THE IMPACT OF MIRNA-146A AND MIRNA-149 GENE POLYMORPHISM ON SUSCEPTIBILITY TO POLYCYSTIC OVARIAN SYNDROME (PCOS)

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Abstract
Background: Polycystic ovary syndrome (PCOS) is considered one of the most prevalent disorders among females, with physiological and environmental factors. Nonetheless, a variety of hereditary illnesses are linked to this syndrome.
The Aim of study: This study is aimed to evaluate the association of miR-149 rs2292832 and miRNA-146a rs2910164 single nucleotide polymorphisms (SNPs) and PCOS
Patients and Methods: this is a case-control study which included 60 women with Polycystic Ovarian Syndrome (PCOS) and 60 aged-matched healthy women. Genomic DNA was extracted from whole blood, the gene fragments corresponding miRNA-146a and miRNA-149 SNPs were amplified with conventional PCR. genotyping was perfumed by (RFLP) Restriction fragment length polymorphism or amplification Refractory Mutation System-polymerase Chain Reaction (ARMS-PCR)
Results: The results indicate that the homozygous genotype (TT) of miR-149 rs2292832 was more frequent in patients (15%) Than Controls (5%) With a Significant difference (OR= 4.36, 95% CI=1.06-17.96, p=0.041). At allelic level, the mutant allele (T) was more frequent in patients than controls (39.17% versus 25.83%) with a significant difference (OR=1.85, 95%CI= 1.07- 3.2, p= 0.028). Similarly, the homozygous genotype (CC) of miRNA-146a rs2910164 was more frequent in patients (16.67%) than controls (3.33%) With a Significant difference (OR= 7.71, 95%CI=1.55-38.28, p=0.013). At allelic level, The mutant allele (C) was more frequent in patients than controls (38.33% versus 20.83%) with a Significant difference (OR=2.36, 95%CI= 1.33- 4.19, p= 0.003).
Key words: PCOS, miR-149 rs2292832 and miRNA-146a rs2910164 gene polymorphism.

INTRODUCTION

Polycystic-ovary-syndrome (PCOS) is the most prevalent, complex and heterogeneous endocrine disorder in women of reproductive ages [1]. It's characterized by symptoms such as chronic anovulation, excess androgenic hormones, and ovarian cysts and metabolic disorders, such as hyperglycaemia, hypertension, and obesity in women[2,3].
This syndrome is a very common infertility condition worldwide as 5-10% of diagnosed infertility cases linked to PCOS, including developing countries [4]. Although the exact etiology has not been clearly determined, it is suspected that this syndrome has major causative factors that include environmental factors, and genetic variants [5].
Many genes are confirmed in their association with some diseases like breast cancer and lung. However, micro RNA genes are less commonly investigated. MicroRNAs are a class of group of small, short (18 to 25 nucleotides) which code regulatory RNAs that mediate post-transcriptional regulatory processes such as regulating protein synthesis through the recognition of specific mRNA sequences and as a regulatory element [6,7].
Previous studies showed that miR-149 rs2292832 and miRNA-146a rs2910164 polymorphism are associated with so many diseases like type 2 diabetes mellitus (T2DM)[8], neuroblasoma risk [9], cancer [10]. Literatures about the association of these SNPs with PCOS as are scarce. Thus, the present study aimed to evaluate the role of miRNA-146a and miRNA-149gene polymorphisms as risk factors for PCOS.

PATIENTS AND METHODS

The Study Population

This case-control study was carried out in Al-Nahrain University's Department of Chemistry and Biochemistry, as well as the Medical Research Unit of the College of Medicine and infertility clinic or center /Al-yarmouk Hospital/Iraq from 1st January 2023 to 31st December 2023. The study population comprises two groups:
Group 1: 60 women with PCOS according to Rotterdam criteria: oligo-anovulation, Ahyperandrogenism, and polycystic

ovaries (≥12 follicles with a diameter of 2–9 mm and/or an ovarian volume >10 mL in one ovary). Those patients will be recruited from Baghdad Medical City.
Group 2: 60 age-matched apparently healthy women with a regular menstrual cycles and no signs or symptoms of PCOS.

