The role of circulating miRNAs in mechanism of action and prediction of therapeutic responses of metformin in polycystic ovarian syndrome

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Objective: To study the involvement of microribonucleic acids (miRNAs) in the pathogenesis of chronic anovulation and mechanism of metformin treatment in polycystic ovary syndrome (PCOS).
Design: Case-control and prospective validation cohort study.
Setting: Tertiary university hospital.
Patient(s): A total of 146 patients with PCOS and chronic anovulation and 20 non-PCOS controls were enrolled. Patients who resumed ovulation after metformin treatment (MET-OV) and remained anovulatory after metformin treatment (MET-AO) were assigned to MET-OV and MET-AO groups, respectively.
Intervention(s): All patients with PCOS received metformin treatment for 6 months.
Main Outcome Measure(s): Baseline and chronological changes in the plasma levels of 14 miRNAs (miR-21, 93, 132, 193b, 221, 222, 223, 27a, 125b, 200b, 212, 320a, 429, and 483) selected by literature review, anthropometric data, and hormonal as well as metabolic profiles were measured. Predictive modeling based on baseline circulatory miRNA levels and clinical parameters was performed to predict ovulation recovery after metformin treatment.
Result(s): No significant differences were observed in the baseline hormonal and metabolic profiles between the MET-OV and MET-AO groups. However, the expression of miR-27a, miR-93, and miR-222 was significantly higher in the MET-OV group than that for the MET-AO and control groups. After 6 months of metformin treatment, the levels of insulin, luteinizing hormone, and 6 circulating miRNAs (miR-21, 27a, 93, 221, 222, and 223) and homeostatic model assessment for insulin resistance decreased significantly in the MET-OV group, but remained unchanged in the MET-AO group. The area under curve, sensitivity, and specificity of the adjusted prediction model, based on miRNA levels and clinical parameters using logistic regression analysis for predicting ovulatory response after metformin treatment, were 0.807, 0.892, and 0.632, respectively.
Conclusion(s): The present study demonstrated a distinct pattern of baseline expression and chronological changes in the levels of several circulatory miRNAs between the MET-OV and MET-AO groups, suggesting that aberrantly overexpressed diabetogenic miRNAs are involved in the pathophysiology of chronic anovulation in PCOS, and their down-regulation might contribute toward the therapeutic effects of metformin. This could provide new insights into the mechanism of action and applicability of individualized metformin therapy in women with PCOS. (Fertil Steril® 2023;119:858–68. ©2023 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: miRNA, PCOS, metformin, predictive modeling, ovulation

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common female endocrinopathy, affecting up to 8% to 13% of reproductive-age women (1) and is characterized by chronic anovulation, clinical and/or biochemical hyperandrogenism (HA), and polycystic ovarian morphology that constitute the 3 diagnostic features as per the Rotterdam criteria (2). In fact, up to 90% of the reproductive-age women with PCOS have chronic anovulation and menstrual irregularity (3), contributing significantly to the health care-related economic burden caused by this disease (4). Although oral contraceptives (OCPs) are recommended as first-line treatment for menstrual irregularity by most international guidelines (1, 5), concerns still exist regarding their long-term use. Increased risks of cardiovascular diseases and thromboembolic events have been associated with the consumption of OCPs in women with multiple risk factors, including obesity, hypertension, type 2 diabetes mellitus (T2DM), and dyslipidemia (6). Further, studies have reported weight gain (7) and worsened lipid profiles (8) in women with PCOS who consume OCPs. Therefore, the long-term risks and benefits of OCP treatment for the metabolism of women with PCOS remain controversial. Metformin, a biguanide approved for treating T2DM, is the most widely investigated and commonly applied insulin sensitizer in treating patients with PCOS who have obesity and are overweight or present metabolic abnormalities. In a recent Cochrane review (9) comparing the effects of metformin versus placebo or no treatment, a significant improvement in ovulation rate and menstrual frequency was observed in the metformin group. However, the percentage of patients showing improved menstrual cyclicity after metformin treatment was only up to 40% to 50 % (9, 10). Half of the patients had persistent oligomenorrhea, and it is still unclear which specific subgroups of patients could restore ovulation after metformin treatment.

Microribonucleic acids (miRNAs) are small noncoding RNAs, 19 to 22 nucleotides, that play a critical role in post-transcriptional gene regulation (11). They inhibit mRNA translation or induce mRNA degradation by binding to the 3′ untranslated region of mRNA targets and participate in multiple physiologic processes including cell proliferation, differentiation, metabolism, aging, and apoptosis (12). Dysregulated miRNA expression is involved in the pathogenesis of numerous diseases, such as cancer (13), autoimmune diseases (14), obesity (15), T2DM (16), and cardiovascular diseases (17). Since the discovery of miRNAs in peripheral blood, there has been an increasing interest in the potential use of the circulating miRNAs as diagnostic and prognostic biomarkers of human diseases and in their use for evaluating responses to specific treatments (11), especially because they are resistant to ribonuclease degradation and remain stable in long-frozen samples (18). Although the involvement of miRNAs in the etiology or pathophysiology of PCOS is still poorly understood, several studies have demonstrated altered expression of certain serum miRNAs in PCOS (19–22), and hence, the application of circulating miRNAs as diagnostic biomarkers is considered promising (19, 23).

