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Investigation of allele frequencies of polymorphic variants in genes that are related to polycystic ovary syndrome

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SUMMARY

OBJECTIVE: Polycystic ovary syndrome is a hormonal disorder that normally affects women of reproductive age in the range of 18–44 years. This study aimed to investigate the allelic frequencies of two polymorphisms, *IRS rs18012781* and *INSR rs1799817*, which are suspected to be involved in polycystic ovary syndrome.

METHODS: The samples were obtained from the patients admitted to the Near East University Hospital, Department of Gynecology and Obstetrics. The samples were divided into two groups: control and polycystic ovary syndrome groups. Blood samples were collected from 55 women in the control group and 65 samples from the patient group. DNA from whole blood was obtained. The allelic frequencies of single-nucleotide polymorphisms were determined using real-time PCR. Results were presented as the heterozygous and homozygous state of the single-nucleotide polymorphisms.

RESULTS: There were no significant differences in the allelic frequencies of the single-nucleotide polymorphisms between the patient and control groups. Further statistical analysis investigating the *INSR* Tm using the Mann-Whitney U test value revealed that there was no difference in the homozygous and heterozygous state of *INSR rs1799817*. The result of this study showed that there was no statistically significant difference between the allelic frequencies of *IRS1 rs1801278* and *INSR rs1799817* between the patient and control groups.

CONCLUSION: These single-nucleotide polymorphisms do not seem to modify the risk of polycystic ovary syndrome, and they cannot be used as a marker in clinical circumstances to evaluate the possible occurrence of polycystic ovary syndrome.

KEYWORDS: Polymorphism, genetic. Polycystic ovary syndrome. Insulin resistance. Genetic testing.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the underdiagnosed and underrated medical conditions that affect women around the world. PCOS is a heterogeneous condition that causes intense endocrine and reproductive malfunction. It commonly affects women of reproductive age in the range of 18–44 years. The gross effects of this condition can lead to hormonal complexities, such as dysfunctional menstrual cycles that may lead to infertility, obesity, hirsutism, and acne. The diagnosis of PCOS is performed following the Rotterdam Criteria and the Androgen Excess Society Criteria[1]. Furthermore, PCOS is associated with metabolic abnormalities as well as obesity[2,3]. However, management of this syndrome varies based on observable symptoms. PCOS is usually not diagnosed unless the patient encounters other related medical challenges, such as alopecia, hirsutism, androgenic acne, or infertility[4].

PCOS is shown to be clustered in families. Numerous studies are carried out to identify the ultimate etiology and the genetic background of PCOS[5]. However, the molecular regulation of this disease is not well understood. All the genes that function in oogenesis and ovulation may have a role in the development of PCOS. Some of these genes are the luteinizing hormone/choriogonadotropin receptor (*LHCGR*), estrogen receptor (*ER*), and androgen receptor (*AR*). In addition to these genes involved in gametogenesis, genes involved in insulin homeostasis, such as insulin receptor substrate 1 (*IRS1*) and insulin receptor (*INSR*), contribute to the pathogenesis of PCOS[6]. Furthermore, recent studies revealed that single-nucleotide polymorphisms (SNPs) may be important for evaluating PCOS susceptibility. Therefore, this study aimed to investigate the allelic frequencies of SNPs within genes associated with PCOS.

METHODS

Sample collection

Ethical approval was granted by the Institution Review Board of Near East University (YDU/2019/67-784). The samples

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required for this study were obtained from patients of Near East University Hospital, Department of Obstetrics and Gynecology. Informed consent was obtained from each patient. Clinical information of the patient was collected, and body mass indexes were reported. The samples to be studied were divided into two groups: the control group consisting of normal ovulation and non-obese women, and the patient group involving non-obese patients with PCOS. Blood samples were collected from 55 women in the control group and from 65 women included in the patient group. DNA from whole blood was obtained. The allelic frequencies of SNPs in two genes associated with PCOS were determined using real-time PCR.

