Original Article

The TNF-α-308G/A Gene Polymorphism and Serum TNF-α Levels in Women With Preeclampsia

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Abstract

Objective: Worldwide, preeclampsia (PE) is a multifactorial disorder reported in 2–5% of pregnancies, which increases mortality during pregnancy. In general, 10–15% of maternal deaths are directly related to PE and eclampsia. One of the susceptibility genes for PE is tumor necrosis factor-α (TNF-α) expressed by most immune cells. TNF-α is a protein involved in various biological processes, including proliferation and apoptosis, as well as the expression of inflammatory genes. The goal of this study was to investigate the role of TNF-α single nucleotide polymorphism (SNP) -308G/A (rs1800629) and their relationship with TNF-α in PE patients.

Materials and methods: The SNP was genotyped in 90 cases and 90 controls. Whole blood was collected from women with PE and normal pregnancy in EDTA containing tubes, and DNA extraction was performed from their blood lymphocytes according to a standard phenol-chloroform procedure. Then, DNA was genotyped by real-time PCR and the polymorphism was detected by TaqMan assay. Serum levels of TNF-α protein were measured by enzyme-linked immunosorbent (ELISA) assay.

Results: TNF-α levels in women with PE were significantly higher than in healthy ones (p<0.001). We did not observe any correlation between allelic outbreak (p=0.3) and TNF-α-308G/A polymorphism (p=0.7) with the incidence of PE.

Conclusion: Although TNF-α-308G/A gene polymorphism does not appear to affect susceptibility to PE, an increased level of serum TNF-α can be used as a predictor for PE during pregnancy. We recommend that more research be conducted on possible factors related to the incidence of PE.

Keywords: Preeclampsia; Tumor Necrosis Factor-alpha (TNF-α); Polymorphism; Real-Time PCR; Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Preeclampsia (PE) is a systemic disorder during pregnancy (1) typically affecting 2–5% of pregnant women, which is a major factor of maternal and fetal mortality (2). Thus far, several theories have been suggested for the pathogenesis of PE. One of the main pathophysiological features of PE is systemic inflammation (3). In PE, the immune responses shift

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to Th1 cellular immunity (4) and T lymphocytes produce pro-inflammatory cytokines (5). Excessive secretion of pro-inflammatory cytokines, especially tumor necrosis factor-α (TNF-α), by activated monocytes and macrophages could cause endothelial activation and damage and result in PE (6). Another feature of PE is hypertension associated with proteinuria. HELLP syndrome is a severe form of PE, which is characterized by hemolysis, increased liver enzymes, and reduced platelet counts (7).

TNF-α is a multifunction inflammatory cytokine implicated in the pathogenesis of many human inflammatory and autoimmune diseases (8). This cytokine is also produced by trophoblast cells during pregnancy (9). Significantly higher plasma TNF-α levels have been observed in women with PE relative to healthy pregnant women (10). Mechanistically, increased TNF-α level induces trophoblast invasion and placentation (11) as well as pro-apoptotic gene expression in human fetal membranes, which accelerates membrane degradation and higher susceptibility to infertility (12). Regulation of TNF-α expression in a developing placenta may have a negative effect on pregnancy survival. TNF-α-308G/A single-nucleotide polymorphism is associated with increasing TNF-α levels in addition to a number of infectious and metabolic diseases (13- 16). Various studies investigating this SNP with PE have reported this correlation (17). However, other studies reported no evidence of an association (5, 18).

Since PE is prevalent in Khuzestan Province of Iran, it is necessary to conduct additional research to discover the factors that trigger and control this disease. Given the conflicting results of previous studies on the correlation of TNF-α-308G/A polymorphism with PE, in this study, we intend to examine this correlation and evaluate serum TNF-α levels in women with PE from Khuzestan Province compared with healthy pregnant women.

