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Polymorphisms in the tumor necrosis factor-alpha gene in Turkish women with pre-eclampsia and eclampsia*

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Abstract

The genetic background predisposing pregnant women to pre-eclampsia/eclampsia (PE/E) is still unknown. The aim of the current study was to investigate whether there is an association between the TNF-alpha-308 and 850 polymorphisms and PE or eclampsia. In this study, 40 cases of eclampsia, 113 cases of PE and 80 normotensive control cases were genotyped for the TNF-alpha-G-308A and C-850 polymorphisms. At position 308, the replacement of Guanine with Adenosine was denoted as TNF2. We found a significant difference between the TNF2 allele frequencies of the eclamptic, pre-eclamptic and normotensive controls. TNF2 (AA) polymorphism frequency was significantly higher among the eclamptics and pre-eclamptics (control : 5%, PE : 13.3%, E : 12.9%). A significantly different genotype distribution of C-850T polymorphism was observed between the PE/E and control groups, with the frequency of the variant TT genotype being significantly reduced in the preeclamptics (PE : 17% ; E : 17.5%) when compared with the control group (24.3%). We have demonstrated an association between TNF-alpha polymorphisms and pre-eclampsia susceptibility. However, it is not known whether C-850T polymorphism has a functional effect on the TNF-alpha gene. In addition, it was not possible to determine whether this polymorphism promotes the progression from PE to eclampsia because of no statistically significant difference between eclampsia and the controls.

KEYWORDS: TNF-alpha, polymorphisms, eclampsia, pre-eclampsia

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Original Article

Polymorphisms in the Tumor Necrosis Factor-alpha Gene in Turkish Women with Pre-eclampsia and Eclampsia

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The genetic background predisposing pregnant women to pre-eclampsia/eclampsia (PE/E) is still unknown. The aim of the current study was to investigate whether there is an association between the TNF-alpha-308 and 850 polymorphisms and PE or eclampsia. In this study, 40 cases of eclampsia, 113 cases of PE and 80 normotensive control cases were genotyped for the TNF-alpha-G-308A and C-850 polymorphisms. At position 308, the replacement of Guanine with Adenosine was denoted as TNF2. We found a significant difference between the TNF2 allele frequencies of the eclamptic, pre-eclamptic and normotensive controls. TNF2 (AA) polymorphism frequency was significantly higher among the eclamptics and pre-eclamptics (control: 5%, PE: 13.3%, E: 12.9%). A significantly different genotype distribution of C-850T polymorphism was observed between the PE/E and control groups, with the frequency of the variant TT genotype being significantly reduced in the preeclamptics (PE: 17%; E: 17.5%) when compared with the control group (24.3%). We have demonstrated an association between TNF-α polymorphisms and pre-eclampsia susceptibility. However, it is not known whether C-850T polymorphism has a functional effect on the TNF-α gene. In addition, it was not possible to determine whether this polymorphism promotes the progression from PE to eclampsia because of no statistically significant difference between eclampsia and the controls.

Key words: TNF-alpha, polymorphisms, eclampsia, pre-eclampsia

Pre-eclampsia (PE) is the most common serious medical disorder of human pregnancy with a worldwide incidence of 2-5%. It continues to be a leading cause of maternal as well as perinatal morbidity and mortality. PE is diagnosed mainly by the new onset of hypertension and proteinuria during the latter half of pregnancy and, if untreated, can progress to eclampsia, a particularly serious condition which can lead to the death of the mother, the baby or both. The biological factors which determine the progression of PE to eclampsia are unknown [1].

