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A NOVEL STUDY FOR THE DETECTION OF PROGRAMMED CELL DEATH-1 GENE VARIANTS USED IN PATHOGENESIS AND CONSEQUENCES OF PCOS IN IRAQI OBESE WOMEN

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ABSTRACT

Background: Apoptosis is a kind of programmed cell death that take place within the granulosa cells of ovary, is an essential part of folliculogenesis. So there is greater incidence of apoptosis in polycystic ovary syndrome women. Programmed cell death 1 PDCD1 play important negative regulatory roles in inflammation. A genetic mutation in PD-1 is associated with the incidence and progression of PCOS. **Objective:** To study the genetic polymorphism of PD-1gene (rs2227982) in Iraqi women with PCOS and explore its role in pathogenesis of the PCOS disease. **Materials and Methods:** Whole blood samples of 80 PCOS obese patients and 80 healthy people were collected in duration from Dec., 2021 to April, 2022, at the gynecological and obstetric teaching hospital, Kerbala health directorate, Iraq. The Rotterdam criteria-2003 was accepted to PCOS females with age range between (18-40 years). Patients with any 2 of the next 3 items can be identified as PCOS: oligo menorrhea / amenorrhea, increase androgen level, ovarian volume >10mL on U/S, and follicles ≥12 with diameter 2-9 mm. Controller group with ages between (18-40 year). They have regular menstruation, with normal ovaries as they were accepted by the gynecologist. BMI and WHR and hormonal status were determined. DNA was extracted and stored at -20 ^{o}C until use. Primers designed for PDCD-1gene (rs2227982) based on NCBI database. Genotypes detected using the *Taq* Man allelic discrimination real-time PCR method. **Results:** The obese PCOS women had a seriously higher frequency of the PDCD-1 GA/GG genotypes (rs2227982) compared with the controls. PD-1(rs2227982) GA genotype significantly associated together with a higher PCOS frequency. P-value (0.0151), OR (2.728) and PDCD-1gene allele frequencies (rs2227982). An allele had significance P-value (0.0237), OR (2.393) with PCOS patient. **Conclusion:** Our findings indicates that the PDCD1 (rs2227982) genotype GA is associated with a susceptibility to obese PCOS women and affect the clinical features and pathogenesis. It may be new possible polymorphic loci for PCOS development.

KEYWORDS: Programmed cell death protein-1, Polycystic ovary syndrome, Single-nucleotide Polymorphism.

INTRODUCTION

Polycystic ovarian syndrome (PCOS) is one of the most common endocrine and metabolic disorders. It affects 5–15% female in reproductive age. The etiopathogenesis of PCOS is complex (genetic, environmental and lifestyle interaction) **(Moghetti and Tosi, 2021).** It is a heterogeneous disorder with unknown etiology. Genetically inherited with the autosomal dominant way and 50% possibilities of inheritance from mother to daughter **(Pelanis *et al.*, 2017).** The PCOS patients will exhibit increase levels of testosterone and other androgen hormones and it lead to the acne, derange menstruation, gain weight which leads to obesity as well as infertility **(Ollila *et al.*, 2017).** PCOS is a polygenic pathology. A recent large genome-wide meta-analysis identified 14 gene loci associated with the observed metabolic, reproductive and pathophysiologic findings in women with PCOS **(Castillo-Higuera *et al.*, 2021)**. In the context of modern lifestyle and environmental influences, such as poor-quality diet, reduced physical activity, environmental chemical exposure, circadian disruption and stress, developmental programming of

these same genomic variants **(Parker and O'Brien, 2021)**. PCOS patients are risky for the emergence of breast, ovarian and endometrial cancers also cardiovascular diseases (atherosclerotic) **(Allen et al., 2021)**. Other signs include obesity, hirsutism, insulin resistance, diabetes mellitus, endothelial dysfunction, and a state of low-grade inflammation, which have possible effected on the pathogenesis of PCOS **(Li *et al.*, 2019).**

