Molecular Basis of Programmed Cell Death-1 Gene and its Association with Encoding Protein Levels of Iraqi Women with Polycystic Ovarian Syndrome

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Abstract:

Background: Tumors, immunological disorders, and inflammation are all significantly reduced by programmed cell death-1 (PD-1) Polycystic ovarian syndrome (PCOS) patients have a uniform clinical feature of chronic inflammation. PD-1 ligand can produce T-cells apoptosis and produce immunosuppressive effects. The level of PD-1 was increased in both CD4+ and CD8+ T cells from infertile patients with PCOS.

Materials and Methods: Whole blood samples of 80 PCOS patients and 80 healthy people were collected from Dec., 2021 to June, 2022 at the gynecological and obstetric teaching hospital, Kerbala health directorate, Iraq with age range between (18 -40) years. The Rotterdam criteria-2003 was accepted to PCOS females. BMI and WHR were determined. DNA was extracted and stored at -20 C until use. Primers designed based on NCBI database. Genotypes detected using the Taq Man allelic discrimination real-time PCR. Hormonal status was determined by cobas e411. Elabscience /USA ELISA kit was used to determine PDCD-1 level in serum.

Results: The PCOS women had a significantly higher frequency of PD-1(rs2227982) GA genotype. P-value (0.0151), OR (2.728) and an allele had significance P-value (0.0237), OR (2.393). A significant elevation of PDCD-1 level (P=0.01) were obtained in PCOS patients group and there is significant association between PD-1 level and PD-1 gene (rs2227982) GA genotype, P value = 0.0002.

Conclusion: The observed data indicate that the PDCD-1 protein significantly increase in PCOS patients. PDCD-1(rs2227982) genotype GA is associated with a susceptibility to PCOS and its pathogenesis. It also demonstrated that PD-1 rs2227982 was significantly associated with GA genotype in PCOS Iraqi patient.

Keywords: Programmed cell death protein-1, Polycystic ovary syndrome, Single-nucleotide Polymorphism. Gene. PDCD-1.

1. Introduction

The cause of the heterogeneous condition of polycystic ovarian syndrome (PCOS) is uncertain. Genetic studies have shown that PCOS is inherited in an autosomal dominant way, with a 50% likelihood of inheritance from mother to daughter [2]. The prevalence of PCOS in the first-degree relative of the individual, which was reported to be between 55 and 60 percent in a number of small families, reinforced the idea that the condition being inherited autosomally dominantly [1]. In PCOS, autosomal dominant transmission is referred to as polygenic disease even though it may be associated with a single gene deficiency. One of the potential causes is that a certain gene may have a predominating effect on any family and trigger the phenotypic expression [3]. Immune checkpoint Programmed Cell Death-1 (PD-1) guards against autoimmunity using two different pathways. First, it causes more antigen-specific T lymphocytes to undergo apoptosis (programmed cell death) [4]. Additionally, it lessens regulatory T-cell apoptosis (anti-inflammatory, suppressive T- cells) [5].PD-1, which is expressed on activated T cells, natural killer and B lymphocytes, macrophages, dendritic cells, and monocytes, inhibits both innate and adaptive immune responses [6]. Granulosa cells supply the nutrients for oocyte and regulate its growth. on the other hand, the central oocyte stimulates growth and differentiation of granulosa cells [7]. These bidirectional mutual actions between the oocyte and granulosa cells have an effect on follicle maturation. According to this, apoptosis

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within the granulosa cells is an essential part of folliculogenesis. So there is greater incidence of apoptosis in between PCOS women [8]. Granulosa cell apoptosis is a complex process which can be triggered by lots of factors which includes inflammatory cytokines [9].

Programmed cell death-1 (PD-1) is crucial for preventing cancers, autoimmune disorders, and inflammation [1]. PCOS patients have a uniform clinical feature of chronic inflammation. The PD-1 is a suppression signal molecule that inhibits activation of T cells [10]. PD-1 ligand, can produce T-cells apoptosis and activate cells to secrete IL-10, that produce immunosuppressive effects [11]. Both CD4+ and CD8+ T lymphocytes from infertile PCOS patients had higher levels of PD-1 [12]. The host is protected from disease by the immune system, which is a defense mechanism made up of numerous biological components. When the immune system of the body is compromised, a number of diseases can result. According to a recent study, immunological systems play a role in the control of PCOS [13].