Gene amplification and genotyping of miR-149 rs2292832 and miRNA-146a rs2910164
Using a ready kit (gSYNCTM DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea), genomic DNA was extracted from whole blood in accordance with the manufacturer's instructions. Two sets of primers were used to amplify gene fragments corresponding miRNA-149 and miRNA-149 SNPs.
Forward primer: 5'-TGT CTT CAC TCC CGT GCT TGT CC-3' and **reverse primer**: 5'-TGA GGC CCG AAA CAC CCG TA-3'. For miRNA-146a, four primers were used, so forward primer: 5'-GTTGTGTCAGTGTCAGACGTC-3', 5'-CCAGCTGAAGAACTGAATTTGAC-3', 5'-TCTACCATACACATCCCCTACA-3' and 5'-CACACTCCTTATACCTTCAGAGC-3'.
In the PCR reaction for both SNPs, there was 2.5 U of Taq polymerase, 250 µM of dNTP (dATP, dCTP, dGTp, and dTTP), and pH 9.0 Tis-HCl. Tracking dye and stabilizer (10 mM, KCl 30 mM, MgCl2 1.5 mM). No specific concentration as well . The PCR condition included initial denaturation 95C° for 5 min, denaturation 94C° for 30 sec. 35 cycles, then annealing60C° for 30 sec and elongation 72C° for 1 min. The PCR products were subjected to 2% gel electrophoresis (figure 1).
The genotyping of miRNA-149 was perfumed by Restriction fragment length polymorphism(RFLP), wherein 0.1 µL of PvuII restriction enzyme (Sibenzyme, Russia) and five µL of PCR products were combined. Next, 1.5 µL of restriction buffer (10X) and 0.1 µL of bovine serum albumin (BSA) were added to the mixture. Finally, the reaction mixture was brought up to 15 µ l using molecular grad water. Finally, mineral oil (20 µl) was added to each tube to prevent evaporation. Finally, the reaction mixture was incubated for three hours in a water bath at 60 degrees Celsius. The limited product was resolved on 2% agarose . (Figure 2).

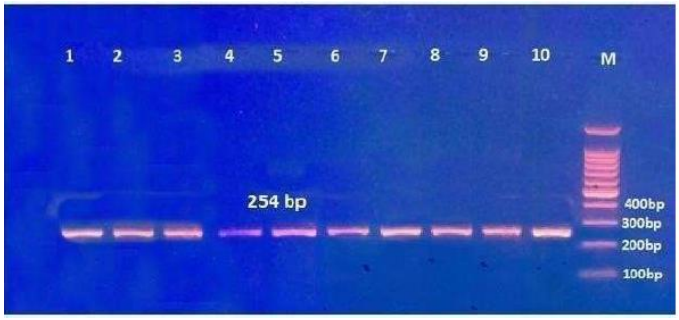

Figure (1): miR-149 rs2292832 C/T gene polymorphism was amplified using a particular pair of primers using conventional PCR and then electrophoresed on a gel. Ethidium bromide was used to stain the PCR result. There were 254 bp in the fragment.

Figure (2 8): MiR-149 rs2292832 C/T gene polymorphism genotyping following PvuII digestion and ethidium bromide staining. Lanes 1, 4, 5, 7, and 9 are CC; lanes 2, 3, 9, 10, 11, and 12 are CT; lanes 6 and 13 are TT genotype; M is a 100 bp DNA maker.

