Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

The Effect of Interleukin-6 and Tumor Necrosis Factor-Alpha Gene Polymorphism and Hormone Replacement Therapy on Polycystic Ovary Syndrome

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ARTICLE INFO

Original paper

Article history:
Received: June 28, 2021
Accepted: September 13, 2021
Published: December 15, 2021

Keywords:
IL-6; TNF-α; SNP; HRT; PCOS

ABSTRACT

PCOS (polycystic ovarian syndrome) is a prevalent and complicated gynecological endocrine disease that affects around 6% to 10% of women of reproductive age. PCOS is marked by oligoanovulation or anovulation, hyperandrogenism, hyperinsulinemia, monthly irregularity, and infertility. This study included 58 Kurdish females with PCOS who went to private clinics at Hawler city. The disease was confirmed by the doctors with laboratory results and US checking. They were at different age groups with different marital statuses. Demographic distribution, hormonal level and hormone replacement therapy were measured. Cytokine gene polymorphisms were evaluated by Single nucleotide polymorphism (SNP). The amplification-refractory mutation system (ARMS) was used to determine the gene polymorphisms. There was a significant change in the hormone levels and the medications as hormone replacement therapy gained best results for impregnation of the patients by Progyluton, Diane35 with metformin. Results of genetic variations in the evaluated cytokines revealed that for IL-6-174GC polymorphism the CC genotype was considered as a risk factor with OR:1.58, CI:0.16-15.36. While for TNF-α the higher producer GG genotype was the most susceptible cause of the disease with OR:1.41, CI: 0.59-3.36. Data of this study indicated the positive relationship between IL-6 -174GC polymorphism with PCOS while no association was detected for TNF-α -308GA.

DOI: http://dx.doi.org/10.14715/cmb/2021.67.5.38

Introduction

PCOS (polycystic ovarian syndrome) is a prevalent and complicated gynecological endocrine disease that affects around 6% to 10% of women of reproductive age. PCOS is marked by oligoanovulation or anovulation, hyperandrogenism, hyperinsulinemia, monthly irregularity, and infertility (1). Dyslipidemia, atherosclerosis, obesity, hirsutism, insulin resistance, and an increased prevalence of type 2 diabetes mellitus are all linked to PCOS. According to recent research, 15 to 55 percent of PCOS patients are diagnosed with nonalcoholic fatty liver disease (NAFLD) (2). Furthermore, 32.7 percent to 44.6 percent of PCOS individuals have metabolic syndrome. Patients with PCOS who also have NAFLD are more likely to develop metabolic syndrome. Although the specific etiological process of PCOS is unknown, there is evidence that genetic factors play a significant role (3).

PCOS is caused by a variety of environmental factors, including alcohol consumption, eating food packaged in plastic, adrenal dysfunction, and obesity. PCOS has an etiology that includes epigenetic alterations as well as various genetic changes that are yet unknown (4).

Some findings demonstrate that a dietary stimulus like glucose can increase inflammatory response in mononuclear cells (MNC) of women with PCOS, regardless of body mass, and that there is a link between inflammation and insulin resistance in PCOS (5).

Inflammation associated with PCOS has a hereditary basis. PCOS has been linked to variations in genes producing numerous pro-inflammatory cytokines and their receptors that have been linked to insulin resistance, obesity, and diabetes. In European groups with similar clinical characteristics for PCOS and associated metabolic problems, SNPs in the genes producing interleukin-6 (IL-6) and its signal transducer, as well as tumor necrosis factor-a (TNF-a), have been linked to PCOS (6).

Interleukin 6 (IL-6) is a multifunctional cytokine that

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plays an essential function in reproductive physiology as a pro-inflammatory and immunomodulatory cytokine. IL-6 is implicated in the development of atherosclerosis, ovarian steroid production, fertilization and implantation, coronary heart disease, osteoporosis, and allergic responses, among other human illnesses and pathophysiological processes (3,7). The IL-6 gene has a 303-bp promoter and is found on chromosome 7p21–24. A frequent single-nucleotide polymorphism (SNP) in the promoter region of IL-6 that results in a change of G>C 174 has been shown to affect the gene's transcription rate (7). A study was conducted on the association between IL–6 –174G/C and PCOS susceptibility. The conclusions, however, are ambiguous and contentious, owing to the diverse demographics and small sample numbers. As a result, we used a meta-analysis to look into the link between the IL–6 –174 G/C (rs1800795) gene and PCOS susceptibility (3).

