

Single nucleotide polymorphisms in binding site of miRNA-135a and targeted gene IRS2 are correlated with multiple clinical features of PCOS: A study in Chinese women

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Abstract.

BACKGROUND: The etiology of polycystic ovary syndrome (PCOS) remains unclear with highly heterogeneous clinical manifestations, recently growing evidence revealing genetic variants play a crucial part in its pathogenesis.

OBJECTIVE: This study aimed to examine the correlation between SNPs in miRNA-135a's binding site of targeted gene IRS2 and clinical manifestations of PCOS in Chinese females.

METHOD: A total of 126 Chinese women with PCOS and 109 healthy women were enrolled, divided into 4 groups based on different clinical features of hyperandrogenemia (HA), insulin resistance (IR), polycystic ovary morphology (PCOM) and obesity. We analyzed 2 single nucleotide polymorphisms (SNPs) of the IRS2 gene (rs2289046 and rs1865434) and clinical features' laboratory measurements such as sex hormone, fasting plasma glucose (FPG), fasting plasma insulin (FINS).

RESULTS: Located in miRNA-135a binding site of IRS2 gene, the rs2289046's triple genotypes distribution showed a significant difference between PCOS/control group and PCOM/non-PCOM group ($P < 0.05$) while the rs1865434's triple genotype distribution showed a significant difference between obesity/non-obesity group ($P < 0.05$).

CONCLUSION: The results revealed the two SNPs as rs2289046 and rs1865434 in the IRS-2 binding region of miRNA-135a have correlations with the clinical features of PCOS in Chinese population.

Keywords: Polycystic ovary syndrome (PCOS), single nucleotide polymorphism (SNP), insulin receptor substrate 2 gene (IRS2 gene), microRNA (miRNA), China

1. Introduction

Polycystic ovary syndrome (PCOS) is a common female endocrine and metabolic disorder that af-

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fects 5–10% women in childbearing age [1] and is characterized by menstrual irregularities, chronic anovulation, obesity, polycystic ovary morphology (PCOM), hyperandrogenemia, insulin resistance (IR) or hyperinsulinemia (HA) [2–5]. PCOS is considered to be a polygenic feature under the influence of environmental factors, which may be ascribed with the interaction of protective and susceptible genomic variants [6]. Although the etiology of PCOS remains unknown, growing evidences showed the point of genetic defects played a crucial role in PCOS pathogenesis.

Insulin resistance (IR) is reported as one of the main pathophysiological characteristics of reproductive and metabolic disorders in PCOS [7]. The IRS-2 gene is located on chromosome 13q34, encoding the insulin receptor substrate protein 2 (IRS-2), which is a cytoplasmic signaling molecule composed of 1354 amino acids. As a molecular adaptor, IRS-2 regulates peripheral glucose metabolism and pancreatic islet β -cell function by mediating the effects of insulin, insulin-like growth factor 1 (IGF-1) action and other cytokines, for having an effect on the phenotype of PCOS [8]. Its key medium is insulin receptor substrate protein, which plays an indispensable role in insulin signal transduction, both as a substrate for insulin receptor tyrosine kinase and as an intermediate for multiple biological regulations of insulin [8]. The principle mechanism of insulin resistance in PCOS is the post-binding defect of insulin signal caused by the decrease of tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) and the increase of serine phosphorylation, affect the classical insulin target and ovarian metabolic pathway. In addition, other factors for the development of IR in women with PCOS are considered to be activation of extracellular signal division/regulation of kinase components in activation of protein kinase pathway, and gene destruction of insulin signal in central nervous system [9].

Single nucleotide polymorphisms (SNPs) in the miRNA-binding sites of target gene are correlated with susceptibility to multiple diseases as multiple miRNA expression profiles are altered in pathophysiology of PCOS [10]. Studies showed that the expression of miR-135a was up-regulated in PCOS's follicular fluid, while the potential target gene IRS-2 was down-regulated [10,11]; miR-135a down-regulates the expression of the IRS2 product by binding to the 3'UTR site to play an crucial role in the mechanism of IR. In this study, we explored the relevance of two SNPs rs2289046 and rs1865434 in miRNA-135a and its target gene IRS-2 binding region with the clinical features of PCOS.