Nevertheless, the use of circulating miRNAs as prognostic biomarkers of therapeutic responses in PCOS has rarely been evaluated. In a small retrospective cohort study (24), the expressions of serum miR-27 and miR-155 were significantly increased after 4 months of anti-androgen hormonal therapy in a Caucasian women with PCOS (n = 7) and HA. The investigators proposed that this might provide a link for the adverse metabolic effects of anti-androgen hormonal therapy with ethinyl-estradiol and cyproterone-acetate on altered lipid profiles because miR-27 has been shown to be involved in cholesterol homeostasis and fatty acid metabolism. In another Danish study (25), the effects of metformin and OCP treatment on the expression of 22 selected circulating miRNAs, previously related to metabolism, were investigated in patients with PCOS. The results showed significant changes in the expression of several miRNAs after metformin treatment (n = 19), but not with OCP treatment (n = 23), suggesting a better metabolic health after metformin treatment. Although the expression of miRNAs appeared to be altered by pharmacologic treatment in PCOS (24–26), whether the levels of circulating miRNAs can be used as prognostic predictors of therapeutic effects has never been investigated.

We hypothesized that the levels of circulating miRNAs could be used as biomarkers for evaluating the therapeutic effects of metformin treatment to predict the ovulation recovery. First, we conducted a case-control study to analyze the expression of 14 selected circulating miRNAs that are related to PCOS, ovulation, steroidogenesis, and/or insulin resistance (IR), and established a predictive model using regression analysis. Second, a separate validation cohort was prospectively recruited to validate the efficacy of the predictive model. Chronological changes in the circulating miRNAs during metformin treatment, along with alterations in anthropometric, endocrinologic, and metabolic profiles, were also investigated. Our results could be applied clinically in precision medicine to predict therapeutic responses in patients with PCOS and to provide mechanistic clues on how the metformin treatment affects ovulation.

MATERIALS AND METHODS

Patient Recruitment and Ethical Approval

This case-controlled clinical study was conducted in a single university hospital with approval from the Research Ethics Committee of the National Taiwan University Hospital (institutional review board no.: 201512199RINB). Between 2008 and 2018, 146 patients with PCOS and 20 non-PCOS controls were enrolled, and written informed consent was obtained from all the patients. Patients with PCOS initially visited our reproductive endocrinology clinic for menstrual irregularities with or without symptoms of HA. The patients did not receive any medical treatment for PCOS within 3 months before enrollment. Furthermore, PCOS was diagnosed according to the 2003 Rotterdam criteria (2), and all recruited patients fulfilled the features of amenorrhea or oligomenorrhea, with the presence of 1 or 2 additional features. Patients with other endocrine and organic

abnormalities were excluded. Hirsutism was defined as a Ferriman–Gallwey score of >8. The control participants had regular menstrual cycles (interval: 25–35 days). They did not have any of the 3 features of the Rotterdam criteria or any known endocrinopathies.

Protocol and Sample Collection

All participants underwent collection of fasting blood samples, anthropometric measurements, pelvic ultrasonography, and history acquisition during the early follicular phase in patients with spontaneous ovulation cycles and randomly in patients with amenorrhea. The processing of fasting blood samples has been described in our previous studies (3, 10). The biochemical assays and the formulas for the homeostasis model assessment-insulin resistance (HOMA-IR) and free androgen index (FAI) were described in the supplemental materials. All patients with PCOS received metformin (Loditon; Standard Chem and Pharm, Tainan, Taiwan) therapy for ≥6 months. The dosage of metformin was 500 mg/day in the first month, followed by 1000 mg/day in the second month, and 1500 mg/day from the 3rd to 6th month. Dosage adjustment was allowed if intolerable gastrointestinal adverse effects were observed. Patients underwent another blood sampling, anthropometric measurements, pelvic ultrasonography, and history acquisition after 6 months of metformin treatment. During the study period, a normal menstrual cycle or successful resumption of ovulation was defined as >4 ovulatory menses within a 6-month interval. Ovulatory menses were defined as vaginal bleeding after either a biphasic basal body temperature chart or elevated serum progesterone level. Patients who resumed ovulation after metformin treatment (MET-OV) and remained anovulatory after metformin treatment (MET-AO) were assigned to the MET-OV and MET-AO groups, respectively.

Isolation of Plasma miRNA, Reverse Transcription and, Quantitative Reverse Transcription Polymerase Chain Reaction

Fourteen circulatory miRNAs that were related to PCOS, ovulation, steroidogenesis, and/or IR in the previous studies (All the related references were listed in Supplemental Table 1, available online) were selected and quantified by reverse transcription polymerase chain reaction (RT-PCR) (including miR-21, 93, 132, 193b, 221, 222, 223, 27a, 125b, 200b, 212, 320a, 429, and 483). The expression levels before and after 6 months of metformin treatment were measured. The miRNAs prioritized for selection are those miRNAs that were shown to be differentially expressed between PCOS and non-PCOS women by genome-based profiling methods in >1 human studies. The miRNAs which were related to both PCOS and IR or PCOS and HA were also prioritized. The methods for extraction and quantitative RT-PCR of the miRNAs were described in the supplemental materials. The TaqMan probe assay IDs and sequences for the quantitative PCR systems used are listed in Supplemental Table 2. The miR-423-5p probe was used as the control for quantification. Fold changes were expressed relative to baseline using the $2^{-\Delta\Delta Ct}$ method (27).