DNA extraction from blood samples

DNA from each sample was extracted using an Invitrogen pure link genomic DNA mini kit (Invitrogen, USA) following the manufacturer's protocol. The concentration of the DNA was measured using NanoDrop (Thermo Scientific, Pittsburg, USA) at a wavelength of 260 nm (OD_{260}). The purity and quality were evaluated by the 230/260 ratio.

PCR amplification

Real-time PCR was conducted in order to identify the allelic frequencies at the particular SNP sites of the polymorphic genes, *IRS1* and *INSR*, which are associated with PCOS. Primer sequences are listed in Table 1. The reaction mixture consisted of 5 µl of master mix (LightCycler 480 SYBR Green, Roche), 0.8 µl of both forward and reverse primer (Table 1, final concentration of 0.25 µM), 0.6 µl of $MgCl_2$, and 0.8 µl of H_2O were included in the reaction mixture. A volume of 2 µl of the extracted DNA was added to each reaction. All the PCRs were set up in a laminar flow hood in order to avoid contamination. The PCR condition is shown in Table 2. The allelic frequencies of the two SNPs within two genes were analyzed using the high-resolution melting (HRM) method, and the thermal cycler software was used to obtain the cycle of threshold (Ct) and melting temperature (Tm) values.

Statistical analysis

Statistical packages for the social sciences (SPSS version 10, Chicago, IL, USA) were used in this study. Descriptive statistics and an independent sample test of the Mann-Whitney U test were performed. The results were considered statistically significant if $p \leq 0.05$.

RESULTS

This study was designed to investigate the allelic frequencies for the polymorphic variant genes that are associated with PCOS. A total number of 120 blood samples were collected. Of these, 65 were diagnosed with PCOS and 55 were included in the control group, who did not present any signs of PCOS. The average age was 20 years and the average body mass index for all the patients and the control group was 17. The PCOS patients were diagnosed by correcting their hormonal levels as well as by vaginal ultrasonography.

For each amplification, the cycle of threshold (Ct) was recorded. Ct indicates the total amount of cycle required for the fluorescent signal to cross the threshold. Likewise, for each amplification, melting temperature (Tm) values were recorded. Tm represents the melting temperature when the DNA is 50% double-stranded and 50% single-stranded. In HRM analysis, following PCR amplification, the amplicons produced are subjected to a gradual melting analysis. This enables the emission of fluorescence that is detected by real-time PCR equipment. These melt curves have different shapes due to the differences in the Tm values.

In this study, a total of 79.3% of the patients were shown to be homozygous for *IRS1 rs1801278*, respectively (Figure 1, Tables 3 and 4). There was no significant

Table 1. Details of primers used.

Primer name	Sequence (forward primer)	Sequence (reverse primer)
IRS1 rs1801278	GGAAGAGACTGGCACTGAGG	CTGACGGGGACAACTCATCT
INSR rs1799817	GGTGAAGACGGTCAACGAGT	AGAAAGGGAAGGGTCAGGAA

Table 2. PCR cycling conditions used in the amplification of *IRS* and *INSR* sites.

PCR conditions	Denaturation	Annealing	Extension	HRM
Temperature/time	95 for 10 min	95 for 10 s	72 for 25 s	95 for 1 h
				40 for 1 h
				65 for 1 s
				97 for 1 s
Cycle	1	40		

Figure 1. (A) PCR-HRM image showing melting curve analysis of the PCR products of the homozygote samples for IRS1 rs1801278. (B) PCR-HRM image showing melting curve analysis of the PCR products of the homozygote samples for different alleles of IRS1 rs1801278.