2. Materials and methods

2.1. Study subjects

Study participants were recruited from the Guangdong Provincial Family Planning Specialist Hospital from June 2014 to December 2016. This study was approved by the Medical Ethics Committee of the Guangdong Provincial Family Planning Specialist Hospital. All participants obtained the informed consent before taken part in this study.

All participants were assigned to the case group and the control group. A total of 126 patients with a diagnosis of PCOS were enrolled in the study. Diagnosis of PCOS was based on the “2003 ESHRE/ASRM guidelines” if more than two of the following criteria were fulfilled: oligo-/anovulation (AO), polycystic ovaries on ultrasonography (USG), and clinical and/or biochemical signs of hyperandrogenism [12]. Disorders with similar clinical features, e.g., congenital adrenal hyperplasia, hypercortisolism, or androgen-secreting tumors, were not considered. Subjects who had received drugs which have known to interfere with hormonal levels within 3 months were also excluded. The control group consisted of 109 healthy women between 20–35 years old with a regular menstrual cycle range of 21–35 days and at least one successful pregnancy history, while no family history of diabetes, endocrine disorders and sonographic signs of PCOS were shown.

Table 1
Target gene-specific primer pairs for the IRS-2 gene

SNPs	Nucleotide change	Primers	Product size (bp)
rs2289046	G > A	Forward: TACCTGCGATGTTTACGTCCAC Reverse: TATTCATCCCCTTCCCAAAGC	198
rs1865434	A > G	Forward: ACTCCAGAGATTGCTCTGTTC Reverse: ACACAGTCATTGCTCAGATCC	191

2.2. Parameters analyzed

Hormone level and metabolic examinations follicle-stimulating hormone (FSH), luteinizing hormone (LH), the LH/FSH ratio, estradiol (E2), prolactin (PRL) and total testosterone were measured by chemiluminescence immunoassay. Before ovarian stimulation, all participants were given a 75 g oral glucose tolerance test and measured the insulin levels. Plasma glucose and insulin were measured by the glucose oxidase method.

In addition, the general information of patients were also collected, i.e., age, body height (m), weight (kg), waist circumference, body mass index (BMI) calculated as weight (kg)/body height² (m²) (obesity is diagnosed when BMI \geq 25 kg/m²) [13,14], menstruation history, mean antral follicular count, baseline prolactin levels and the HOMA index which was calculated as insulin (μ U/mL) \times glucose (mmol/L)/22.5 (HOMA \geq 2.69 is defined as insulin resistance) [14].

2.3. SNP genotyping

Peripheral blood (2 ml) was collected from all participants. Genomic DNA was isolated by using a TIANamp Genomic DNA kit (TIANGEN Biotech (Beijing) Co., Ltd.). The primer design synthesis and SNP typing were performed with the assistance of Shanghai Jereh Bioengineering Co. The multiple single-base extension (SNapShot) method was used to analyze the polymorphisms of the SNPs. Two SNP sites for the IRS-2 gene, rs2289046 and rs1865434 were analyzed. Total volume of the amplification reaction was set for 15 μ l, containing 1 μ l genomic DNA, 1.5 μ l 10xbuffer, 1.5 μ l MgCl₂ (25 mmol), 0.3 μ l dNTP (10 mmol), 0.15 μ l primer (10 μ mol), 0.3 μ l Taq enzyme (5 u/ μ l), and DNase-free sterile-filtered water. The sequence-specific primers were used to perform Polymerase chain reaction (PCR) amplification (Table 1). The thermal cycle program consisted of an initial denaturation at 94°C for 3 min, followed by amplification step of 35 cycles of 15 s at 94°C and 15 s at 55°C; final step consisted of 3 min at 72°C.

2.4. Statistical analysis

The database was created using EpiData 3.0 software. Analyses were performed using SPSS Statistics version 22.0. The SNPs were analyzed for deviation from Hardy-Weinberg equilibrium (HWE). We calculated the genotype and allelic frequencies of target SNPs. The χ^2 test was performed to calculate the genotype distribution in case and control group respectively to evaluate the accordance with Hardy-Weinberg equilibrium (HWE). The odds ratios (ORs) between different genotypes among different characteristic-dependent groups and 95% confidence intervals (95% CI) were used with 2 \times 2 comparisons. Student's *T* test and multiple regression analysis were also performed to evaluate the statistical significance of odds ratios and resolve interference among different genotypes.

