Original Paper

Role of Serum miRNAs in the Prediction of Ovarian Hyperstimulation Syndrome in Polycystic Ovarian Syndrome Patients

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Key Words

Abstract
Background: Polycystic ovarian syndrome (PCOS) causes a significantly increased risk of ovarian hyperstimulation syndrome (OHSS). Here, we focused on the altered expression of serum miRNAs and their predictive value for OHSS in PCOS patients. **Methods:** We used the TaqMan low density array followed by individual quantitative reverse transcription-polymerase chain reaction to identify and validate the expression of serum miRNAs in PCOS patients likely to develop severe OHSS. **Results:** The miR-16 and miR-223 expression levels were significantly reduced in the patients who were likely to develop severe OHSS than in the control subjects who were likely to develop mild or no OHSS. The sensitivity and specificity of the basal LH, basal LH/FSH, and body mass index (BMI) as OHSS predictors were also evaluated. miR-16 was the most efficient for OHSS prediction as it yielded the highest AUC. Logistic binary regression analyses revealed a positive association of miR-223 and BMI. **Conclusion:** Serum miRNAs are differentially expressed in PCOS patients likely to suffer from severe OHSS. We identified and validated two serum miRNAs that have potential for use as novel noninvasive biomarkers to accurately predict OHSS before controlled ovarian hyperstimulation (COH) for PCOS patients.

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Introduction

Ovarian hyperstimulation syndrome (OHSS) is an iatrogenic complication of controlled ovarian hyperstimulation (COH) used in assisted reproduction treatment (ART). Approximately 3–6% of patients develop OHSS. This condition is characterized by a variety of manifestations including ascites, pleural hemorrhage, hemoconcentration, and oliguria [1, 2]. The risk factors reported for the development of OHSS in patients undergoing in-vitro fertilization (IVF) include young age, the presence of PCOS, an explosive response to gonadotropin stimulation (*e.g.* rapid increase of serum estradiol [E_2] levels), and the development of multiple follicles (>20) before induction of ovulation [3, 4]. Therefore, of all women who undergo IVF, those with PCOS are characterized by a significantly enlarged cohort of early growing and recruitable follicles leading to a significantly increased risk of OHSS [5–7].

Although the predictive value of many predictors for OHSS are presented [8], the E_2 level and number of follicles are determined near the completion of COH and it is not particularly easy to accurately predict OHSS prior to COH for IVF cycles, using only age and body mass index (BMI). Furthermore, no specific biomarkers are currently available for predicting OHSS in women with PCOS.

miRNAs are a class of small non-coding RNAs that function as translational repressors and are involved in many important biological processes [9, 10]. A recent study found an abundance of miRNAs in human serum/plasma; these miRNAs have attracted considerable attention because they possess unique features (stability, easy detection, and disease specificity) [11]. Plasma/serum miRNAs have been verified to be an independent predictive system for different diseases,

Here, we hypothesized that serum miRNA could serve as a biomarker for predicting OHSS in women with PCOS before COH is initiated. To address this hypothesis, we systematically screened serum miRNAs by using Taqman low density array (TLDA) chips, followed by individual quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays. Individual qRT-PCR assays were also carried out to validate them in the large sample.

Materials and Methods

Study Design and Study Population

We designed a multistage retrospective nested case-control study to determine whether serum miRNA profiling could predict OHSS. Patients with PCOS undergoing IVF or IVF/intracytoplasmic sperm injection (ICSI) from February 2010 to July 2013 were recruited to this study. 384 patients diagnosed with PCOS were enrolled in this study. PCOS was defined by credentialed gynecologists according to the revised 2003 Rotterdam criteria, which requires the presence of at least two of the three following indicators: anovulation/oligoovulation, signs of clinical and/or biochemical hyperandrogenism, and polycystic ovaries on ultrasonography after exclusion of specific identifiable disorders (congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's syndrome, thyroid dysfunction, and hyperprolactinemia) [12, 13]. All the patients have been treated with Diane-35 for 3-6 months and the serum Testosterone of all the patients were all decreased to normal. Routine blood samples were obtained on day 3 of the stimulation cycle prior to gonadotropin administration, and the sera were isolated within 4 hours and stored at –70°C until analysis. This study was approved by the Institutional Review Board of Nanjing Medical University and written informed consents were obtained from each participant prior to the start of the study.

