ORIGINAL RESEARCH

Clinical Value and Mechanism Exploration of Serum miR-379 in Obesity-Polycystic Ovary Syndrome

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Objective: As a common endocrine and metabolic disorder, polycystic ovary syndrome (PCOS) is mostly associated with an obese phenotype. The present research focuses on the clinical significance of miR-379 in obesity-PCOS and attempts to elucidate its potential mechanisms.

Methods: Healthy individuals (n = 46), obesity-PCOS (n = 92), and non-obesity PCOS (n = 31) subjects were enrolled. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to examine the level of serum miR-379. The receiver operating characteristic (ROC) curve and logistic regressions were applied to reveal the diagnostic significance. Dual luciferase reporters were performed to validate the targeting relationships. And cell count kit (CCK-8) assay was used to detect cell proliferation.

Results: Serum miR-379 was highly expressed in PCOS patients ($P < 0.05$), in especially obesity-PCOS patients. Higher miR-379 was associated with greater body mass index (BMI), higher bioavailable testosterone (bT), and greater insulin resistance (IR). Additionally, miR-379 was an independent risk factor for the development of obesity-PCOS. The sensitivity of miR-379 in identifying patients with obesity-PCOS from healthy or non-obesity-PCSO patients was 81.52% and 72.83%, and the specificity was 86.96% and 80.65%. Semaphorin 3 A (SEMA3A) was identified as a target of miR-379 and was reduced in the patients with obesity PCOS ($P < 0.05$). Inhibition of miR-375 reduced KGN proliferation, but this reduction was partially restored by silencing of SEMA3A ($P < 0.05$).

Conclusion: Elevated miR-379 assists the diagnosis of obesity-PCOS and regulates the proliferation of KGN by targeting SEMA3A engaged in disease development.

Keywords: obesity, PCOS, miR-379, diagnostic, SEMA3A

Introduction

Polycystic ovarian syndrome (PCOS) is a heterogeneous disease, characterized by the symptoms of polycystic ovary, hyperandrogenemia, menorrhagia, amenorrhea, and other manifestations. PCOS could last the entire life of a woman and frequently occurs at childbearing age. Women with PCOS are prone to obesity, over half of PCOS patients show signs of obesity, and obesity in turn affects the onset and development of PCOS. Additionally, there is a vicious cycle between fat deposition and hyperandrogenemia in PCOS patients, which induces endocrine disturbance.[1] It was also believed that obesity could influence leptin levels and lead to corresponding resistance, which inhibits ovulation by affecting the interaction of insulin-like growth factors, follicle-stimulating hormone, and other factors.[2] Therefore, obesity PCOS has become an important research hot point. However, the diagnosis criteria of obesity PCOS have been constantly revised in controversy and there are no definite conclusions yet, due to the unclear etiology, and pathogenesis. The diagnosis of obesity PCOS is always confirmed after the exclusion of hyperandrogenemia- or ovarian dysfunction-related diseases. Therefore, it is an urgent need to explore reliable and effective diagnostic biomarkers for obesity PCOS.

MicroRNAs (miRNAs) could bind with proteins and therefore negatively regulate the transcription and translation of target mRNAs. The function of miRNAs has been widely reported, especially for their roles in human diseases, miRNAs

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have been considered critical regulators in disease development. Previous studies speculated miRNAs were involved in the onset and regulate the progression of PCOS, where several potential biomarkers were identified, such as miR-199a-5p,[3] miR-98-3p,[4] and miR-130b-3p.[5] The identified candidate miRNAs always showed abnormal expression and could regulate cellular processes during PCOS development. Recently, a number of studies have been devoted to constructing expression profiling in PCOS aiming to explore more potential biomarkers, which lacked data confirmation. For example, Xiao et al established a miRNA expression profile in 75 PCOS women. The differentially expressed miRNAs were demonstrated to be correlated with the menstrual cycle, antral follicle count, and hormone level and showed significance in discriminating PCOS patients.[6] A previous sequencing study identified several hyperandrogenism-related abnormally expressed miRNAs in the follicular fluid of PCOS women, where miR-379 was included.[7] miR-379 has been demonstrated to be involved in various endocrine diseases, such as insulin resistance,[8] and was correlated with fat deposition and obesity-induced kidney injury and liver injury.[9–11] Therefore, miR-379 was speculated to also play a role in screening obesity PCOS and serve as a biomarker.