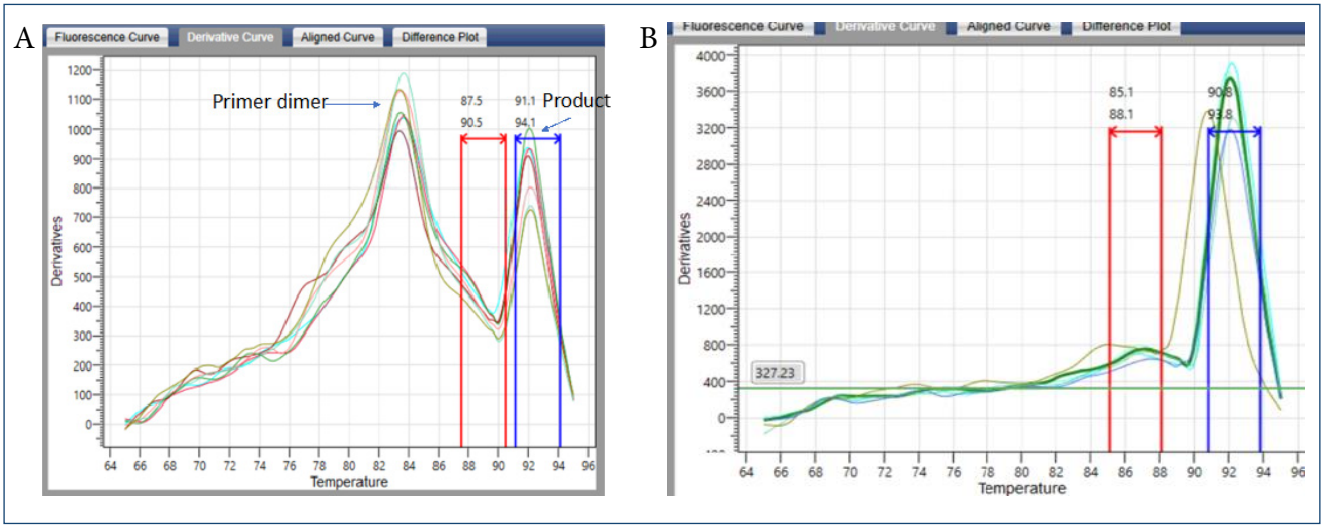

Table 3. Heterozygosity status with the Ct and Tm values for *IRS1 rs1801278* and *INSR rs1799817* in polycystic ovary syndrome patients.

Patients code	IRS1 Ct	IRS1 Tm	Heterozygosity	INSR Ct	INSR Tm	Heterozygosity
1	27.13	91.5	Homozygous	23.7	85.8	Homozygous
2	25.22	91.4	Homozygous	23.04	86.2	Homozygous
3	25.7	92.1	Homozygous	25.8	86	Homozygous
4	30.98	78.9	Homozygous	27.2	86	Homozygous
5	31.37	78	Homozygous	27.8	87.4	Homozygous
6	24.99	91.4	Homozygous	26.1	84.6	Homozygous
7	29.44	83.6	Homozygous	29.94	87.36	homozygous
8	28.22	83.3	Homozygous	36.7	86.3	Heterozygous
9	22.8	91.2	Heterozygous	22.5	85.7	Homozygous
10	32.75	92	Homozygous	28.9	86.7	Homozygous
11	29.7	78.7	Homozygous	24.26	86.4	Homozygous
12	25.85	91.8	Homozygous	23.1	86.1	Homozygous
13	29.33	91.2	Homozygous	23.1	85.4	Homozygous
14	25.48	91.3	Homozygous	23.49	85.8	Homozygous
15	31.31	76.5	Homozygous	22.75	86.1	Homozygous
16	28.02	91	Heterozygous	23.4	86	Homozygous
17	22.27	92.3	Heterozygous	23.58	85.9	Homozygous
18	31.99	77.9	Homozygous	22.1	90.1	heterozygous
19	30.29	79	Homozygous	26.3	85.8	Homozygous
20	23.93	91.6	Homozygous	24.2	86.5	Homozygous
21	30.52	78.1	Homozygous	24.05	86.1	Homozygous
22	22.2	92.19	Heterozygous	23.6	85.7	Homozygous
23	30.47	79.1	Homozygous	23.36	86.1	Homozygous
24	29.36	78.9	Homozygous	23.7	85.9	Homozygous

Continue...