TNF gene producer and coding site diversity might alter a cytokine's secretary responsiveness. In the human ovaries, TNF-a governs granulose cellular proliferation, follicular growth, ovulation and luteolysis, steroidogenesis, and prostaglandin production, among other biological properties. Modifications in completely impervious TNF-a tiers and release out from corpus luteum have been documented during the menstrual cycle (8). Genetic differences change the engagement location of particular transcriptional regulators, which affects transcriptional control. Multiple promoter polymorphisms have been linked to pathological diseases such as insulin resistance, adiposity, preeclampsia, endometriosis, and PCOS. In premenopausal persons, a significant single nucleotide polymorphism (SNP) at position -308 (rs1800629) of the TNF-a gene has indeed been tied to modified promoter activity and varied plasma levels of TNF-a (3). In immature follicles from the human ovary, TNF-a has been seen to suppress follicle-stimulating hormone (FSH)-induced estradiol production. Those twin TNF-a pathways may be linked to ovarian steroidogenesis issues and metabolic syndrome. TNF-a has been linked to obesity, glucose intolerance, and premature ovarian failure, all of which are characteristics of PCOS (9).

Materials and methods

This study included 58 Kurdish females with PCOS who went to private clinics at Hawler city. The disease was confirmed by the doctors with laboratory results and US checking. They were at different age groups with different marital statuses. A questionnaire form was filled for them by direct interview with them. After their agreement, the blood samples were collected. In addition to patients, 30 healthy Iraqi Kurdish subjects were included as a control sample in the study. From each participating subject, 5 ml of venous blood was drawn using a disposable syringe, and distributed into two aliquots. The first aliquot (3 ml) was dispensed into a plain tube and it was centrifuged (15 minutes at 3000 rpm) in a temperature-controlled centrifuge (4°C). The separated serum was distributed into 3 aliquots in Eppendorf tubes, which were frozen at -20˚C until an assessment of hormones and cytokine serum levels. The second aliquot (2 ml) was transferred to an EDTA tube and frozen at -20˚C until DNA extraction for genotyping of cytokine gene polymorphisms.

The genomic DNA was isolated and extracted from the venous blood of the studied samples according to the manufacture's protocol. The primer sequences were as follows IL -6 generic primer, 5′-GCC TCA GAG ACA TCA CCA GTC C-3′, IL -6 (G) Allele Primer 5′-CCC CTA GTT GTG TCT TGC G-3′, and IL -6 (C) Allele Primer 5′-CCC CTA GTT GTG TCT TGC C-3′. And for TNF-α-308 generic primer 5'-TCC TCC CTG CTC CGA TTC CG-3' , TNF-α A allele primer 5'-CAA TAA GTT TTG AGG GGC ATG A-3' and G allele primer 5'-CAA TAA GTT TTG AGG GGC ATG G-3'. The PCR reaction was carried out in a thermal cycler (PX2) with the following program for IL-6-174. The samples were placed in a 20 µL reaction volume containing 40 ng genomic DNA, 1.5 mM dNTPs, 25 mM MgCl2, 1 µL of 10 pmol of each primer, and 0.4 units of Taq polymerase (Fermentas, Maryland, USA) in 1X Reaction Buffer. Cycling conditions were as follows: 1 minute at 95ºC, followed by 10 cycles of 15 seconds at 95°C, 50 seconds at 58°C, 40 seconds at 72℃, followed by 20 cycles of 20 seconds at 95℃, 50 seconds at 54°C and 50 seconds at 72°C, with 5 minutes at 72℃ as the final extension, 230bp. And for TNF-α-308, a primary 4-min denaturation at 95°C, followed by 35 30-s cycles at 95°C, 60°C, and 74°C. The final extension step was at 74°C for 6 min, 104bp. The amplified products were analyzed on a 2% agarose gel.