This study focused on the expression and significance of miR-379 in obesity PCOS aiming to identify a novel biomarker candidate for the early detection and risk prediction of obesity PCOS.

Patients and Methods

Patients

A total of 123 PCOS patients were enrolled in this study, including 92 obesity patients (BMI $\geq$ 25 kg/m^2) and 31 non-obesity patients (BMI < 25 kg/m^2). Additionally, another 46 healthy individuals were enrolled as the control group.

The diagnosis criteria of PCOS[12,13] were: 1) dilute or anovulation; 2) hyperandrogenemia; 3) ultrasound results showed that the number of follicles with a diameter of 2–9 mm $\geq$ 12 in one or both sides of ovarian or the volume of ovarian $\geq$ 10 mL; 4) two of the above 3 terms and other hyperandrogenic causes were excluded.

The healthy individuals were non-pregnant women with regular menstrual cycles, normal ovulation, and normal endocrine hormone levels. Patients with ovarian tumors, uterine fibroids, endometriosis, abnormal uterine bleeding, thyroid and adrenal diseases, hypertension, diabetes, hyperprolactinemia, androgen tumors, and other diseases were excluded. Healthy individuals had no pregnancy or hormone use for at least three months before enrollment. All study subjects had signed informed consent and the study had obtained approval from the Ethics Committee of Beijing Tongren Hospital Affiliated to Capital Medical University.

Sample Collection

Fasting venous blood samples were collected on the 2nd-4th day of the menstrual day from all participants. Samples of amenorrhea patients were collected during the period when B ultrasonography showed no dominant follicles. Collected samples were centrifuged at 3000 r/min for 10 min after standing at room temperature for 1 h to obtain the serum. Isolated serum samples were stored at −80°C for the following analyses.

Cell Culture

KGN cell line, a nonluteinized human granulosa cell, was obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in the DMEM culture medium. The DMEM culture medium was supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 IU/mL penicillin. Cell culture was performed at 37°C.

Cell Transfection

Cultured cells were transfected with miR-379 inhibitor for its knockdown using Lipofectamine 2000 (Invitrogen, USA). Cell transfection was performed at room temperature and transfected cells were available for the following experiments after 48 h of transfection.

Real-Time Quantitative PCR

Total RNA of serum samples and cell lines was isolated with TRIzol reagent (Invitrogen, USA) and its concentration and purity were evaluated. Then, cDNA was reversed from extracted RNA with the cDNA synthesis kit (Invitrogen, USA). PCR quantification was performed on the 7500 PCR system (Applied Biosystem, USA) with the help of the SYBR Green master mix according to the manufacturer's protocols. The relative expression of miR-379 was calculated with the $2^{-\Delta\Delta Ct}$ method with miR-39 (for miR-379) and GAPDH (for SEMA3A) as the internal references.

Cell Proliferation Assay

Transfected cells were seeded into 96-well plates triplicate supplied with a completed culture medium and incubated for a certain period of time. CCK-8 reagent was added to each well at time intervals of 12, 24, 48, 72, and 96 h. Absorbance at 450 nm was measured after 2 h of adding CCK-8 and the OD450-time curve was plotted to evaluate cell growth.

Dual-Luciferase Reporter Assay

Wild-type and mutant-type Semaphorin 3 A (SEMA3A) luciferase reporter vectors were established by cloning corresponding sites predicted by the online database into the pGL3 vector (Promega). Established vectors were co-transfected with miR-379 mimic or inhibitor into KGN cells. The luciferase activity of SEMA3A was detected with Dual-Luciferase Assay System (Promega, USA) with Renilla as the internal reference.

