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CIRCULATING MIR-132, MIR-146A, MIR-222, AND MIR-320 EXPRESSION IN DIFFERENTIAL DIAGNOSIS OF WOMEN WITH POLYCYSTIC OVARY SYNDROME

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Abstract

Purpose. The aim of the study was to investigate whether the circulating miR-132, miR-146a, miR-222, and miR-320 levels are used in the differential diagnosis of women with polycystic ovary syndrome (PCOS) and healthy women.

Methods. This prospective case-control study included 50 women with PCOS and age- and body mass index- matched 50 healthy controls. The hormone and lipid profiles, levels of microRNAs (miRNAs), and parameters of carbohydrate metabolism were measured.

Results. Expression levels of miRNAs were assessed using the two-step quantitative real-time polymerase chain reaction. Circulating miR-132, miR-146a and miR-222 levels were significantly downregulated in the PCOS group compared with the control group. The miR-320 levels did not differ between the two groups. Free testosterone was negatively correlated with miR-132, miR-146a and miR-222. Insulin was negatively correlated with miR-132 and miR-146a.

Conclusions. The results of the study revealed that miRNA expression, may suggest a possible distinction between healthy women and PCOS patients. miR-132, miR-146a, and miR-222 may have key functions in the pathogenesis of PCOS.

Keywords: Polycystic ovary syndrome, miR-132, miR-146a, miR-222.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder, affecting 5–10 % of women in reproductive age (1). Patients with PCOS demonstrate a broad spectrum of clinical manifestations and increased risk for insulin resistance, diabetes, metabolic syndrome, dyslipidemia, and cardiovascular disease (2). Genetic predisposition, environmental and epigenetic factors are known to contribute to the pathogenesis of PCOS. However, the molecular mechanisms underlying pathogenesis of PCOS have not been fully elucidated.

MicroRNAs (miRNAs) are small regulatory RNA molecules that modulate posttranscriptional gene regulation by binding to specific messenger RNA targets. These molecules play important roles in several physiological processes including cell-cycle, differentiation, apoptosis, metabolism, and development (3, 4). Alterations in expression of miRNAs may be related with numerous diseases, including cancer, endometriosis, and diabetes (5). Therefore, it has been speculated that for various diseases miRNAs may be used as biomarkers for the diagnosis or specific targets for the gene therapy.

Studies have shown a difference in the expression of some miRNAs between PCOS subjects and healthy controls (5-7). Emerging evidence has suggested that miRNAs may be involved in the occurrence of PCOS and may be used as diagnostic marker for PCOS. Insulin resistance and hyperandrogenism appear to be the major players in the etiology of PCOS. Recent studies pointed out that miR-146a and miR-320 participated in chronic inflammation and insulin sensitivity (8-10).

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miR-222 have been implicated in insulin signaling and glucose metabolism (11). In addition, miR-320 has been reported to account for hyperandrogenemia through suppression of estrogen production (12). A previous report demonstrated that miR-132 and miR-146 suppress the release of progesterone, estradiol and testosterone (13). Moreover, miR-132 and miR-320 have been revealed to be the targets of genes associated with the etiology of PCOS (14). In light of these considerations, it may be hypothesized that there may be a relationship between PCOS and miR-132, miR-146a, miR-222, and miR-320.

Understanding the association between miRNAs and PCOS may provide new insights to the pathogenesis, diagnosis and treatment of PCOS. Therefore, the present study was undertaken to investigate whether circulating miR-132, miR-146a, miR-222, and miR-320 are differentially expressed in women with PCOS.

MATERIALS AND METHODS

Ethical approval

The ethical approval obtained from the Ethics Committee of Istanbul University Cerrahpasa Medicine School (No: 83045809/604.01/02-6805, Date: January, 07, 2016). The study was carried out in the Department of Obstetrics and Gynecology of Istanbul Education and Research Hospital. The written informed consent was taken from all participants.