Results and discussion

The characteristics results of the study found that the age of the patients was ranged from (18-46) and they were as different marital statuses 43(74.13%) were married and 15(25.87%) of them were single. Another symptom was heurism when most of the patients (69%) were had heurism. Menstrual cycle was another factor considered in this study, most of the patients had irregular menstruation (31%), and only (69%) had a regular menstrual cycle. Body mass index was taken from the patients, the results were (6.9%, 31%, 51.7% and 10.3%) for underweight, normal weight, overweight and obesity respectively table (1).

Table 1. Percentage of patient's characters of the studied group

Characters	No. (%) of patients
Age	18-46 years
Social Status	Married =43 (74.13%) Single=15 (25.87%)
Hair Situation	Yes=40 (69%) & No=18 (31%)
Misstate Cycle	Regulars=18 (31%) & Irregulars=40 (69%)
BMI	Underweight =4 (6.9%)
	Normal weight =18 (31%)
	Overweight=30 (51.7%)
	Obesity =6 (10.3%)

Noticeably there was an increase in hormone levels in PCOS patients when compared to the control group. The level of FSH and LH hormones was significantly increased in patients, ($p \leq 0.05$). the results were also the same for LH:FSH ratio, when there was an elevation in the ration with p=0.05 table (2).

Table 2. The relation of LH, FSH hormone between control and PCOS patients

Hormones Evaluation	Mean±SD Healthy Control (mIU/L)	Mean±SD PCO (mIU/L)	P value
FSH	9.576 ± 1.249	5.884 ± 0.9762	0.0327
LH	11.86 ± 1.098	8.439 ± 0.8122	0.0228
LH:FSH Ratio	1.255 ± 0.1356	1.705 ± 0.1702	0.0402

Different regimen of medications was used to treat this disease, particularly some of the medications that were used to treat the symptoms within the condition. Especially to treat the hormonal imbalance and the existence of the male hormone activity such as acne or excessive hair growth. High amounts of androgens obstruct the proliferation of eggs as well as their liberation. Medications should be used to encourage ovulation and regulate menstruation. Table 3 show the different type of medications used by the doctors to treat the patients. Good results were obtained after the medication was given and the patients almost had normal menstruation and they got impregnated within a different period, ranging from 3-9 months. Others do not respond well to the medication and they were recommended to another type of therapy rather than medication for example surgery. Besides the medications, doctors recommended changing and regulating the lifestyle of the patients like a healthy diet, physical activity, etc…

Table 3. Medications used to treat PCOS patients and their dosages

Medications	Dosage	Duration
Progyluton	500mg daily	1-2 month
Diane35	4mg daily	4-8 months
Diane35+ Metformin	500-100 mg daily	2-3 weeks
Myo inositol	600-2400 mg daily	2-3 months

Results of the study showed different numbers and frequencies in all three genotypes for both patients and control groups regarding numbers and percentages for observed and expected values. There were no significant differences in the genotypes (GG, GC, and CC) in patients and controls, according to Hardy-Weinberg equilibrium. In the association between IL-6-174 genotypes or alleles in the studied groups, the GG genotype is related to the PCOS disease with OR:1.35 and 95% CI:0.56-3.27. While in CC genotype the ratio was higher and this genotype was considered as a risk factor for PCOS with OR:1.58 and 95% CI:0.58-2.19 (Table 4 and 5).

Table 4. Statistical evaluations of associations between *IL6*$_{-174}$ genotypes or alleles and PCOS patients

IL6$_{-174}$ Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
GG	1.35	0.16	Not significance	0.56-3.27
GC	0.67	0.13	Not significance	0.27-1.67
CC	1.58	0.01	Not significance	0.16-15.36
G	1.12	0.08	Not significance	0.58-2.19
C	0.89	0.02	Not significance	0.46-1.73

Table 5. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *IL6*-$_{174}$ genotypes and alleles in PCOS patients and controls