Statistical Analysis

Pairwise comparison was performed on the basic information of study subjects using Student's t-test ($P < 0.05$) and ROC analysis was employed to evaluate the potential of miR-379 in the early diagnosis of PCOS. The association of miR-379 with the clinicopathological features of PCOS patients were evaluated by the Chi-square test. The logistic regression analysis was conducted to assess the risk factors for obesity-PCOS. Experimental data were expressed as mean ± SD and analyzed by one-way ANOVA ($P < 0.05$).

Results

Subject's Demographic Characteristics and Hormonal Indicators Comparisons

As illustrated in Table 1, BMI and LH were significantly higher in the obesity-POCS group than in the healthy group ($P < 0.05$). Compared to the healthy group, LH, bT, FINS, and HOMA-IR were markedly elevated in the obesity-POCS

Table 1 Clinical Characteristics and Sociodemographic Characteristics of PCOS Patients and Healthy People

	Healthy Group (n = 46)	Obesity-POCS Group (n = 93)	Non-Obesity PCOS Group (n = 31)	P value		
				P1	P2	P3
Age (years)	27.50 ± 3.25	27.32 ± 3.24	27.52 ± 3.27	0.753	0.983	0.766
BMI (kg/m^2)	23.52 ± 2.04	27.74 ± 3.65	24.26 ± 2.29	<0.001	0.140	<0.001
FSH (U/L)	5.25 ± 0.93	3.07 ± 0.74	3.28 ± 0.77	<0.001	<0.001	0.184
LH (mIU/mL)	5.62 ± 1.14	13.15 ± 1.72	12.02 ± 1.22	<0.001	<0.001	0.001
E2 (pmmol/L)	196.43 ± 13.43	198.34 ± 6.39	197.97 ± 4.14	0.258	0.540	0.760
bP (nmol/L)	2.03 ±0.59	2.09 ± 0.33	2.03 ± 0.34	0.465	0.988	0.389
bT (nmol/L)	1.65 ± 0.35	1.95 ± 0.27	1.86 ± 0.50	<0.001	0.025	0.198
FINS (uIU/mL)	7.88 ±0.58	23.34 ± 1.55	10.16 ± 1.39	<0.001	<0.001	<0.001
Glu (mmol/L)	4.84 ± 0.63	4.78 ± 0.75	4.80 ± 0.79	0.8624	0.789	0.901
HOMA-IR	1.70 ± 0.25	4.96 ± 0.87	2.17 ± 0.50	<0.001	<0.001	<0.001

Notes: P1, obese PCOS group vs Control group; P2, non-obese PCOS group vs Control group; P3, obsess PCOS group vs non-obese PCOS group. Date was presented as mean ± SD.
Abbreviations: BMI, body-mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; BP, progesterone; Glu, glucose; bT, bioavailable testosterone; HOMA-IR, homeostasis model assessment of insulin resistance; FINS, fasting plasma insulin.

group and the non-obesity POCS group, and FSH was typically abrogated ($P < 0.05$). Among them, the levels of LH, FINS, and HOMA-IR were significantly higher in the obesity-POCS group than in the non-obesity-PCOS group ($P < 0.05$). In contrast, E2, bP, and Glu were non-statistically different in the three groups ($P > 0.05$).

miR-379 Was Remarkably Elevated in the Patients with Obesity-PCOS

To explore the clinical significance of miR-379 in obesity-PCOS patients, the levels of miR-379 were first examined. Compared with the control group, miR-379 was significantly higher in PCOS patients, with miR-379 significantly higher in the obesity-PCOS group than in the non-obesity-PCOS group ($P < 0.001$, Figure 1A). Subsequently, based on the mean value of miR-379 in obesity-PCOS patients were divided into miR-379 high expression group (n = 49) and miR-379 low expression group (n = 44). Additionally, patients with increased miR-379 expression exhibited higher BMI ($P = 0.024$), bT ($P < 0.001$), and HOMA-IR ($P = 0.014$, Table 2) levels, which are of greater concern. The findings suggest that elevated miR-379 may be a function miRNA in obesity-PCOS.

