Polymorphism of tumor necrosis factor-alpha 308 G/A gene in Iraqi patients with polycystic ovarian syndrome

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Objective This study was designed to understand the etiological molecular role of tumor necrosis factor-alpha (TNF-α) 308 polymorphism as pro-inflammatory cytokine in the pathogenesis of this syndrome.

Methods Genetic evaluation of 70 women with PCOS occur when the promoter region of the gene was amplified by polymerase chain reaction (PCR) and the presence or absence of the polymorphism at -308 was determined and compared with 30 healthy control women. In addition to the routine investigations and hormonal assay, this study includes the measurement of body mass index (BMI), lipid profile and glucose level in both patients and control group.

Results It was found that TNF-α, 34.28% of (AA) allele, 2.85% of (AG) allele and 62.85% of (GG) allele this percentage detected in PCOS patients and compared with 20% of (AA) allele, 80% of (GG) allele with no (AG) alleles found in healthy control group (P > 0.05).

Conclusion This study appeared no significant association between TNF-α gene polymorphism with PCOS. The relation of TNF-α 308 polymorphism with clinical and biochemical parameters examined and it found significantly linked with androgen excess in patients with PCOS as compared with control group. No correlation found for other clinical or biochemical measurements in PCOS and non PCOS groups.

Keywords TNF-α, PCOS, polymorphism, allele

Introduction

Hormone investigation (FSH, LH and E2) is necessary and FSH above 40 IU/l and estradiol under 50 pmol/l in women aged below 40 years approve the diagnosis.1 Both primary and secondary forms of ovarian failure are biochemically described by low levels of gonadal hormones (like, estrogens) and extraordinary gonadotropins (LH and FSH) (hypergonadotropic amenorrhea). The elevation of FSH is frequently more marked than that of LH and, an FSH value >30 IU/l is indicative of ovarian failure.1, 2

Polycystic ovarian syndrome (PCOS) is a pro-inflammatory called hyperandrogenic anovulation. It is an endocrine-metabolic disturbance, which has features of multiple hormonal imbalances that produce short- and long-term consequences on women health.3 It’s affecting about 9–18% of studied populations.4 Polycystic ovary including granulose cell proliferation, follicular development, ovulation and luteolysis, steroidogenesis, and prostaglandin biosynthesis. Variations in the levels of immune-reactive TNF-α throughout the menstrual cycle and its secretion from the corpus luteum have been reported.6

Polymorphisms affect transcriptional regulation by modifying the binding site of specific transcription factors. Several polymorphisms in the promoter region associated with pathological conditions such as insulin resistance, obesity, pre-eclampsia, endometriosis and PCOS. Functional single nucleotide polymorphism (SNP) at position -308 (rs1800629) of TNF-α gene has an evidence to be related to altered promoter activity with different plasma levels of TNF-α in healthy women.4

The A allele of rs1800629 polymorphism has been detected to be involved with increasing adiposity, insulin-dependent diabetes and development of insulin resistance. Polymorphism that guanine substituted by adenine at position -308 in the promoter region of the TNF-α gene has been shown to increase transcriptional activity of the TNF-α gene in vitro.9

Tumor necrosis factor TNF-α is a pro-inflammatory cytokine secreted and expressed in adipose tissue and many sites in the body, and it thought to play an important role in insulin resistance by decreasing the tyrosine kinase activity of the insulin receptor, hyper-androgenism.11
TNF-α may be connected to androgen metabolism. In a Spanish study, lean hyperandrogenic women had slightly higher serum TNF-α concentration than the lean controls, and the carriers of the TNF-α -308. A variant had higher androgen concentrations than those with the -308 G allele only in the whole study population.12 The presence of the TNF2 has been related to both insulin resistance development and increasing adipose tissue.13

TNF-α found to inhibit follicle-stimulating hormone (FSH)-induced estradiol secretion in small follicles from the human ovary. These two mechanisms of TNF-α may correlate the ovarian steroidogenesis problems with those of insulin resistance.

In view of the strong evidence implicating TNF-α in adiposity, insulin resistance and anovulation and all represent features of PCOS.14

This study was designed to understand the etiological molecular role of TNF-α 308 polymorphism as pro-inflammatory cytokine in the pathogenesis of this syndrome. This study examined the association of TNF-α 308 G/A gene polymorphism with PCOS and its clinical, anthropometric parameters in Karbala city of Iraq.

