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Original Article

Association of miR-146a rs2910164 and miR-222 rs2858060 polymorphisms with the risk of polycystic ovary syndrome in Iranian women: A case–control study

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ABSTRACT

Objective: Today, many single nucleotide polymorphisms in microRNA genes are known to alter the microRNA expression levels or processing causing susceptibility of several human diseases. The present study aimed to investigate the association of microRNA-146a (rs2910164) and microRNA-222 (rs2858060) polymorphisms with susceptibility to polycystic ovary syndrome (PCOS) in an Iranian population.

Materials and methods: This case–control study was performed on 205 patients with PCOS and 205 normal women as the control group. After DNA extraction, Tetra-amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) was used to detect the polymorphisms. The association between genotypes and the risk of PCOS was examined by odds ratios (OR) and 95% of confidence intervals (CIs).

Results: Our results showed that there are significant differences in CG genotype frequencies between case and control groups regarding miR-146a rs2910164 polymorphism (OR = 2.03, CI = 1.3–3, $P = 0.001$). In a dominant model for the C allele, CC + CG genotypes were associated with PCOS risk (OR = 2, 95% CI = 1.3–2.9, $P = 0.001$) and the C allele increased the risk of PCOS (OR = 1.6, 95% CI = 1.1–2.1, $P = 0.004$). Furthermore, a positive association was observed between miR-222 CG genotype and the risk of PCOS (OR = 2.2, 95% CI = 1.1–4.1, $P = 0.02$). These results were evident after adjustment for age and body mass index.

Conclusion: The present results suggest that the miR-146a rs2910164 and miR-222 rs2858060 polymorphisms are associated with an increased risk of PCOS. Therefore, both polymorphisms could play an important role as a genetic risk factor for development of PCOS in the Iranian population.

Introduction

The polycystic ovary syndrome (PCOS) is a complex and heterogeneous endocrine disorder characterized by chronic anovulation, polycystic ovary (PCO) and hyperandrogenism [1,2]. The reported prevalence of PCOS ranges between 2.2% and 26% in various countries [3], depending on the criteria used for its definition and the method used to define each criterion [4]. Despite the high prevalence, a definitive endocrine marker for PCOS is not fully understood [5]; But Compelling evidence has suggested that genetic risk factors can play an important role in the pathogenesis of this syndrome [6].

Different studies have suggested that expression altering in many genes leads to the genetic abnormality in PCOS [7–9]. In recent years, microRNAs (miRNAs) have emerged as master regulators of gene expression in mammals [10]. The small noncoding RNA molecules serve as post-transcriptional suppressors of gene expression, and are involved in many cellular processes such as proliferation, differentiation, apoptosis and metabolism [11]; so, may play an important role in the control of reproductive endocrine functions especially the process of folliculogenesis and oocyte

Abbreviations: PCOS, polycystic ovary syndrome; T-ARMS-PCR, Tetra-amplification refractory mutation system polymerase chain reaction; MAPK, mitogen-activated protein kinase; JAK-STAT, Janus kinase/signal transducers and activators of transcription; SNP, single nucleotide polymorphism; BMI, body mass index.

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maturation [12]. The alternation in miRNA regulation networks can cause disruptions of the normal cellular activity leading to increased risk of diseases [13]. Evidence suggests that miRNA expression is altered in PCOS patients in comparison with healthy women and as such may be considered as biomarkers of diagnosis in this syndrome [14]. Resent study showed that the expression levels of miR-146a and miR-222 were significantly increased in PCOS patients with respect to the healthy women [5]. Also, miR-146a has been reported to be differentially expressed in ovary tissues of PCOS [13]. Finally, Bioinformatics analyses indicated that the genes targeted by miR-146a and miR-222 were found to be involved in apoptosis, cell cycle and endocrine pathways, such as MAPK, Wnt and Jak-STAT signaling pathways. These data indicated that miR-222 and miR-146a may be involved in the pathogenesis of PCOS [5].

Some polymorphisms in pre-miRNAs, flanking regions or target sites have been demonstrated to affect miRNA function and related to certain physiological processes or related with diseases [15]. Identification of these polymorphisms can help to elucidate disease pathogenesis, and this knowledge can be used to improve prognosis for women with a particular disorder, such as PCOS [16]. So, our study was aimed to find out the impact of microRNA-146a (rs2910164) and microRNA-222 (rs2858060) polymorphisms on susceptibility to PCOS in a sample of the Iranian population.