Table 3. Continuation.

Patients code	IRS1 Ct	IRS1 Tm	Heterozygosity	INSR Ct	INSR Tm	Heterozygosity
25	26.5	92.02	Homozygous	22.4	85.6	Homozygous
26	No result	No result	No result	35.4	86.4	Homozygous
27	31.96	78.4	Homozygous	24.77	85.6	Homozygous
28	29.85	79.1	Homozygous	23.35	86.1	Homozygous
29	No result	No result	No result	22.3	86	Homozygous
30	No result	No result	No result	28.1	86.2	Homozygous
31	29.48	78.5	Homozygous	27.7	85.9	Homozygous
32	31.8	78.7	Homozygous	27.5	86	Homozygous
33	No result	No result	No result	23.5	86	Homozygous
34	30.91	78.5	Homozygous	26.6	85.7	Homozygous
35	29.65	91.7	Homozygous	26.9	86.2	Homozygous
36	32.07	78.4	Homozygous	26.7	86.5	Homozygous
37	31.99	85.62	Homozygous	23	86.3	Homozygous
38	31.68	86.04	Homozygous	25.8	87.3	Homozygous
39	30.42	91.6	Homozygous	25.77	87.1	Homozygous
40	24.47	83.6	Homozygous	23	85.9	Homozygous
41	No result	No result	No result	24.1	85.7	Homozygous
42	27.2	91.74	Homozygous	22.63	85.7	Homozygous
43	25.85	91.6	Homozygous	23.5	85.8	Homozygous
44	25.15	91.5	Homozygous	23	86	Homozygous
45	No result	No result	No result	23.7	86.6	Homozygous
46	25.85	90.7	Heterozygous	23	84.9	Homozygous
47	27.4	91.6	Homozygous	24.3	84.6	Homozygous
48	31.77	82	Homozygous	29.34	87.1	Homozygous
49	23.88	91	Homozygous	23.3	84.9	Homozygous
50	24.15	91.9	Heterozygous	24.1	84.8	Homozygous
51	26.35	91.6	Homozygous	28.89	87.1	Homozygous
52	24.04	83.8	Homozygous	23.15	86	Homozygous
53	25.7	91.6	Homozygous	26.41	61.3	Homozygous
54	25.98	91.5	Homozygous	25.36	61.7	Homozygous
55	25.83	91.6	Homozygous	27.72	61.5	Homozygous
56	26.6	91.1	Homozygous	22.8	85.9	Homozygous
57	25.65	92.1	Homozygous	23.4	85.9	Homozygous
58	26.36	92.2	Homozygous	22.9	85.9	Homozygous

difference between the homozygote and heterozygote status of *IRS1* rs1801278 when the patient group was compared with the control group. Furthermore, the heterozygosity of *INSR* rs1799817 did not show any significant difference between the patient group and the control group (p=0.059, Tables 3 and 4).

DISCUSSION

PCOS is one of the most common endocrine disorders affecting women of reproductive age. PCOS is one of the leading causes of infertility. Genetic and environmental factors tend to influence the complexity of PCOS (Figure 2). A number of studies have shown that genes and proteins are overexpressed

Table 4. Heterozygosity status with the Ct and Tm values for *IRS1 rs1801278* and *INSR rs1799817* in control group.