Elevated miR-379 is a Potential Diagnostic Biomarker for Obesity-PCOS

miR-379, BMI, FSH, bT, and HOMA-IR were included in logistic regression to explore potential risk factors for obesity-PCOS. As illustrated in Table 3, in addition to BMI (HR=7.362, 95% CI: 2.441–22.202, $P < 0.001$), miR-379 (HR=7.172, 95% CI: 2.415–21.300, $P < 0.001$) was also a potential risk factor for the development of obesity-PCOS. ROC curves were employed to assess the diagnostic significance. The AUC of ROC was 0.9414 and the sensitivity and specificity of miR-379 levels to identify obesity-PCOS patients from the healthy group was 81.52% and 86.96% at a cut-off value of 1.295. More importantly, miR-379 levels could significantly differentiate obesity-PCOS patients from non-obesity PCOS patients when the cut-off value was 1.465, and the sensitivity was 72.83% and 80.65% with AUC of 0.8084 (Figure 1B). Overall, miR-379 may be a potential diagnostic biomarker for obesity-PCOS patients.

SEMA3A is a Target Gene of miR-379 in Obesity-PCOS

To further explore the molecular mechanism of miR-379 in obesity-PCOS, downstream targets were attempted to be searched. Bioinformatics revealed a partial binding sequence of SEMA3A to miR-379 (Figure 2A). The dual-luciferase report assay confirmed that miR-379 mimic significantly inhibited the luciferase activity of WT-SEMA3A, while miR-379 inhibitor significantly promoted the luciferase activity of WT-SEMA3A ($P < 0.05$), but neither of them had a significant effect on the luciferase activity of MT-SEMA2A ($P > 0.05$, Figure 2B). What's more, SEMA3A was weakly-expressed in
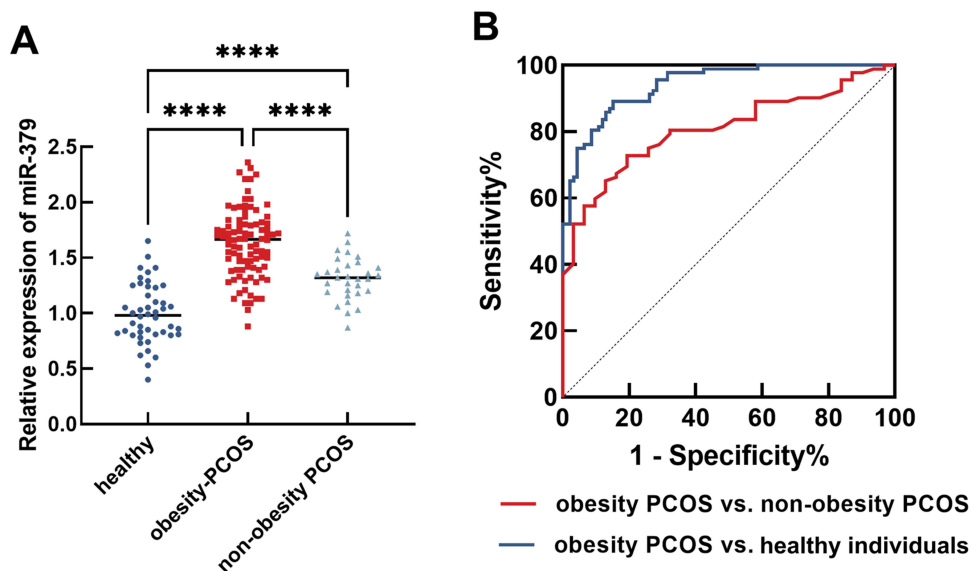

Figure 1 Elevated miR-379 is a potential diagnostic biomarker for obesity-PCOS. (**A**) The expression of miR-379 in three groups was detected by RT-qPCR. (**B**) ROC was conducted to analyze the diagnostic value of miR-379 in identifying obesity-PCOS patients from the healthy or non-obesity-PCOS group. ****$P < 0.0001$.