Patient selection and study design

This prospective case-control study included 50 consecutive subjects with PCOS and age- and body mass index (BMI)- matched 50 healthy controls, who were admitted to the gynecology department between January 2016 and June 2017. The women who met at least two of the following criteria: oligomenorrhea; clinical and/or biochemical hyperandrogenism; and polycystic ovaries after exclusion of other etiologies (e.g., Cushing's syndrome, androgen-secreting tumors, and congenital adrenal hyperplasia) were diagnosed as PCOS according to the revised Rotterdam criteria (15). Control subjects had no evidence of chronic anovulation or hyperandrogenism. The exclusion criteria for all subjects included women who had undergone treatment with drugs known to interfere with sex hormone secretion and metabolism (e.g. antiandrogens, oral contraceptives, ovulation induction agents, and antidiabetics) during previous 6 months, hypothyroidism, hyperprolactinemia, hypertension, pregnancy, and age less than 18 or more than 35 years. Oligomenorrhea was defined as <8 cycles per year. Polycystic ovaries were defined as the presence of ≥12 antral follicles in each ovary measuring 2–9 mm in diameter, and/or an ovarian volume >10 mL in ≥1 ovary. Clinical hyperandrogenism was defined as Ferriman-Gallwey score of >8 (16). Biochemical hyperandrogenism was defined as elevated serum free testosterone levels. Ultrasound examinations were performed with a 4-9-MHz transvaginal (or transrectal for virgin women) transducer (Voluson E6 General Electric, Milwaukee, WI, USA).

Anthropometric and biochemical measurements, demographic characteristics, and ultrasound examination of participants were recorded. The blood samples were obtained from patients at early morning, after an overnight fast. Total testosterone, sex hormone binding globulin (SHBG), free testosterone, androstenedione, dehydroepiandrosterone sulphate (DHEA-S), 17-hydroxyprogesterone (17-OHP), follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), anti-mullerian hormone (AMH), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL) and triglyceride (TG), fasting glucose (FG), and insulin levels were measured on the Olympus AU 800 analyzer by commercial kits. The body weight and height of participants were measured and BMI was calculated as kg/m^2. The degree of insulin resistance was examined by using homeostatic model assessment insulin resistance (HOMA-IR) according to the formula: HOMA-IR = [FG (mg/dL) × insulin (μIU/mL)] / 405. Insulin resistance was considered HOMA-IR >2.5 (17). During routine blood sampling, 5 cc of blood was taken from patients for microRNA analysis. The collected blood was allowed to clot at room temperature and then centrifuged. The serum was stored at -80° C until assayed.

miRNA analysis

Total RNA was extracted from serum samples using mirVana RNA Isolation Kit (miRNeasy kit (Qiagen, CA)). All isolation protocols were conducted according to the manufacturers' instructions, without further modifications. The concentrations and purities of RNA were estimated using NanoDrop spectrophotometer, and values of 1.7–2.0 were considered indicative of relatively pure RNA. To estimate the expression miRNA levels a two-step RT-PCR assay was used. Firstly total RNA samples were transcribed into cDNA using the miScript Reverse Transcription kit (Qiagen, Valencia, CA),

then cDNAs amplified and the expression of miR-132, miR-146a, miR-222 and miR-320 analyzed with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using specific TaqMan® Small RNA Assays (Applied Biosystems, Carlsbad, CA). RNU43 (TaqMan® Small RNA Controls from Applied Biosystems) was used as a small RNA endogenous control. The relative expression levels of miR-132, miR-146a, miR-222 and miR-320 were calculated using the 2−ΔΔCT method. Each sample was tested in triplicate.

Statistical analysis

All data were analyzed using SPSS software (SPSS Inc, Chicago, IL) for Windows version 22.0. The results are expressed as means ± SD unless otherwise stated. Distribution of the variables was measured by Kolmogrov-Smirnov test. Independent quantitative values were assessed using the independent t-test and Mann-Whitney U test. Qualitative values were analyzed by chi-square test or Fischer test. Spearman's correlation test was used for correlation analysis. $p < 0.05$ was considered statistically significant.