Materials and methods

Study groups: This case-control study enrolled subjects who were referred to two hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). The subjects included 90 women with mild PE as the target group and 90 healthy pregnant women for the control group. PE was described based on the classification criteria of National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (19). The inclusion criteria were pregnant women with PE who had elevated blood pressure (>140/90 mmHg) and proteinuria of ≥300 mg in a 24-hour urine sample (19, 20). We excluded all PE cases who had a history of chronic hypertension, illegal drug or cigarette use, diabetes mellitus, abortion, renal and cardiovascular diseases, chorioamnionitis, activated autoimmune disease, and PE (21, 22). The control group consisted of healthy pregnant women with a history of successful parturition who had no record of pregnancy-related complications such as chronic kidney disease, high blood pressure, or bleeding before pregnancy. Participants of both groups signed informed consent forms to participate and this study was approved by Ethics Committee of Ahwaz Jundishapur University (IR.AJUMS.REC.1394.460). Table 1 lists the characteristics of the two groups.

Blood samples: We collected 5 ml of peripheral blood from PE patients and control group during the third trimester of gestation. The samples were collected in two sterile tubes: one containing EDTA (F.L. Medical, Torreglia, Italy) for DNA extraction and the other without anti-coagulation for serum separation to determine the TNF-α concentration. Blood samples from PE group were obtained before treatment with antihypertentsive drugs.

DNA extraction and TaqMan real-time PCR: Genomic DNA samples were extracted from leukocytes of whole blood samples within the EDTA tubes by using standard phenol-chloroform procedure. Then, the concentration and purity of genomic DNA samples were measured by ultraviolet spectrophotometry at 260/280 nm in a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, NC, USA).

Table 1: Clinical characteristics of PE and control groups

Characteristics	PE group	Control group	Significance (p-value)
	Range (mean ± SD)	Range (mean ± SD)	
Age (years)	22–34 (29.21 ± 3.43)	21–33 (29.01 ± 3.35)	NS
Blood collection (weeks)	31–41 (34.24 ± 1.83)	32–41 (35.14 ± 1.47)	NS
Systolic BP (mm Hg)	142–157 (145 ± 4.9)	112–134 (121 ± 8)	<0.05
Diastolic BP (mm Hg)	93–107 (96 ± 7.6)	70–85 (78 ± 8)	<0.05
Proteinuria (g/24 hours)	1.12–1.39 (1.43 ± 0.7)	Absent	-

PE; preeclampsia, BP; blood pressure, NS; not significant

Samples with a concentration of >10 μg/μl and purity of ≥1.8 were used for real-time PCR.

TNF-α-308G/A polymorphisms in PE patients and the control group were defined by Taq Man real-time genotyping using a specific probe. Genotyping of TNF-α-308G/A polymorphism was performed with TNF-α forward primer 5′-AGGCAATAGGTTTTGAGGGCCAT-3′ and reverse primer 5′-TCCTCCCTGCTCCGATTCCG-3′ (23). Optimal reaction conditions included an initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s, 60°C for 60 s, 72°C for 60 s, and a final extension step at 72°C for 10 min in a Step One Real-time PCR system (Applied Biosystems, Foster City, CA, USA) depending on the melting temperature of the primer and the fragment length. These reactions were performed in a final volume of

5 μl. The components of each reaction included 2.4 μl TaqMan Universal PCR Master Mix (*Takara*, Shiga, *Japan*); 0.4 μl each of the forward and reverse primers (SinaClon, Tehran, Iran) with a concentration of 10 pM; 0.07 μl of the probes (Applied Biosystems) with a concentration of 10 pM; 0.43 μl DNase/ RNase free water; 2 μl DNA template with a concentration of 20 ng/ml; and 0.1 μl Rox Dye (Takara). In this study, both FAM and VIC fluorescent materials were used to identify the two polymorphisms with alleles G and A.