Table 2
Demographic, clinical and hormonal characteristic of the study population

Parameters	Controls ($n = 109$)	PCOS ($n = 126$)	t	P values
Age (years)	30.97 ± 5.45	28.61 ± 4.11	3.77	< 0.01
Weight (kg)	54.73 ± 7.05	59.34 ± 9.96	-4.13	0.01
BMI (kg/m^2)	19.18 ± 6.22	23.50 ± 3.58	-6.39	0.06
FSH (mIU/ml)	4.70 ± 1.02	5.15 ± 1.65	-2.53	< 0.01
LH (mIU/ml)	3.83 ± 1.42	7.88 ± 4.61	-9.36	< 0.01
LH/FSH	0.85 ± 0.36	1.74 ± 1.94	-5.06	< 0.01
E2 (pmmol/L)	144.95 ± 47.40	167.39 ± 270.89	-0.91	0.36
PRL (mIU/L)	328.18 ± 114.29	339.54 ± 116.03	-0.76	0.45
T (nmol/L)	0.95 ± 0.50	2.38 ± 1.41	-10.60	< 0.01
HOMA_IR	1.60 ± 0.55	4.27 ± 2.37	-12.27	< 0.01

3. Results

3.1. Basic features of the participants in this research

Clinical, hormonal and demographic features of women with PCOS and controls are summarized in Table 2. The demographic features of cases and controls were comparable, showing a statistically significant difference ($p < 0.05$). Testosterone, FSH and LH concentrations in serum among women with PCOS were significantly higher than controls: FSH 5.15 versus 4.70 mIU/ml; LH 7.88 versus 3.83 mIU/ml; T 2.38 versus 0.95 nmol/L ($p < 0.05$). The levels of HOMA-IR and LH/FSH were significantly higher in PCOS patients than in control subjects ($P < 0.05$). E2) and PRL showed no difference with prominence between case and control groups.

3.2. Genotype frequencies of IRS-2 gene

The distribution of genotypes at the genetic loci IRS-2 rs2289046 and rs1865434 are in accordance with HWE in both control and PCOS samples ($p > 0.05$). Genotype counts and frequencies in PCOS and control groups are shown in Table 3. In the genotype comparison, the rs2289046 genotypes (AG and AA) showed significantly lower frequencies in the PCOS versus the control (61.9% vs. 70.6%, $p = 0.012$, OR = 0.349; 15.1% vs. 20.2%, $p = 0.020$, OR = 0.298). Furthermore, homozygous mutant allele (GG) was not detected in controls in IRS-2 gene rs1865434 polymorphism, potentially due to the little sample size of participants. Based on the Chi-square test, the rs1865434 GG genotype showed significantly higher frequency in the PCOS compared to the control (4% vs. 0%, $p = 0.000$, OR = 1.066), but no differences with prominence were found in the genotype frequency of the rs1865434 AG between same comparison groups.

3.3. Genotype frequencies distribution of IRS-2 gene in accordance with clinical manifestations of PCOS

Based on different phenotypes and features of PCOS, a total of 235 females were divided into several subgroups: PCO morphology (PCOM) and non-PCOM on pelvic ultrasound; HA and non-HA; IR and non-IR; obesity and non-obesity on BMI.

3.3.1. Polycystic ovaries were diagnosed according to the Rotterdam criteria with presenting follicles diameter and ovarian volume in sonography. A total of 235 subjects were divided into PCOM group and

Table 3
Genotypes distribution in PCOS ($n = 126$) and control ($n = 109$) subjects

SNP	Genotype	PCOS	Control	χ^2	P	OR (95% CI)
rs2289046	G/A					1 ^a
	GG	29 (23)	10 (9.2)			
	AG	78 (61.9)	77 (70.6)	6.339	0.012	0.349 (0.159–0.766)
rs1865434	AA	19 (15.1)	22 (20.2)	5.422	0.02	0.298 (0.116–0.766)
	A/G					1 ^a
	AA	76 (60.3)	64 (58.7)			
	AG	45 (35.7)	45 (41.3)	0.404	0.417	0.842 (0.495–1.431)
	GG	5 (4)	0	4.092	0	1.066 (1.008–1.127)

OR: odds ratio; CI: confidence intervals; ^areference genotype.