Both the etiology and pathogenesis of PE/E (eclampsia) are poorly understood. PE/E is primarily a disease of first pregnancy, and prior exposure to paternal antigens appears to have a protective effect, leading to the widely held view that there is involvement of the immune system, at least in part [1]. A complex genetic basis is suspected with a

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widely held view being that both maternal and paternal genes contribute to susceptibility [2]. Recent genome scans in Icelandic [2] and Australian/New Zealand [3] populations have so far confirmed linkage to a locus on chromosome 2, with as yet no obvious positional candidates identified. Endothelial cell dysfunction is considered to play a key role in the pathophysiology of PE/E [4]. This contention is supported by morphological, biochemical and functional observations consistent with endothelial damage or activation [5]. Inflamatory cytokines have been shown to upregulate the gene expression of numerous molecules in endothelial cells signaling their activation [6]. A multifunctional cytokine, tumor necrosis factor α (TNF-α), is the principal immune mediator derived from macrophages and lymphocytes. It has been implicated in the transcriptional regulation of the genes for the potent vasoconstrictors platelet-derived growth factor and endothelin-1 [1, 7, 8]. TNF-alpha has also been shown to induce the expression of plasminogen activator inhibitor-1 in cultured human endothelial cells [9]. Increased concentrations of endothelin-1 and plasminogen activator inhibitor-1 have been found in the plasma of PE patients, and serum from PE patients upregulates platelet-derived growth factor gene expression in cultured endothelial cells [10]. These studies suggest that one mechanism by which inflammatory cytokines such as TNF-α may affect endothelial cell function in PE is through the upregulation of molecules that have profound effects on the vasculature. The gene for TNF-α resides within the class 3 region of the major histocompatibility complex, and several polymorphisms in the promoter region of the TNF-α gene have been described ($-$1,032, $-$863, $-$857, $-$850, $-$575, $-$375, $-$308, $-$274, $-$243, $-$237, $-$162) [11]. G-308A and C-850T transition polymorphisms within the TNF-α gene promoter have been associated with a negative outcome in some diseases, including PE. The association of the T allele of the C-850T polymorphism was found with PE, but its association with eclampsia is unknown. The majority of studies investigating the functional significance of TNF-α promoter polymorphisms have focused on the biallelic G to A transition at position $-$308. The genetic variation on position $-$308 results in 2 allelic forms, in which the presence of guanine (G) defines the common variant variation, TNF1 (GG), and the presence of adenine (A) defines the less common variant, TNF2 (AA) [12]. The genetic variation on position $-$850 results in 2 allelic forms, in which the presence of Cytosine (C) defines the common variant, CC, and the presence of Thymidine (T) defines the less common variant, TT. Taken together, these reports suggest that the role of TNF-α in the development of PE/E is evident but not completely understood.

The aim of this study was to investigate whether there is an association between the TNF-α-G-308A and C-850T polymorphisms and PE or eclampsia.

Materials and Methods

Written approval was obtained from the Ethics Committee of Cukurova University Hospital and informed consent from all patients and controls before peripheral blood samples were taken. Information was collected retrospectively about 113 preeclamptic, 40 eclamptic pregnancies of primiparous women and 80 (70 for C-850T and 80 for G-308A polymorphism were analysed) normotensive control women with no history of preeclampsia who delivered at Cukurova University Hospital between June 2004 and August 2005.

Peeclampsia was defined as the development of hypertension and new-onset proteinuria ($>$300 mg of urinary protein in 24h) in women with no protienuria at baseline. Diagnosis was based on clinical assessment, using the criteria of the Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy, 2000 [13]. Pregnant women were considered to have severe PE if they had either (1) an increase in systolic blood pressure of greater than or equal to 25 mmHg above baseline and/or an increase in diastolic blood pressure of greater than or equal to 15 mmHg above baseline, or (2) a persistent systolic pressure of greater than or equal to 140 mmHg and/or a diastolic pressure of greater than or equal to 90 mmHg. These levels had to occur on at least 2 occasions greater than 0.3 g/l in a 24-hour specimen, or the dipstick proteinuria score had to be greater than or equal to 2+ in a random urine collection. Women who met these criteria and who had experienced either convulsions or unconsciousness in the perinatal period were classified as having had eclampsia.

Because PE/E is typically a disease of first pregnancies [1, 14, 15], this study was limited only to primigravid patients. Women with preexisting hypertension, chronic renal disease or autoimmune disorders such as systemic lupus erythematosus known to predispose them to PE were excluded.