To interpret the results, the type of allele was determined according to the dye attached to the probe. If there was one signal, the sample was considered as a homozygote, while the existence of two signals indicated the heterozygote form of polymorphism (Figure 1).
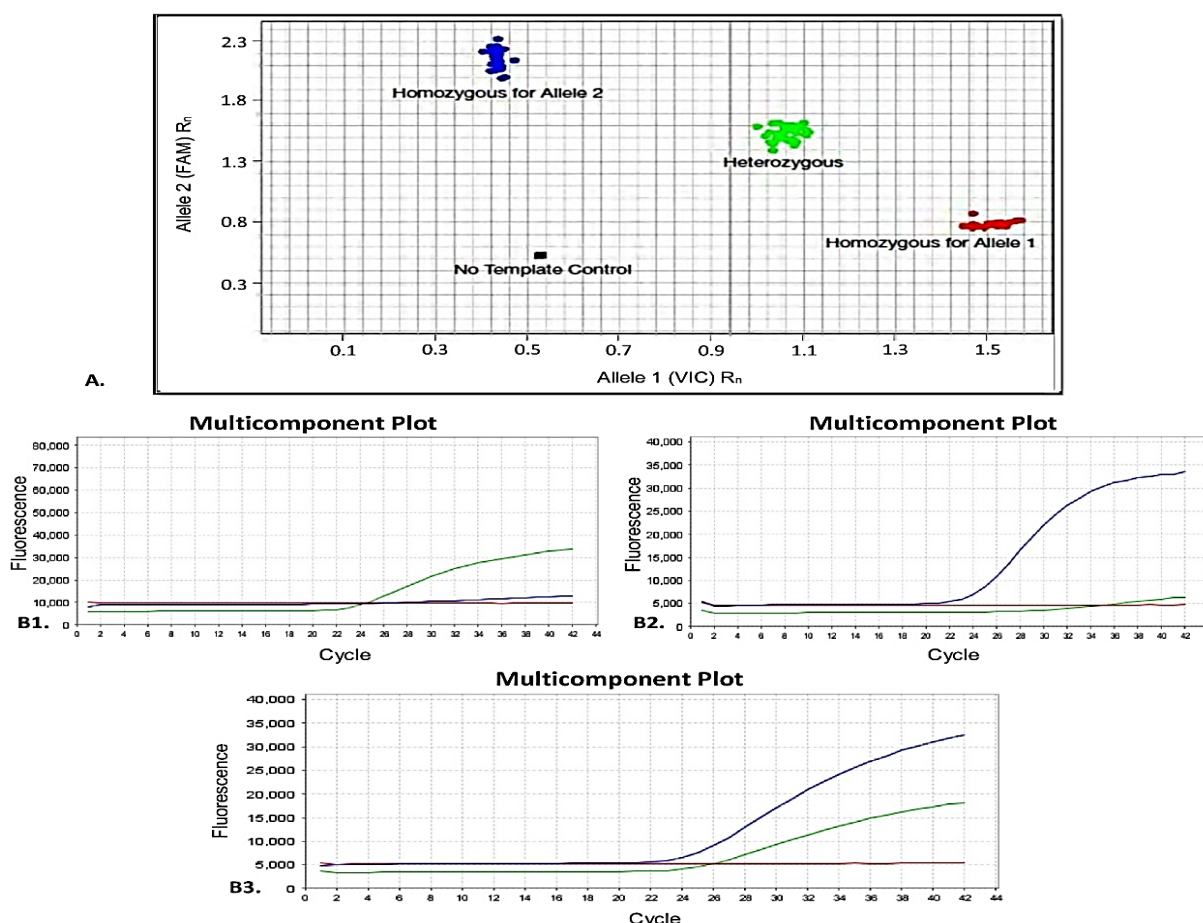

Figure 1: (A) An overview of the allelic discrimination plot according to real-time PCR, in which the VIC signal and FAMTM signal are considered to be allele 1 (G allele) and allele 2 (A allele), respectively. The genotype of the red and blue samples are homozygous for alleles 1 and 2, respectively, while the genotype of the green samples are heterozygous for allele 1-allele 2 (B) The blue color indicates the wild allele, green the mutant allele, and red the background Rox. B1, B2, and B3 belong to the homozygous mutant, homozygous wild-type, and heterozygote samples, respectively.

Quantification of tumor necrosis factor-α (TNF-α) concentration in serum: Evaluation of TNF-α concentration in serum samples was done by a specific enzyme-linked immunosorbent assay (ELISA) technique. For ELISA, we used a kit containing a conjugated enzyme with HRP anti-human IgG according to manufacturer's directions (TNF-α kit; Ebioscience, CA, USA). The color density that depended on the concentration of TNF-α protein in the serum samples was measured at 450 nm with an ELx800™ ELISA Reader (BioTek, Winooski, VT, USA) and the serum levels of TNF-α were calculated based on standard curves. Minimum detection sensitivity was up to 0.7 pg/ml and TNF-α concentrations were presented as pg/ml.

