60˚C for 40 sec. U6 mRNA was used as the internal control, and a no-template control was used as a negative control.

The qPCR mixture contained 10.0 μl EvaGreen qPCR Master Mix, 2.0 μl cDNA template, 0.5 μl forward and reverse primer each, and 7.0 μl nuclease-free water in a total volume of 20 μl (Herogen Biotech). qPCR was performed in an ABI Prism 7500 Sequence Detection System with the following thermocycling conditions: 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec. For quantitative results, the relative expression levels of each miRNA are presented as a fold change using the $2^{-\Delta\Delta Cq}$ method (27). Each sample was assayed in duplicate, and the average was used for the analysis.

Functional enrichment analyses. The functions of the differentially expressed miRNAs were analyzed using bioinformatics analysis. Related biological pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg) and gene set enrichment using Gene Ontology (GO) (http://www.geneontology.org/). The-log (P-value) was used as the enrichment score, which indicated the significance of the correlation.

Statistical analysis. All data were analyzed using SPSS version 17.0 (IBM Corp.). The normality of the distribution of continuous variables was assessed using the Kolmogorov-Smirnov test. Data are presented as the mean ± standard deviation, or median (range). Differences in the means of two groups of data were compared using a Student's t-test, and the medians of two groups were compared using a Mann-Whitney U test. Spearman's correlation coefficients were calculated to evaluate the relationship between miRNA levels and other variables in both groups. The optimal cut-off points for the miRNA and AMH levels to distinguish between the two groups were evaluated using receiver operating characteristic (ROC) analyses after calculating the area under the curve (AUC), given the maximum sum of the sensitivity and specificity (the Youden Index) for the significance test. P<0.05 was considered to indicate a statistically significant difference.

Results

Patient characteristics. The metabolic characteristics based on the samples of the two groups are provided in Table I. There were no significant differences observed in age, testosterone levels, and high-density lipid cholesterol levels (P>0.05). The levels of AMH, DHEA-S, triglyceride, serum total cholesterol, low-density lipoprotein cholesterol, fasting blood glucose, and insulin in the serum of the PCOS group were significantly higher than those of the control subjects (P<0.001 and P<0.05). In contrast, PCOS patients showed lower levels of sex hormone-binding globulin than controls (P<0.001). PCOS patients also showed significantly higher FAI and HOMA insulin resistance values compared to those of the control group (P<0.001). FSH and LH levels of first-trimester women that served as control individuals were very low or undetectable and are not listed. High glucose and insulin levels are important causes of type 2 diabetes mellitus and insulin resistance in PCOS patients, and high androgen levels are one of the characteristics of PCOS. Overall, abnormalities of the metabolic characteristics in PCOS patients contributed gradually to the development of the disease, which affected their health and quality of life.

Table II. Relative expression levels of miR-151a-5p, miR-223-3p, and miR-4488 in PCOS patients were compared with the CON group, MPS group and AUB group.

Variable	PCOS, n=107[b]	Control, n=101[b]	MPS, n=10[b]	AUB, n=10[b]	P-value
miR-151a-5p	1.92 (1.36-3.20)	0.48 (0.33-0.91)	0.02 (0.01-0.05)	0.01 (0.01-0.04)	<0.01[a]
miR-4488	3.42 (1.73-4.89)	0.51 (0.32-0.95)	2.03 (1.04-4.81)	2.36 (1.67-3.09)	<0.01[a]
miR-223-3p	47.01 (36.25-70.03)	68.83 (45.78-100.9)	0.20 (0.01-0.32)	0.35 (0.18-0.51)	<0.01[a]

[a]P≤0.01. [b]Data are presented as the median (range). PCOS, polycystic ovary syndrome; CON, control; MPS, menopausal syndrome; AUB, abnormal uterine bleeding; miR, microRNA.

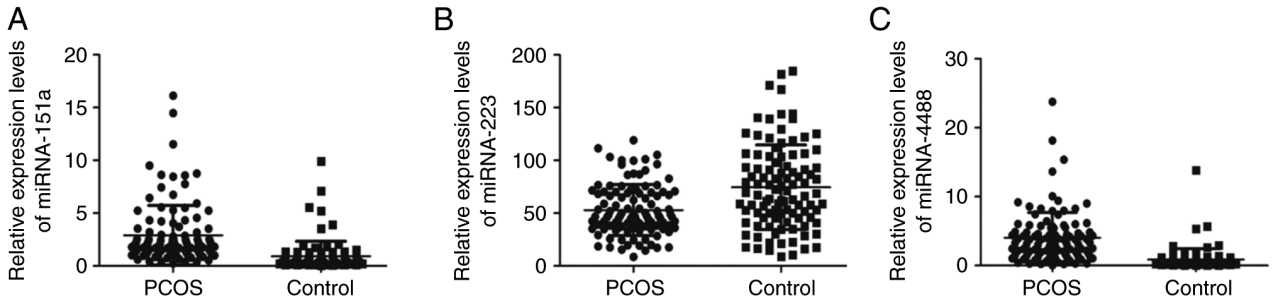

Figure 1. Relative expression levels of the miRNAs of interest in PCOS patients compared with the CON group. Relative expression of (A) miR-151a-5p, (B) miR-223-3p, (C) and miR-4488 in the PCOS patients (n=107) compared with the CON group (n=101). PCOS, polycystic ovary syndrome; CON, control; miRNA/miR, microRNA.