Groups			*IL6*-$_{174}$ Genotype or Allele					H-W P ≤
			GG	GC	CC	*G*	*C*	
PCOS (N=58)	Observed	No.	37	18	3	92	24	Not significant
		%	63.8	31	5.2	79.3	20.7	
	Expected	No.	36.5	19	2.5	Not Estimated		
		%	62.9	32.8	4.3			
Controls (N=30)	Observed	No.	17	12	1	46	14	Not significant
		%	56.7	40	3.3	76.7	23.3	
	Expected	No.	17.6	10.7	1.6	Not Estimated		
		%	58.7	35.7	5.3			

About the results for TNF-A-308, the results were as follows for the three genotypes (GG (32 and 14), GA (25 and 15) and AA (1and 1) for patients and control groups respectively. The results of observed and expected values were in disagreement with the Hardy-Weinberg equilibrium, for both groups. Genotypes in TNF-A-308 were as GG with relative risk OR:1.41 and 95% CI:59-3.36 and AA genotype as a protective factor with OR:0.51 and 95% CI:0.03-8.13 (Tables 6 and 7).

Table 6. Statistical evaluations of associations between *TNF*-$_{308}$ genotypes or alleles and PCOS patients

TNF-$_{308}$ Genotype or Allele	Statistical Evaluations			
	Relative Risk	Etiological or Preventive Fraction	Fisher's Exact Probability	95% Confidence Intervals
GG	1.41	0.16	Not significance	0.59-3.36
GA	0.76	0.12	Not significance	0.32-1.81
AA	0.51	0.02	Ref.	0.03-8.13
G	1.3	0.18	Not significance	0.69-2.46
A	0.77	0.07	Not significance	0.41-1.45

Table 7. Observed numbers and percentage frequencies and Hardy-Weinberg (H-W) equilibrium of *TNF*-$_{308}$ genotypes and alleles in PCOS patients and controls

Groups			TNF-$_{308}$Genotype or Allele					H-W P ≤
			GG	GA	AA	G	A	
PCOS (N=58)	Observed	No.	32	25	1	89	27	Not significance
		%	55.2	43.1	1.7	76.7	23.3	
	Expected	No.	34.14	20.72	3.14	Not Estimated		
		%	58.9	35.7	5.4			
Controls (N=30)	Observed	No.	14	15	1	43	17	Not significance
		%	46.7	50	3.3	71.7	28.3	
	Expected	No.	15.41	12.18	2.41	Not Estimated		
		%	51.4	40.6	8			

PCOS is a prevalent endocrine condition characterized by a number of genetic and epigenetic changes. Polymorphisms in cytokine genes may have a role in the induction of PCOS (10). IL-6, a common combinatorial cytokine, has been shown to alter fertilization, implantation, and ovulation processes, all of which are impaired in women with PCOS. Many genetic research has been carried out to look into the links between the IL6 rs1800795 polymorphism and PCOS risk, however, the results have been equivocal (3). Because of the limited sample size, single research does not have enough statistical power to confirm the link between rs1800795 and PCOS risk. They conducted a meta-analysis in a large sample size (512 patients with PCOS and 606 controls) to evaluate the correlations between PCOS susceptibility and the IL-6 174 G/C (rs1800795) functional polymorphism to investigate the unclear relationship between these two entities (11). The findings showed that IL-6 rs1800795 was linked to a lower incidence of PCOS in all of the groups examined. The findings showed that the IL-6 rs1800795 polymorphism is a PCOS susceptibility protective factor. Because of the previously mentioned issue, a prior meta-analysis with just four studies including 351 cases and 464 controls was unable to confirm a connection between these two elements (12). Furthermore, in a retrospective study of

studies that adhered to the HWE under the allele model, recessive model, and dominant model, they failed to find a link between the IL-6 rs1800795 polymorphism and PCOS susceptibility. However, we discovered a statistically significant association between studies that followed the HWE under the allele model and dominant model in our research (3). A further meta-analysis discovered a significant association between the IL-6 rs1800795 polymorphisms and the risk of PCOS in the allelic, homozygous, heterozygous, and dominant models; however, the results were inaccurate because some original data from the included studies were incorrect when they were extracted (13). In healthy women, for particular, there were 35 G/C genotypes, but in the research stated above, there were only 25. The number of C/C genotypes in PCOS patients was 56, compared to 36 in the research stated above (14). These errors, according to the researchers, made the results untrustworthy. Because of the tiny sample size, no definitive conclusion could be made. The goal of this research was to provide solid evidence for the link between IL6 rs1800795 polymorphisms and PCOS risk (15). Although the P-value under the recessive model was modest, the data showed that the IL-6 polymorphism rs1800795 likely has a protective impact on PCOS. One probable explanation for this link is that the C allele causes a decrease in IL-6 production, which aids in the normalization of ovarian function (11).