DNA was extracted from peripheral blood lymphocytes by using a standard salting out extraction method modified from Miller's method [16]. In the method, 0.5 ml of peripheral blood was treated with 1 ml of erythrocyte lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 1% Triton X-100) at room temperature for 5 min. In order to collect leukocytes, the mixture was centrifuged at 4,000 rpm for 5 min and the leukocyte pellet was dissolved with 0.5 ml of the lysis buffer, and then centrifuged and rinsed with physiological tampon (0.075 M NaCl, 0.025 M EDTA). The cells were incubated in lysis buffer containing 250 μl of TE-9 (500 Tris-HCl pH9.0, 20 mM EDTA, 10 mM NaCl), 100 μl of 10% SDS and 20 μl of proteinase-K (10mg/ml) at 65 ℃ for 1h. At the end of the incubation, 150 μl of 6 M salt solution was added, shaken strongly and centrifuged at 15,000 rpm for 10 min. Supernatant was taken to a new tube and DNA was concentrated by adding 1 ml of absolute ethanol. After the centrifugation at 15,000 rpm for 5 min, the DNA pellet was rinsed with 1 ml of 75% ethanol, dried by inverting the tube and dissolved in 200 μl of TE buffer (1 mM Tris-HCl, 1 mM EDTA, pH7.5).

The G-308A and C-850T polymorphisms in the promoter of the TNF-α gene were genotyped by using a PCR-RFLP method as previously described [1, 12, 17–19]. The G-308A variants were genotyped by using primers designed to incorporate the polymorphic site at position −308 relative to the TNF-α transcription start site [12] (Primers for G-308A polymorphism: F-5'-GGGACACACAAG CATCAAGG-3'; R-5'-AATAGGTTTTGA-GGGCCATG-3'). This variant (A at −308) creates an *NcoI* (MBI Fermentas, Lithuania) restriction site and can be differentiated by size (126 for the TNF1 allele and 142 for the TNF2 allele) on a 12% polyacrylamide gel. The PCR product (131bp) of C-850T polymorphism was amplified with primers FM850 (mismatch) and R850 [18] (Primers for C-850T polymorphism: F-5'-AAGTCGAGTATGGGGACCC CCCGTTAA-3'; R-5'-CCCCAGTGTGTGGCCATA TCTTCTT-3'). Subsequently, the 131bp PCR product was digested with *HincII* (MBI Fermentas, Lithuania) restriction enzyme and subjected to 12% polyacrylamide gel electrophoresis. In the case of a C allele at position −850, *HincII* digestion produces 106bp and 25bp fragments, whereas the 131bp fragment remains undigested when the T allele is located at this position. Statistical analyses for comparing individual allele and genotype frequencies were carried out using the chi-square test (two-sided asymptotic p values) with SPSS 10.0 software and the level of statistical significance was defined as $p < 0.05$.

Results

The clinical characteristics of the eclamptics, pre-eclamptics and controls are shown in Table 1. All women with eclampsia and pre-eclampsia and the controls were primigravid. They were used for comparison to exclude the confounding effect of parity on pregnancy outcome. In this study, there was a significant difference between control-eclamptics and control-pre-eclamptics. On average, deliveries occured 4–5 weeks earlier in the pre-eclamptic and eclamptic women than in the controls.

The genotyping data for the G-308A and C-850T polymorphisms are presented in Tables 2 and 3. We found a difference among the TNF2 allele frequencies of the eclamptics, pre-eclamptics and controls. No homozygous TNF2 genotype was found in the PE/E and control groups. In the heterozygotes (TNF1/TNF2 = GA) the genotype frequency increases from control to eclamptic and pre-eclamptic individuals with frequencies in the order of 5, 12.9, and 13.3%, respectively. A χ^2 analysis in a 2×2 table showed a significant difference between control-pre-eclamptics ($\chi^2 = 16.287$ and $p < 0.001$) and control-eclamptics ($\chi^2 = 5.022$ and $p = 0.025$) (Table 2).