Materials and methods

Study population

A detailed description of the study population has been reported in our previous study [17]. This case–control study was conducted on 205 healthy women (controls) and 205 PCOS patients (cases) who were recruited from the Shiraz fertility center, Iran. The case and control women were matched for age (±5).

The diagnosis of PCOS was based on the criteria proposed by the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group [18], and control group consisted of women without any related endocrine disorders, except those having simple overweight/obesity.

Written informed consents were obtained from the case and control women before collecting blood samples. Ethical approval of this study was obtained from the Research Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Genotyping

Genomic DNA was extracted by salting-out method as described previously [19]. Genotype determination was done by a tetra amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) for rs2858060 and rs2910164 polymorphisms. Tetra-primer ARMS-PCR is a procedure originally developed for the analysis of single nucleotide polymorphisms (SNP), which employs two primer pairs to amplify the two different alleles of an SNP in a single PCR reaction [20]. The primer sequences designed for T-ARMS-PCR is shown in Table 1.

The PCR reaction mixture contained 50–100 ng DNA, 0.5 μL dNTP 10 mM, 0.75 μL $MgCl_2$ 50 mM for rs2910164 and 1 μL $MgCl_2$ 50 mM for rs2858060, 1 μL of FO and RO (10 pm/μL) and 1.5 μL of FI and RI primers (15 pm/μL) for rs2910164; 1.5 μL of each FO and RO (15 pm/μL) and 1 μL of each FI and RI (10 pm/μL) primers for rs2858060, 0.3 U Taq DNA polymerase 5 U/μL (Cinnagen, Iran) in a 25 μL mixture.

For PCR amplification, an initial denaturing cycle at 95 °C for 5 min, followed by 30 amplification cycles (denaturing at 95 °C for 30 s, annealing 30 s at 57 °C for rs2910164, 30 s at 54 °C for rs2858060 and extension at 72 °C for 30 s) and a final extension at 72 °C for 10 min was performed. The PCR products were verified on 2% agarose gel containing safe stain (Fig. 1). To confirm the genotyping results, one product from each genotype was examined by DNA sequencing.

Table 1
The primers used for T-ARMS-PCR.

Polymorphism	Primers	Sequence (5′–3′)
miR-222 rs2858060	FI (G allele)	TGTATTATCCTCAGTTCGTAAGAG
	RI (C allele)	GTAAATTGCAGTTAAAAAATTCTTACG
	FO	TATCGAAAATAGCATTCCTCTTAAC
	RO	AAATCTCTATACTTCTACAGCATACA
miR-146a rs2910164	FI (G allele)	GTTGTGTCAGTGTCAGACGTC
	RI (C allele)	CCAGCTGAAGAACTGAATTTGAC
	FO	TCTACCATACACATCCCCTACA
	RO	CACACTCCTTATACCTTCAGAGC

Statistical analysis

Data processing and statistical analysis were performed by using the SPSS version 22. Statistical analysis of allele and genotype frequencies between PCOS patients and controls was processed using the Chi-square test. The association between genotypes and PCOS was assessed by computing the odds ratio (OR) and 95% confidence intervals (CI) by logistic regression analysis. A P value < 0.05 was considered statistically significant.

Results

Totally 410 Iranian women, including 205 PCOS cases with a mean age of 31.2 ± 5.5 years and 205 controls with a mean age of 28.5 ± 5 years were examined in this study. A significant difference was observed between the groups with respect to mean age ($P < 0.001$). Table 2 shows the anthropometric parameters of the women with PCOS and controls. Our findings showed significant differences in weight ($P = 0.004$) and BMI ($P = 0.007$) between case and control groups.

The genotype and allele frequencies of miR-146a rs2910164 polymorphism were shown in Table 3. The genotype frequencies of the control subjects did not show significant deviation from Hardy–Weinberg equilibrium ($\chi^2 = 0.19$, df = 2, $P = 0.66$). There was a significant difference in the distribution of CG genotype between PCOS patients and controls (OR = 2.03, 95% CI = 1.3–3, $P = 0.001$). Also, the C allele frequency in PCOS patients was significantly higher than control group (OR = 1.6, 95% CI = 1.1–2.1, $P = 0.004$). Moreover, in the dominant effect of the C allele (comparison between CC + CG vs. GG), CC + CG genotypes significantly increased the risk of PCOS (OR = 2, 95% CI = 1.3–2.9, $P = 0.001$). After adjustment for age and BMI, genotypes in three co-dominants, dominant and recessive model did not show any remarkable change in ORs. Table 4 shows miR-222 rs2858060 genotype and allele frequency in case and control groups. The genotypes in the control group were in Hardy–Weinberg equilibrium ($\chi^2 = 1.3$, df = 2, $P = 0.24$). A significant difference was observed in CG genotype frequency between PCOS patients and control group (OR = 2.1, 95% CI = 1.1–4.1, $P = 0.02$). The associations remained unaltered after adjusting miR-222 genotypes for BMI and age.