The patients participating in this study followed a long GnRH agonist protocol that began with daily subcutaneous injections of 0.1 mg leuprolide acetate (LA Lupron, Takeda Pharmaceutics, Stolberg, Germany) on Day 21 of the pre-stimulation cycle (long protocol). The GnRH agonist lupron was continued until the day of human chorionic gonadotropin (hCG) administration. The patients received subcutaneous injections of 150 IU of hCG/day (Gonal-F; Serono, Bari, Italy), for five days, after which the dose was adjusted to stimulate follicular development according to the ovarian response. The resultant ovarian response was monitored by transvaginal ultrasound and serum E2 levels. When two or more follicles reached a maximum diameter of 18 mm, 5000 IU of hCG was administered. Transvaginal oocyte retrieval was performed 34–36 h after hCG injection.

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Patients were divided into two groups based on the possible severity of OHSS: those who would suffer from severe OHSS were placed in the OHSS group, and those without or with only mild signs of OHSS were placed in the control group. The study was divided into two phases. In phase I (biomarker discovery), we randomly pooled the sera of ten women from the OHSS group and those of ten matched controls, and used the samples to identify the differential miRNA expression profile by TLDA chip assays between the two groups. By comparing the relative expression level of serum miRNAs, significantly up- or downregulated miRNAs were preliminarily selected for further analysis in the next phase. In phase II of this experiment, the initially screened miRNAs were verified by qRT-PCR assay of samples from 30 and 70 women from the OHSS and control groups, respectively. Depending on the results of this phase, receiver operating characteristic (ROC) curve-based risk assessment analysis was conducted to assess the sensitivity and specificity of serum miRNA for predicting OHSS.

Classification of OHSS

The criteria for OHSS classification defined by Navot et al. [14] were utilized to assess the relative severity of OHSS. Moderate OHSS was characterized by abdominal distension and discomfort, nausea, evidence of ascites, and an ovarian size of 8–12 cm on ultrasonography. Severe OHSS consisted of clinical ascites with or without pleural effusion, edema/anasarca, oliguria, serum creatinine of 1.0–1.5, creatinine clearance of 50 mL/min, liver dysfunction, 45% hematocrit, and white blood cell count of 15 000. Only patients with moderate or severe OHSS were considered as having OHSS and were placed in the OHSS group.

Serum preparation and RNA extraction

On day 3 of the stimulation cycle prior to gonadotropin administration, 5-mL venous blood samples were collected from each participant in procoagulant drying tubes. Whole blood was separated by centrifugation at 4,000 rpm for 10 min, followed by centrifugation at 12000 rpm for 15 min to completely remove cell debris.

The total RNA in the serum was quantified as described previously with some modifications [15]. Briefly, Trizol reagent (Invitrogen, Carlsbad, CA) was used to denature the serum, and the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA) was used for RNA collection and purification according to the manufacturer's protocol. After the initial denaturizing step, we routinely spiked in synthetic *C. elegans* miR-39 (cel-mir-39, 5'-UGACAGAAGAGAGUGAGCAC-3'; Takara, Japan) to a final concentration of 500 pmol/mL for all samples in order to control variations in RNA extraction and/or purification procedures [16]. Equal volumes of serum were processed for each sample.

TLDA chip assays and qRT-PCR

In the discovery stage, we carried out TLDA chip screening using the pooled serum samples of ten OHSS and ten control subjects. Then, we performed individual qRT-PCR assay on these samples to verify the number of differentially expressed miRNAs. Subsequently, a validation was conducted to confirm the results from the discovery stage. For validation, 30 cases and 70 controls were tested.