Similar results were obtained by comparing the allele frequencies. There was a significant difference between both the control-preeclamptics ($\chi^2 = 15.87$ and $p < 0.001$; 2×2 table) and control-eclamptics allele frequencies ($\chi^2 = 4.893$ and $p = 0.027$; 2×2 table). Two χ^2 analyses between the eclamptic and pre-eclamptic individuals for comparing the genotype and allele frequencies did not show a significant difference, respectively ($\chi^2 = 0.007$ and $p = 0.933$;

Table 1 Clinical characteristics of the eclamptic, pre-eclamptic and unaffected women

Characteristic [s]	Controls mean N=70	Eclampsia group mean N=40	*P* Value For C and E	PE group mean N=112	*P* Value for C and PE
Age (years)	28.5	31.9	<0.001	30.1	<0.001
Pregnancy BMI (kg/m^2)	22.8	25	<0.001	24.9	<0.001
Systolic BP (mmHg)	129	163	<0.001	162	<0.001
Diastolic Bp (mmHg)	83	104	<0.001	103	<0.001
Gestational age at delivery (weeks)	38.7	33.7	<0.001	34.7	<0.001

BP, blood pressure; E, eclampsia; PE, pre-eclampsia; C, control.

Table 2 Genotype and allele distribution of the G-308A polymorphism in the TNF-α gene in eclamptic, pre-eclamptic and control patients

	Genotype frequencies							Allele frequencies				
	GG (TNF1)		GA (TNF1/TNF2)		AA (TNF2)		sum	GG (TNF1)		AA (TNF2)		sum
	n	%	N	%	n	%		n	%	n	%	
Eclamptic	34	87.2	5	12.9	–	–	39	73	93.6	5	6.4	78
PE	98	86.7	15	13.3	–	–	113	211	93.4	15	6.6	226
Controls	76	95	4	5	–	–	80	156	97.5	4	2.5	160
χ^2=16.287, p<0.001 (2×2 with controls for PE)*								χ^2=15.87, p<0.001 (2×2 with controls for PE)*				
χ^2=5.022, p=0.025 (2×2 with controls for E)*								χ^2=4.893, p=0.027 (2×2 with controls for E)*				

*The significant χ^2 statistics between controls, pre-eclamptics and eclamptics

Table 3 Genotype and allele distribution of the C-850T polymorphism in the TNF-α gene in eclamptic, pre-eclamptic and control patients

	Genotype frequencies							Allele frequencies				
	CC		CT		TT		sum	C		T		sum
	n	%	n	%	n	%		n	%	n	%	
Eclamptic	16	40	17	42.5	7	17.5	40	49	61.3	31	38.8	80
PE	68	60.7	25	22.3	19	17	112	161	71.9	63	28.1	224
Controls	20	48.4	33	31.9	17	24.3	70	73	52.1	67	48	140
χ^2= 57.609, p<0.001 (2×3 with controls for PE)*								χ^2=34.951, p<0.001(2×2 with controls for PE)*				

*The significant χ^2 statistics between controls and pre-eclamptics

$\chi^2 = 0.006$ and $p = 0.936$; 2×2 tables).

A different genotype distribution of C-850T polymorphism was observed between the PE/E and control groups, with the frequency of variant TT genotype being reduced in the preeclamptic (17%) and eclamptic groups (17.5%) compared with the control (24.3%). A χ^2 analysis in a 2×3 table showed a significant difference between the control and preeclamptic samples with $\chi^2 = 57.609$ and $p < 0.001$ (Table 3). There was no significant difference between the control and eclamptic patients ($\chi^2 = 2.770$ and $p = 0.250$). The frequency of variant T allele was reduced in the preeclamptic (28.1%) and eclamptic groups (38.8%) compared with the control (48%). The same results with genotype frequencies were obtained by comparing the allele frequencies of the PE and controls. We found a significant difference between the control and pre-eclamptic allele frequencies ($\chi^2 = 34.951$ and $p < 0.001$; 2×2 table), while we found no significant difference between the control and eclamptics ($\chi^2 = 2.659$ and $p = 0.103$; 2×2 table).