Prediction of Putative miRNA Target Genes and Pathway Analysis

TargetScan Human release 7.2 web site (http://www.targetscan.org/vert_72/) and miRBase release 22.1 (http://www.mirbase.org/) were used to identify putative miRNA target genes. Gene ontology (GO) and pathway enrichment analysis of the predicted genes were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov/) and Panther 16.0 (http://pantherdb.org/). The GO analysis covered 3 domains: biologic process, cellular component, and molecular function. The recommended cutoff P value for the significance of correlated pathways was .05.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software version 9.00 (San Diego, CA, USA) and the Statistical Program for Social Sciences (SPSS version 17; SPSS Inc., Chicago, IL, USA). The statistical significance of differences between groups was assessed using a two-tailed unpaired Student's t-test or one-way analysis of variance for continuous variables and χ^2 test for categorical variables, as appropriate. The Bonferroni post hoc test was applied when the results of analysis of variance revealed statistical significance. A paired-sample t test was used to assess differences in repeated measurements before and after treatment. R language was used for predictive modeling with logistic regression and receiver operating characteristic (ROC) curve analysis. Univariate logistic regression analysis and ROC curves were plotted for each miRNA individually to determine their discriminating effects in the MET-AO and MET-OV groups. The sensitivity and specificity of discriminating MET-AO and MET-OV groups were assessed using the area under the ROC curve (AUC) and 95% confidence interval (CI). The univariate logistic regression analysis revealed that 4 miRNAs (miR-93, miR-222, miR-223, and miR-429) had significant discriminating ability between the MET-AO and MET-OV groups, with the lower bound of the 95% CI for AUC curve being >0.5. To improve the predictive efficacy, the expression levels of these 4 miRNAs were included in the multiple logistic regression analysis and further adjusted for 4 clinical parameters, including age, body mass index (BMI), menstrual interval (expressed as days), and the presence of HA (expressed as 0 or 1). The rationale for variable selection in the multiple logistic regression model is described in the supplemental materials. All results are expressed as mean ± standard deviation. Statistical significance was set at $P<.05$, and all statistical tests were two-sided.

RESULTS

Baseline Characteristics of the Recruited Population

A total of 75 patients with PCOS who received metformin treatment for ≥6 months were recruited for plasma miRNA

TABLE 1

Chronological changes of hormonal and metabolic profiles before and after metformin treatment.

	MET-OV PCOS (n = 37)			MET-AO PCOS (n = 38)				
	Before treatment	After treatment	*P* value[a]	Before treatment	After treatment	*P* value[a]	Control (n = 20)	*P* value[b]
Baseline characteristics								
Age (y)	24.5 (4.9)			24.4 (5.4)			25.8 (2.3)	NS
Interval (d)	169 (112)[c,d]	35 (5)	< .0001	240 (123)[e,d]	236 (118)	NS	29.9 (1.8)[c,e]	< .0001
Hormone profiles								
Total testosterone (ng/mL)	0.71 (0.37)[c]	0.44 (0.25)	< .0001	0.62 (0.26)[e]	0.47 (0.2)	< .0001	0.32 (0.12)[c,e]	< .0001
Sex hormone-binding globulin (nmol/L)	40.3 (25.5)[c]	40.3 (25.2)	NS	34.3 (20.2)[e]	34.8 (18.2)	NS	57.3 (24.2)[c,e]	.003
Free androgen index (%)	7.85 (4.63)[c]	5.04 (3.31)	.001	8.87 (7.0)[e]	7.03 (7.7)	.013	2.4 (1.8)[c,e]	< .0001
Dehydroepiandrosterone sulfate (μg/mL)	276.4 (105.5)[c]	302.1 (139.9)	NS	276.1 (126.8)[e]	294.9 (127.2)	NS	100.2 (36.9)[c,e]	< .0001
Follicle-stimulating hormone (mIU/mL)	6.66 (1.55)	6.11 (0.29)	NS	6.23 (2.04)	6.02 (1.82)	NS	7.1 (1.8)	NS
Luteinizing hormone (mIU/mL)	12.1 (6.4)[c]	7.9 (6.0)	< .0001	11.9 (5.4)[e]	11.4 (5.8)	NS	4.8 (2.4)[c,e]	< .0001
Metabolic profiles								
Weight (kg)	65.2 (17.1)	62.7 (15. 9)	< .0001	62.9 (16.2)	60.8 (14.9)	< .0001	55.2 (5.7)	.057
BMI (kg/m^2)	25.4 (6.8)	24.3 (6.1)	< .0001	24.4 (5.7)	23.6 (5.2)	< .0001	21.8 (1.6)	.064
Waist circumference (cm)	87.8 (15.6)[c]	84.4 (13.5)	.0001	84.8 (12.1)[e]	82.2 (10.5)	.005	74.0 (6.5)[c,e]	.001
Hip circumference (cm)	95.1 (13.5)	92.7 (11.9)	.007	92.7 (11.4)	90.3 (10.4)	.01	88.7 (5.5)	NS
Glucose (mg/mL)	83.6 (7.8)	83.4 (6.1)	NS	83.2 (5.1)	82.9 (6.3)	NS	84.8 (7.3)	NS
Insulin (IU/mL)	9.07 (9.20)	6.21 (7.33)	.036	8.93 (9.48)	9.03 (9.86)	NS	7.7 (13.4)	NS
Homeostatic model assessment for insulin resistance	1.98 (2.12)	1.35 (1.72)	.037	1.87 (2.01)	1.88 (2.07)	NS	1.04 (0.59)	NS
Total cholesterol (mg/dL)	182.0 (31.8)	171.2 (34.6)	.039	202.5 (45.7)	194.4 (44.0)	NS	183.8 (27.8)	NS
Low-density lipoprotein-cholesterol (mg/dL)	101.1 (26.3)	99.6 (27.9)	NS	117. 2 (40.8)	113.6 (40.7)	NS	87.8 (33.5)	NS
High-density lipoprotein-cholesterol (mg/dL)	53.8 (12.6)	52.4 (9.3)	NS	57.9 (12.6)	57.0 (11.8)	NS	58.0 (9.1)	NS
Triglyceride (mg/dL)	86.3 (51.5)	83.3 (53.6)	NS	88.5 (51.7)	95.8 (55.4)	NS	61.9 (21.8)	NS

BMI = body mass index; MET-AO = anovulatory after metformin treatment; MET-OV = ovulation after metformin treatment; PCOS = polycystic ovary syndrome.

[a] *P* value stands for comparison between before treatment and after treatment and was analyzed using a paired *t* test.