Table 2 Correlation Between miR-379 Levels and Clinical Features in Obesity Patients

Parameters	Cases No. (n = 93)	miR-379 Expression		P
		Low (n = 44)	High (n = 49)	
BMI (kg/m^2)				0.024
< 28	52	30	22	
≥ 28	41	14	27	
FSH (U/L)				0.186
< 3.1	49	20	29	
≥ 3.1	44	24	20	
bT (nmol/L)				< 0.001
< 2	52	33	19	
≥ 2	41	11	30	
FINS (uIU/mL)				0.393
< 23	38	20	18	
≥ 23	55	24	31	
HOMA-IR				0.014
< 5	51	30	21	
≥ 5	42	14	28	

Abbreviations: BMI, body-mass index; FSH, follicle-stimulating hormone; bT, basic testosterone; IR, insulin resistance; FINS, fasting plasma insulin.

Table 3 Logsitic Employed to Analyze Risk Factors for the Development of Obesity PCOS

Characteristics	Multivariate Analysis		
	HR	95% CI	P
miR-379	7.172	2.415–21.300	<0.001
BMI	7.362	2.441–22.202	<0.001
FSH	0.507	0.192–1.342	0.171
bT	0.556	0.211–1.460	0.233
HOMA-IR	0.573	0.216–1.517	0.262

Abbreviations: PCOS, polycystic ovary syndrome; BMI, body mass index; FSH, follicle-stimulating hormone; bT, bioavailable testosterone; HOMA-IR, homeostasis model assessment of insulin resistance.

obesity-PCOS patients than in non-obesity-PCOS patients and healthy ($P < 0.05$, Figure 2C). SEMA3A expression was also negatively correlated with SEMA3A levels in obesity-PCOS patients (r = −0.6957, Figure 2D).

The Inhibition of KGN Proliferation by Reduced miR-379 Was Typically Attenuated by Silencing SEMA3A