In the discovery stage, we used TLDA Chips (Applied Biosystems Inc, CA, USA) to screen differentially expressed miRNAs from the two pooling samples. A total of 960 µL of serum was used from each sample pool. Megaplex RT reactions and pre-amplification reactions were run according to the manufacture's protocol. Then, 75 µL of 0.1× TE was added to the PreAmp product, and 9 µL of diluted PreAmp product was used to run the RT-PCR reactions by dispensing 100 µL of the PCR reaction mix into each port of the TaqMan MicroRNA Array. The default PCR procedure was used and analyzed using RQ manager software (Applied Biosystems Inc). The ΔCT and ΔΔCT values were calculated using the following mathematical formula: $\Delta CT = CT_{sample} - CT_{U6}$, $\Delta\Delta CT = \Delta CT_{case} - \Delta CT_{control}$. Finally, the ΔΔCT value was normalized against the cel-miR-39.

Then, we used TaqMan microRNA probes (Applied Biosystems Inc.) to perform qRT-PCR assay according to the manufacturer's instructions [17]. An equal volume of sample was used for each step from serum purification to qRT-PCR. The total RNA was reverse-transcribed to cDNA by using a TaqMan microRNA RT kit and stem-loop RT primers (Applied Biosystems Inc.). RT-PCR was performed using the TaqMan PCR kit on an ABI 7900 real-time PCR system (Applied Biosystems). The reactions were initiated in a 384-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We assigned equal number of patients and controls in one plate and run the RT-PCR assay for target miRNAs and cel-miR-39 simultaneously. All reactions, including the no-template controls, were run in triplicate. The CT values were

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determined using fixed threshold settings. The relative expression levels of target miRNAs were normalized by the spike-in cel-miR-39 and determined by the equation $2^{-\Delta Ct}$, in which $\Delta CT = CT_{sample} - CT_{cel-39}$

Statistical analysis

All statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, USA). Inter-group differences in the demographic and clinical characteristics were estimated by the Student's *t* test. The Mann-Whitney *U* test was used to compare the relative expression level of the maternal serum miRNAs between the two groups. For each miRNA, an ROC curve was to evaluate its discriminating effects. The sensitivity and specificity of detection were assessed by the area under the ROC curve (AUC) and 95% confidence interval (CI). To improve the diagnostic rates of OHSS, we combined the expression levels of the two miRNAs using multiple logistic regression analysis to perform the above risk assessment. All P values less than 0.05 were considered statistically significant, and all the statistical tests were two-sided.

Results

Participant characteristics

The participant characteristics are summarized in Table 1. As shown, the cases and controls were well-matched on age, basal estradiol (E2), basal testosterone (T), basal FSH, fasting insulin, and duration of infertility. The values of basal LH, basal LH/FSH, estradiol on the hCG day, number of oocytes, and number of good embryos were significantly higher in the OHSS group than in the control group (P < 0.05), and the values of BMI, gonadotropin dose, and stimulation days of gonadotropin were significantly lower in the OHSS group than in the control group (P < 0.05).

Phase I: Biomarker discovery

The purpose of this study was to screen the potential of serum miRNAs as biomarkers for predicting OHSS in patients before undergoing COH. TLDA was used to identify the differentially expressed miRNAs in serum samples. We calculated the mean expression level and employed fold changes of the included miRNAs. On the basis of both scientific and applicable consideration, miRNAs in the OHSS group that were >twofold upregulated or <0.5-fold downregulated relative to the control group were selected as the candidates for the next stage of validation. Preliminary screening revealed that three miRNAs (miR-146a, miR-30c, and miR-191) were upregulated and five (miR-16, miR-223, miR-212, miR-451, and miR-92a) were downregulated. In phase II, the differential expression levels of these eight miRNAs were validated.