[b] *P* value stands for comparison of before treatment conditions between MET-OV, MET-AO, and control groups using analysis of variance with Bonferroni post hoc analysis. The same letters represent significant differences between the 2 variables.

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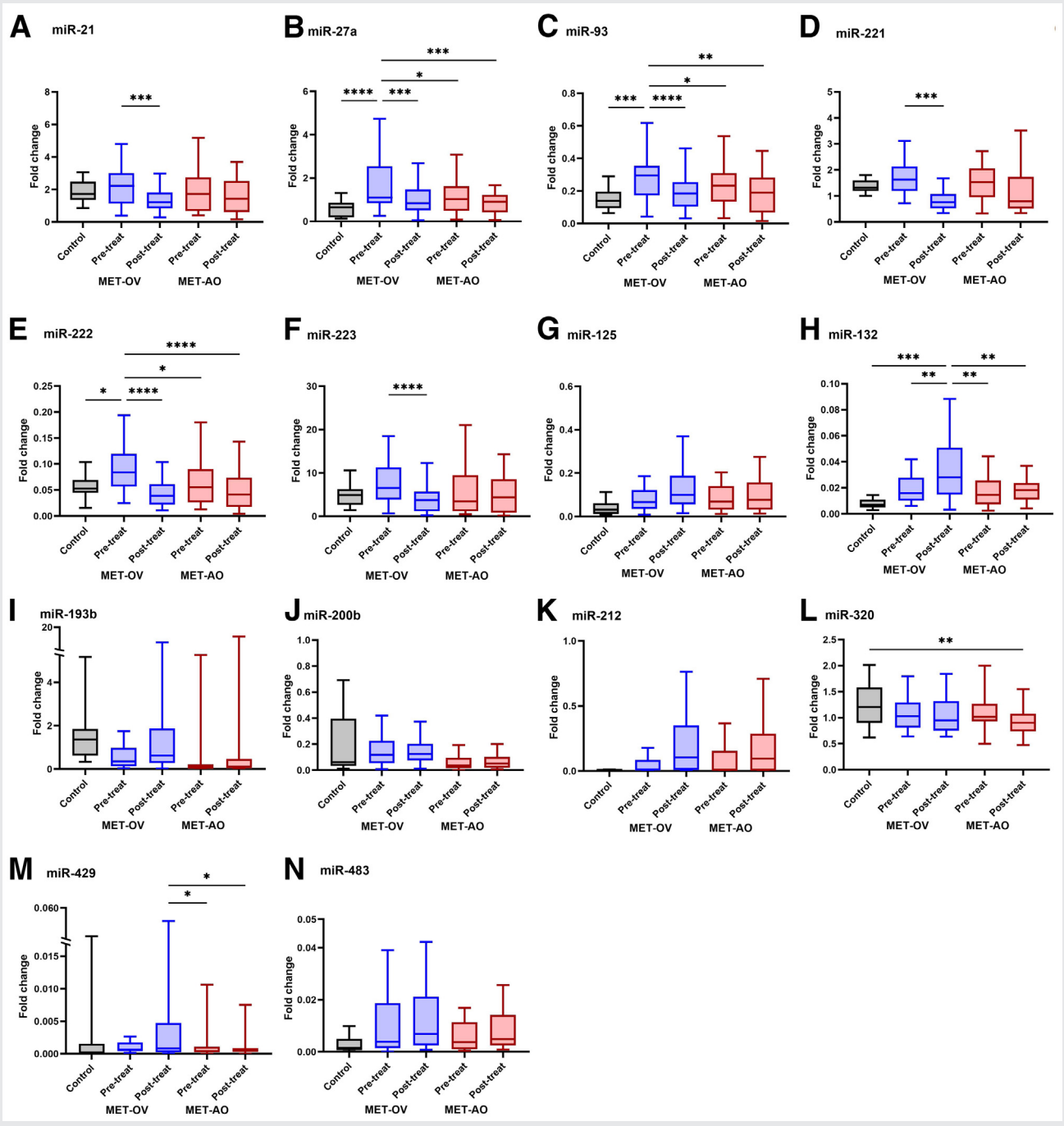
FIGURE 1



The chronological changes of plasma levels of 14 selected miRNAs measured by quantitative RT-PCR is shown as box-and-whisker plots. Twenty non-PCOS controls and 75 anovulatory patients with PCOS were evaluated. Thirty-seven patients with PCOS resumed ovulation after 6-month metformin treatment in the MET-OV group, whereas 38 patients remained anovulatory in the MET-AO group. The box extends from the 25th to 75th percentiles and the whiskers were calculated with Tukey's method (GraphPad Prism software version 9.00). *:$P<.05$, **:$P<.01$, ***:$P<.001$, ****:$P<.0001$.