Co-transfection of si-SEMA3A with miR-379 inhibitor was performed in the KGN cell. As illustrated in Figure 3A, miR-379 levels were typically suppressed by miR-379 inhibitor ($P < 0.05$) but had no effect on SEMA3A expression ($P > 0.05$). Furthermore, miR-379 inhibitor typically increased the level of SEMA3A, but this increase was attenuated by silencing of SEMA3A ($P < 0.05$, Figure 3B). Meanwhile, CCK-8 confirmed that inhibition of miR-379 significantly impaired KGN proliferation, but this impairment was partially restored by silencing of SEMA3A ($P < 0.05$, Figure 3C).
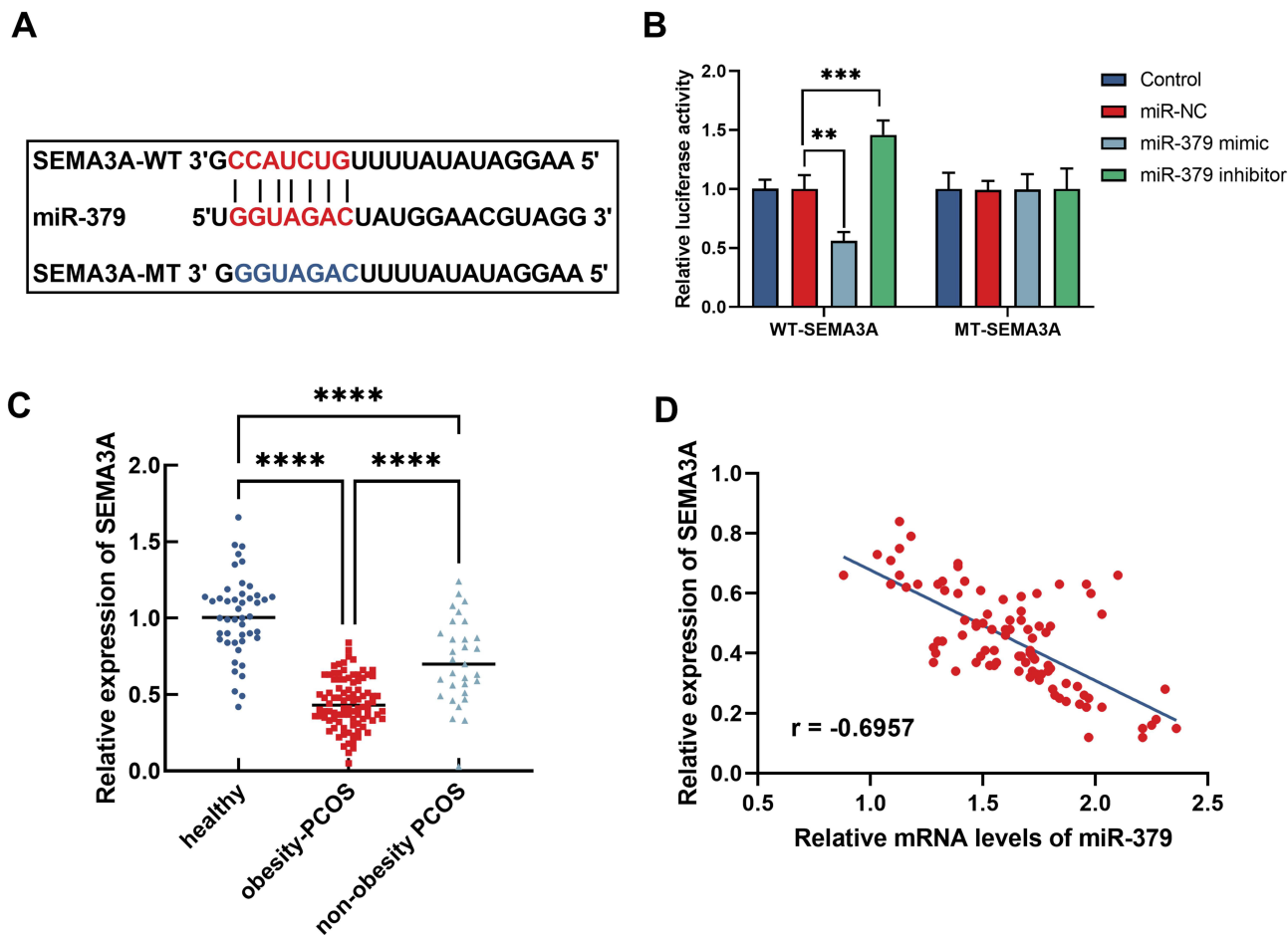
Figure 2 SEMA3A is a target gene of miR-379 in obesity-PCOS. (**A**) Sequence fragment of SEMA3A bound to miR-379. (**B**) Dual luciferase reporter validates targeted binding between miR-379 and SEMA3A. (**C**) Expression of SEMA5A in the three groups was detected by RT-qPCR. (**D**) Correlation analysis between miR-379 and SEMA3A in obesity-PCOS patients. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$.
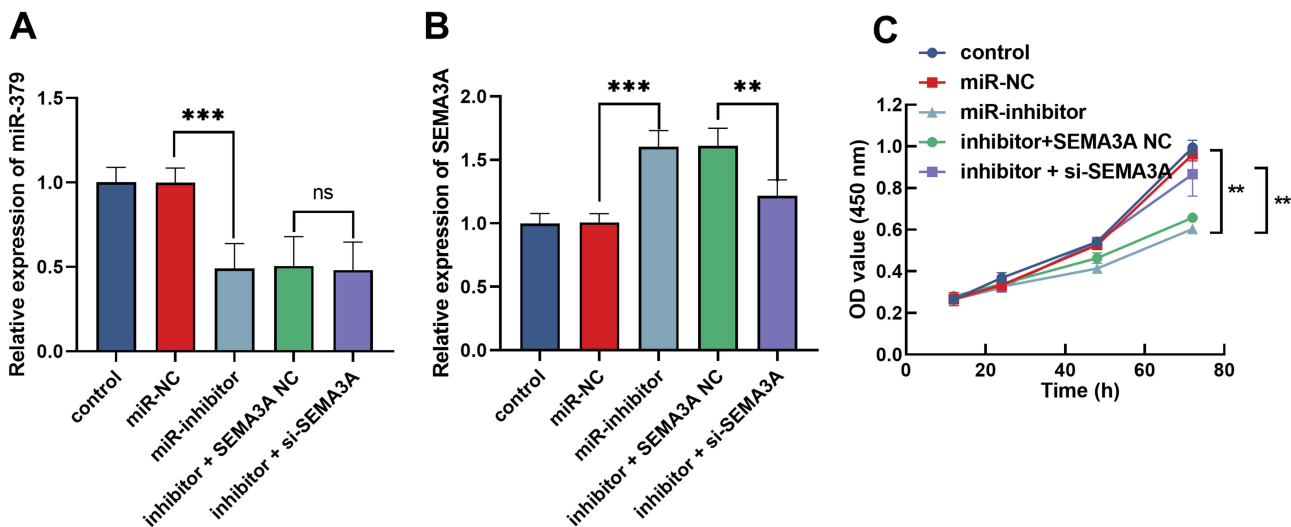