Materials and Methods

This study was carried out during December 2014 and November 2015 in a total number of 100 women within the reproductive age (18–45 years old). Seventy women patients out of 100 were attended from gynecological and obstetric hospital in Karbala Province/Iraq, and they all diagnosed by their physicians as PCOS (depending on Rotterdam criteria). The patient should meet any two of the following three criteria.

(i) oligomenorrhea or amenorrhea for at least 6 months
(ii) clinical and/or biochemical signs of hyperandrogenism
(iii) polycystic ovaries on ultrasound and they compared with thirty healthy control women with comparable age.

Thirty, healthy, regular-menstrual cycle women, fertile control group with age range 18–45 years were included. The history recorded for the infertility type (primary, secondary), menstrual history, history of previous medical condition, drug history in addition to biochemical, hormonal, ultrasound records.

Exclusion criteria include postmenopausal women (age >45 years). Other disorders that may affect menstrual regularity and hyperandrogenism such as adrenal tumor, congenital adrenal hyperplasia, Cushing syndrome, thyroid dysfunction, and pituitary disease related to hyperprolactinemia, cause infertility other than PCOS (female and/or male factor).

Serum hormonal analysis of LH, FSH, and total testosterone were measured by using MiniVidas instrument. Serum levels of glucose, total cholesterol, triglycerides, and high-density lipoprotein-cholesterol (HDL-C) were measured by a commercially available kit (all kits from Biomerieux, SA, France) and measured by using UV-Visible spectrophotometer. Low-density lipoprotein-cholesterol (LDL-C) and very low-density lipoprotein-cholesterol (VLDL-C) levels were calculated using Friedwald formula.

Genomic DNA was isolated from blood samples using a DNA purification kit (Bioneer-Korea). The TNF-α 308 G/A was genotyped using ARMS-PCR. Primers were derived from genomic polymorphism of TNF-α gene is:

Forward inner allele (A allele): 5’ TGGAGG CAATAG GTTTTG AGG GGG CAG GA-3’
Reverse inner primer (G allele): 5’ TAG GAC CCT GGA GGCTGAAACCGGTACC-3’

Forward outer primer: 5’ ACCCAA ACA CAG GCC TCA GGA CTC ACA-3’
Reverse outer primer: 5’ AGT TGG GGA CAC GCA AGC ATG AAG GTA-3’

PCR was carried out in a 20 μl premix tube mixture containing; added 0.2 μl from outer F primer, 0.2 μl from outer R primer, 2 μl of inner F (A) primer and 2 μl of inner R (G) primer 5 μl of DNA product and complete the volume up to 20 μl with distilled water. The PCR run cycle conditions are indicated below:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time</th>
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<tr>
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<tr>
<td></td>
<td>95°C</td>
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<td>Stage 2</td>
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<tr>
<td>Stage 3</td>
<td>72°C</td>
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Electrophoresis by using agarose gel stained by ethidium bromide used for validity of genotype of PCR, ladder (100 bp) used as standard for alleles comparison.

Statistical Package for Social Sciences (SPSS) version 22 software program was utilized to perform data analysis. Numeric variable were presented as Mean ± SE (Standard Error), Mean ± SD (Standard Deviation), while nominal variables were expressed as number and percent. Chi-square test was used for comparison of frequency for genotype analysis and it is also used for describing the correlation between genetic alleles with other numeric variables. Pearson’s correlation coefficient was used to study correlation between numeric variable in a specified group (for patients and control group, respectively). P value was considered significance when it is less than 0.05.

Results

**Tumor Necrosis Factor-α (-308 G/A) Gene Polymorphism**

The genotype distribution occurs both in patients and control and according to allelic distribution as, homozygous (either AA or GG allele found), and heterozygous mutation (presence of AG alleles). It has been found that there is no significant
The correlation of genetic mutations of TNF-α -308 G/A allele polymorphism with age, BMI and other biochemical parameters in patients and control groups.