Discussion

MicroRNAs constitute the most abundant class of small RNAs in the ovary [21]. They bind to the 3′ untranslated region of the mRNA,

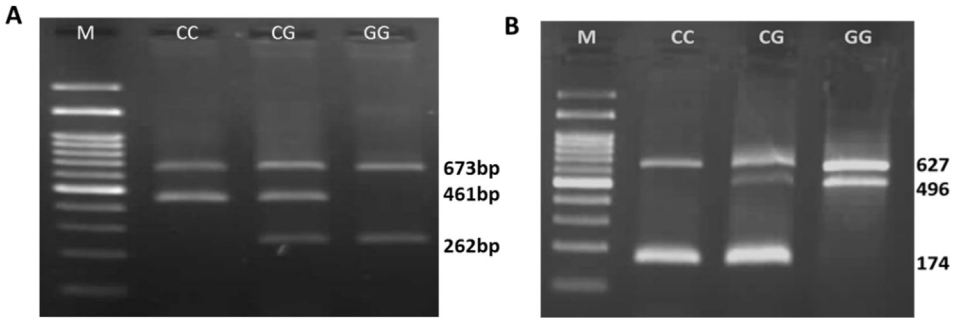

Fig. 1. Eectrophoresis pattern of T-ARMS-PCR products for detection of miR-222 rs2858060 C/G polymorphisms (A): the product sizes were 262 bp for G allele, 461 bp for C allele, and 673 bp for control band. miRNA-146a rs2910164 G/C (B): the product sizes were 496 bp for G allele, 174 bp for C allele, and 627 bp for control band. M: 100 bp DNA ladder.

Table 2
Anthropometric characteristics in PCOS patients and control group.

	PCOS (mean ± SD)	Control (mean ± SD)	*P*
Age (years)	31.2 ± 5.5	28.5 ± 5	<**0.001**
Height (cm)	161.7 ± 6.7	161.7 ± 7.3	**0.98**
Weight (kg)	69.6 ± 14.5	65.9 ± 11	**0.004**
BMI (kg/m^2)	26.5 ± 5	25.1 ± 4.6	**0.007**

SD, standard deviation, BMI, body mass index.
Independent sample *t*-test was used to analyze the difference between case and control, *P*-value < 0.05 is considered as statistically significant.

with either leading to its cleavage or inhibiting the translational machinery [22]. A number of microRNAs have been reported to be differently expressed in the ovary tissues of PCOS patients, such as miR-146a, miR-132, miR-22, miR-141, miR-200c, and miR-21 [13]. Recently, Long et al. have found that serum miR-146a and miR-222 are differentially expressed between PCOS and healthy subjects and could act novel non-invasive biomarkers for diagnosis of PCOS [5]. miR-146a was reported to suppress the release of progesterone, androgens, and estrogens. It was hypothesized that miR-146a act as

Table 3
Frequency distribution of miR-146a genotypes in PCOS patients and controls.

rs2910164 polymorphism	PCOS (%)	Control (%)	[a]OR (95% CI)	*P*	[b]OR (95% CI)	**P*
Co-dominant						
GG	78 (38)	113 (55.1)	1		1	
CG	112 (54.6)	80 (39)	2.03 (1.3–3)	0.001	2.05 (1.3–3.2)	0.003
CC	15 (7.3)	12 (5.9)	1.8 (0.8–4)	0.15	1.5 (0.58–3.8)	0.41
Dominant						
GG	78 (38)	113 (55.1)	1		1	
CC + CG	127 (62)	92 (44.1)	2 (1.3–2.9)	0.001	1.97 (1.2–3.1)	0.004
Recessive						
GG + CG	190 (92.6)	193 (94.1)	1		1	
CC	15 (7.3)	12 (5.9)	1.27 (0.6–2.9)	0.55	1.03 (0.4–2.6)	0.9
Allele						
G	268 (65)	306 (75)	1			
C	142 (35)	104 (25)	1.6 (1.1–2.1)	0.004		

[*P-value < 0.05 is considered as statistically significant.]
CI, confidence interval; OR, odds ratio.
[a] Logistic regression analysis.
[b] Adjusted for age and BMI.