Table 4
Genotype frequencies of rs2289046 and rs1865434 polymorphisms in PCOM ($n = 107$) and non-PCOM ($n = 128$) subjects

SNP	Genotype	Non-PCOM	PCOM	χ^2	P	OR (95% CI)
rs2289046	G/A					1 ^a
	GG	11 (8.6)	28 (26.2)			
	AG	92 (71.9)	63 (58.9)	10.922	0.001	0.269 (0.125–0.580)
	AA	25 (19.5)	16 (14.9)	7.399	0.007	0.251 (0.098–0.642)
rs1865434	A/G					1 ^a
	AA	72 (56.3)	68 (63.5)			
	AG	56 (43.7)	34 (31.8)	8.391	0.004	0.643 (0.375–1.103)
	GG		5 (4.7)	5.108	0.024	1.074 (1.009–1.142)

OR: odds ratio; CI: confidence intervals; ^areference genotype.

non-PCOM group. The two SNPs (rs2289046 and rs1865434) genotype distributions of IRS-2 gene in the PCOM and non-PCOM groups are shown in Table 4. In the comparison of genotype distributions, the rs2289046 genotypes (AG and AA) and rs1865434AG genotype showed significantly lower frequencies in the PCOM compared to the non-PCOM group (58.9% vs. 71.9%, $p = 0.001$, OR = 0.269; 14.9% vs. 19.5%, $p = 0.007$, OR = 0.252; 31.8% vs. 43.7%, $p = 0.004$, OR = 0.643); whereas rs1865434GG genotype showed significantly higher frequency in the PCOM versus the non-PCOM group (4.7% vs. 0%, $p = 0.024$, OR = 1.074).

3.3.2. All 235 subjects were divided into the HA group ($T > 1.97$ nmol/L) and the non-HA group ($T \leq 1.97$ nmol/L) according to blood testosterone (T) concentration. Genotype counts and frequencies in HA and non-HA groups are as shown in Table 5. We compared the rs2289046 genotypes frequencies and the rs1865434 genotypes frequencies, and no differences with prominence were found between same comparison groups.

3.3.3. A total of 235 subjects were divided into IR ($HOMA > 2.69$) and non-IR ($HOMA \leq 2.69$) groups based on HOMA-IR values [15]. Genotype counts and frequencies in IR and non-IR groups are as shown in Table 6. In the IR group, the rs2289046 AA genotype showed significantly lower frequency compared to the non-IR group (11% vs. 21.5%, $p = 0.042$, OR = 0.340), while no significant differences between the two groups of the rs2289046 AG genotype frequency. Meanwhile, no differences with prominence were found between different rs1865434 genotypes in all participants.

3.3.4. According to BMI, all 235 subjects were divided into the obesity ($BMI > 25$) and non-obesity ($BMI \leq 25$) groups. In the genotype comparison as shown in Table 7, no significant differences between

Table 5
Genotype frequencies of rs2289046 and rs1865434 polymorphisms in the HA ($n = 156$) and non-HA ($n = 79$) groups

SNP	Genotype	Non-HA	HA	χ^2	P	OR (95% CI)
rs2289046	G/A					1 ^a
	GG	29 (18.6)	12 (15.2)	2.306	0.129	0.539 (0.264–1.102)
	AG	106 (67.9)	49 (62)			
rs1865434	AA	21 (13.5)	18 (22.8)	1.764	0.184	0.483 (0.192–1.213)
	A/G					1 ^a
	AA	90 (57.7)	50 (63.3)	0.803	0.067	0.771 (0.437–1.362)
	AG	63 (40.4)	27 (34.2)			
GG	3 (1.9)	2 (2.5)	0.039	0.738	1.200 (0.194–7.423)	

OR: odds ratio; CI: confidence intervals; ^areference genotype.