According to other studies, patients with PCOS have excessive numbers of leukocytes, endothelial dysfunction, and a disturbance of the proinflammatory cytokines [14]. They also have a chronic low-grade inflammation condition. Human preovulatory follicles contain significant numbers of immunocompetent cells, such as T-cells, B-cells, macrophages, and dendritic cells [15]. T-cells, which make up the majority of lymphocytes, perform a number of biological tasks, the majority of which are connected to the body's cellular immune response. They have the ability to directly kill target cells or to increase and broaden the immunological effect by releasing lymphatic factor [16].

2. Materials and Methods

In this case-control work, 80 patients of women with PCOS are included, and another 80 women without PCOS as a control in childbearing age at the reproductive fertility consultant of gynecological and obstetric teaching hospital, Kerbala health directorate / Kerbala - Iraq during the period between Dec., 2021 to June, 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 [15]. 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. The following formula was used to determine body mass index: BMI = Weight (kg) / Height (m2). Normal BMI level is (20-24.9) kg/m2 and (25-29.9) kg/m2 for overweight. When BMI ≥ 30kg / m2, the woman is considered as obese [16]. The WHR diagnostic standard for obesity is 0.85 for women [17]. The blood sample 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, FSH and prolactin 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 and Elabscience/USA ELISA kit uses the Sandwich-ELISA principle was used to determined PDCD-1 level in serum. 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 of molecular studies concerning PD-1 gene. The Kerbala Health Directorate and Kerbala College of Medicine's ethical research commission approved the study's protocol. Approval also taken from administration of gynecological and obstetric teaching hospital and from each patient after explaining the nature and purpose of study. Each blood sample had its genomic DNA extracted using the Add Prep Genomic DNA Extraction Kit 10023 Addbio/Korea. The genomic 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.

The graph pad Prism 9.0.0 was used for all statistical calculations. It was released on October 28, 2020. In order to represent the significance of the analyzed groups, the genotype and allele frequencies of PDCD1 were compared between healthy controls and patients with PCOS using probability values (P values), odds ratios (OR), and 95 percent confidence intervals (CI). The extremely significant value in statistical analysis is (P 0.01), while the significant value is (P 0.05).

3. Results

Based on inclusion and exclusion criteria, 160 women were involved in this study, the women involved 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 indicated 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.

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

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

The results shown in Table 2 used statistical unpaired T-test for age, BMI and WHR as well as using statistical Mann Whitney test. Significant elevations in LH concentrations (p<0.0001), LH/FSH ratio (p<0.0001), free testosterone levels (p<0.0001), prolactin level (p<0.0001) and PDCD-1 level (p=0.01) were prevailed in the PCOS patients group when compared with the control group. However, FSH was noticed significantly decreased during a comparable evaluation (p=0.01).

Table 2: The biochemical parameters measured for the registered patients and the control group.

Biochemical parameters	Control N= 80 Mean ± SD	Patients N= 80 Mean ± SD	P –value
	29.1 ± 5.175	26.87 ± 5.3	0.05
Age, year BMI (kg/m^2)	23.3 ± 1.156	32.5 ± 6.357	<0.0001
WHR LH (m.iu/ml) FSH	0.777 ± 0.0143	0.912 ± 0.0563	<0.0001
(m.iu/ml) LH/FSH ratio	106 ± 0.555	11.89 ± 3.188	< 0.0001
Free testosterone	6.73 ± 0.65	5.36 ± 1.36	0.01 <
(pg/ml) Prolactin	0.986 ± 0.041	2.414 ± 0.379	0.0001 <
(ng/ml) PDCD-1	2.97 ± 1.812	18.7 ± 14.98	0.0001 <
(ng/ml)	12.19 ± 2.92	16.14 ± 4.05	0.0001
	0.28 ± 0.09	0.34 ± 0.1	0.01

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 homozygote (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.

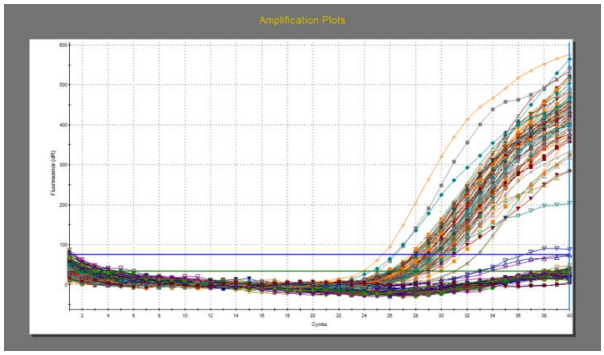


Fig. 1: Detection of PD-1gene (G>A) (rs2227982) gene polymorphism by RT-PCR technique. PCR products with two possible genotypes (GA or GG).