Patients code	IRS1 Ct	IRS1 Tm	Genetic conditions	INSR Ct	INSR Tm	Genetic conditions
1	No result	No result	No result	23.5	87.5	Homozygous
2	27.72	83.8	Homozygous	24.5	85.4	Homozygous
3	29.94	82.6	Homozygous	22.7	86.3	Homozygous
4	30.74	78.6	Homozygous	23.2	85.9	Homozygous
5	31.1	78.4	Homozygous	24	90.94	Heterozygous
6	25.88	92.1	Homozygous	29.29	87.23	Homozygous
7	26.5	92.26	Homozygous	23.2	85.9	Homozygous
8	32.27	78.5	Homozygous	24.1	85.9	Homozygous
9	27.3	91.47	Homozygous	22.83	85.8	Homozygous
10	27.2	92	Homozygous	23.01	85.9	Homozygous
11	26.09	92	Homozygous	33.15	87.3	Homozygous
12	26.21	91.4	Homozygous	28.76	87	Homozygous
13	30.35	82.38	Homozygous	27.1	87.56	Homozygous
14	25.92	91.6	Homozygous	25.69	62.8	Heterozygous
15	21.91	91.4	Homozygous	25	84.8	Homozygous
16	24.4	91.7	Homozygous	24.7	85	Homozygous
17	25.3	92.4	Homozygous	24.9	84.5	Homozygous
18	24.5	92.2	Homozygous	24.1	85.6	Homozygous
19	24.86	91.9	Homozygous	22.4	85.7	Homozygous
20	35.17	77.9	Homozygous	23	84.8	Homozygous
21	24.67	91.4	Homozygous	25	84.7	Homozygous
22	23.42	91.3	Homozygous	23.3	84.7	Homozygous
23	25.92	91.4	Homozygous	23.9	84.2	Homozygous
24	28.74	91.6	Homozygous	23.2	85	Homozygous
25	26.01	91.7	Homozygous	25.91	62.54	Homozygous
26	25.02	91.8	Homozygous	25.36	63.9	Heterozygous
27	25.11	99.9	Homozygous	26.7	63	Homozygous
28	25.38	91.6	Homozygous	24.95	62.9	Heterozygous
29	26.8	91.7	Homozygous	26.41	63.3	Homozygous
30	29.35	83.2	Homozygous	24.96	85.6	Homozygous
31	30.33	83.2	Homozygous	27.88	87.56	Homozygous
32	20.99	87.6	Heterozygous	29.41	86.84	Homozygous
33	No result	No result	No result	29.5	87.36	Homozygous
34	30.13	83.9	Heterozygous			Homozygous
35	26.8	91.8	Homozygous	28.59	87.3	Homozygous
36	25.85	91.8	Homozygous	31.16	87.2	Homozygous
37	25.46	91.6	Homozygous	26.47	86.9	Homozygous
38	22.55	91.7	Heterozygous	23.61	62.6	Homozygous
39	26.8	91.4	Homozygous	28.17	86.9	Homozygous
40	25.63	91.5	Homozygous	29.22	86.7	Homozygous
41	No result	No result	No result	No result	No result	No result
42	25.55	91.6	Homozygous	29.06	87.1	Homozygous
43	30.63	82.8	Homozygous	28.7	87.62	Homozygous
44	29.51	82.8	Homozygous	23.6	87.2	Heterozygous
45	21.49	84.1	Homozygous	22.99	86	Homozygous
46	29.96	83.3	Homozygous	23.27	86.1	Homozygous
47	22.97	83.9	Homozygous	22.32	85.8	Homozygous

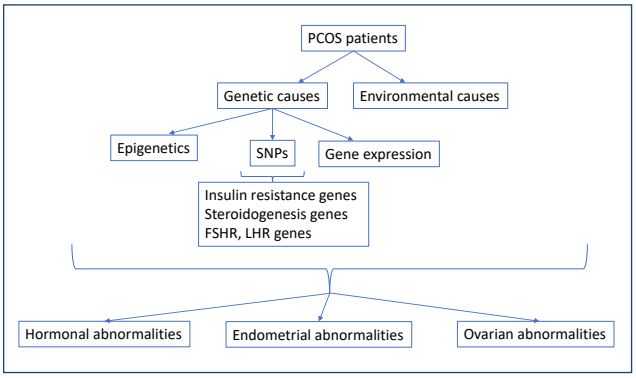

Figure 2. Flowchart diagram of causes of polycystic ovary syndrome.