RESULTS

The anthropometric measurements, demographic characteristics, and biochemical values of the groups are presented in Table 1. Age and BMI were similar among the groups. AMH, insulin, HOMA-IR, free testosterone, DHEA-S, and androstenedione levels were significantly higher in the PCOS group than in the control group. FG, FSH, LH, E2, total testosterone, 17-OHP, TSH, prolactin, TG, HDL, LDL and TC levels did not differ among the groups.

The results of miRNA analysis of the groups are shown in Table 2. The levels of miR-132 were significantly decreased in the PCOS group compared with the control group. The ratio of miR-132 down-regulated subjects was significantly higher in PCOS group than in the control group ($P < 0.001$). The PCOS group had significantly lower levels of miR-146a and miR-222 compared with the controls ($P = 0.005$, $P = 0.013$, respectively). The ratio of miR-146 and miR-222 down-regulated subjects was higher in women with PCOS with respect to healthy controls. However, the differences between two groups were not significant. miR-320 levels and the ratio of miR-320 were not

Table 1. Demographic characteristics, clinical and biochemical parameters in PCOS and control groups

	CONTROL GROUP		PCOS GROUP		
Variable	**Mean±SD**	**Median**	**Mean±SD**	**Median**	***p***
Age (year)	23.9 ± 4.1	23.5	22.7 ± 3.5	22.0	0.101[m]
Height (m)	1.6 ± 0.1	1.6	1.6 ± 0.1	1.6	0.189[m]
Weight (kg)	64.6 ± 11.2	64.5	66.9 ± 13.4	64.0	0.622[m]
BMI (kg/m²)	24.3 ± 4.7	23.8	25.5 ± 5.2	23.9	0.306[m]
AMH (ng/mL)	5.2 ± 3.1	4.7	9.6 ± 5.0	9.5	0.000 [m]
FBG (mg/dL)	90.2 ± 7.1	91.0	89.1 ± 8.2	88.5	0.469[t]
Insulin (µIU/mL)	7.5 ± 4.8	6.5	9.9 ± 5.3	8.6	0.012[m]
HOMA-IR	1.7 ± 1.2	1.5	2.2 ± 1.3	1.9	0.030[m]
FSH (mIU/mL)	6.7 ± 2.1	7.0	6.3 ± 1.9	6.2	0.221[m]
LH (mIU/mL)	8.4 ± 5.7	6.6	10.4 ± 6.9	8.5	0.100[m]
E2 (pg/mL)	83.6 ± 91.9	47.0	50.4 ± 33.7	44.0	0.121[m]
Free Testosterone (pg/mL)	1.6 ± 0.6	1.5	2.5 ± 1.4	2.3	0.000[m]
Total Testosterone (nmol/L)	56.4 ± 21.2	56.6	63.8 ± 23.0	61.8	0.148[m]
SHBG (nmol/L)	87.5 ± 73.1	81.3	49.6 ± 24.8	46.0	0.000[m]
DHEA-S (mcg/dL)	241.2 ± 99.1	228.5	281.6 ± 101.6	264.9	0.047[t]
17-OHP (ng/mL)	0.9 ± 0.8	0.6	0.8 ± 0.6	0.6	0.975[m]
Androstenedione (ng/mL)	1.4 ± 0.6	1.3	1.8 ± 0.6	1.6	0.002[m]
TSH (µIU/mL)	2.3 ± 1.0	2.2	2.0 ± 0.8	1.9	0.273[m]
PRL (ng/mL)	17.0 ± 7.9	15.4	15.0 ± 5.8	14.0	0.309[m]
TG (mg/dL)	88.1 ± 51.3	79.0	88.3 ± 36.1	78.0	0.452[m]
HDL (mg/dL)	58.5 ± 11.5	57.0	54.2 ± 10.1	51.0	0.067[m]
LDL (mg/dL)	106.6 ± 28.1	105.9	107.7 ± 28.4	104.6	0.823[m]
Total Cholesterol (mg/dL)	182.7 ± 32.4	186.5	177.9 ± 33.4	169.0	0.406[m]

BMI: Body Mass Index; AMH: Anti-Müllerian Hormone; FBG: Fasting Blood Glucose; HOMA-IR: Homeostatic Model Assessment Insulin Resistance; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; E2: Estradiol; SHBG: Sex-Hormone Binding Globulin; DHEA-S: Dehydroepiandrosterone Sulphate; 17-OHP: 17-Hydroxy Progesterone; TSH: Thyroid Stimulating Hormone; PRL: Prolactin; TG: Triglyceride; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein. [m]Mann-Whitney U test / [t] t test.