Statistical analysis: Statistical analysis was done using SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons among parameters of various TNF-α genotypes with allele distributions in control and PE samples were performed by Hardy-Weinberg equilibrium test and Fisher' s exact test. The difference in expression of TNF-α gene between control and PE groups was assessed for statistical significance with Mann-Whitney U test. Additionally, the relationship between serum TNF-α levels and other variables was evaluated by chi-square and ANOVA. Graph Pad Prism 5 Demo software was used to produce the image and $p < 0.05$ was considered as significance level.

Results

Table 2 shows the distribution of genotypes and alleles of TNF-α-308G/A (rs1800629) polymorphism. The control and PE group had a markedly higher frequency of GG genotype than GA and AA genotypes.

Table 2: The genotypes frequency of TNF-α -308G/A gene polymorphism (rs1800629) in PE and control groups

Genotypes and alleles	PE[a,b] Group n (%)	Control group n (%)
Genotypes		
Homozygote (GG)	69 (76.7)	72 (80.0)
Heterozygote (GA)	17 (18.9)	16 (17.8)
Homozygote (AA)	4 (4.4)	2 (2.2)
Alleles		
G allele	155 (86.1)	160 (88.9)
A allele	25 (13.9)	20 (11.1)

Comparison between PE and control groups:
[a]Genotypes: $P = 0.7$; Fisher's exact test.
[b]Alleles: $P = 0.3$; Fisher's exact test.
TNF-α; Tumor Necrosis Factor-α; PE, preeclampsia.

The frequency of AA genotype was lower than the other two genotypes. Comparing the distribution of -308G/A gene polymorphism between PE and control groups showed no significant difference ($p = 0.7$). The frequency of G allele was the highest in both PE and control groups. No significant difference was seen between the two groups when we evaluated the allele frequencies of -308G/A ($p = 0.3$).

Figure 2 shows the evaluation results of serum TNF-α levels in PE (mean: 4.40 pg/ml) and control (mean: 3.29 pg/ml) groups. The data distribution was normal; therefore, we compared mean serum TNF-α levels between the two groups using independent samples test. Comparison of TNF-α expression between PE patients and control group showed a significantly higher serum level of TNF-α in PE patients relative to control group ($p < 0.001$).
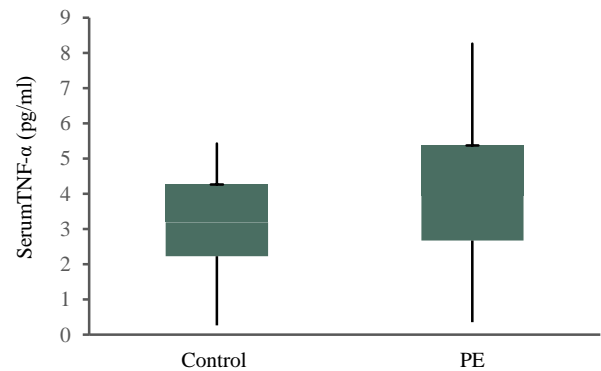

Figure 2: The serum levels of tumor necrosis factor-α (TNF-α) were measured using the enzyme-linked immunosorbent assay (ELISA). The serum TNF-α level in PE patients was significantly higher than the control group (p<0.001).

Discussion

PE is a systemic disorder during pregnancy (1) that affects the quality of pregnancy. It is characterized by pathophysiological features such as hypertension, proteinuria, and systemic inflammation. As the susceptibility gene for PE, TNF-α is expressed by most immune cells. It is a protein involved in various biological processes, including proliferation and apoptosis, as well as inflammatory gene expression (24).

In the present study, we assessed the association between -308G/A polymorphism in promoter region of TNF-α gene with susceptibility to PE. We observed no significant correlation between this single nucleotide polymorphism (SNP) and PE. Numerous studies have evaluated the association of

-308G/A polymorphism with various diseases, including coronary artery disease (25), metabolic syndrome (26), insulin resistance (27), type II diabetes (28), and rheumatoid arthritis (29).