Expression of miRNA in controls and PCOS patients. During the screening stage, two miRNAs (miR-151a-5p and miR-4488) with upregulated expression and one miRNA (miR-223-3p) with downregulated expression were identified. Next, the expression levels of these miRNAs in the validation groups were quantified using RT-qPCR. The miR-151a-5p levels in the PCOS group were 4x that in the control subjects. Compared to control subjects, the expression levels of miR-4488 in the PCOS group were increased by ~570% (P<0.001; Fig. 1A and C). The relative expression of miR-223-3p in the PCOS group was reduced ~31% vs. control subjects (P<0.001; Fig. 1B).

To demonstrate the specificity of exosomal miR-4488, miR-151a-5p, and miR-223-3p to PCOS, the expression of these miRNAs in patients with MPS and AUB were also detected. The results provided in Table II revealed that the expression levels of the three miRNAs differed significantly in patients with MPS and AUB compared with those in the PCOS and control groups (P<0.01). Moreover, the levels of these miRNAs were several times higher in the PCOS group than in the MPS and AUB patients. This supports the relative specificity of these three miRNAs in PCOS vs. other gynecological diseases.

Correlations between miRNA levels and other clinical parameters in PCOS patients. To evaluate the possible association between miRNA levels and glycometabolic parameters, samples from the PCOS and control groups were studied to determine any correlations (Fig. 2). The results revealed that the miRNAs and clinical parameters were weak-moderately correlated with various parameters. miRNA-151a-5p and miRNA-4488 were primarily positively correlated with AMH, FSH, and LH, and miRNA-223-3p was negatively correlated with FSH, LH, and DHEA-S. In addition, the parameters measured showed notable correlations with each other including FSH, LH, AMH, triglyceride, low-density lipoproteins, glucose, and insulin resistance (Fig. 2). The results of the Spearman's correlation analyses are shown in Table SI.

ROC curve analyses. ROC analyses were performed to evaluate the value of differentially expressed miRNAs in discriminating PCOS patients from control subjects. The highest AUC value was observed for miR-4488 (AUC=0.889; P<0.001), followed by miR-151a-5p (AUC=0.871; P<0.001). The lowest value was observed for miR-223-3p (AUC=0.664; P<0.001) (Fig. 3). Accordingly, miR-4488 had a sensitivity and specificity of 81.3 and 91.1%, respectively. miR-151a-5p showed 88.8% sensitivity and 78.2% specificity, whereas miR-223-3p showed 86.9% sensitivity and 45.5% specificity (Fig. 3).

Among miR-151a-5p and miR-223-3p, miR-4488 had an AUC of 0.889 which increased the sensitivity to 89.7% and slightly increased the specificity to 93.1%. Evaluation of AMH yielded an AUC value of 0.926 which increased further when combined with the three miRNAs (AUC=0.967; P<0.001), with a sensitivity and specificity of 91.6 and 93.1%, respectively, indicating this may be a more potent diagnostic tool. The results of the ROC curve analysis for miRNA and clinical measurement discrimination are shown in Table SII.

Functional analysis of predicted targets of miR-151a-5p, miR-223-3p, and miR-4488. GO and KEGG pathway analyses were performed using TarBase to identify the predicted