Table 2. microRNA levels in PCOS and control groups

	CONTROL GROUP		PCOS GROUP		
	Mean±SD	**Median**	**Mean±SD**	**Median**	**p**
miR-132	3.1 ± 6.1	1.5	1.0 ± 2.1	0.4	**0.000**[m]
miR-146a	3.9 ± 10.1	1.8	1.9 ± 4.3	0.6	**0.005**[m]
miR-222	3.0 ± 4.5	1.2	1.8 ± 3.5	0.4	**0.013**[m]
miR-320	5.5 ± 13.2	1.0	5.2 ± 9.3	2.6	0.689[m]
	n	**%**	**n**	**%**	**p**
miR-132					
Downregulated	21	42.0%	33	66.0%	
Normal	1	2.0%	8	16.0%	**0.000**[X²]
Upregulated	28	56.0%	9	18.0%	
miR-146a					
Downregulated	21	42.0%	28	56.0%	
Normal	2	4.0%	2	4.0%	0.360[X²]
Upregulated	27	54.0%	20	40.0%	
miR-222					
Downregulated	20	40.0%	27	54.0%	
Normal	2	4.0%	2	4.0%	0.360[X²]
Upregulated	28	56.0%	21	42.0%	
miR-320					
Downregulated	22	44.0%	19	38.0%	
Normal	4	8.0%	1	2.0%	0.261[X²]
Upregulated	24	48.0%	30	60.0%	

[m]Mann-Whitney U Test/ [X²]Chi-Square Test.

Table 3. Correlation between demographic characteristics, clinical and biochemical parameters with serum microRNA levels by Spearman Correlation

	miR-132		**miR-146a**		**miR-222**		**miR-320**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (Year)	0.077	0.443	0.047	0.645	0.079	0.436	-0.056	0.582
BMI (kg/m²)	-0.165	0.101	-0.128	0.203	-0.188	0.060	-0.174	0.083
AMH (ng/mL)	-0.179	0.075	-0.136	0.178	-0.158	0.117	0.025	0.802
FBG (mg/dL)	0.114	0.259	0.069	0.497	0.049	0.626	-0.082	0.418
Insulin (µIU/mL)	**-0.241**	**0.016**	**-0.201**	**0.045**	-0.174	0.083	-0.184	0.066
HOMA-IR	**-0.202**	**0.043**	-0.181	0.072	-0.152	0.131	-0.179	0.075
FSH (mIU/mL)	0.155	0.124	0.165	0.101	0.159	0.114	0.181	0.072
LH (mIU/mL)	-0.094	0.350	0.092	0.363	-0.044	0.664	0.072	0.478
E2 (pg/mL)	-0.001	0.992	-0.036	0.722	-0.001	0.996	-0.131	0.193
Free Testosterone (pg/mL)	**-0.295**	**0.003**	**-0.253**	**0.011**	**-0.290**	**0.003**	-0.116	0.250
Total Testosterone (nmol/L)	-0.073	0.469	-0.054	0.596	-0.084	0.404	-0.022	0.827
SHBG (nmol/L)	**0.244**	**0.014**	0.181	0.072	**0.220**	**0.028**	0.187	0.062
DHEA-S (mcg/dL)	-0.143	0.157	-0.109	0.280	-0.125	0.216	-0.114	0.259
17-OHP (ng/mL)	-0.053	0.600	-0.103	0.309	-0.072	0.474	-0.155	0.125
Androstenedione (ng/mL)	-0.132	0.191	-0.148	0.141	-0.140	0.164	0.008	0.938
TSH (µIU/mL)	-0.030	0.765	0.084	0.407	0.081	0.423	-0.057	0.571
PRL (mIU/mL)	0.010	0.924	-0.163	0.106	-0.030	0.766	-0.141	0.163
TG (mg/dL)	-0.063	0.532	-0.057	0.574	-0.043	0.673	-0.112	0.265
HDL (mg/dL)	0.166	0.098	0.079	0.437	0.092	0.365	0.018	0.858
LDL (mg/dL)	0.012	0.902	0.019	0.851	-0.007	0.945	0.044	0.663
Total Cholesterol (mg/dL)	0.072	0.477	0.041	0.684	0.037	0.714	0.028	0.778