Apoptosis is a kind of programmed cell death that take place in multicellular organisms and within the granulosa cells in ovary is an essential part of folliculogenesis ***(*Regan *et al*., 2018)**. So there is greater incidence of apoptosis in polycystic ovary syndrome PCOS women **(Wang *et al*., 2019).** Follicles composed of granulosa and theca cells with oocytes. The oocytes surrounded by the granulosa cells, providing them with nutrients and regulators during oocytes maturation. Apoptosis in granulosa cell is the main cause of follicular atrophy which include degeneration of DNA. PCOS women display more maturing and following atrophic follicles that not pass into dominant follicles, with abnormal apoptosis of granulosa cells in PCOS folliculogenesis. That lead to anovulation and infertility **(Huang *et al.*, 2021).** Programmed cell death-1cytokin (PD-1; PDCD1) play very important inhibitory functions in inflammation and autoimmune diseases as well as tumors. They are significantly implicated in decreasing response to inflammation and immune avoidance to tumor (Han et al., 2021). The PD-1 gene is discovered on chromosome no. 2 in the q37.3 zone. There are >30 single nucleotide polymorphism sites (SNPs) in different gene zones include intron, exon, promoter, and 3´UTR zone **(Ghorbani *et al.*, 2019, Karami *et al.*, 2020)**. The PD-1 cytokine and its ligands, PD-L1 and PD-L2 are given inhibitory signals to keep T-cell tolerance and activation as well as immune-mediated tissue damage. lack of its role can overset the role of T cells **(Mi et al., 2021))**. Although, if the PD-1 gene polymorphisms (rs2229782) are associated with the incidence and pathogenesis of PCOS has not been recorded **(Han *et al.*, 2021)**.

The current study aimed to estimate the relation between PD-1 genetic variants (rs2227982) and various anthropometric characteristics with PCOS pathogenesis in Iraqi women.

MATERIALS AND METHODS

This study is a case-control study involves 80 PCOS patients. and 80 non-PCOS women as a control in childbearing age at the Reproductive Fertility consultant of gynecological and obstetric teaching hospital, Kerbala health directorate Iraq and College of Medicine, University of Kerbala Iraq during the duration from Dec., 2021 to April, 2022. An exhaustive interview gathering personal and family history, blood pressure, demographic information and laboratory examination was carried out. The Rotterdam criteria-2003 was presumed to 80 PCOS females with ages ranged between (18-40) years. Patients with any 2 of the next 3 items can be recognized in diagnosis: oligomenorrhea or amenorrhea, increase androgen levels, ovarian volume > 10mL on U/S, and follicles ≥12 with diameter 2-9 mm **(Park *et al*., 2022).** Controller group has 80 ladies which ages reached between (18-40 years). They have regular menstruation, with normal ovaries as they were detected by the gynecologist. Body mass index were calculated from the following equation: BMI = Weight (kg) / Height (m^2). Normal BMI level is (20-24.9) kg/m^2 and (25-29.9) kg/m^2 for overweight. When BMI ≥ 30kg / m^2, the woman is considered as obese **(Eweis *et al*., 2021).** The WHR diagnostic standard for obesity is 0.85 for women **(Ahn *et al.*, 2020).** The volume withdrawn from each patient was 5.0 mL divided into two parts, 3.0 mL was used for serum separation and used for hormonal assays. The hormonal levels of each of LH and FSH were measured by the chemiluminescent automated immunoassay system (Cobas e411, Roche diagnostic, Germany). Free Testosterone level was measured by Competitive Enzyme Immunoassay using Monobind/USA ELISA kit. The remaining 2.0 milliliters of blood specimen that have been placed in EDTA tube. Then this tube saved by freezing at -30˚C till investigation PD-1 gene polymorphism. The protocol for study was certified by the ethical research commission of College of medicine, University of Kerbala and Kerbala Health Directorate. Approval also taken from administration of gynecological and obstetric teaching hospital and from each patient after explaining the nature and purpose of study. Genomic DNA was extracted from each blood sample using (Add Prep Genomic DNA Extraction Kit 10023 Addbio/Korea). The DNA was stored at -20 ^oC until use. The SNP of PDCD-1 (rs2227982) was genotyped using conventional genotyping Real-Time PCR. a GoTaq® Probe qPCR Master Mix (Promega, USA), Primers and probeS Macrogen/Korea and Stratagene Real-Time thermal cycler (Stratagene, USA) were used.

All statistical analyses were performed with the graph pad Prism 9.0.0 was released on October 28, 2020. Genotype and allele frequencies of PDCD1was compared between the healthy controls and patients with PCOS using the probability value (P value), Odds Ratios (OR) and 95% Confidence Intervals (95% CI) were compute for each analysis to express the significance connecting the studied groups. In statistical analysis the highly significant value is ($P < 0.01$) and the significant value is ($P < 0.05$).

RESULTS

Based on inclusion and exclusion criteria, 160 women were involved in the last data analysis, the women involved within the study finally were with an age ranged between (18-40) years and the mean ± SD of them were 26.78 ± 5.3 years. The results of this study were displayed in Table 1. They incorporate the mean ± SD of the patients with and without hirsutism and those with primary or secondary infertility and (regular or

irregular) menstruation pattern. It is clear that the two groups are almost well matched, thus obtained results could be estimable.