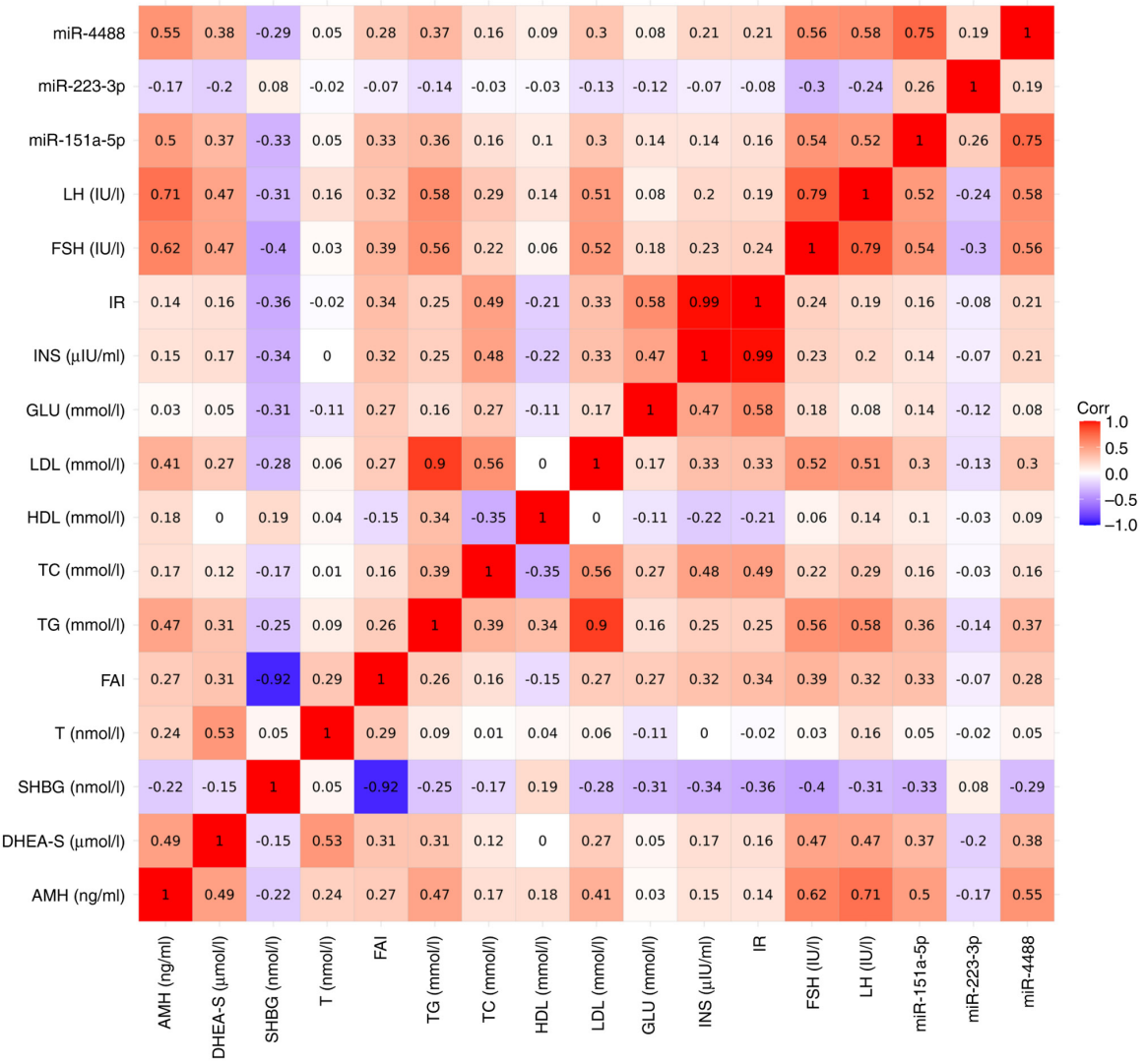

Figure 2. Correlation matrix of the clinical parameters and levels of miR-151a-5p, miR-223-3p, and miR-4488. miR, microRNA.

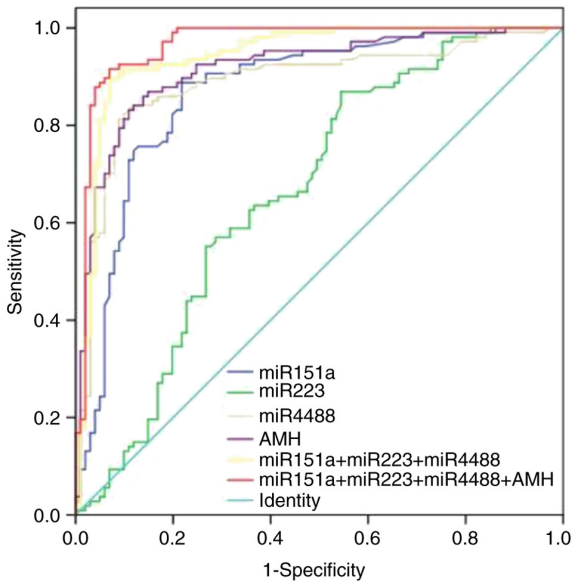

Figure 3. Receiver operating characteristic curve analyses to determine the discriminative significance of miR-151a-5p, miR-223-3p, miR-4488, and AMH, and for prediction of polycystic ovary syndrome. miR, microRNA; AMH, anti-Müllerian hormone.

conserved targets and biological functions potentially influenced by the three miRNAs. In the GO analysis, the number of target genes corresponding to GO entries was determined, and the enrichment score was regarded as the-log (P-value); the target genes of the three analyzed miRNAs are summarized in Fig. 4A-C. For the biological process enrichment, the most significant term was organelle organization, which was significantly enriched in the independent targets of both miR-223-3p and miR-4488. In the cellular component, the term with the most genes was 'protein complex', and the most significantly enriched term was 'cytosol'. For molecular functions, the most significantly enriched term was 'binding'. Thus, the three mRNAs were involved in metabolic processes, growth, and development.

The KEGG pathway analysis identified the top 15 significantly enriched pathways (Fig. 4D). The three miRNAs were involved in multiple signaling pathways such as those regulating the pluripotency of stem cells, transcriptional miscegenation in cancer, the cGMP-protein kinase A signaling pathway, proteoglycans in cancer, and the MAPK signaling pathway, which is related to cancer, hormone secretion, and pluripotency of stem cells.