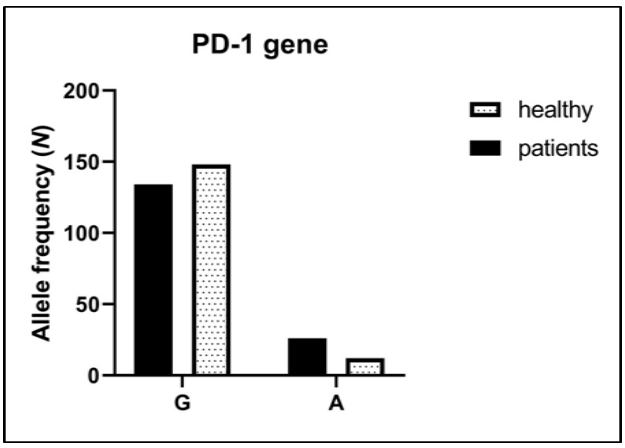
Allele frequencies for 160 women involved in our study of PD-1gene (rs2227982), in PCOS patient group G allele 134 (84%) and A allele 26 (16%), while in control grope G allele 148 (93%) and A allele 12 (7%) of SNP rs2227982 in the PD-1gene. The genotyping results of current study were displayed in Table 3 using Fisher's exact test.

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

rs2227982 (G/A)		Study groups Patients, *N*(%)	Healthy, *N*(%)	OR	CI 95%	*P-value*
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) when compared with the controls. PDCD-1 (rs2227982) GA genotype which 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), while A allele had significance p-value (0.0237), OR (2.393), CI 95% 1.197 to 4.815with PCOS patient as showed in Fig. 2.

Unpaired T test used to evaluate the association between PDCD-1 protein level and PDCD-1 (rs2227982) genotypes as indicated in table 4 and figure 3. The current study result showed that there is significant association between PD-1 level and PD-1 gene (rs2227982) GA genotype, P value = 0.0002, CI95% GA: 0.37 – 0.86 and CI95% GG: 0.29 – 0.38. While there is non-significant association between PD-1 level and genotype GG of PD-1 gene (rs2227982) in PCOS patients group.



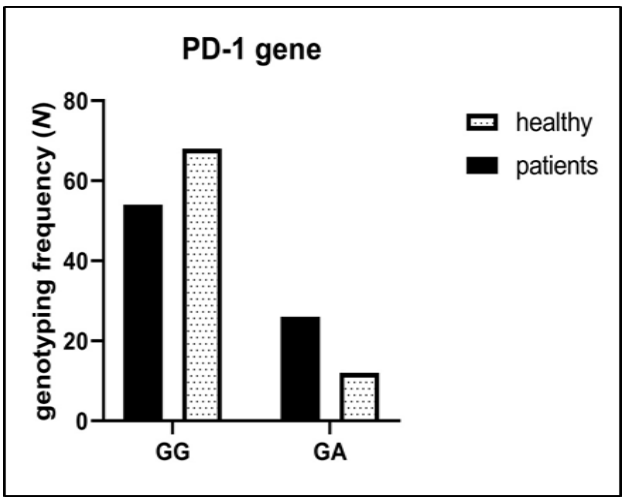


Fig. 2: Genotyping Frequency and Allele Frequency of PDCD-1 gene (rs2227982) with two possible genotypes (GA or GG).

Table 4: Association of PDCD-1 level with PDCD-1 gene (rs2227982) in the registered PCOS patients and control group

Genotype	PCOS and controlMean ± SD	CI 95%	P value
GA	0.61 ± 0.4	0.37 – 0.86	0.0002
GG	0.34 ± 0.1	0.29 – 0.38	NS