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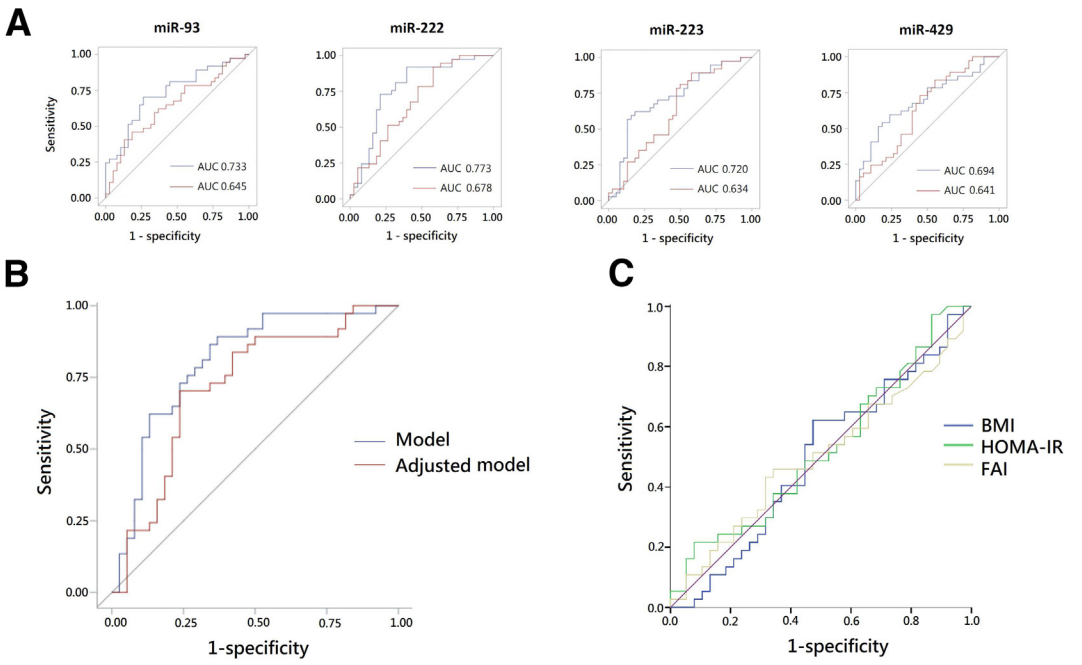
analysis. All patients with PCOS fulfilled the criteria for oligomenorrhea. Among them, 37 patients successfully resumed ovulation after 6 months of metformin treatment (designated as the MET-OV group), whereas the other 38 remained anovulatory (designated as the MET-AO group). Both the MET-OV and MET-AO groups exhibited aberrant hormonal and metabolic profiles, which included a significantly larger waist circumference (WC), higher serum levels of total

FIGURE 2



Variable	*Sensitivity*	*Specificity*	*Youden Index*	*AUC*	*95% CI*	*P value*
Univariate regression analysis						
miR-93	0.459	0.816	0.275	0.645	0.519-0.771	0.031
miR-222	0.919	0.421	0.340	0.678	0.556-0.800	0.008
miR-223	0.892	0.421	0.313	0.634	0.506-0.762	0.046
miR-429	0.838	0.447	0.285	0.641	0.514-0.767	0.036
BMI	0.216	0.895	0.137	0.523	0.391-0.655	0.735
HOMA-IR	0.459	0.658	0.117	0.500	0.367-0.633	1.000
FAI	0.622	0.514	0.136	0.510	0.376-0.643	0.884
Multivariate regression analysis						
Model	0.703	0.763	0.466	0.722	0.602-0.841	<0.001
Adjusted model*	0.892	0.632	0.523	0.807	0.704-0.909	<0.001

- BMI: body mass index; HOMA-IR: homeostatic model assessment for insulin resistance; AUC: area under curve; CI: confidence interval
- *Adjusted model was adjusted for the interval of menstrual cycle (MC interval), age, BMI and hyperandrogenism (HA).
- Model Y = log (p/(1-p)) = -1.8255 + 4.1106 * **miR-93** + 18.0284 * **miR-222** - 0.1152 * **miR-223** + 145.8 * **miR-429**
- Adjusted model Y = log (p/(1-p)) = -2.0454 + 0.0242 * **Age** + 0.0219 * **BMI** + 0.1168 * **HA** - 0.00689 * **MC interval** + 3.6416 * **miR-93** + 25.4973 * **miR-222** - 0.1253 * **miR-223** + 163.0 * **miR-429**

Predictive modeling based on baseline circulating miRNAlevels or clinical parameters for successful resumption of ovulation after 6-months of metformin treatment among patients with polycystic syndrome, who were anovulatory, was established using logical regression analysis.
(A) Univariate logistic regression analysis of the discriminative effects of the 4 miRNAs applied in predictive modeling. (B) Among the 14 selected miRNAs, miR-93, -222, -223, and -429 exhibited significant discriminative ability between the MET-AO and MET-OV groups and were included for modeling. The model was further adjusted for 4 clinical parameters, including age, body mass index, menstrual interval (expressed as days), and the presence of hyperandrogenism (expressed as 0 or 1). The rationale for variable selection in the multiple logistic regression model is described in the supplemental materials. The adjusted model exhibited a trend of better predictability, but the difference between the 2 models did not reach statistical significance (P=.063). (C) Predictive modeling based on any single clinical parameter (body mass index , homeostatic model assessment for insulin resistance, or free androgen index) did not predict the resumption of ovulation after metformin treatment alone. MET-AO = anovulatory after metformin treatment; MET-OV = ovulation after metformin treatment; miRNA = microribonucleic acid.