TNF-α gene was classified according to allelic distribution into homozygous, heterozygous, or absence of mutation alleles. The age variable has been subdivided into age interval groups (<25 years, 25–34 years, 35–45 year) when correlated with TNF-α (allele A,G) in both patients and control groups and statistically using Chi square test. There was no significant correlation found between age and TNF-α for both alleles in patients group. BMI has been divided according to severity of increase body weight into five categories: normal (<25 kg/m²), over weight (25–29.9 kg/m²), obese (30–34.9 kg/m²), very obese (35–39.9 kg/m²), morbid weight (≥40 kg/m²).

No significant correlation has been found between BMI and TNF-α gene in patients while significant correlation seen in control group. Other biochemical parameters have been classified according to their normal value as follows:

Total cholesterol (<200, >200 mg/dl), triglyceride (<150, >150 mg/dl), HDL-C (<50, >50 mg/dl), LDL-C (<160, >160 mg/dl), glucose (<110, >110 mg/dl) testosterone (<0.8, ≥0.8 mg/dl) and LH/FSH ratio (≤0.5, >0.5). And according to this classification TNF-α -308 G/A gene has been correlated with each parameter by using Chi square test as shown in Table 2.

High significant correlation has been found between TNF-α gene and total testosterone hormone in PCOS group while no significant correlation found in control group. No significant correlation seen for other biochemical parameters and TNF-α gene in both patients and control groups.

**Discussion**

Polycystic ovarian syndrome can be described as an oligogenic disorder has pro inflammatory state as the interaction of a number of genetic and environmental factors may determine the heterogeneous, clinical, and biochemical phenotype of this syndrome.15

It was believed that there are no particular genes accepted to play a significant role in the etiology of this syndrome due to absence of specific diagnostic criteria that depends on PCOS diagnosis with failure to identify a complete knowledge about the pathophysiology of this disorder, also the age ranging interval for women must be studying via reproductive period only, and due to the study design population that contain limited patients number with only one or two genes variants that included in the study.16

TNF-α 308 G/A polymorphism variant was chosen to be studied in this group population which is directly affecting the gene expression among these variants.17

This study finds no association of this gene polymorphism with PCOS and no difference seen between patients and healthy control group with predominant allele A distribution with 34.28% in contrast to allele G with 2.85%. Turkish, Chinese studies concluded the same result by finding no significant difference in allelic distribution in patients and control group population.18

South Indian study reported the same finding that suggests no association of this polymorphism and PCOS. On the other hand the same study confirms an association of -1031T/C

![Fig. 1 Detection of TNF-α 308 A/G gene polymorphism by ARMS-PCR. PCR products with three possible genotypes (AG, AA, or GG).](image-url)
which is other type of variant polymorphism of TNF-α gene with this syndrome.19

TNF-α is a significant source of genetic variability. There are many single nucleotide polymorphisms in the regulatory region of the TNF-α gene, and some of them have been suggested to play a role in the pathogenesis of insulin resistance, type 2 diabetes mellitus, and obesity. Insulin resistance and obesity represent the major pathogenic causes of PCOS. The variants that are studied up to date are -308 G/A, −1196 C/T, −1125 G/C, −1031 T/C, −863 C/A, −857 C/T, −316 G/A, −238 G/A, −163 G/A. The polymorphisms in the TNF-α gene do not seem to have a key role in the pathogenesis of PCOS.20

A study in 2002 on −850 C/T polymorphism in Caucasian population show no significant correlation with PCOS disorder,21 while other Indian study show TNF-α contributes to the clinical, biochemical manifestations of PCOS, and −C850T TNF-α gene polymorphism is associated with PCOS and could be used as a relevant molecular marker to identify women with risk of developing PCOS in studied population.22

The first study on the -1031(T/C) polymorphism of TNF-α gene in PCOS was in Korean population and concluded that the -1031(T/C) polymorphism of TNF-α gene is associated with PCOS patients.23