Table 1. Comparison of clinical features between the two groups

	OHSS (n=30)	Control (n=70)	*P* value
Age (years)	27.93±3.84	27.70±3.44	0.765
Body mass index (kg/m2)	21.87±2.93	24.39±3.67	**0.001**
Fasting Insulin	14.34±2.76	13.76±3.65	0.145
Infertility duration	3.48±2.37	3.96±2.32	0.237
Basal serum FSH concentrations (U/L)	6.07±1.53	6.48±1.43	0.201
Basal serum LH concentrations (U/L)	7.78±4.11	5.22±2.22	**0.003**
Basal LH/FSH	1.28±0.57	0.83±0.39	**0.000**
Basal serum E2 concentrations (U/L)	43.35±18.11	42.39±25.43	0.852
Basal serum T concentrations (ng/ml)	0.52±0.17	0.49±0.18	0.464
Dose of gonadotrophins (U)	1459.4 ±364.96	1638.2±453.25	**0.016**
No. of stimulation days	9.1±1.63	9.74±2.20	**0.044**
Oestradiol on day of HCG (pg/ml)	10373.1±2747.16	3250.4±964.12	**0.000**
Number of oocytes	18.10±2.62	8.23±2.72	**0.000**
Number of good embryos	11.3±8.43	4.33±2.59	**0.000**

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Table 2. Serum miRNAs during the validation stage. $^a\Delta CT = C_{Tsample} - C_{TCel-39}$; $^b\Delta\Delta C_T = \Delta C_{Tcase} - \Delta C_{Tcontro\backslash}$ from TLDA data; ^{c}Mann-Whitney U test

| miRNA		Control				OHSS				$\Delta\Delta CT$ ^{b}	P value ^{c}
	N	ΔCT ^{a}	mean	SD	Median	N	ΔCT ^{a}	mean	SD	Median		
miR-146a	70	10.44	3.60*10-5	5.60*10-5	3.41*10-5	30	9.09	9.18*10-5	7.62*10-5	8.89*10-5	-1.35	0.091
miR-16	70	4.49	2.23*10-3	3.06*10-3	2.44*10-3	30	8.14	1.77*10-4	1.95*10-3	1.74*10-4	3.65	0.000
miR-212	70	11.58	1.63*10-5	1.43*10-5	1.59*10-5	30	12.22	1.05*10-5	9.12*10-4	1.01*10-5	0.64	0.889
miR-223	70	4.05	3.02*10-3	7.07*10-3	3.84*10-3	30	7.13	3.58*10-4	5.06*10-3	3.09*10-4	3.08	0.012
miR-451	70	9.13	8.92*10-5	2.89*10-5	7.98*10-5	30	10.34	3.86*10-5	2.12*10-5	3.76*10-5	1.20	0.678
miR-30c	70	10.05	4.72*10-5	3.72*10-5	3.89*10-5	30	9.87	5.34*10-5	3.98*10-5	4.91*10-5	-0.18	0.784
miR-191	70	9.74	5.85*10-5	3.85*10-5	4.97*10-5	30	9.68	6.10*10-5	4.80*10-5	5.78*10-5	-0.06	0.882
miR-92a	70	6.31	6.30*10-4	4.85*10-4	5.79*10-4	30	7.31	3.15*10-4	5.85*10-4	9.85*10-3	1.00	0.589
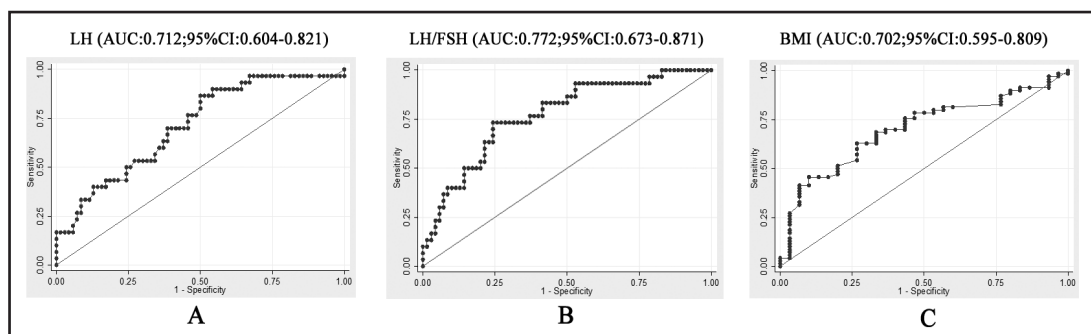
Fig. 1. ROC curve analysis using three clinical characteristics to distinguish between women with polycystic ovarian syndrome (PCOS) who will develop severe ovarian hyperstimulation syndrome (OHSS) and control patients with PCOS who will develop negligible or mild symptoms of OHSS. (A) LH, (B) LH/FSH, (C) BMI.