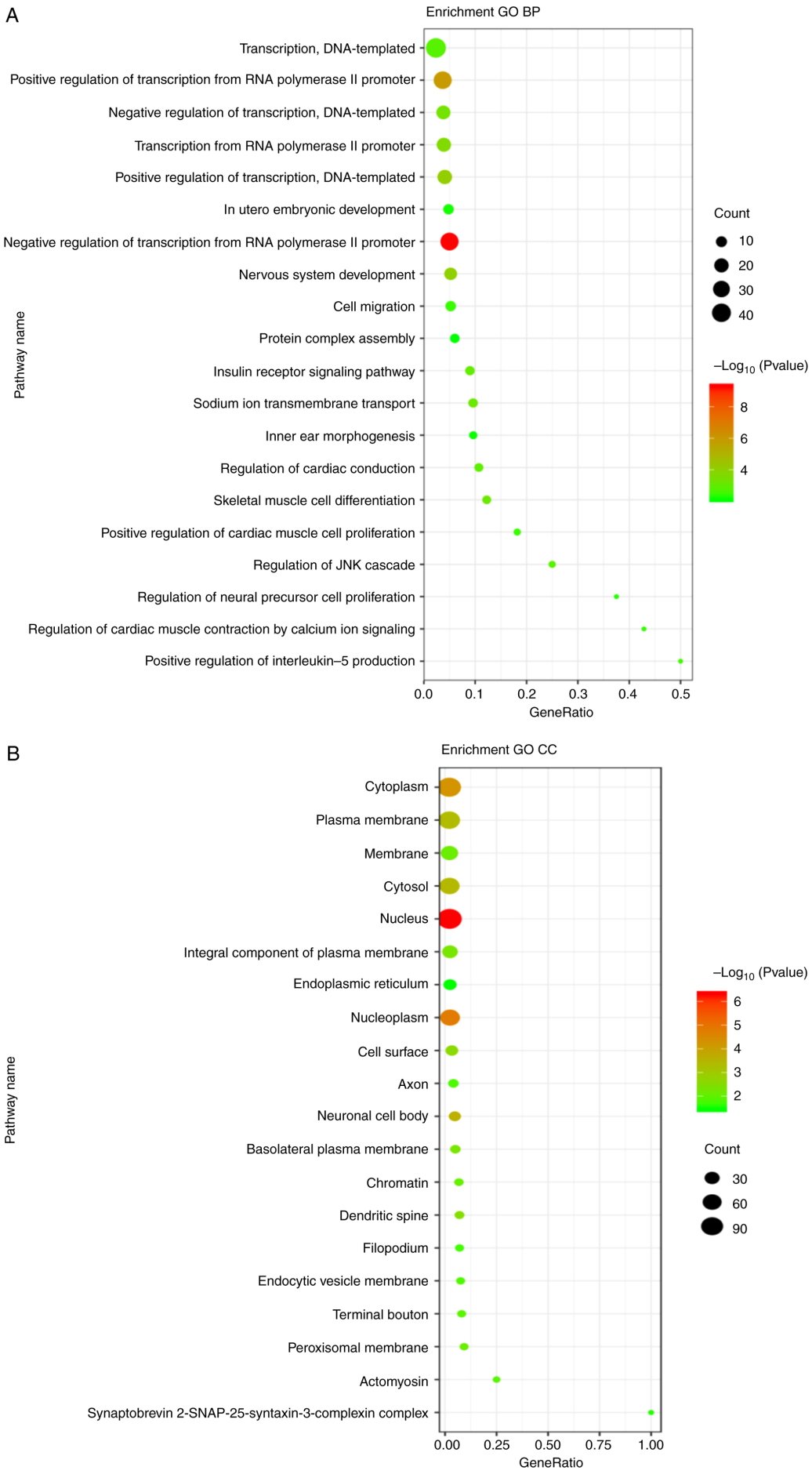

Figure 4. Continued.