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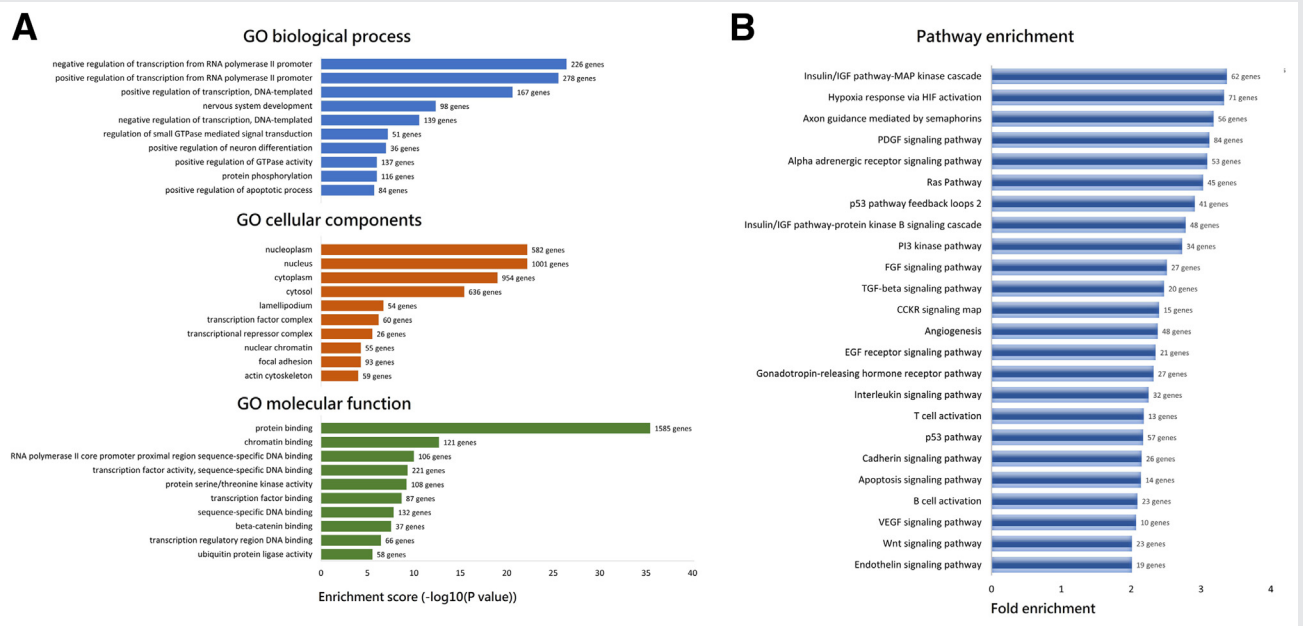
FIGURE 3



The top 10 enriched gene ontology processes and 24 significantly enriched pathways (defined as false discovery rate<0.05) of 2804 targeted genes of 4 miRNAs (miR-93, miR-222, miR-223, and miR-429). These miRNAs showed significant predictability on the resumption of ovulation by 6-month metformin treatment which was analyzed using DAVID and Panther.

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testosterone, dehydroepiandrosterone sulfate, luteinizing hormone (LH), and lower serum levels of sex hormone-binding globulin (Table 1) than that of the control group. No statistically significant differences were observed in the baseline hormonal and metabolic profiles and most of the demographic and anthropometric characteristics between the MET-OV and MET-AO groups, except a significantly longer menstrual interval in the MET-AO group (Table 1).

Different Patterns of Chronological Changes in the Serum Insulin and LH Level Between the MET-OV and MET-AO Groups

A significant decrease was observed in the total testosterone level, FAI, weight, BMI, and WC in the MET-OV and MET-AO groups, revealing the therapeutic effects of metformin on HA and obesity (Table 1). The degree of improvement was similar between the 2 groups, suggesting that the pathogenesis of chronic anovulation in the MET-AO group cannot be alleviated by improving HA and obesity. Although the serum insulin and LH levels as well as HOMA-IR significantly decreased after metformin treatment in the MET-OV group, no change was observed in these parameters after metformin treatment in the MET-AO group. In general, drug compliance of the participants in both MET-OV and MET-AO groups were similar. There were no significant differences in the mean doses of the prescribed metformin between the 2 groups at the start of the study (MET-OV vs. MET-AO: 534 ± 168 mg vs. 520 ± 90 mg, $P = .652$), or at 6 months (1344 ± 273 mg vs. 1389 ± 270 mg, $P = .493$). Overall, 5.4% (2/37) and 7.9% (3/38) of patients in the MET-OV and MET-AO groups, respectively, experienced intolerable gastrointestinal adverse events and had a dose reduction from 1500 to 1000 mg. There was no significant difference in the proportion of participants with dosage adjustments between the groups.

Significantly Different Baseline Levels and Chronological Changes in Circulating miRNAs After Metformin Treatment Between the MET-OV and MET-AO Groups

The relative expression levels of circulating miRNAs before and after metformin treatment are shown in Figure 1. Among the 14 measured miRNAs, the expression of miR-27a, 93, and 222 was significantly higher in the MET-OV group than in the MET-AO group. After metformin treatment, the levels of circulating miR-21, 27a, 93, 221, 222, and 223 were significantly decreased in the MET-OV group. In contrast, the expression levels of these miRNAs remained unchanged in the MET-AO group.

The baseline circulatory levels of miRNAs and the effects of metformin on miRNAs were evaluated after stratifying the patients according to the presence of HA and IR. The presence of HA was defined as biochemical (total testosterone > 7 ng/mL) and/or clinical HA (hirsutism or alopecia). The presence of IR was defined as a HOMA-IR level >2 (28). The baseline expressions of miR-21, 27a, 93, 221, 222, and 223 were significantly higher in the IR subgroup (n = 22) than that of the non-IR subgroup (n = 53) and were attenuated after metformin treatment in both subgroups (Supplemental Fig. 1,

available online). There were no significant differences in the baseline expressions of most miRNAs between the HA (n = 60) and non-HA (n = 15) subgroups, except for a higher expression of miR-221 in the HA subgroup (Supplemental Fig. 2). The expressions of miR-21, 27a, 93, 221, 222, 223 decreased after metformin treatment in both subgroups, although these were statistically significant only in the HA subgroup, which may be because of the smaller case number in the non-HA subgroup.