Phase II: Validation of differential miRNA expression levels

We carried out qRT-PCR on 30 and 70 samples from the OHSS and control groups, respectively, to validate the putative markers identified in the discovery phase. Using cel-miR-39 as the normalization control, our data demonstrated that two of the eight serum miRNAs (miR-16 and miR-223) were significantly downregulated in the women of the OHSS group than controls (P = 0.000 and P = 0.012, respectively). The circulating levels of the other six miRNAs did not differ between the two groups (Table 2).

Prediction value of clinical characteristics for OHSS

To evaluate the sensitivity and specificity of the basal LH, basal LH/FSH, and BMI for the prediction of OHSS, we established ROC curves and calculated the AUC in each case (Fig. 1A, 1B, and 1C). We assessed the discriminating the effect of basal LH, basal LH/FSH, and BMI at the cutoff values of 5.79, 0.852, and 23.51, respectively, at which the largest Youden's index (sensitivity + specificity – 1) was defined as the optimal diagnostic point. The sensitivity and specificity of the basal LH, basal LH/FSH, and BMI were 70.00% and 61.43%, 83.33% and 58.57%, 62.86% and 73.33%, respectively (data not shown).

Prediction value of serum miRNAs for OHSS

To evaluate the sensitivity and specificity of the serum miRNA signature individually and in combination for the prediction of OHSS, we further established ROC curves and calculated the AUC in each case (Fig. 2A and 2B), and the AUC of miR-16 was 0.848 which yielded the largest AUC in all the potential predictors. We assessed the effect of miR-16 and miR-223 at cutoff values of 0.0019 and 0.0020, respectively, at which the largest Youden's index was defined as the optional diagnostic point. The sensitivity and specificity of miR-16 and miR-223 were found to be 80.00% and 74.29%, and 86.67% and 67.14%, respectively. Multiple logistic regulation analysis of the two miRNAs revealed an AUC of 0.836, and the sensitivity and specificity increased to 83.33% and 67.14%, respectively (Fig. 2C). The results indicated that miR-16, which yielded the largest AUC, could adequately serve as a biomarker for the
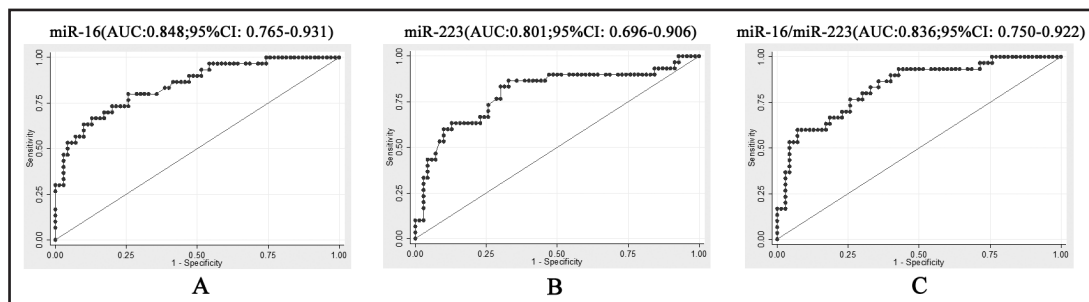
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Fig. 2. ROC curve analysis using two miRNAs to distinguish between women with PCOS who will develop severe OHSS and control patients with PCOS who will develop negligible or mild symptoms of OHSS. (A) miR-16, (B) miR-223, and (C) receiver operating characteristic (ROC) curve of the combination of the two miRNAs using multiple logistic regression analysis. The combination of the two miRNAs (miR-16 and miR-222) yielded the largest area under the ROC curve (AUC).