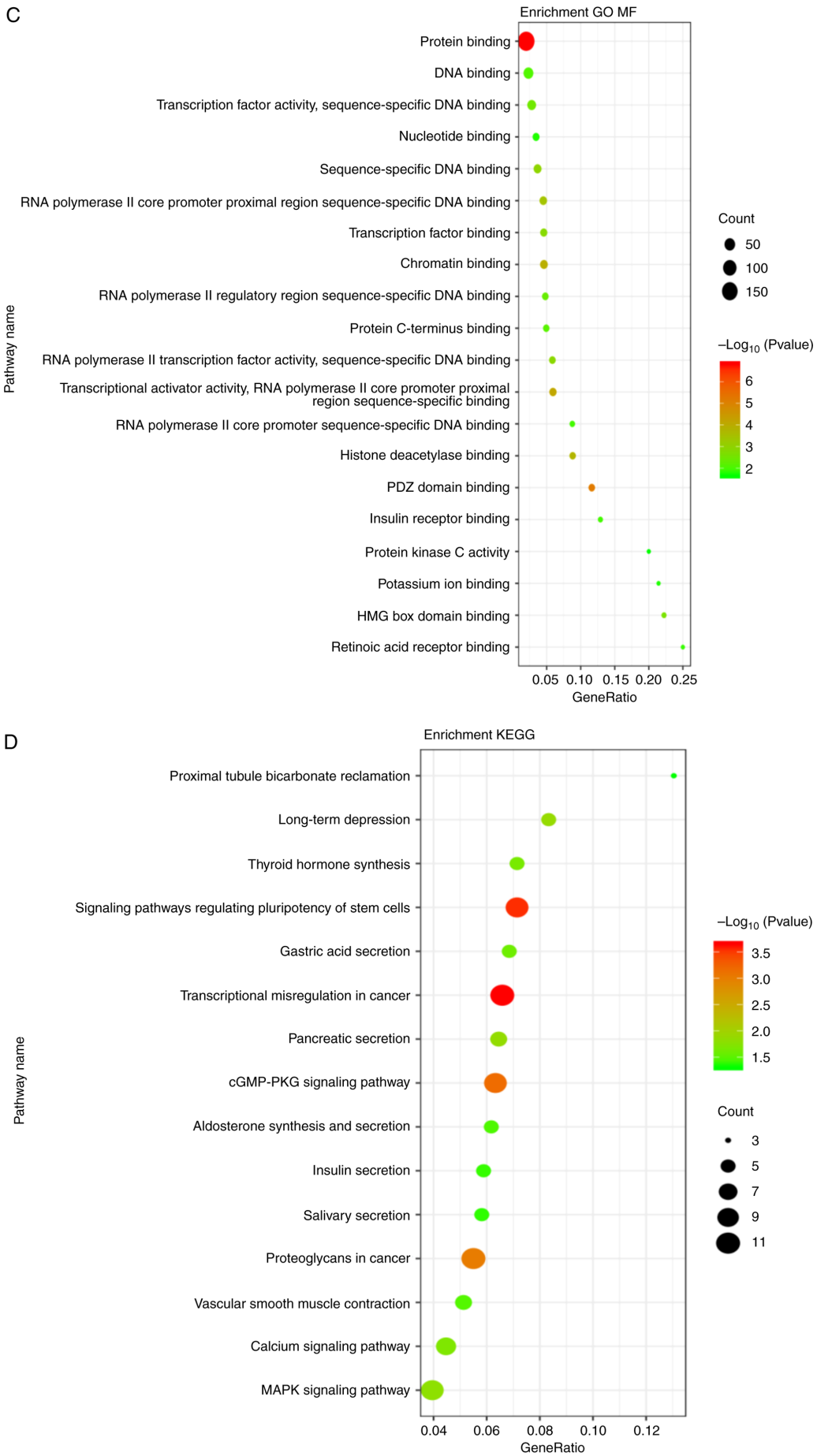

Figure 4. (A-C) GO analyses and (D) KEGG annotations associated with the target genes of differentially expressed miRNAs. GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA.

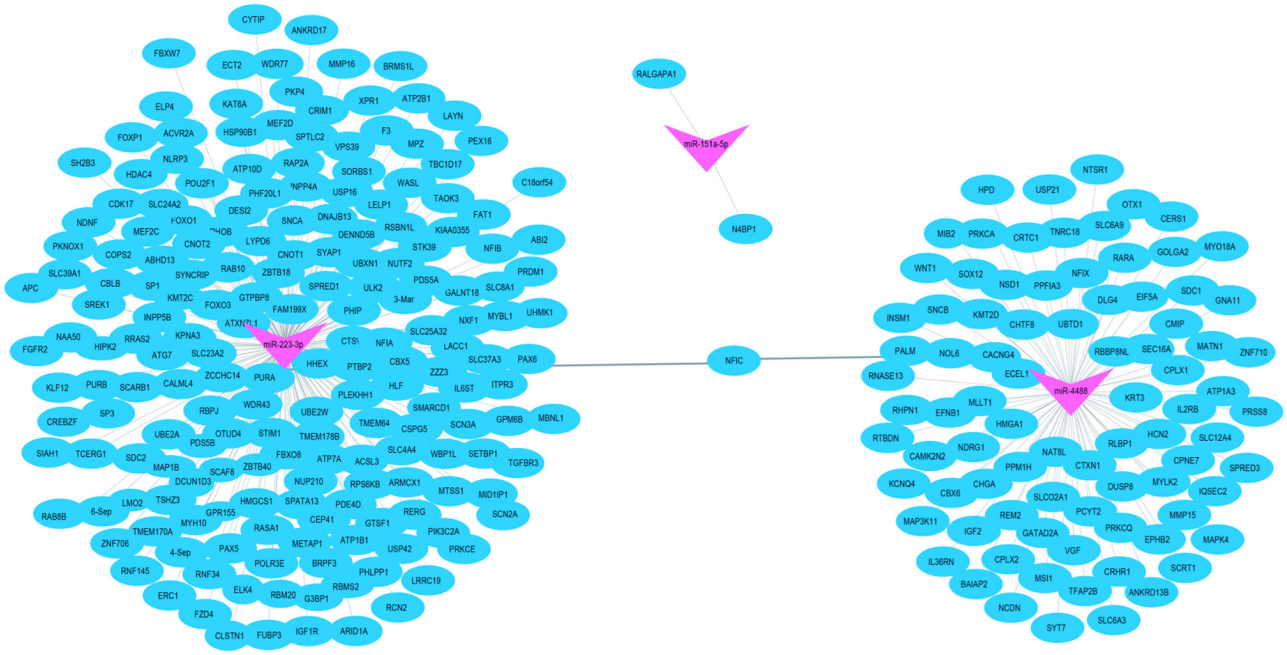

Figure 5. Mapping of the target genes related to miR-151a-5p, miR-223-3p, and miR-4488, visualized using Cytoscape software. The pink arrows indicate the miRNAs, and the blue nodes represent the target genes. miRNA/miR, microRNA.