Table 6
Genotype frequencies of rs2289046 and rs1865434 polymorphisms in the IR ($n = 91$) and non-IR ($n = 144$) groups

SNP	Genotype	Non-IR	IR	χ^2	P	OR (95% CI)
rs2289046	G/A					1 ^a
	GG	20 (13.9)	19 (20.9)	0.974	0.324	0.702 (0.347–1.421)
	AG	93 (64.6)	62 (68.1)			
	AA	31 (21.5)	10 (11)	4.120	0.042	0.340 (0.131–0.878)
rs1865434	A/G					1 ^a
	AA	85 (59)	55 (60.4)	0.324	0.246	0.853 (0.492–1.476)
	AG	58 (40.3)	32 (35.2)			
	GG	1 (1)	4 (4.4)	3.316	0.063	6.182 (0.673–56.770)

OR: odds ratio; CI: confidence intervals; ^areference genotype.

Table 7
Genotype frequencies of rs2289046 and rs1865434 polymorphisms in the obesity ($n = 47$) and non-obesity ($n = 188$) groups

SNP	Genotype	Non-obesity	Obesity	χ^2	P	OR (95% CI)
rs2289046	G/A					1 ^a
	GG	32 (17)	7 (14.9)	0.394	0.53	1.333 (0.542–3.281)
	AG	120 (63.8)	35 (74.5)			
	AA	36 (19.2)	5 (10.6)	0.519	0.471	0.635 (0.183–2.200)
rs1865434	A/G					1 ^a
	AA	110 (58.5)	30 (63.8)	1.221	0.024	0.675 (0.336–1.358)
	AG	76 (40.4)	14 (29.8)			
	GG	2 (1.1)	3 (6.4)	4.086	0.044	5.500 (0.879–34.430)

OR: odds ratio; CI: confidence intervals; ^areference genotype.

the two groups of rs2289046 genotypes frequencies while rs1865434 AG genotype showed significantly lower frequency in subjects with obesity compared to the non-obesity group (29.8% vs. 40.4%, $p = 0.024$, OR = 0.675), and rs1865434 GG genotype showed significantly higher frequency in subjects with obesity versus the non-obesity group (6.4% vs. 1.1%, $p = 0.044$, OR = 5.500).

4. Discussion

PCOS is considered with highly heterogeneous clinical manifestations and unclear etiology syndrome of ovarian dysfunction [16]. Hyperandrogenism (HA) and polycystic ovary morphology (PCOM) are

known as cardinal features [17], while other clinical manifestations like menstrual irregularities, insulin resistance (IR), and obesity, appear additionally. A meta-analysis showed that Asian and American women with PCOS presented a higher prevalence of T2DM with 4.4- and 4.7- fold increasing versus to those women without PCOS [7]. Insulin resistance occurs in PCOS patients with a variable range of 40–70% [18,19]. Chen et al. showed that dysregulated glucose metabolism coexists among 23.7% of the Chinese patients with PCOS [20]. Li et al. indicated impaired β -cell function with common finding IR in PCOS women and regular glucose tolerance with BMI > 25.545 kg/m² [21]. Besides, a meta-analysis revealed that IRS-1 Gly972Arg polymorphism (rs1801278) was correlated with PCOS in the Caucasian ethnicity, while IRS-2 Gly1057Asp polymorphism (rs1805097) was associated with PCOS in the Asian ethnicity, leading to the conclusion of the controversial-remained parts of these polymorphisms in the pathogenesis of PCOS [22]. Whereas, polymorphisms in the IRS-2 gene may contribute to developing type 2 diabetes and other metabolic traits, such as polycystic ovary syndrome and obesity [23].

In this survey, we detected the two SNPs in IRS-2 gene SNP among women with PCOS and performed a parallel with the genotype frequencies with healthy women without PCOS. The rs2289046 genotype and the rs186534 genotype showed a significant association with PCOS and other laboratory measurements suggested that the IRS-2 gene polymorphism was correlated with the evolvement of PCOS and/or metabolic disorders; however, the genotypes were not associated with HA. The rs2289046 genotype (AA and AG) displayed a negative correlation with the development of PCOS and PCOM; meanwhile, the rs2289046 AA genotype was negatively associated with IR; and the rs1865434AG genotype was negatively associated with PCOM and obesity, make a consequence to a strong protective effect. However, the rs1865434 GG genotype showed a positive correlation to PCOS, PCOM and obesity and was a strong risk factor with a higher odds ratio. This series of findings indicated that the rs2289046 and rs186534 genotypes were associated with the development and clinical manifestations of PCOS, except for HA. Among the genotypes being analyzed, rs2289046 genotype (AA and AG) and rs1865434 genotype (AG and GG) showed significant associations with laboratory measurements, including FSH, LH, LH/FSH and HOMA-IR. Different genetic variants may contribute to the differences in serum T concentrations between the PCOS and controls.