BMI: Body Mass Index; AMH: Anti-Müllerian Hormone; FBG: Fasting Blood Glucose; HOMA-IR: Homeostatic Model Assessment Insulin Resistance; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; E2: Estradiol; SHBG: Sex-Hormone Binding Globulin; DHEA-S: Dehydroepiandrosterone Sulphate; 17-OHP: 17-Hydroxy Progesterone; TSH: Thyroid Stimulating Hormone; PRL: Prolactin; TG: Triglyceride; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein.

different between the two groups.

Free testosterone showed a significant negative correlation with all measured miRNAs, except miR-320 (Table 3). SHBG was positively correlated with miR-132 and miR-222 levels. Insulin was negatively correlated with miR-132 and miR-146a levels ($r = -0.241$, $p = 0.016$; $r = -0.201$, $p = 0.045$, respectively). HOMA-IR was negatively correlated with miR-132 ($r = -0.202$, $p = 0.043$). There was no significant correlation between assessed miRNAs and AMH.

DISCUSSION

The results of the present study demonstrated that circulating miRNAs may have ability to distinguish between PCOS patients and controls. We identified three (miR-132, miR-146a, and miR-222) circulating miRNAs with increased levels in PCOS patients compared with control subjects independent of age and BMI. In addition, clinical variables related to PCOS, such as insulin, free testosterone, and HOMA-IR were correlated with identified miRNAs suggesting their involvement in the pathophysiology of PCOS.

miRNAs are short non-coding RNAs that act as an epigenetic regulator of gene expression. They existed in cellular microenvironment and also found in extracellular fluids such as serum, plasma, urine, semen, and follicular fluid. Studies have declared that miRNAs may serve as promising potential biomarkers for metabolic disorders, cardiovascular disorders, and several cancers as they are resistant to nucleases, abundant in serum, and stable over freeze-thaw cycles (8, 18-20). Besides, alterations in expression of miRNAs have shown in serum, adipose tissue, theca cells, granulosa cells, and follicular fluid of women with PCOS (5). In recent studies, their effects on pathophysiology of PCOS have been investigated and important relationships were determined (21,22). However, only few studies have been conducted on circulating miRNA expression in women with PCOS.

miR-132 expression has been revealed to be significantly decreased in the follicular fluid of PCOS patients (6). Moreover, High Mobility Group AT-Hook 2 (HMGA2) which has been reported as PCOS-related gene is the target of miR-132 (14). Similarly, we observed lower expression levels of miR-132 in PCOS subjects. It is reasonable to suggest that miR-132 might have participated in the pathophysiology of PCOS via affecting HMGA2 gene expression. Bioinformatic analysis indicated that targets of miR146a and miR-222 are involved in metastasis, cell cycle, apoptosis and endocrine pathways. Evidence also demonstrates that miR-146a expression increased in PCOS patients (7). Moreover, increased expression of miR-146a has been indicated in a PCOS mouse model (23). On the other hand, a recent report showed lower miR146a expression levels in follicular fluid of PCOS subjects (24). Another study proposed that miR-146a gene variation could increase the risk of PCOS through increased expression of miR-146a (25). Contrarily, we determined lower miR-146a levels in the PCOS group. The controversy of results might be secondary to differences in ethnicity and heterogeneity of the syndrome. Estrogen receptor 1 (ESR1) and PTEN genes which are the targets of miR-222 regulate insulin signaling and glucose metabolism as well as steroidogenesis, respectively (14). It has been observed that serum levels of miR-222 were higher in PCOS patients. The authors also suggested that miR-222 with miR-146a may be used as novel non-invasive biomarkers in PCOS (7). Conversely, decreased miR-222 expression was found in the current study. This could attributed presence of the heterogeneity within group samples.