Genotyping of miRNA-146a was performed by ARMS-PCR (figure 3)
Figure (3): gel electrophoresis of miR-146a rs2910164 G/C gene polymorphism amplified with specific pair of primers using ARMS-PCR. The PCR product was stained with ethidium bromide. Lanes 4,7,9 and 11: GG genotype, lanes 1,3,5,6,8 and 10: GC genotype, lanes 2: CC genotype, M: molecular marker

Statistical Analysis
The statistical software SPSS 25.0 (SPSS, Chicago) was used to perform the analyses. The mean and standard deviation of continuous data were shown and the Student t-test was used for analysis. Chi-square analysis was performed on categorical variables that were expressed as numbers and percentages. The Chi-square test was also utilized to look at how SNPs deviated from the Hardy-Weinberg equilibrium (HWE). Genetic polymorphism and PCOS were found to be associated by binary logistic regression. Calculating the odds ratio (OR) and associated 95% confidence interval (CI) was done using the test results. A difference that was considered to be statistically significant occurred with a p-value less than 0.05.

Result
The study population's demographic characteristics
Table 1 shows the demographic characteristics of the study population. Themean age of women with PCOS was 26.98±7.22 years, which was very close to that of controls (25.07±6.39 years) with no significant difference. Likewise, the two groups were comparable in terms of weight, height and BMI with no significant differences. Family history of PCOS was very common among patients (46.67%) compared with controls (5%) with a highly significant difference. The mean duration of the PCOS in affected women was 4.18±2.33 years.

Table 11: The study population's demographic characteristics

Variables	Controls (n=60)	Patients (n=60)	p- value
Age, years			
Mean±SD	26.98±7.22	25.07±6.39	0.126
Range	14-52	16- 44	
Weight, kg			
Mean ±SD	70.43±6.64	72.84±7.72	0.070
Range	56-85	52-89	

Height			
Mean±SD	163.57±3.51	164.17- 4.84	0.438
Range	154-174	150-176	
BMI, kg/m^2			
Mean ±SD	26.33±2.39	27.01±2.54	0.132
Range	22.15-33.30	22.68-32.85	
Family history of PCOS			
No	3(5%)	32(53.33%)	**<0.001**
Yes	57(95%)	28(46.67%)	

Molecular Assay

The distribution of different genotypes in both SNPs was in accordance with HWE in the patient population as well as the control group.

miR-149 rs2292832 C/T

Digestion with PvuII restriction enzyme revealed three genotypes: CT, CT and TT.

The homozygous genotype (TT) was more frequent in patients (15%) than controls (5%) with a significant difference (OR= 4.36, 95%CI=1.06-17.96, p=0.041). At allelic level, the mutant allele (T) was more frequent in patients than controls (39.17% versus 25.83%) with a significant difference (OR=1.85, 95%CI= 1.07- 3.2, p= 0.028) as shown in table 2.

Table 2: Genotypes and alleles of **miR-149 rs2292832 C/T** in women with PCOS and controls

miR-149 rs2292832 C/T	**Controls (n=60)**	**Patients (n=60)**	***P*-value**	**OR(95%CI)**
Genotypes				
CC	32(53.33%)	22(36.67%)	0.089	1.0
CT	25(41.67%)	29(48.33%)	0.179	1.68(0.88-3.16)
TT	3(5%)	9(15%)	0.041	4.36(1.06-17.96)
HWE	0.498	0.912		
Dominant model				
CC+CT	57(05%)	51(85%)	0.081	1.0
TT	3(5%)	9(15%)		3.35(0.86-13.07)
Recessive model				
CC	32(53.33%)	22(36.67%)	0.068	1.0
TT+CT	28(46.67%)	38(63.33%)		1.97(0.95-4.09)
Alleles				
C	89(74.17%)	73(60.83%)	0.028	1.0
T	31(25.83%)	47(39.17%)		1.85(1.07-3.2)

miR-146a rs2910164 G/C

ARMS-PCR was used to amplify and genotyping of this polymorphism. The PCR product was undergone gel electrophoresis which revealed three genotypes GG, GC and CC.

The homozygous genotype (CC) was more frequent in patients (16.67%) than controls (3.33%) with a significant difference (OR= 7.71, 95%CI=1.55-38.28, p=0.013). This polymorphism seems to act in both dominant and recessive model. At allelic level, the mutant allele (C) was more frequent in patients than controls (38.33% versus 20.83%) with a significant difference (OR=2.36, 95%CI= 1.33- 4.19, p= 0.003) as shown in table 3.