Predictive Modeling for the Resumption of Ovulation After Metformin Treatment

To verify the usefulness of predictive modeling based on the baseline circulating miRNA levels for successful resumption of ovulation by metformin treatment, logistic regression and ROC analysis were conducted. The results showed that 4 miRNAs (miR-93, 222, 223, and 429) had significant discriminating abilities between the MET-OV and MET-AO groups (Fig. 2A). The expression levels of these 4 miRNAs were included in the multiple logistic regression analysis, and the AUC of the final predictive model was 0.722 (95% CI, 0.602−0.841) (Fig. 2B). When the models were further adjusted for 4 clinical parameters (i.e., age, BMI, menstrual interval, and presence of HA), the AUC was 0.807 (95% CI, 0.704−0.909). None of the clinical parameters, including BMI, FAI, and HOMA-IR, could be applied alone to predict ovulation resumption after metformin treatment (Fig. 2C). A separate validation cohort was prospectively recruited from the outpatient clinic at our hospital to verify the effectiveness of the prediction model. A total of 71 anovulatory women with PCOS were recruited. After 6 months of metformin therapy, 40 women with PCOS became ovulatory and 31 women remained anovulatory. The AUC curve of the adjusted model was 0.665 (95% CI, 0.533−0.797), indicating a fair discrimination capacity of our predictive model.

Gene Ontology and Pathway Enrichment Analysis

Targeted genes of the 4 miRNAs (miR-93, 222, 223, and 429) that showed significant predictability for the resumption of ovulation under metformin treatment were predicted using TargetScan. A total of 2804 predicted miRNAs gene targets were identified and added for further GO and pathway enrichment analyses using DAVID and Panther. The top 10 enriched GO processes and 24 significantly enriched pathways (defined as false discovery rate<0.05) are listed in Figure 3. The target genes enriched in the biologic process category were mainly involved in cellular biosynthetic and metabolic processes, nervous system development, signal transduction, and biologic regulation. The significantly enriched Panther pathways included the insulin or insulin growth factor pathway (fold enrichment [FE]: 3.37), gonadotropin-releasing hormone (GnRH) receptor pathway (FE: 2.32), transforming growth factor beta signaling pathway (FE: 2.47), phosphoinositide 3-kinases pathway (FE: 2.73), hypoxia response via hypoxia inducible factor activation (FE: 3.33), and axon guidance mediated by semaphorins (FE: 3.18) (Fig. 3B). The predicted target genes in the significantly enriched Panther pathways are listed in Supplemental Table 3.

DISCUSSION

The present study is the first to show that differentially expressed circulator miRNAs can be applied to predict the therapeutic responses to metformin administration in women with PCOS, and the levels of miRNAs may be more sensitive than any single clinical parameter (including BMI, FAI, and HOMA-IR) in predicting the response to resuming ovulation within 6 months of metformin treatment initiation. Alongside our previous publication (10), our results might provide new insights into the therapeutic policies of PCOS that metformin could be offered to the patients with normal androgenic and metabolic features but altered miRNA expression levels to improve ovulation and the menstrual cycle. Moreover, the resumption of ovulation after metformin treatment was accompanied by changes in the expression of several circulating miRNAs, which were not altered in patients who remained anovulatory after receiving metformin treatment. This suggests that miRNA regulation may be involved in the biologic mechanisms by which metformin modulates ovulation.

The underlying pathophysiology of anovulation in PCOS may be multifactorial, and the mechanism by which metformin improves ovulation remains unclear. Traditionally, metformin has been mainly offered to patients with PCOS having high metabolic risks, such as obesity or impaired glucose tolerance, to improve their metabolic outcomes. The alleviation of HA and hyperinsulinemia by metformin therapy has been proposed to further improve ovulation and menstrual cycle (29). In our study, significant improvements were noticed in both endocrinologic and metabolic phenotypes, including serum androgen levels, weight, BMI, and WC after metformin treatment in both MET-AO and MET-OV groups. However, as shown in our study, the improvement in HA and obesity was not necessarily accompanied by an improvement in ovulation. This should reflect the heterogeneous pathogenesis of PCOS and that the underlying mechanisms of anovulation might not simply be caused by HA or obesity among patients, especially in the MET-AO group.

The resumption of ovulation in the MET-OV group was accompanied by decreased levels of insulin, HOMA-IR, LH, whereas these parameters were unchanged in patients with PCOS who remained anovulatory after metformin treatment. This indicates toward the pathologic roles of hyperinsulinemia and dysregulation of hypothalamic-pituitary function in ovulatory dysfunction in this subgroup. Nevertheless, the exact causal relationship between modulation of the hypothalamus-pituitary-ovary axis and resumption of ovulation by metformin requires further research. Interestingly, although baseline insulin levels and HOMA-IR were similar between the MET-AO and MET-OV groups, metformin treatment significantly decreased the insulin levels and HOMA-IR in the MET-OV group, but not in the MET-AO group. The

clinical responses to metformin therapy have been shown to vary widely (30). Several genes and single nucleotide polymorphisms have been reported to modulate the pharmacokinetic and pharmacodynamic responses of metformin (31), and it is possible that the patients in the MET-OV group are more susceptible, genetically, to the insulin-modulating effects of metformin. Further pharmacogenomic analyses would be necessary to provide insights into the mechanisms of metformin action and the identification of novel drug targets.

In this study, the baseline expression levels of miR-27a, 93, and 222 were higher in the MET-OV group than that of the MET-AO and control groups. Furthermore, the plasma concentrations of miR-21, 27a, 93, 221, 222, and 223 significantly declined after metformin treatment in the MET-OV group but remained unchanged in the MET-AO group. This suggests that these abnormally overexpressed miRNAs might be related to the pathophysiology of anovulation in the MET-OV group, and down-regulation of these miRNAs may be involved in the biologic mechanisms by which metformin modulates ovulation. In our subgroup analysis, the baseline expressions of miR-21, 27a, 93, 221, 222, and 223 were also significantly high in women with PCOS complicated by increased IR (Supplemental Fig. 1). Incidentally, these miRNAs were also reportedly downregulated after metformin in both IR and non-IR subgroups, per published literature (32). These aberrantly overexpressed miRNAs were shown to be involved in the dysregulation of numerous downstream protein targets of the insulin signaling pathways (33). MiR-27a has been reported to have considerable effect in promoting IR through the peroxisome proliferator-activated receptor-γ-mediated phosphatidylinositol 3-kinase/protein kinase B (Akt) signaling pathway in adipocyte culture and in a high dietary-fat-treated mice model (34). The up-regulation of miR-93 in the adipocyte inhibits *GLUT4* gene expression, which is associated with IR in PCOS (35). Increased expressions of miR-222 in the serum of patients with PCOS had been reported (36) and was proposed to be related to T2DM and insulin sensitivity (37). More studies are needed to delineate the exact roles of these miRNAs in the initiation and development of IR in PCOS. With accumulation of more evidence, these miRNAs may see use as prognostic tools for IR-related complications of PCOS, or as novel targets for preventive or therapeutic measures.