Consistent with our findings, previous paper concluded that serum levels of miR-320 were similar between PCOS patients and controls (7). Another paper indicated that expression levels of miR-132 and mir-320 were lower in follicular fluid of PCOS patients compared with controls, however miR-320 was observed to be up-regulated in a research using PCOS follicular fluid and granulosa cells (6, 12). The different results could arise from the sources of miRNA and differences in the ethnicity of subjects with PCOS. The target gene network analysis has been revealed that Ras-related protein Rab-5B (RAB5B) gene which has been identified as PCOS-related gene, is predicted to be the target of miR-320 (6, 14). A recent report observed that the single nucleotide polymorphisms (SNPs) of the rs11550558, rs705700, and rs11171718 loci of the RAB5B gene which may be miR-320 binding sites are associated with the PCOS risk. The authors determined that plasma miRNA-320 levels were significantly lower in PCOS patients than that in the control group. Moreover, they declared that the rs11550558 SNP was associated with PCOS risk only in subjects with age ≥31.1 years and rs11550558, rs1045435, and rs11171718 SNPs were significantly associated with PCOS risk only in women with BMI ≥23.8 kg/m^2 (26). Inconsistent results with our study may be explained the differences in distribution of the patients in terms of age and BMI of the two study populations.

Ovarian steroid production is critical for the normal follicular development and ovulation. Abnormalities in ovarian steroidogenesis and local steroid-mediated signaling lead to PCOS through abnormal follicle growth, excess ovarian androgen production, and anovulation. Transfections with miR-132, miR-222 and miR-320 mimics regulated estradiol concentration in the steroidogenic human granulosa-like tumor cell line (6). Also, miR-132 and miR-146 were shown to inhibit progesterone, estradiol and testosterone secretion (13). A negative correlation between miR-146a and testosterone was demonstrated in PCOS (7). Likewise, miR-132 and miR-146a were found to be negatively correlated with free testosterone in the present study. Therefore, pathologic consequences of intra-ovarian actions could be modulated by miR-132 and miR-146 *via* regulating ovarian steroidogenesis. We also observed a correlation between miR-222 and miR-320 and testosterone. Although the mechanism of miRNA effect on the control of steroidogenesis remains unknown, these findings may represent one of the possible directions for future investigations.

Hyperandrogenism and insulin resistance are two key physiopathological characteristics that underpin PCOS. Moreover, a recent study showed that insulin may affect LH, SHBG, and testosterone levels in PCOS (27). Another study indicated that low adiponectin levels may play a role in the development of insulin resistance in PCOS patients (28). It has been revealed that increased miR-222 expression is associated with type 2 diabetes and gestational diabetes mellitus (GDM) (29). miR-320 has been found to be decreased in the diabetic population and played a key role in regulating insulin resistance (29, 30). Furthermore, increased miR-222 expression was demonstrated to be positively associated with insulin in PCOS (7). However, we did not find a significant correlation between miR-320, miR-222 and insulin levels. This discrepancy may be explained by the wide spectrum of clinical appearances of PCOS. miR146a participated in insulin resistance in type 2 diabetic patients (8). The current report showed that miR-146a was negatively correlated with insulin and miR-132 was negatively correlated with both insulin and HOMA-IR. Based on the obtained results, it should be speculated that there might be a strict association with miR-132 and PCOS, so that miR-132 draws attention as a potential research area to elucidate PCOS etiology.

The altered miRNA expression in women with PCOS *versus* controls may suggest a possible discrimination between healthy women and PCOS patients. However, the exact mechanism of changes in circulating miRNAs and whether altered miRNA expression profile is the cause or consequence of PCOS is still a large concern.

In conclusion, miR-132, miR-146a, and miR-222 may have key functions in the pathogenesis of PCOS. Further studies are required to examine whether these miRNAs constitute a valuable tool for diagnosis and treatment of PCOS.

Conflict of interest

The authors declare that they have no conflicts of interest.

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