The results of this study were displayed in Table 2 using statistical unpaired T-test; age, BMI and WHR as well as using statistical Mann Whitney test. Significant elevations in LH concentrations ($p<0.001$), LH/FSH ratio ($p<0.001$) and free testosterone levels ($p<0.001$) were prevailed in the PCOS patients group when contrasted with the control group. However, FSH was noticed significantly decreased during a comparable evaluation ($p=0.01$).

Table 1: Demographic parameters of the registered patients and the control.

Demographic parameters	Control N=80 Mean ± SD	Patients N=80 Mean ± SD
Menstruation Pattern (regular)	80	17
Menstruation Pattern (Irregular)	------	63
With Hirsutism	------	67
Without Hirsutism	80	13
Primary infertility	--------	52
Secondary infertility	-----	28

Table 2: Biochemical parameters of the registered patients and the control.

Biochemical parameters	Control N= 80 Mean ± SD	Patients N= 80 Mean ± SD	P –value
Age, year	29.1 ±5.175	26.87 ± 5.3	0.05
BMI(kg/m^2)	23.3 ± 1.156	32.5 ± 6.357	<0.0001
WHR	0.777 ± 0.0143	0.912 ± 0.0563	<0.0001
LH, m.iu/ml	106 ± 0.555	11.89 ± 3.188	< 0.0001
FSH, m.iu/ml	6.73 ± 0.65	5.36 ± 1.36	0.01
LH/FSH ratio	0.986 ± 0.041	2.414 ± 0.379	< 0.0001
Free testosterone, pg/ml	2.97 ± 1.812	18.7 ± 14.98	< 0.0001

The subjects that enrolled in present study were classified into tow genotypes, for PD-1gene (G>A) (rs2227982): one homozygous for the G allele (GG) wild type and one heterozygous (GA). Out of 80 patients, there were 26 heterozygous (GA) genotypes (32.5%) and 54(GG) genotypes (67.5%). While in 80 control group there was 12 heterozygous (GA) genotypes (15%) and 68 (GG) genotypes (85%) of SNP rs2227982 in the PD-1gene. Fig. 1 summarize genotyping of study subjects according to polymorphism of (G>A) (rs2227982) of the PD-1gene.

Allele frequencies for 160 women that involved in our study of PD-1gene (rs2227982) in patient group G allele134 (84%) and A allele 26 (16%).while in control grope G allele 148 (93%) and A allele 12 (7%) of SNP rs2227982 in the PD-1gene. The genotyping results of current study were displayed in Table 3 using Fisher's exact test.

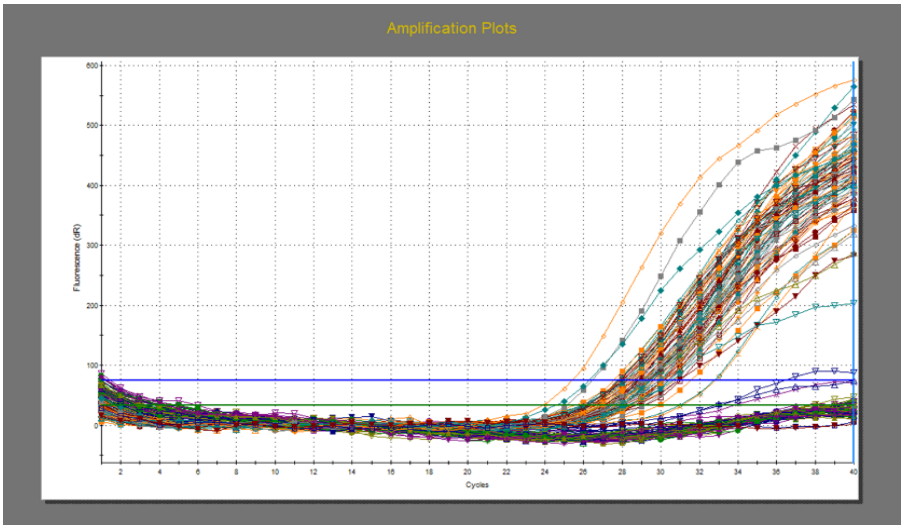

Fig. 1: Detection of PD-1gene (G>A) (rs2227982) gene polymorphism by RT-PCR technique. PCR products with two possible genotypes (GA or GG).