On the basis of our results, we suggest that IL6 cannot be regarded as a candidate gene for PCOS. The existence of the IL6 polymorphism appears to be connected with clinical features of PCOS, despite the fact that it is not linked to the condition's development. Women harboring a least one mutant allele of IL6 were more likely to have a pathological OGTT result, increased serum T levels, and a higher BMI (16).

The TNF—308 G/A nucleotide variation was picked to be examined in just this particular demographic because it has a significant impact on the expression of genes (7). The study revealed no link in between gene polymorphism and PCOS, and no distinction between patients and healthy controls, with allele A accounting for 34.28 percent of the population vs allele G accounting for 2.85 percent. There was no considerable variation in allelic diversity between sufferers and the control subjects populace in Turkish and Chinese research (17).

TNF-α is a major contributor of genetic variation. The regulatory region of the TNF-a gene has numerous single nucleotide polymorphisms, many of which have been linked to the development of insulin resistance, type 2 diabetes, and obesity. The main pathogenic factors of PCOS are hyperglycemia and obesity (18).

According to a study, carriers of the -308 A allele had higher levels of serum androgen and 17-hydroxyprogesterone before and after stimulation with the GnRH analogue leuprolide, implying that, irrespective of obesity and insulin resistance, the TNF-system may have a role in the pathophysiology of hyperandrogenism (3). To regard TNF-α as a key contributing element to the development of hyperandrogenism, specific techniques to identify the connection between the TNF-system and androgen excess are necessary (19). These data suggest that phenotypic clinical characteristics in PCOS patients may be influenced by TNF-α gene variation. This analysis revealed a substantial disparity between the sufferers and the comparison group when testing total cholesterol, LDL-C, and HDL-C. Numerous research that were required to determine a significant relation respectively dyslipidemia and PCOS by attempting to find a relationship with obesity and others that show that perturbation in plasma lipids associated with PCOS irrespectively of BMI as in the Romanian given situation and the implications the increase in total cholesterol, LDL-C, and decrease in HDL-C. (20).

For the TNF-α and IL-10 genes, there were no or very few differences in allele or genotype frequencies across groups. Out of a total of 217 study patients, Yun et al. (2011) included 144 healthy women as controls (21).

TNF-α, a multipurpose proinflammation cytokine, regulates a variety of diseases. It can be found in human ovarian follicular fluid as well as oocytes and granulose cells. TNF-α is thought to be linked to ovarian apoptosis, anovulation, and enhanced ovarian steroid production. (21). The TNF-α (rs1799724) C/T polymorphism findings likewise revealed that the majority of female patients and controls were homozygous for CC, with around 12% of controls and 14% of PCOS patients having the heterozygous (CT) condition as well as the wild type allele (CC) (22). Overall, there was no significant difference between the PCOS and control groups. When we compared our findings to those described in the literature, we found that our findings were consistent with one research

that concluded that numerical combinations of TNF genetic variants had no association with PCOS (23). On other hand, Yun et al. (2011) conducted research in which 217 PCOS patients and 144 female controls (healthy women) were investigated (21). The -1031(T/C) polymorphism of the TNF-α gene was compared to PCOS in a Korean population, and the results revealed a substantial connection between PCOS (P-value = 0.0003, OR = 2.53) and the -1031(T/C) polymorphism in the TNF-α gene promoter region. Furthermore, in comparison to PCOS patients, the C allele was less common in controls (24). Thathapudi et al. (2014) found in an Indian population that the TNF system may have a role in the etiology of HA, Ob, and IR in PCOS patients, irrespective of the TNF-α -C850T (rs1799724) polymorphism. (22).