Table 4
Frequency distribution of miR-222 genotypes in PCOS patients and controls.

rs2858060 polymorphism	PCOS (%)	Control (%)	[a]OR (95% CI)	*P*	[b]OR (95% CI)	**P*
Co-dominant						
GG	17 (8.3)	30 (14.6)	1		1	
CG	134 (65.4)	107 (53.2)	2.1 (1.1–4.1)	0.02	2.2 (1–4.5)	0.04
CC	54 (26.3)	68 (33.2)	1.3 (0.67–2.7)	0.4	1.3 (0.6–2.9)	0.47
Dominant						
GG	17 (8.3)	30 (14.6)	1		1	
GG + CG	188 (91.7)	175 (85.4)	1.8 (0.97–3.4)	0.06	1.8 (0.9–3.7)	0.09
Recessive						
GG + CG	151 (73.7)	137 (67.8)	1		1	
CC	54 (26.3)	68 (33.2)	0.7 (0.5–1.1)	0.12	0.7 (0.43–1.1)	0.14
Allele						
G	168 (41)	167 (41)	1			
C	242 (59)	243 (59)	0.99 (0.7–1.3)	0.94		

[*P-value < 0.05 is considered as statistically significant.]
CI, confidence interval; OR, odds ratio.
[a] Logistic regression analysis.
[b] Adjusted for age and BMI.

physiological suppressors of general secretory activity [23]. miR-222 has been shown a marked increase in Type 2 Diabetes and its potential relevance in insulin sensitivity [24]. Furthermore, bioinformatics analysis indicated that the predicted targets function of the two miRNAs mainly involved in the metastasis, cell cycle, apoptosis and endocrine [5]. These reports indicated that miR-146a and miR-222 may serve as novel biomarkers for diagnosis of PCOS. In this study, we showed for the first time a positive association of miR-146a rs2910164 and miR-222 rs2858060 polymorphisms with the risk of PCOS. Due to significant differences in age and BMI between the patient and control groups, genotypes were adjusted for age and BMI and the ORs did not increase.

The miR-146a rs2910164 can result in a change from a G:U pair to a C:U mismatch in the stem structure of the miR-146a precursor and a reduced production of mature miR-146a [25]. Up to now, miR-146a rs2910164 polymorphism has been mainly studied for its association with a several diseases; Positive association of this variant was identified with gastric cancer [26], malignant melanoma [27], oral squamous cell carcinoma [28], head and neck cancer [29], and autoimmune diseases [30]. In confirmation of the impact of miR-146a rs2910164 polymorphism in the pathogenesis of diseases, the current study showed that there is a significant association between rs2910164 polymorphism and the risk of PCOS.

We observed an increased risk of PCOS for the subjects with the miR-146a CG genotype. Consistent with our results, other studies have succeeded to show this association with some other diseases. For example; Yamashita et al. (2013) showed that individuals with the CG genotype have a twofold increase in susceptibility to malignant melanoma [27]. A further functional study on rs2910164 G/C polymorphism showed that GC heterozygotes differed from both GG and CC homozygotes by producing mature microRNAs that modulated genes mainly involved in the regulation of apoptosis, leading to an exaggerated DNA-damage response in the heterozygotes [31]. Moreover, in our study, the C allele was significantly increased in the patients compared with controls. Jazdzewski et al. showed that the C allele of G/C polymorphism within the pre-miR-146a sequence reduced the production of mature miR-146a compared with the G allele [32]. It confirmed that the rs2910164 polymorphism could functionally affect the miR-146a expression levels.

Furthermore, our statistical analysis revealed a significant association between the miR-222 rs2858060 polymorphism and the risk of PCOS. The rs2858060 was genotyped because bioinformatics analysis suggested that it included a putative transcription factor binding site [33]. Our results showed that CG genotype significantly increases the risk of PCOS. To date, there is no study regarding the effect of the rs2858060 polymorphism in pathogenesis of human diseases; therefore, the present study was the first research to assess the association of miR-222 rs2858060 polymorphism and PCOS.

In conclusion, our study showed for the first time that miR-146a rs2910164 and miR-222 rs2858060 polymorphisms play a role in the development of PCOS in the Iranian population. Larger studies with different ethnicities are needed to validate our results.

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Conflict of interest statement

No competing financial interests exist.

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