Table 3. Gene ontology (GO) biological process and molecular function annotations associated with the gene targets of differentially expressed miRNAs (P < 0.05). The key GO biological processes are described for each individual miRNA and include the number of gene targets represented in each classification, and the related BioCarta pathways of the predicted miRNA gene targets are shown

MicroRNA	No. of target gene	GO biological processes	No. of gene	*P* value	Biocarta Pathway	No. of genes	*P* value
hsa-miR-16	622	regulation of transcription, DNA-dependent	58	5.46E-46	Growth Hormone Signaling Pathway	5	1.43E-06
		transcription	51	6.24E-36	Regulation of ck1/cdk5 by type 1 glutamate receptors	4	1.77E-06
		protein amino acid phosphorylation	25	1.41E-24	Regulation of BAD phosphorylation	4	1.22E-05
		development	36	1.43E-21	VEGF, Hypoxia, and Angiogenesis	3	1.75E-04
		signal transduction	41	3.44E-20	AKT Signaling Pathway	4	1.22E-05
		protein transport	22	1.34E-19	mTOR Signaling Pathway	4	3.77E-06
hsa-miR-223	237	regulation of transcription, DNA-dependent	24	8.55E-21	Cyclin E Destruction Pathway	2	2.13E-04
		cell adhesion	12	3.86E-13	IFN alpha signaling pathway	2	3.63E-04

prediction of OHSS, but the combination of both miRNAs resulted in OHSS prediction with greater sensitivity.

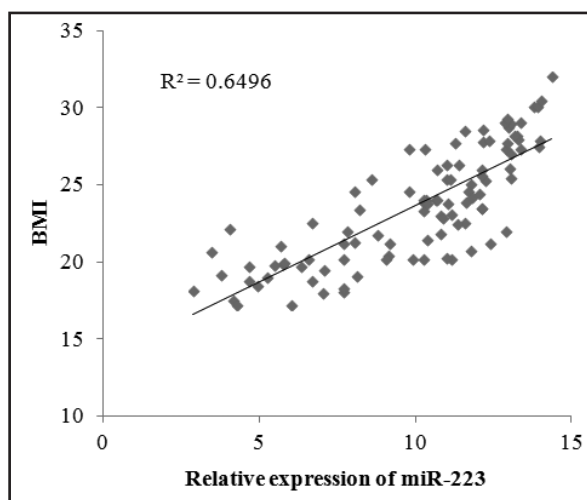*Functional classification of differentially expressed miRNAs*

Enrichment of biological processes in terms of Gene Ontology categories with respect to the target genes was determined using the DAVID Bioinformatics Resources. As shown in Table 3, the gene targets of miR-223 were involved in the many biological processes such as the insulin receptor signaling pathway, regulation of body size, and positive regulation of glucose import.

In order to obtain a list of pathways that are potentially controlled by the differentially expressed miRNAs, we used a web-based computational tool, DIANA-miRRath, to analyze some of the common signaling pathways. The computational tool estimates the impact of co-expressed miRNAs in biological pathways. As shown in Table 3, genes targeted by miR-16 were found to be involved in the vascular endothelial growth factor (VEGF), hypoxia, and angiogenesis pathways.

Correlation between miR-223 and BMI

Spearman's analysis was used to evaluate the association between miR-223 and BMI. The results revealed that the miR-223 level was strongly and positively associated with the BMI (R = 0.83; P = 0.000; Fig. 3).

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Fig. 3. Scatter plots of the serum miR-223 level vs. body mass index (BMI) in PCOS patients.