Obesity or overweight affects 60–70% of women with PCOS, and obesity is linked to insulin resistance. Insulin resistance is found in many slim women with PCOS, according to several research (25). A deficiency in insulin binding to its receptor or alterations in insulin signal transmission are two processes that contribute to insulin resistance (26). These women's ovaries, on the other hand, retain a normal insulin response. Insulin's effect on the ovary via the IGF-1 receptor provides a partial explanation for this process. As a result of anticipatory hyperinsulinemia, this binding occurs when insulin concentrations are high. Furthermore, insulin's effect on the ovary employs the inositol glycan system as a signal mediator, which is different from the system triggered by tyrosine phosphorylation of the receptor in other tissues (27). In certain PCOS women from the United States and Greece, urine inositol clearance increased. It decreases inositol availability in the tissues. This pathway may have a role in insulin resistance in PCOS women (28). By engaging in thecal and granulosa cells, hyperinsulinemia promotes ovarian steroidogenesis directly. Insulin promotes thecal cell proliferation, increases androgen production mediated by LH, and enhances cytochrome P450 expression of LH and IGF-1 receptor *in vitro* studies. Because the enzymes involved in ovarian steroidogenesis and adrenal steroidogenesis are similar, several investigations have suggested that insulin can serve as a direct stimulant of adrenal steroidogenesis (29,30). Metformin, an insulin-sensitizing medication, dramatically decreases the synthesis of 17OHP, T, and A in PCOS women in response to ACTH (31).

Medications for the polycystic ovarian syndrome (PCOS) can help you control your symptoms and reduce your risk of long-term health issues including diabetes and heart disease. You and your doctor should discuss your objectives so that a treatment plan may be devised. If you want to get pregnant but are experiencing difficulties, for example, your therapy will focus on assisting you in conceiving. If you wish to treat PCOS-related acne, your therapy will focus on skin issues (30).

Eating healthily and exercising frequently are two of the most effective methods to deal with PCOS. PCOS affects a large number of women who are overweight or obese. Losing just 5% to 10% of your body weight will help to alleviate certain symptoms and make your periods more regular. It may also aid in the management of blood sugar levels and ovulation issues. Your doctor may advise you to avoid starchy or sugary meals since PCOS can cause high blood sugar. Conversely, consume foods and meals high in fiber, which will gently boost your blood sugar level. Staying active also aids with blood sugar and insulin management. Additionally, exercising every day will assist you in losing weight (31).

For women who don't want to get pregnant, birth control is the most frequent PCOS therapy. Hormonal birth control, such as tablets, a skin patch, a vaginal ring, injections, or a hormonal IUD (intrauterine device), can help you get your periods back on track. Acne and unwanted hair growth are also treated with hormones. These birth control techniques may help reduce your risk of endometrial cancer, which affects the uterus' inner lining (32). Taking only a hormone called progestin might help you regain control of your periods. It doesn't stop pregnancies or cure acne or undesirable hair growth. It can, however, reduce the risk of uterine cancer. Metformin (Fortamet, Glucophage) is an insulin-lowering medication. It can aid weight loss and may help you avoid developing type 2 diabetes. It could also help you become more fertile (33-35).

Conclusion

Results of the present study found that there might be an association between IL-6-174 and TNF-α-308 SNPs and susceptibility to polycystic ovary syndrome. IL-6 CC low producer and TNF-α GG high producer were among the most susceptible for having the

disease. In light of the complexities and confusion surrounding the etiology of such disease, cytokines play a critical role in the disease's course and effects. The disease will be studied more for its exact etiology and development triggers, like cytokine SNPs and combination of their serum level.

Acknowledgments

None.

Interest conflict

The authors declare no conflict of interest.

Author's contribution

Sarhang Hasan Azeez conceived of the presented idea. Carried out the statistical analysis.

Ismail Bilal Ismail sample collection and interview with the patients. worked out almost all of the technical details

Suhaila Nafia Darogha wrote the paper with input from all authors.

All authors discussed the results and contributed to the final manuscript.

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