Figure 3 miR-379 affects the proliferation of KGN by regulating SEMA3A. (**A**) Effect of miR-379 inhibitor and si-SEMA3A on miR-379 levels in KGN detected by RT-qPCR. (**B**) Effect of miR-379 inhibitor and si-SEMA3A on SEMA3A levels in KGN detected by RT-qPCR. (**C**) CCK-8 assay was employed to examine the proliferation of KGN cells function of miR-375 and SEMA3A. ***$P < 0.001$, **$P < 0.01$.

Discussion

More than half of patients with PCOS are reported to have concomitant signs of obesity. The co-morbidity between obesity as a risk factor for the development of PCOS has long been observed in the clinical setting.[14] Moderate weight loss (5%) has been reported to significantly improve the reproductive, hyperandrogenic, and metabolic characteristics of PCOS.[15] The pathological changes of PCOS are complex, and the exact pathogenesis and etiology remain unclear, while genetic mutations and epigenetic alterations are among the main cause of PCOS pathogenesis. IR is reported to be the pathological basis of obese PCOS, where reduced insulin sensitivity leads to islet dysfunction and excessive estrogen secretion by the ovaries. Additionally, obesity affects the production of hormones and metabolites associated with follicular development, which in turn affects egg quality.[16] Therefore, obesity plays an integral role in the pathogenesis of PCOS. In the current study, we enrolled healthy control, non-obesity-PCOS, and obesity-PCOS. The results showed that androgen levels and glucose metabolism index levels were significantly higher in the patients with obesity-PCOS than in the non-obesity-PCOS and controls, and they showed more serious endocrine disorders and metabolic abnormalities. And this is consistent with the findings of Yang et al.[17]