Discussion

It is well known that PCOS patients who undergo COH are at a high risk of OHSS. Thus far, however, there are no specific predictors to predict OHSS in PCOS patients. Several studies have reported that human serum/plasma can serve as a class of novel promising noninvasive biomarkers for diseases [18–20]. In our previous study, we used a two-stage experiment to investigate the role of serum miRNAs in predicting GDM in the early second trimester and found that the miRNA (mir-29a and mir-222 and miR-132) signatures showed aberrant expression prior to abnormalities in the serum glucose level [17].

To our knowledge, this is the first study comparing the expression patterns of serum miRNAs in PCOS women who have or will develop severe OHSS and in women with negligible or mild symptoms of the disease. Our aim was to identify potential candidate miRNAs that are differentially expressed in the two study samples to identify potential candidates that can serve as predictors for severe OHSS. Our findings indicate that the candidate serum miRNAs levels prior to gonadotropin administration could provide useful information to direct the application of mild, patient-friendly COH protocols to avoid moderate and severe OHSS in PCOS patients.

The traditional determinants for OHSS prior to gonadotropin stimulation for all IVF patients appear to include only subject age, lean habitus, and signs of PCOS (both hormonal and ultrasonographic characteristics). In this study, we found that the predictive values of lean habitus and subject age for OHSS were somewhat limited (the sensitivity of PCOS to predict OHSS). In addition, the ratio of the serum basal LH/FSH level (hormonal characteristic of PCOS) appeared to be a more efficient predictor of OHSS than subject age and BMI for PCOS patients since the ROC_{AUC} for it featured an area of 0.772. Furthermore, our research is the first study to reveal a serum miRNA signature for predicting OHSS prior to gonadotropin administration and demonstrate that the mir-16 and mir-223 signatures may serve as novel biomarkers for predicting OHSS in patients with PCOS; similar results were obtained in the ROC analysis. Our data demonstrate that miR-16 might be a more efficient predictor of OHSS than basal LH/FSH in PCOS patients since the ROC_{AUC} for miR-16 was 0.848.

OHSS is a self-limiting disorder with a broad spectrum of clinical manifestations related to increased capillary permeability and fluid retention mediated by many inflammatory mediators such as VEGF [21]. hCG treatment increases the production of VEGF in endothelial cells, and the growth factor in turn increases vascular permeability [22]. Recently, VEGF was predicted to be targeted by miR-16, and miR-16 had been verified to modulate angiogenesis by regulating the VEGF-A expression [23]. Taking into consideration the crucial role of VEGF in the increased endothelial/vessel permeability and development of OHSS [24–26] and our result which showed lower serum miR-16 levels in PCOS women who develop severe OHSS than in women who develop mild or no OHSS, miR-16 is implicated in the development of

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OHSS in women with PCOS and VEGF and angiogenesis pathways might be more susceptible in OHSS group than in the control group.

MiR-223 has been reported to be upregulated in the adipose tissue in PCOS, and it has been found to be overexpressed only in PCOS patients with insulin resistance (IR) but not in non-PCOS women with IR [27]. Therefore, miR-223 might be specifically associated with IR in PCOS women. Although we did not find any significant difference in the fasting insulin between the two groups in our study, the BMI was significantly lower in the OHSS group than in the control group. Our result revealing the positive association between miR-223 and BMI in PCOS women further verified the role of miR-223 in regulating the IR in PCOS.

Biological variability is a common challenge in any study using a limited sample size in such discovery-related array analyses because patient variability can simply lower or mask many important qualitative and quantitative differences. Hence, a combined group of several biomarkers would be more beneficial and reliable than a single miRNA as marker or indicator of the disease process and its onset. Therefore, here we propose to combine the miRNAs and clinical characteristics as potential biomarker candidates that can be useful in the prognosis/diagnosis of the development of severe OHSS and for monitoring IVF therapy.

Larger, multicenter joint collaborative studies on patient groups are necessary to confirm our findings. The positive outcomes of these studies can potentially improve the sensitivity and specificity of the predictors for OHSS, as well as provide a direction for novel therapeutic interventions.

Acknowledgments

Disclosure Statement

All authors have no conflicts of interest to declare.

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