The attenuated expression of these miRNAs may further modulate various target genes that regulate downstream metabolic or neuroendocrine pathways, eventually improving ovulation. In our in vitro experiments using mouse pituitary cell lines, which suggest possible pathologic roles of these aberrantly overexpressed miRNAs, we have found that the expression of LH could be augmented after treatment with miR-27a mimics (data not shown). In contrast, the expression of LH was reduced by metformin treatment via the inhibition of GnRH receptor-mediated mitogen-activated protein kinase 3/1 phosphorylation, as described in another in vitro experiment performed in rat pituitary cells (38). These results are in agreement with our pathway analysis, which show that the downstream target genes of these miRNAs were enriched in the insulin or insulin growth factor–mitogen-activated protein kinase signaling pathways and the GnRH receptor pathway. The accumulated evidence supported the interplay between the insulin signaling pathways and neuroendocrine regulation of ovulation, which may be modulated by miRNAs and metformin.

CONCLUSIONS

The present study demonstrated a distinct pattern of baseline expression and chronological changes in several circulatory miRNAs among different therapeutic responses to metformin treatment in patients with PCOS. Patients who resumed ovulation after metformin treatment showed overexpression of several miRNAs involved in the insulin signaling pathway before treatment. Metformin treatment downregulated these miRNAs, which was accompanied by decreased insulin and LH levels and HOMA-IR. On the other hand, in patients with PCOS who remained anovulatory after metformin treatment, HA and obesity improved significantly, but ovulation did not resume with no significant influence on HOMA-IR and levels of insulin, LH, and circulatory miRNAs. These results suggest that aberrantly overexpressed miRNAs may be involved in the therapeutic effects of metformin treatment on hyperinsulinemia and anovulation. Moreover, differentially expressed circulating miRNAs may be used to predict the therapeutic responses to metformin treatment to improve ovulation in women with PCOS. This could provide new insights into the mechanism of action and applicability of metformin therapy in patients with PCOS.

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El papel de los miARN circulantes en el mecanismo de acción y predicción de respuestas terapéuticas de metformina en el síndrome de ovario poliquístico.

Objetivo: Estudiar la participación de los ácidos microrribonucleicos (miARN) en la patogenia de la anovulación crónica y el mecanismo del tratamiento con metformina en el síndrome de ovario poliquístico (SOP).

Diseño: Estudio de cohortes de validación prospectiva, casos y controles.

Lugar: Hospital universitario de tercer nivel.

Paciente(s): Se incluyeron un total de 146 pacientes con SOP y anovulación crónica y 20 pacientes control sin SOP. Las pacientes que reanudaron la ovulación después del tratamiento con metformina (MET-OV) y permanecieron anovulatorias después del tratamiento con metformina (MET-AO) fueron asignadas a los grupos METOV y MET-AO, respectivamente.

Intervención(es): Todos los pacientes con SOP recibieron tratamiento con metformina durante 6 meses.

Medida(s) de resultado principal: cambios cronológicos y de referencia en los niveles plasmáticos de 14 miARN (miR-21, 93, 132, 193b, 221, 222, 223, 27a, 125b, 200b, 212, 320a, 429 y 483) seleccionados por revisión bibliográfica, se midieron datos antropométricos y perfiles hormonales y metabólicos. Se realizó un modelo predictivo basado en los niveles de miARN circulatorios basales y los parámetros clínicos para predecir la recuperación de la ovulación después del tratamiento con metformina.

Resultado(s): No se observaron diferencias significativas en los perfiles hormonales y metabólicos iniciales entre los grupos MET-OV y MET-AO. Sin embargo, la expresión de miR-27a, miR-93 y miR-222 fue significativamente mayor en el grupo MET-OV que en los grupos MET-AO y de control. Después de 6 meses de tratamiento con metformina, los niveles de insulina, hormona luteinizante y 6 miARN circulantes (miR-21, 27a, 93, 221, 222 y 223) y la evaluación del modelo homeostático para la resistencia a la insulina disminuyeron significativamente en el grupo MET-OV , pero se mantuvo sin cambios en el grupo MET-AO. El área bajo la curva, la sensibilidad y la especificidad del modelo de predicción ajustado, en función de los niveles de miARN y los parámetros clínicos mediante análisis de regresión logística para predecir la respuesta ovulatoria después del tratamiento con metformina, fueron 0,807, 0,892 y 0,632, respectivamente.

Conclusión(es): El presente estudio demostró un patrón distinto de expresión inicial y cambios cronológicos en los niveles de varios miARN circulatorios entre los grupos MET-OV y MET-AO, lo que sugiere que la sobreexpresión diabetogénica aberrante. Los miARN están involucrados en la fisiopatología de la anovulación crónica en el SOP y su regulación a la baja podría contribuir a los efectos terapéuticos de la metformina. Esto podría proporcionar nuevos conocimientos sobre el mecanismo de acción y la aplicabilidad de la terapia individualizada con metformina en mujeres con SOP.