In a study on Caucasian women, the mutant A allele of -308G/A polymorphism was higher in those with PE than the control group. Also, the carriers of this allele were at higher risk for intra-uterine growth disruptions of the embryo (18). This finding contrasted with the results of our study where we did not observe any correlation between -308G/A polymorphism and PE. In another study, Pazarbasi et al. showed that the mutant A allele in 308 promoter region of TNF-α increased susceptibility to PE (30). In another research on pregnant Finnish women, different allelic haplotypes of two polymorphisms in TNF-α promoters G-308A and C-850T were investigated. After genotyping, it was observed that the prevalence of haplotypes A (mutant allele -308) and C (natural allele -850) in PE was higher than in control group in their study, the G and T haplotypes were more prevalent in control group than PE group which indicated a protective role of these two alleles in susceptibility to PE (31). In contrast to our study, it has been shown that the occurrence of G and A alleles of -308G/A polymorphism has an essential role in increasing the risk of PE, which could be attributed to multi-factorial nature of this disease. The serum levels of TNF-α increased in PE patients relative to the control group This relationship could reveal the role of this molecule in pathogenesis and symptoms of PE (32). Also, Pinheiro et al. demonstrated that plasma levels of TNF-α, IL-6, and IFN-γ in patients with severe PE were higher than those in healthy pregnant women (33).

Another research has affirmed that TNF-α polymorphisms, especially -1031C/A and -376G/A, are significantly related with PE. In addition, the results of one study indicated that the major G allele of -308G/A SNP had a protective effect on systolic blood pressure and that higher diastolic blood pressure can be observed in carriers of minor A allele (34). In another investigation on PE in pregnant Iranian women, IL-lβ (C+3954T) and TNF-α (G-308A) polymorphisms in promoter regions of IL-1β and TNF-α were examined. It was observed that systolic and diastolic blood pressures in PE patients were associated with -308G/A polymorphism and with the occurrence of different alleles (p = 0.001) but not correlated with IL-1β +3954 polymorphism. On the other hand, TNF-α-308G/A mutant allele was involved in increasing PE susceptibility, while the IL-1β +3954 mutant allele affected the exacerbation of PE (35). These findings were not consistent with the findings of the present study.

Other studies have revealed an association between TNF-α polymorphism and elevated blood pressure during pregnancy. In one study, women who were carriers of homozygote form of A allele were more likely to have hypertension during the third trimester of pregnancy (36). Blood pressure in pregnancy, also known as pregnancy toxicity, is affected by different factors such as leptin, the renin-angiotensin system, bradykinin, and concentration of various ions. Results of another research indicated a significant correlation of TNF-α -308G/A polymorphism and susceptibility to PE under the allele model. According to the evidence of this study, it was demonstrated that carriers of TNF-α (308A) allele were susceptible to PE. This finding was especially noted among Caucasian, Iranian, and primipara women (37). In contrast, the role of TNF-α polymorphisms was investigated in placenta of pregnant women with PE. Interestingly, the TNF-α -308G/A genotype was remarkably higher in controls relative to PE women, and this genotype could be a protective factor for PE susceptibility. This study for the first time showed evidence of an association between placental *TNF-α* -308G/A genotype and *TNF-α* -238A allele with a reduced risk for PE (38). Zubor et al. examined the association between G and A alleles of -380G/A polymorphism with other symptoms of PE, which included renal failure, capillary permeability, involvement of other organs, and PE severity. They observed that the A allele polymorphism promoted the risk of disease and that an association existed with elevated serum levels of TNF-α, sVCAM, and fibronectin in PE patients. Besides, the severity of proteinuria in carriers of A allele (GA, AA) was markedly higher than homozygous GG subjects (p<0.01). In contrast to our study, they revealed that, there was a significant difference in prevalence of alleles and percentage of genotypes between control and PE groups. However, they reported that the levels of serum TNF-α in patients with PE increased relative to the control group, which was consistent with our research (39).

Conclusion

In our research, we have not observed any association between the occurrence of the mutant A allele and the incidence of PE signs such as hypertension which

could indicate that hypertension, among other symptoms, is affected by factors other than TNF-α. Considering the variation in the expression of a single allele and similarity in the serum level of the protein, the differences between our results with other studies could be related to regulation of gene expression at mRNA level. Given the contradictions and ambiguities in previous studies due to the relationship between TNF-α polymorphism with the incidence and severity of PE and given the increased prevalence of this disease in pregnant women, we recommend that additional studies be conducted on other polymorphisms in the gene of this and other related cytokines.

Conflict of Interests

The authors declare they have no conflict of interest.

Acknowledgments

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