Table 4 shows a comparison of our results with those of previous reports. The genotype and allele distributions of the C-850T polymorphism differed significantly between the pre-eclampsia group and the control group ($p = 0.003$ and $p = 0.003$, respectively), as previously reported (Heiskanen *et al.*, 2002). The genotype and allele distributions of the G-308A polymorphism differed significantly between the eclampsia

Table 4 Comparison of the genotype and allele frequencies of the TNF-α gene promoter C-850T and G-308A polymorphisms among women with eclampsia, pre-eclampsia and healthy pregnant controls at 4 studies

		TNF C-850T (%)					TNF G-308A (%)				
		Genotype frequencies			Allele frequencies		Genotype frequencies			Allele frequencies	
		CC	CT	TT	C	T	GG TNF	GA TNF1/TNF2	AA TNF2	G	A
Heiskanen *et al*. 2002;	Control	83.5	13.9	2.6	90.4	9.6					
	PE	93.2	4.5	2.3	95.5	4.5					
Kaiser *et al*. 2004	Control						67.0	29.0	4.0	81.5	18.5
	PE						60.7	36.1	3.3	78.7	21.3
	E						43.1	51.0	5.9	68.6	31.4
Saarela *et al*. 2005	Control						81.7	18.3	0.0	90.9	9.1
	PE						72.9	24.1	3.0	85.0	15.0
Our results	Control	48.4	31.9	24.3	52.1	48.0	95.0	5.0	0.0	97.5	2.5
	PE	60.7	22.3	17.0	71.9	28.1	86.7	13.3	0.0	93.4	6.6
	E	40.0	42.5	17.5	61.3	38.8	87.2	12.9	0.0	93.6	6.4

P-values for each study:

1. The frequency of the T allele in the control group was 9.6%, whereas in the preeclamptic group it was 4.5%. $p = 0.030$ and 0.030 for the genotype and allele data of PE-Control statistics respectively (Heiskanen *et al*., 2002).
2. A significant difference between control and eclamptic samples for the genotype and allele data of heterozygotes (TNF1/TNF2) with $p = 0.025$ (Kaiser *et al*., 2004).
3. In the case of G-308A polymorphism, there were no statistically significant differences in the genotype distribution ($p = 0.080$). The frequency of the A allele was 15% in the preeclamptics and 9.1% among the controls ($p = 0.046$) (Saarela *et al*., 2005).
4. We found a significant difference between the control-preeclamptics ($p < 0.001$ and $p < 0.001$) and control-eclamptics ($p = 0.025$ and $p = 0.027$) for the genotype and allele data of heterozygotes (GA) respectively. We also found a significant difference between the control-preeclamptics ($p < 0.001$ and $p < 0.001$) for the genotype and allele distribution of the C-850T polymorphism in the TNF-α gene.

group and the control group ($p = 0.025$ and $p = 0.025$, respectively), as Kaiser *et al.* reported. The genotype and allele distributions of the G-308A polymorphism did not differ significantly between the pre-eclampsia group and the control group ($p = 0.046$ and $p = 0.08$, respectively), as Saarela *et al.* (2005) reported.

Discussion

This study analysing eclampsia and PE patients in a Turkish population with respect to the TNF-α -G-308A and C-850T promoter polymorphisms showed a significantly increased incidence of the TNF2 (AA) allele and a reduced variant T allele with PE and eclampsia.

Although we found no association between body mass index, systolic bp, diastolic bp and gestational age at delivery of preeclamptics and eclamptics, elevated prepregnancy body mass index and waist circumference have been strongly associated with pre-eclampsia in previous studies [20–29]. In our study, they are statistically related to the diseases compared with the controls. Women with preeclampsia and eclampsia were of higher socioeconomic status, and this likely biased the relationship between body mass index and preeclampsia toward the null in our study. Obesity is associated with chronic inflammation and oxidative stress [29, 30]. Thus, elevated body fat may trigger excessive cytokine production among genetically susceptible pregnant women [29].