The mapping analysis using Cytoscape software indicated that miR-151a-5p, miR-223-3p, and miR-4488 may influence the expression of certain genes (such as N4BP1, PURA, and ECEL1) that were primarily enriched in several pathways associated with mRNA binding activity, single-stranded DNA-binding proteins, cellular responses to ultraviolet radiation, and negative regulation of viral genome replication. These genes were predicted to be located in the cytosol and nucleolus, and to be regulated by neuropeptides and peptide hormones. Interestingly, there were no common genes among the three miRNAs (Fig. 5), suggesting that these three miRNAs may play independent roles in the regulation of biological processes through different pathways.

Discussion

Here, for the first time, it was shown that exosomal miRNAs with increased/decreased expression, namely miR-151a-5p, miR-4488, and miR-223-3p, were associated with the risk of PCOS. To evaluate the diagnostic value of the miRNAs for PCOS, ROC curves and AUCs were used, revealing that a combination of the three miRNAs and AMH may offer a potent diagnostic tool to distinguish between patients with PCOS and controls, with a sensitivity and specificity of 91.6 and 93.1%, respectively. This suggests that exosomal miRNA expression patterns in PCOS samples differ from those in controls.

The experimental results demonstrated that serum miRNAs are differentially expressed in PCOS. Compared with other gynecological diseases such as MPS and AUB, the expression of miR-151a-5p, miR-4488, and miR-223-3p was relatively specific to PCOS. The increased fasting blood glucose and insulin levels found in the PCOS samples could partly explain the changes in the miRNAs; miRNA-151a-5p, miRNA-4488, and miRNA-223-3p were notably correlated with other parameters, particularly AMH, FSH, and LH, which were significantly associated with the upregulated miRNAs. In addition, bioinformatics analyses showed that miR-151a-5p, miR-4488, and miR-223-3p are related to insulin metabolism processes. Further analysis showed that glycometabolic and hormone profiles had a weak-moderate correlation with miRNAs. Of note, the changes in the number of individuals for subsequent miRNA validation and correlation analysis are not expected to have an impact on the present findings and conclusions.

Previous studies have shown the involvement of miRNAs in the pathophysiological mechanism of PCOS. For example, one study showed that miR-222, miR-164a, and miR-30c were highly expressed in PCOS patients, and miR-222 was strongly positively correlated with serum insulin, whereas miR-164a was negatively correlated with serum testosterone (28). In addition, researchers observed decreased serum expression levels of miR-320 and showed downregulated expression when treated with TGF-1, and increased insulin resistance in PCOS subjects, while inconsistent expression was observed in follicular fluid in PCOS and increased expression of granulosa cells (9,29,30). Interestingly, Mohammad, Naji *et al* (31) found that there was no significant change in the expression of miRNAs in the serum samples from PCOS individuals. In contrast to the significant decrease in follicular fluid, the levels of miR-93 and miR-21 were significantly increased in granuloma cells compared with those in normal androgenic patients. Furthermore, serum miR-21, miR-27b, and miR-103 levels were associated with PCOS, metabolic disorders, and low-level inflammation. However, it has been suggested that the expression profile of serum miRNAs does not necessarily reflect local changes in the ovaries (9). As such, interpreting miRNA expression in PCOS requires careful consideration of various confounders, and further exploration of the miRNA profiles is needed to

understand the causality and changes in correlation between miRNAs and glycolipid metabolism, and to clarify how miRNAs change over time as the disease progresses.

Numerous miRNAs are expressed in the ovaries and regulate granulosa cell proliferation and apoptosis, follicular growth, atresia, ovulation, luteinization and spermatogenesis, and play an important role in ovarian disorders such as PCOS (32,33). Manuela *et al* (18) compared the differences in miRNA expression between follicular fluid exosomes and plasma in women of reproductive age and found that the expression of 37 miRNAs was upregulated in follicular fluid, 32 of which existed in exosomes and may be involved in several key signaling pathways of follicular development and egg cell maturation, such as the WNT, MAPK, ErbB, and TGF-ß1 signaling pathways.

Using a bioinformatics approach, it was found that miR-4488 and miR-223-3p targeted numerous genes. The target genes of miR-4488 and miR-223-3p were involved in various biological processes, among which the most important were metabolic, growth, and developmental processes, which likely affect the occurrence and development of PCOS. In addition, these genes were associated with multiple pathways such as insulin secretion, and the cGMP-PKG and MAPK signaling pathways; the latter is one of the two best-characterized insulin signaling pathways.