Table 3: PDCD-1(rs2227982) (G/A) genotypes frequency and allele frequency distribution in patients and healthy groups.

rs2227982 (G/A)		Study groups		OR	CI 95%	*P-value*
		Patients, *N*(%)	Healthy, *N*(%)			
Genotypes	GG	54(67.5)	68(85)	References group		
	GA	26(32.5)	12(15)	2.728	1.230 to 5.977	0.0151*
	AA	0(0)	0(0)			
Total		80(100)	80(100)			
Allele frequencies						
G allele		134(84)	148(93)	References group		
A allele		26(16)	12(7)	2.393	1.197 to 4.815	0.0237*
Total		160(100)	160(100)			

The results demonstrated that the obese patients with PCOS had a seriously higher frequency of PDCD-1 GA/GG genotypes (rs2227982) compared with the controls. PDCD-1 (rs2227982) GA genotype was related significantly with a higher frequency of PCOS p-value (0.0151), OR (2.728), CI 95% 1.230 to 5.977 and PDCD-1gene allele frequencies (rs2227982) A allele had significance p-value (0.0237), OR (2.393), CI 95% 1.197 to 4.815 with PCOS patient.

DISCUSSION

Various clinical and hormonal biomarkers and genetic polymorphisms of different single nucleotide polymorphism (SNPS) have been studied in PCOS Iraqi women **(Al-Tu'ma *et al,* 2020 and Al-Amami, *et. al.*, 2020).** Demographic parameters was taken and revealed obvious increase in hirsutism, irregular menstruation pattern and primary infertility **(Fatema *et al.*, 2021).** The LH values, LH/FSH ratio and prolactin values increased significantly in PCOS women compared to control group while a significant decrease in FSH values were found, these results agrees with study done by **(Ibrahim and Alobaidi, 2021)** Serum levels of LH, testosterone, and prolactin were found to be significantly elevated in PCOS patients at Kalar General Hospital, while serum FSH levels were found to be significantly lower. The results of the present study discovered that the level of free testosterone is elevated in PCOS patients in comparable with controller group and this agrees with **(Luo *et al.*, 2021).** Excessive secretion of androgens from the adrenal gland will produce inhibitory effect on hypothalamus-pituitary-ovarian axis, leading to disorder in releasing rhythm of Gonadotropin-releasing hormone and increasing the levels of LH, then increasing androgen production from the ovaries, leading to hyperandrogenemia. This increase in LH concentration may increase androgen biosynthesis from theca cells of ovaries, while the comparative FSH diminish follicular maturation **(Zeng *et al.*, 2020).** BMI in patients (32.5 ± 6.357 kg/m^2) displayed obesity which existing in varying degrees in PCOS women and exacerbate endocrine disorders and metabolic disorders in PCOS patients **(Dadachanji *et al.*, 2021).**

The current study, established that in PCOS patients the Waist–Hip ratio(WHR) was higher than in the controller group, indicating that the fat cumulated in the PCOS patients abdomen in spite of they had thin figures **(He and Li, 2021).**WHR has been extremely association with androgen levels that mean central obesity has an important role in PCOS **(Zeng *et al.*, 2020).**

The present study is a novel study that identified PDCD-1 gene polymorphisms (rs2227982) correlation with progression and pathogenesis of PCOS disease. PDCD-1gene (rs2227982) were detected in heterozygous genotypes (GA) and A allele for allele frequency in PCOS patients, this demonstrates the positive association with PCOS risk and pathogenesis in Iraqi population and could be used as possible biomarkers to prognosticate the risk of PCOS. While, many studies results detected that PD-1 gene (rs2227982) was act as a risk factor in various cancers for examples, leukemia**,** gastric adenocarcinoma **(Tang *et al*., 2017),** esophageal squamous cell carcinoma **(Zhou *et al.*, 2016)** and ovarian cancer **(Tan *et al.*, 2018).** Rs2227982 locates in the PD-1gene on exon-5 and its polymorphism lead to a nonsynonymous mutation which produce an amino acid exchange (Alanine to Valine) through protein biosynthesis, which affect the PD-1cytokine function **(Huang *et al.*, 2019).** The immune system is consisting of numerous of biological structures that protect the body from disease so it is a defense system. when the immune system is disabled, it can produce several diseases. New studies have revealed that immune system mechanisms are included in polycystic ovary syndrome. PCOS patients were established to be immersed in a chronic low-grade inflammation condition, which include increase leukocytes, dysfunction of endothelium, and the proinflammatory cytokines disturbance. Large numbers of immunocompetent cells, including T and B cells, dendritic and macrophages cells, have been present in human preovulatory follicles **(Li *et al.*, 2019).**

CONCLUSION

This study suggests for the first time that the PDCD-1 (rs2227982) genotype GA is significantly associated with a susceptibility to PCOS and affect the pathogenesis of PCOS in Iraqi ladies with obesity and it may be considering as new possible polymorphic loci for PCOS. This will help in screen susceptive populations at the

genetic level and will provide theoretical motives for diagnosis and treatment of patients with PCOS.

Also the observed results indicated that there is grand increase in the mean of various hormones which are free testosterone and LH in obese PCOS women as compared with control, while there is significant decrease in FSH values was obtained.

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