Insulin resistance (IR) is a collaborative metabolic character of both obesity and PCOS. It was widely documented that women with PCOS, independent of obesity, are insulin resisted and have compensatory hyperinsulinemia [24]. Obesity sensitizes thecal cells to LH stimulation and amplifies functional ovarian hyperandrogenism by upregulating ovarian androgen production [25]. Furthermore, insulin might enhance androgenicity through the increased levels of free (biologically available) testosterone accordingly by suppressing production of sex hormone-binding globulin (SHBG) within the liver [26]. These factors lead to disturbances in follicle development and maturation, formatting small follicles and inducing follicular atresia, resulting in clinical manifestations of PCOS (menstrual irregularity or amenorrhea, polycystic ovarian enlargement, obesity, infertility).

Polycystic ovaries are the morphological ovarian phenotype in women with the PCOS. Diamanti et al. reported the prevalence rates of PCOM in the order of 60–80% and a positive relationship was found between the androgens levels, insulin resistance index, and PCOM [24]. Carmina et al. reported a positive correlation between insulin resistance indices and ovarian volume in 326 women with PCOS [27]. In multiple cell types, IRS-2 is involved in preserving insulin action whereas reduction in IRS expression and/or function may cause development of β -cell failure, diabetes, obesity from insulin resistance [28]. Pablo et al. conducted a case-control study including 452 metabolic syndrome subjects and observed that genetic variants in IRS-2 gene were associated with variables insulin response to different fatty acids and influence glucose metabolism [29]. Our results showed that the rs2289046 genotype (AA and AG) and

the rs1865434 AG genotype displayed a negative association with the development of PCOS and PCOM, which indicated IRS-2 SNPs may be associated with PCOM characteristics. The mechanisms underlying the observed correlation for this polymorphism were required to be clearly clarified. We hypothesized that the rs2289046 polymorphism might decrease the stability of the binding region of the IRS2 gene mRNA to miR-1306-5p, increase the stability of the binding region to miR-323b-3p and miR-532-3p, and generate the binding site with miR-582-5p. The rs2289046 (G > A) polymorphism might result in loss of the binding site to miR-29a-5p, possibly promoting PCOM formation.

In women genetically predisposed to the development of PCOS, weight-gain and obesity often make consequence to clinical and biochemical characters. Obesity is associated with PCOS in between 38% or 88% of cases [30]. A case-control association study showed that IRS-2 SNP (rs1865434) was significantly higher in 273 Caucasian with PCOS paralleled to 173 controls ($P = 0.032$, OR = 0.58, 95% CI = 0.35–0.95), which suggested rs1865434 polymorphism was associated with PCOS [31]. In 934 Hispanic children study, IRS-2 variants (rs2289046 and rs1865434) were associated with BMI, body fat mass, and waist circumference [32]. Our results showed that rs1865434 genotype frequencies were significantly different in subjects with obesity compared to the non-obesity group ($p < 0.05$), which confirmed that the IRS-2 SNPs might be associated with the obesity. However, our study did not perform functional studies to clarify the part of the IRS-2 gene in PCOS.

5. Conclusion

rs2289046 genotype (AA and AG) and rs1865434 AG genotype may have protective effects on PCOS development, while rs1865434 GG genotype may be a risk factor for the evolvement of PCOS. Our data-dependent correlation between genotype, disease, biochemical and hormonal variables may support a role for IRS-2 gene polymorphisms in the pathogenesis of PCOS. Further investigations are still required to be performed in a larger number of subjects to clarify the roles of IRS-2 genetic variants in patients with PCOS.

Acknowledgments

The authors would like to thank all participants in this study. This work was supported by the Guangzhou Science and Technology Innovation Project (Grant number: 201804010003) and Guangdong Medical Research foundation (Grant number: A2020467).

Conflict of interest

None to report.

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