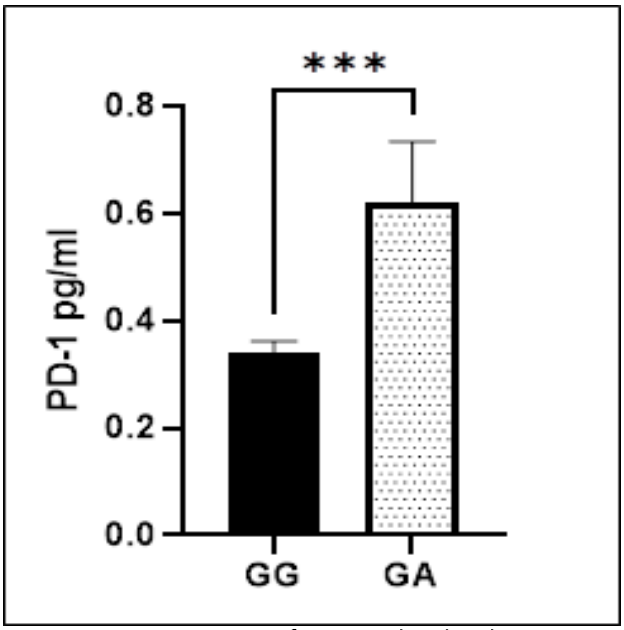


Figure 3: Association of PDCD-1 level with PDCD-1 gene (rs2227982) in the registered PCOS patients and control group

4. Discussion

Various clinical and hormonal biomarkers and genetic polymorphisms of different single nucleotide polymorphism (SNPS) have been studied in PCOS Iraqi womenv [18]. Demographic parameters was taken and revealed obvious increase in hirsutism, irregular menstruation pattern and primary infertility [20]. 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 et al. [21] 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. According to the current study's findings, PCOS patients have higher levels of free testosterone than the control group, which is consistent with other observation performed previously [22, 23]. 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 [24]. PD-1 interacts with two ligands, PD-L1 and PD-L2, to block antiviral T cell responses. It showed that the expression of PD-1 was significantly higher in CD4+ or CD8+ T cells from the PCOS group with infertility than it was in cells from the control group [25]. It was concluded that the immune pathogenesis in the ovary of PCOS patients with infertility may be influenced by T cell malfunction, which may be an immunological characteristic [26]. These findings imply that one of the fundamental reasons for the pathophysiology of PCOS may involve chronic inflammation [25, 27]. Rui Han's study demonstrated that PD-1 levels in the serum of PCOS patients were lower than those in the control group [10].

BMI in patients (32.5 ± 6.357 kg/m2) displayed obesity which existing in varying degrees in PCOS women and exacerbate endocrine disorders and metabolic disorders in PCOS patients [28].

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 [29].WHR has been extremely association with androgen levels that mean central obesity has an important role in PCOS [24].

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. Also the current study demonstrated that rs2227982 Single Nuclear Polymorphism (SNP) of Programmed Cell Death – 1 was reported significantly associated with GA heterozygous genotype in the registered control and PCOS Iraqi patient's groups [30, 31], while there is non- significant association between PDCD-1(rs2227982) and GG genotype in healthy control and PCOS patients group. When the G allele of PD-1 gene is mutated, genotype GA is a risk factor for occurrence of pcos. While, GG genotype consider a protective factor for the occurrence of PCOS disease. which indicates that the PD-1 gene polymorphism is related to the pathogenesis of PCOS [10].

Many studies founded that PD-1 gene (rs2227982) was act as a risk factor in various cancers for examples, leukemia, gastric adenocarcinoma [32], esophageal squamous cell carcinoma [33] and ovarian cancer [34]. 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 [35]. 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. Human preovulatory follicles have been found to include significant numbers of immunocompetent cells, including T and B cells, dendritic cells, and macrophages [12].

5. Conclusion

In conclusion, 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 aid in genetically screening susceptible populations and offer theoretical justifications for PCOS patient diagnosis and therapy. Also the observed results indicated that there is grand increase in the mean of Programmed Cell Death-1 protein in PCOS patients with significant association between PD-1 level and PD-1 gene (rs2227982) GA genotype in the registered PCOS and control group. Various hormones which are free testosterone, Prolactin and LH in obese PCOS women are significantly increase also as compared with control group, while there is significant decrease in FSH values was obtained.

Research Highlights

What is the current knowledge?

✓ PCOS is a heterogeneous condition inherited by autosomal dominant way.

✓ Immune check point PD-1 is crucial for preventing cancer, autoimmune disease and inflammation.

✓ PCOS is a chronic low- grade inflammation condition.

What is new here?

✓ PD-1 protein increase in PCOS patients.

✓ PD-1 gene (rs2227982) GA genotype associated with PCOS susceptibility and pathogenesis.

✓ There is positive association between PD-1 gene (rs2227982) GA genotype and PD-1 level in PCOS patients.

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Funding Sources:

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Ethical Statement:

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.

Competing Interest

The authors advertise that they have no conflict of interest.

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