Table 3: miR-146a rs2910164 G/C 's genotypes and alleles for women with PCOS and controls

miR-146a rs2910164 G/C	**Controls (n=60)**	**Patients (n=60)**	***P*-value**	**OR(95%CI)**
Genotypes				
GG	37(61.67%)	24(40%)	0.023	1.0
GC	21(35%)	26(43.33%)	0.100	1.91(0.88-4.13)
CC	2(3.33%)	10(16.67%)	0.013	7.71(1.55-38.28)
HWE	0.636	0.518		
Dominant model				
GG+GC	58(96.67%)	50(83.33%)	0.028	1.0
CC	2(3.33%)	10(16.67%)		5.8(1.12-27.73)
Recessive model				
GG	37(61.67%)	24(40%)	0.019	1.0
GC+CC	23(38.33%)	36(60%)		2.41(1.16-5.02)
Alleles				
G	95(79.17%)	74(61.67%)	0.003	1.0
C	25(20.83%)	46(38.33%)		2.36(1.33-4.19)

Discussion:

In this study, we investigated the effect of two SNPs in PCOS women in comparison to healthy controls and found a significant association of miR-164a rs2910164, and miR-149 rs2292832 with PCOS under the allelic, dominant, co-dominant, and recessive models.

Results of this study suggest that these miRNAs could contribute to the pathogenesis of PCOs and the results of this study came in the same line with other Several studies that reported genetic variants of the miR which associated with PCOs as in a study conducted by Li *et al*., [11]. Who calculated a significant association exist between PCOS and specific haplotype combinations and particular SNPs in miR-126, miR-146a, miR-196a2, and miR-499.

For miR-149 rs2292832, the TT genotype increase significantly in women with PCOs compared with control group also the T allele ratio increased significantly in PCOs. Up to available knowledge, unfortunately, there are no previous researches investigate role of miR-149 rs2292832 in PCOs and this study is considered one of the few in its field that shows the significant relation between them, also there is a very few and brief studies that related the SNPs polymorphism in miR-149 rs2292832 with other diseases as in a study done by Zhang *et al*. [12] who found that TT genotype or T allele carriers of miR-149 gene rs2292832 polymorphism increased the risk of Gastric Cancer.

Another study published by Sung *et al.* [13] demonstrated the association between miR-149 and the risk of coronary artery disease where the results showed the CT rate was higher in the patient group compared to the healthy group, also the results of the current study agree with Jeon *et al*. [14] who found that miR-149/ T ratio affect ischemic stroke pathogenesis as the results estimated high prevalence of CT genotype and T ratio in patients comparing with control.

For miR-146a rs2910164 the results observed an increased risk of PCOS for the subjects with the miR-146a rs2910164 CC genotype also the same results showed a significant increasing in C allele in patients comparing to control and this result agrees with what he found by Hosseini *et al.* [15] in study conducted to estimate the association of miR-146a rs2910164 and miR-222 rs2858060 polymorphisms with the risk of PCOs in Iranian women as their results showed the increase in miR-146a rs2910164 polymorphism increasing in risk of PCOS for the subjects with the miR-146a and a significant increasing in C allele in the patients in comparison with controls.

Jazdzewski et al. [16] demonstrated that the mRNA-146a rs2910164 polymorphism might effectively influence the miR-146a expression levels by demonstrating that the C allele of G/C polymorphism within the pre-miR146a sequence inhibited the synthesis of mature miR-146a compared with the G allele.

The estimation of this study that indicate an increased risk of developing the disease in women who appear higher than others are also consistent with what was published by another study [17] that examined CC genotype of miR-146a gene variation.

Collectively, these data indicate the importance of miR-149 rs2292832 miRNA-146a rs2910164 as risk factors for PCOS. Further studies with other miRNA genes are required in order to clarify the role of miRNAs in PCOS.

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