In Iraqi population from Kerbala province, TNF-α 308 G/A polymorphism show correlation with total testosterone level in PCOS in contrast to normal control group and no significant correlation with other biochemical parameters in patients and control group. A study show that the carriers of -308 A alleles showed increased serum androgen and 17-hydroxyprogesterone levels before and after stimulation with the GnRH analogue leuprolide suggest that the TNF-system might contribute to the pathogenesis of hyperandrogenism, independent of obesity and insulin resistance However, finding the precise mechanisms to detect the relationship between the TNF-system and androgen excess is required to consider TNF-α as a significant contributing factor to the development of hyperandrogenism.24 These findings may indicate that TNF-α gene polymorphism might be a modifying factor for phenotypic clinical features in PCOS patients.20 This study show that significant difference found in measuring total cholesterol, LDL-C and HDL-C between patients and control group. The elevation of total cholesterol, LDL-C and decreased in HDL-C was explained by many studies that done to find a correlation between dyslipidemia and PCOS by finding a relation with obesity and others show that disturbance in lipid parameters associated with PCOS regardless BMI as in Romanian study.24

Lipid profile assay is important investigations for PCOS women that has related to the metabolic consequences for these patients and this study show that significant difference found in measuring total cholesterol, LDL-C and HDL-C between patients and control group. The elevation of total cholesterol, LDL-C and decreased in HDL-C was explained by many studies that done to find a correlation between dyslipidemia and PCOS by finding a relation with obesity and others show that disturbance in lipid parameters associated with PCOS regardless BMI as in Romanian study.24

Studies show difference in the prevalence of metabolic syndrome that associated with PCOS in Italian women, the prevalence of obesity and hypertriglyceridemia was markedly less than in USA women due to difference in diet habits in USA and Italy. Also, in the USA, alterations in HDL-cholesterol were linked to a higher proportion of saturated fat in the diet.25 Other study reports lipid abnormalities have been shown in PCOS patients with higher androgenic phenotype as compared with other PCOS subgroups.

The conflicted results also suggested by other study indicates the association of hyperlipidemia with PCOS while other found no significant differences in lipids or lipoprotein concentrations between PCOS patients and controls.26 These variations in results may be linked to the difference in ethnicity and different dietary habits.

Other complication that associated with PCOS is insulin resistance that plays significant role in the pathology of PCOS and represent the major cause for patients to have type II DM. Most methods that used to evaluate insulin resistance are expensive, complicated operations, and need time.27

In this study, fasting serum glucose level was used as indication for insulin resistance and it was found to be within normal range and there is no significant difference found between patients and control group. This finding indicates no insulin resistance found in this case study group. A study done in UAE reported the same result.28

LH show significant increase in patients in contrast to control group and it represent the main factor affecting the pathology of PCOS by inducing ovarian receptors to produce higher amount of androgen. Mean concentration of LH is 8.94 ± 0.42, normal level of LH was found in patients diagnosed with PCOS, and this explained by the elevation in pulse frequency or episodic release of LH.29

Androgen level in patients with polycystic ovary show significant elevation and its elevation is reflected clinically as acne, hirsutism that bother many women and detected biochemically by elevation of total testosterone. This may be attributed to increased synthesis of testosterone precursors due to a dysregulation of cytochrome P<sub>450</sub>(P<sub>450</sub>c17α), the rate-limiting enzyme in androgen biosynthesis in the theca cells of the ovary in PCOS.30

Some patients with PCO who complain from hirsutism, acne with normal level of total testosterone and this explained by knowing that free testosterone has the responsibility for the clinical signs of male androgenic characteristics but it is difficult to be detected by laboratory techniques due to inadequate assay sensitivity to measure low testosterone concentration and other technical aspects. It was believed that 1–2 % of testosterone circulates in its free and biologically active form, while the remaining amount bounded tightly to SHBG (65%) and weakly to albumin (33%) and any alteration of albumin levels or any factor modifying SHBG will affect total testosterone levels. Hyperinsulinemia and obesity, two common factors in PCOS, will decrease SHBG.31

It was reported that androgen and LH concentrations were increased in both obese and non-obese women with PCOS, while FSH was slightly lower in the normal weight women with PCOS as compared to the normal weight controls.25 This study found no correlation of hormonal disturbances with increasing BMI in women suffering from PCOS.
Conclusion
There is no significant association between TNF-α gene polymorphism with PCOS. There is a significant correlation between TNF-α 308 polymorphism with some clinical and biochemical parameters examined and androgen excess in patients with PCOS as compared with control group. No correlation found for other clinical or biochemical measurements in PCOS and non PCOS groups.

Conflict of Interest
None.

References