Differentially expressed miRNAs are present in the serum, follicular fluid, GCs, and adipose tissue of PCOS patients.[18] miRNAs can be engaged in the development of PCOS by regulating insulin sensitivity, androgen synthesis, and follicular development.[19] Such as miR-182,[20] miR-664a-3p,[21] and miR-24.[22] MiR-379 is identified as an obesity-associated miRNA that regulates the development of obesity through multiple signaling pathways or metabolic modalities, thereby influencing the development of disease. Abdollahi et al found reduced obesity in miR-379 knock mice on a high-fat diet and that miR-379 regulates IR and obesity by suppressing adipogenesis in endoplasmic reticulum stress.[8] Additionally, miR-379 accelerates obesity-induced kidney injury through endoplasmic reticulum stress.[10] As an obesity-related metabolic syndrome, NAFLD can be typically ameliorated by the lack of miR-379 through the regulation of steatosis and hyperlipidemia.[11] Furthermore, miR-379 has been shown to be a key regulator of glucose metabolism[23] and lipid metabolism.[24]

More notable was the discovery of miR-379 in an investigation of dysregulated miRNAs associated with hyperandrogenemia in the follicular fluid of PCOS.[7] Therefore, we suspect that miR-379 may be implicated in the progression of PCOS and may be associated with obesity-PCOS. To test our speculation, we examined the expression of miR-379 in the subjects. The results confirmed that miR-379 levels were elevated in PCOS patients compared with healthy controls. Among those, miR-379 levels were higher in obesity-PCOS patients. Hyperandrogenism and hyperinsulinemia are the main pathological causes of PCSO, and they are associated with abnormal obesity.[25] In the current study, we found that high miR-379 expression was associated with larger BMI, higher androgenic, and greater IR in PCOS patients. Further exploration of the potential clinical value of mIR-379 in obesity-PCOS revealed that miR-379 is a potential risk factor for obesity PCOS along with BMI. miR-379 can also identify obesity-PCOS patients from healthy individuals or non-obesity-PCOS patients, demonstrating a high diagnostic significance. It is suggested that miR-379 is expected to provide favorable assistance in the early identification and early intervention management of obesity-PCOS. The significant association of miR-379 with obesity, however, whether it could differentiate obesity-PCOS patients and obesity non-PCOS patients has not been disclosed in the present study, which needs further investigation with expanding study subjects.

SEMA3A is a secreted protein in the semaphoring family that has been found to disrupt hormone secretion through its multiple molecular mechanisms. SEMA3A and its receptor mutations were greatly enriched in 982 severely obese patients.[26] SEMA3A was also found that estrogen-induced the expression of SEMA3A in osteoblasts.[27] Additionally, the targeted binding of miR-379 to SEMA3A was demonstrated in autoimmune uveitis.[28] The targeted binding of SEMA3A to miR-379 was identified in our study. And SEM3A3 was abnormally reduced in obesity-PCOS and was negatively correlated with miR-379 levels. Therefore, we speculate that miR-379 may be engaged in the progression of obesity-PCOS by regulating SEMA3A. KGN was reported to be closely associated with oocytes and its survival and proliferation may be the cause of PCOS.[29] To further confirmed our conjecture, we validated the commonly used cell line KGN[30] in an in vitro PCOS model construct. We found no significant effect on SEMA3A levels when miR-379 was suppressed, but significantly promoted SEMA3A levels, and this promotion was partially eliminated by silencing of SEMA3A,

suggesting that SEMA3A may be a downstream gene of miR-379. Inhibition of miR-379 significantly impaired KGN proliferation, but this impairment was partially restored by silencing SEMA3A.

All in all, current results indicate that miR-379 is typically elevated in obesity-PCOS and could function as a potential diagnostic biomarker for it. Mechanistically, miR-379 may weaken SEMA3A levels regulating PCOS progression.

Data Sharing Statement

The data used and analyzed can be obtained from the corresponding author under a reasonable request.

Ethics Approval and Consent to Participate

The experimental procedures were all in accordance with the guideline of the Ethics Committee of Beijing Tongren Hospital Affiliated to Capital Medical University and has approved by the Ethics Committee of Beijing Tongren Hospital Affiliated to Capital Medical University. This study complies with the Declaration of Helsinki. A signed written informed consent was obtained from each patient.

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Disclosure

The authors declare that they have no competing interests.

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