Our findings of a significantly increased incidence of the TNF2 allele and an approximately equal incidence of the TNF1 with controls is in contrast with Chen *et al.* (1996), who found a significantly higher incidence of the TNF1 allele in PE patients compared with controls [31]. However, some researchers found no significant difference in the allele frequencies of the G-308A polymorphism between PE/E and controls [1, 32–34]. The relationship between the G-308A polymorphism and PE has recently been described by Haggerty *et al.* (2005) [29]. Analysing eclampsia and PE separately, we found that the genotype frequencies of the TNF2 in our pre-eclamptic and eclamptic groups was significantly higher than the controls, while no significant difference was found between the eclamptic and PE patients. In agreement with our findings, the study undertaken by Kaiser *et al.* (2004) did not differentiate between eclamptic and PE patients, but they analysed only G-308A promoter polymorphism in their study [1]. In the study of Saarela *et al.* (2005), the less frequent A allele (TNF2) of the G-308A polymorphism was found to be associated with an increased risk of pre-eclampsia in Finnish women [35], but there were no statistically significant differences in the genotype and allele distribution between the pre-eclamptics and controls. The presence of the allele A leads to expression of the TNF2 variant and is correlated with the PE. With reporter genes under the control of the 2 allelic TNF promoters (TNF1 and TNF2), TNF2 is a much stronger transcriptional activator than the common allele (TNF1) [36] and could contribute to the upregulation of various genes involved in PE disease. The significant increase in the TNF2 allele in eclamptic patients could indicate that a higher expression of TNF-α promotes the progression from PE to eclampsia.

It is generally accepted that PE and eclampsia are expressions of the same syndrome, with eclampsia being the fulminating form of PE. Their pathology is common, and PE is a mild case of eclampsia. In our study, the T allele occurred at a lower frequency in PE than in eclampsia. While the T allele is PE<eclampsia<normal, the CC genotype is PE >normal>eclampsia. Our data does not imply that the T allele exerts a protective effect against this pathology. On the other hand, according to the results of PE<normal for the T allele, Heiskanen *et al.* (2002) reported that the C-850T allele may be protective against the development of PE [19]. A lower incidence of the T genotype compared with the controls found in PE in present study is not the first example, but it is the first in eclampsia. In this study, there was a significant difference between the control and pre-eclamptic genotypes for C-850T polymorphism, while there was no significant difference between control and eclamptics [19]. The genotype and allele distributions of the C-850T polymorphism differed significantly between the pre-eclampsia group and the control group as previously reported (Heiskanen *et al.*, 2002).

Hayashi *et al.* (2005) demonstrated no significant increase in TNF-α levels in the placenta in pre-eclampsia despite a significant increase in the serum

TNF-α levels [37]. These findings suggest that TNF-α in the placenta is not a key cytokine that interferes with normal trophoblast invasion into the myometrium in pre-eclampsia, and that sources other than the placenta may be contributing to the elevated levels of TNF-α found in the circulation of pre-eclamptic patients. Freeman *et al.* (2004) found that TNF-α levels were increased by 33% between the first and third trimeters in pre-eclamptic pregnancies. They concluded from this study that pre-eclampsia is associated with short and long-term changes in inflammatory status [38].

The primary strength of our study is that we examined 2 polymorphisms in the promoter region of the TNF-α gene in a group of Turkish pre-eclamptic/eclamptic women, which allowed us to gain an understanding of whether population susceptibility for PE/eclampsia in this population was different from those in other populations. A weakness of our study is that the data observed was obtained from a limited population (Turkish women). The main limitation of the study is the lack of circulating cytokine measures that are needed to prove a functional relationship between polymorphisms, elevated cytokines, and PE/eclampsia.

In summary, we have demonstrated an association between TNF-α polymorphisms and pre-eclampsia susceptibility. It is not known whether C-850T polymorphism has a functional effect on the TNF-α gene, but we could not say that this polymorphism promotes the progression from PE to eclampsia. It is true that susceptibility in Turkey for PE/eclampsia may be different from other cases. There have been some additional reports on TNF-α polymorphism and PE/eclampsia. A possible reason for the inconsistency among these reports may be a genetic basis that causes different susceptibilities among different populations. The mechanisms behind this finding remain to be determined.

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