or underexpressed in samples from PCOS patients. One of the examples of these is the androgen receptors that have been shown to be overexpressed in the endometrial samples obtained from PCOS patients. Furthermore, MKI67, BCL2/BAX, and FASLG/FAS were overexpressed in endometrium samples of PCOS patients. Moreover, these expression patterns were positively correlated with the levels of fasting insulin and negatively correlated with the levels of DHEA-S. Thus, endometrial homeostasis is associated with the levels of fasting insulin and DHEA-S that can also be correlated with the expression of the mentioned proteins[7]. Studies have shown that PCOS patients have high resistance to insulin. Insulin receptor genes, such as *IRS1* and *INSR*, are among the key genes that can be associated with the pathogenesis of PCOS. *IRS1* and *INSR* polymorphisms have tendencies to participate in problems with insulin signaling[8]. In this study, we aimed to investigate the heterozygosity status of two SNPs within *IRS1* and *INSR* genes that may have an increased risk of PCOS.

In this study, the homozygosity and heterozygosity status of the SNPs within *IRS1* and *INSR* genes were analyzed using Tm. The statistical differences were investigated using the Mann-Whitney U test. The results of this study showed that the heterozygosity and homozygosity status of *INSR* showed no significant difference between the control and patient groups. Similarly, another study reported no significant differences between the SNPs investigated within the *IRS1* and *INSR* genes in 48 Iranian women diagnosed with PCOS and 52 women in the control group. Furthermore, the same research group reported no association of *IRS1* polymorphism in PCOS patients and controls in Spain[9]. Similarly, genome-wide association studies have failed to show the link between the polymorphism of *IRS1* and PCOS in Han Chinese population[10]. Moreover, further studies reported no association between SNPs within *INS*, *INSR*, *IRS1*, *IRS2*, *PPAR-G*, and *CAPN10* genes and PCOS[11].

In contrast, one study reported significantly different frequencies of *IRS1* Gly972Arg polymorphism in PCOS and the control subjects in Turkish population[12]. Furthermore, another study was conducted to determine the frequency of polymorphism for the *IRS1* at codon 972 in women with PCOS in South Italy, consisting of 65 women with PCOS and 27 age-matched healthy women. They reported that there was a significant difference in the frequencies of Gly972Arg present in PCOS patients compared to controls. Furthermore, Tang et al. performed a comprehensive meta-analysis consisting of more than 4,000 subjects and reported that the AA/GA genotype of *IRS1* rs1801278 increased the susceptibility of PCOS when compared to the homozygote GG genotype. Similarly, a meta-analysis by Ruan et al. reported that the presence of the A allele significantly increased the risk of PCOS. They further recorded no significant association observed in the *IRS2* Gly1057Asp polymorphism[13].

Further studies also investigated the genotypes of *INSR* rs1799817 in relation to susceptibility of PCOS in Saudi Arabia, reporting that the homozygous allele was significantly higher compared to the control group[14]. Similarly, Chen et al.[15] also reported a significant difference in *INSR* rs1799817 genotypes in nonobese patients with PCOS compared to the controls.

CONCLUSION

Allelic frequencies of genes *IRS1* rs1801278 and *INSR* rs1799817 involved in PCOS recorded no significant difference in the study population. One of the limitations of this study was the small sample size. It is a possibility that, with the increased number of PCOS patients and control group, the heterozygosity status may have shown significant differences. Furthermore, we were able to only analyze a small number of polymorphisms. It would have been ideal to have a genome-wide investigation using next-generation sequencing (NGS) techniques. However, the results of this study are crucial to associate the insulin-related genetic polymorphisms with the PCOS patients. Furthermore, one of the most important strengths of this study was the statistical analysis techniques used. The results of this study form the basis of future studies, especially in newly developed countries where newer technologies such as NGS are out of reach.

AUTHORS' CONTRIBUTIONS

ARA: Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. BO:

Conceptualization, Data curation, Formal Analysis, Resources, Software, Validation, Visualization, Writing – review & editing. **ACO:** Conceptualization, Data curation, Formal Analysis, Resources, Software, Validation, Visualization, Writing – review & editing. **PT:** Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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