A recent study reported that plasma exosomal miRNAs are involved in the proliferation and differentiation of insulin target cells causing insulin resistance in women with PCOS (34). Reproductive cellular processes were involved and the p38 MAPK protein was found to be expressed in oocytes and granular cells, which is central to regulating oocyte maturation and fertilization (35-37). Through the p38 MAPK and protein kinase A signaling pathways, FSH and cAMP were interrelated and promoted the expression of AMH (38). Thus, the differentially expressed miRNAs enriched in this pathway may directly target the ovaries, leading to substantial changes in patients with PCOS.

Both environmental and genetic factors contribute to the etiology of PCOS (39), which exhibits a range of symptoms such as oligomenorrhea, amenorrhea, infertility, obesity, hirsutism, alopecia, acne vulgaris, and insulin resistance (1-3). Recent studies have reported that PCOS and depression share certain similar clinical symptoms that can further affect the quality of life (40,41).

In conclusion, as research on PCOS is still in its relative infancy, an urgent direction for future studies is to increase sample sizes and perform more functional analyses to confirm the etiological, diagnostic, prognostic, and therapeutic significance of miRNAs in PCOS. Collectively, the present study should encourage further research and improve our understanding of this topic.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Science and Technology Commission of Hangzhou (grant no. 20150633B22) and the Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (grant no. 2020KY763).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XS designed the study and analyzed and interpreted the data. YL performed the experiments, analyzed the data and drafted and revised the manuscript. BX and ZW performed the experiments. YC and MD acquired and interpreted the data. All authors have read and approved the final version of the manuscript. XS and YL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All participants were recruited from the Hangzhou Women's Hospital. Written informed consent was obtained from the patients or their legal guardians. All experimental protocols were approved by the Medical Ethics Committee of the Hangzhou Women's Hospital (Hangzhou, China) and performed in accordance with relevant guidelines and regulations [(2015) Scientific research medical Review approval no. (002)-02].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dunaif A: Insulin resistance and the polycystic ovary syndrome: Mechanism and implications for pathogenesis. Endocr Rev 18: 774-800, 1997.
2. Yildiz BO, Bozdag G, Yapici Z, Esinler I and Yarali H: Prevalence, phenotype and cardiometabolic risk of polycystic ovary syndrome under different diagnostic criteria. Hum Reprod 27: 3067-3073, 2012.
3. Vitek W, Hoeger K and Legro RS: Treatment strategies for infertile women with polycystic ovary syndrome. Minerva Ginecol 68: 450-457, 2016.
4. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM and Breakefield XO: Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers. Nat Cell Biol 10: 1470-1476, 2008.
5. Ohshima K, Inoue K, Fujiwara A, Hatakeyama K, Kanto K, Watanabe Y, Muramatsu K, Fukuda Y, Ogura S, Yamaguchi K and Mochizuki T: Let-7 microRNA family is selectively secreted into the extracellular environment via exosomes in a metastatic gastric cancer cell line. PLoS One 5: e13247, 2010.
6. Sun Z, Shi K, Yang S, Liu J, Zhou Q, Wang G, Song J, Li Z, Zhang Z and Yuan W: Effect of exosomal miRNA on cancer biology and clinical applications. Mol Cancer 17: 147, 2018.
7. Hossain MM, Cao M, Wang Q, Kim JY, Schellander K, Tesfaye D and Tsang BK: Altered expression of miRNAs in a dihydrotestosterone-induced rat PCOS model. J Ovarian Res 6: 36, 2013.

8. Sirotkin AV, Lauková M, Ovcharenko D, Brenaut P and Mlyncek M: Identification of microRNAs controlling human ovarian cell proliferation and apoptosis. J Cell Physiol 223: 49-56, 2010.
9. Sørensen AE, Wissing ML, Salö S, Englund AL and Dalgaard LT: MicroRNAs related to polycystic ovary syndrome (PCOS). Genes (Basel) 5: 684-708, 2014.
10. Ambros V: MicroRNAs: Tiny regulators with great potential. Cell 107: 823-826, 2001.
11. Bartel D: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
12. Gallo A, Tandon M, Alevizos I and Illei GG: The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. PLoS One 7: e30679, 2012.
13. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, *et al*: Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 3: e3694, 2008.
14. Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter P, Benoit Y, Espeel M and D'Herde K: Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. Hum Fertil (Camb) 17: 90-98, 2014.
15. Da Silveira JC, Veeramachaneni DN, Winger QA, Carnevale EM and Bouma GJ: Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: A possible new form of cell communication within the ovarian follicle. Bio Reprod 86: 71, 2012.
16. Sang Q, Yao Z, Wang H, Feng R, Wang H, Zhao X, Xing Q, Jin L, He L, Wu L and Wang L: Identification of microRNAs in human follicular fluid: Characterization of microRNAs that govern steroidogenesis in vitro and are associated with polycystic ovary syndrome in vivo. J Clin Endocrinol Metab 98: 3068-3079, 2013.
17. Simons M and Rapose G: Exosmes-vesicular carriers for intercellular communication. Curr Opin Cell Biol 21: 575-581, 2009.
18. Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzì P, Rizzari S, Maugeri M, *et al*: Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: Bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. Fertil Steril 102: 1751-1761.e1, 2014.
19. World Medical Association: World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects. JAMA 310: 2191-2194, 2013.
20. Xiong W, Lin Y, Xu L, Tamadon A, Zou S, Tian F, Shao R, Li X and Feng Y: Circulatory microRNA 23a and microRNA 23b and polycystic ovary syndrome (PCOS): The effects of body mass index and sex hormones in an Eastern Han Chinese population. J Ovarian Res 10: 10, 2017.
21. Xiao CC, Liu YF, Wang TF, Xu MB, Du HL, Han YH, *et al*: International clinical practice guideline of chinese medicine climacteric syndrome. World J Tradit Chin Med 7: 276-279, 2021.
22. Gynecologic Endocrinology Subgroup, Chinese Society of Obstetrics and Gynecology, Chinese Medical Association: Guideline on diagnosis and treatment of abnormal uterine bleeding: 2022 revisions. Zhonghua Fu Chan Ke Za Zhi 57: 481-490, 2022 (In Chinese).
23. Vermeulen A, Verdonck L and Kaufman JM: A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 84: 3666-3672, 1999.
24. Albareda M, Rodríguez-Espinosa J, Murugo M, de Leiva A and Corcoy R: Assessment of insulin sensitivity and beta-cell function from measurements in the fasting state and during an oral glucose tolerance test. Diabetologia 43: 1507-1511, 2000.
25. Rana S, Yue S, Stadel D and Zöller M: Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. Int J Biochem Cell Biol 44: 1574-1584, 2012.
26. Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G and Hivroz C: TCR activation of human T cells induces theproduction of exosomes bearing the TCR/CD3/zeta complex. J Immunol 168: 3235-3241, 2002.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Long W, Zhao C, Ji C, Ding H, Cui Y, Guo X, Shen R and Liu J: Characterization of serum microRNAs profile of PCOS and identification of novel non-invasive biomarkers. Cell Physiol Biochem 33: 1304-1315, 2014.
29. Yin M, Wang X, Yao G, Lü M, Liang M, Sun Y and Sun F: Transactivation of miR-320 by miR-383 regulates granulosa cell functions by targeting E2F1 and SF-1 proteins. J Biol Chem 289: 18239-18257, 2014.
30. Ling HY, Ou HS, Feng SD, Zhang XY, Tuo QH, Chen LX, Zhu BY, Gao ZP, Tang CK, Yin WD, *et al*: Change in microRNA (miR) profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes. Clin Exp Pharmacol Physiol 36: e32-e39, 2009.
31. Naji M, Aleyasin A, Nekoonam S, Arefian E, Mahdian R and Amidi F: Differential Expression of miR-93a and miR-21 in granulosa cells and follicular fluid of polycystic ovary syndrome associating with diferent phenotypes. Sci Rep 7: 14671, 2017.
32. Li Y, Fang Y, Liu Y and Yang X: MicroRNAs in ovarian function and disorders. J Ovarian Res 8: 51, 2015.
33. Deswal R and Dang AS: Dissecting the role of micro-RNAs as a diagnostic marker for polycystic ovary syndrome: A systematic review and meta-analysis. Fertil Steril 113: 661-669.e2, 2020.
34. Jiang X, Li J, Zhang B, Hu J, Ma J, Cui L and Chen ZJ: Differential expression profile of plasma exosomal microRNAs in women with polycystic ovary syndrome. Fertil Steril 115: 782-792, 2021.
35. Bost F, Aouadi M, Caron L and Binétruy B: The role of MAPKs in adipocyte differentiation and obesity. Biochimie 87: 51-56, 2005.
36. Wu W, Zhang J, Zhao C, Sun Y, Pang W and Yang G: CTRP6 regulates porcine adipocyte proliferation and differentiation by the AdipoR1/MAPK signaling pathway. J Agric Food Chem 65: 5512-5522, 2017.
37. Fan HY and Sun QY: Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biol Reprod 70: 535-547, 2004.
38. Taieb J, Grynberg M, Pierre A, Arouche N, Massart P, Belville C, Hesters L, Frydman R, Catteau-Jonard S, Fanchin R, *et al*: FSH and its second messenger cAMP stimulate the transcription of human anti-Mullerian hormone in cultured granulosa cells. Mol Endocrinol 25: 645-655, 2011.
39. Ajmal N, Khan SZ and Shaikh R: Polycystic ovary syndrome (PCOS) and genetic predisposition: A review article. Eur J Obstet Gynecol Reprod Biol X 3: 100060, 2019.
40. Cooney LG, Lee I, Sammel MD and Dokras A: High prevalence of moderate and severe depressive and anxiety symptoms in polycystic ovary syndrome: A systematic review and meta-analysis. Hum Reprod 32: 1075-1091, 2017.
41. Kolhe JV, Chhipa AS, Butani S, Chavda V and Patel SS: PCOS and Depression: Common links and potential targets. Reprod Sci 29: 3106-3123, 2022.

Copyright © 2023 Liu et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.