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Original Article

Transforming growth factor beta-1 (TGF-β1) gene single nucleotide polymorphisms (SNPs) and susceptibility to pre-eclampsia in Iranian women: A case–control study

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ABSTRACT

Objectives: Pre-eclampsia (PE) is a disorder of pregnancy characterized by high blood pressure and proteinuria. Transforming growth factor beta-1 (TGF-β1) is an important replicated PE candidate gene, and few studies have evaluated the direct association of TGF-β polymorphisms and risk to PE. The aim of this study was to investigate the association between three SNPs of TGF-β1 and serum level of this cytokine in PE patients and controls.
Design and methods: In this study the polymorphisms of the TGF-β1 gene at the coding region, and positions 29T→C (Leu 10 Pro), 74G→C (Arg 25 Pro) and 788C→T (Thr 263 Ile) were studied in 123 PE and 120 normal subjects using PCR-restriction fragment length polymorphism PCR-(RFLP) and amplification refractory mutation system (ARMS)-PCR methods. Moreover, serum TGF-β1 was determined by enzyme-linked immunosorbent assay (ELISA) technique.
Results: At positions 74G→C and 29T→C the genotypes and allele frequencies showed no significant differences between PE patients and normal controls ($P = 0.3$ and $P = 0.5$ respectively). While in the case of position 788C→T both genotypes and allele frequencies were significantly different between PE patients and controls ($P = 0.02$). Haplotype analysis on three polymorphic sites showed no significant differences between PE and control individuals ($P = 0.8$). TGC and CGC haplotypes were the most frequent in both studied groups. The mean serum TGF-β1 level was significantly higher (62.73 ng/ml) in PE patients compared with pregnant (47.01 ng/ml) and non-pregnant (40.68 ng/ml) control groups ($P = 0.0001$).
Conclusions: The results of this study suggest that TGF-β1 gene 788C→T polymorphism is an important factor mediating the casual pathway of preeclampsia.

Introduction

Preeclampsia (PE) is a common systemic obstetric disorder and affecting about 3–5% of pregnancies. PE results from imbalance between factors produced by the placenta and maternal adaptation to them [1]. It occurs after the 20th week of gestation and originates in the placenta and

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causes variable maternal and fetal problems. Since termination of pregnancy cures the disease, preeclampsia is a placenta-dependent disorder with both local intrauterine and systemic signs and symptoms. There are two broad classes of pre-eclampsia: maternal and placental, although many cases are a mix of the two [1–4]. Recent studies have described several other immune cells involved in the process of pre-eclampsia, expanding the Th1/Th2 paradigm into the Th1/Th2/Th17 and regulatory T cell (Treg) paradigm, introducing Treg as regulators of Th17 lymphocytes and other immune cell types involved in the feto-maternal tolerance [5]. Normal pregnancy requires a shift to a Th2 type of immunity, at least directed toward the fetus, while some pregnancy complications, such as preeclampsia, could be due to a skewed Th1 type of immunity [6]. TGF-β1 is a 25-kDa homodimeric and multifunctional cytokine secreted in a latent form by many cell types, including peripheral blood mononuclear cells and Treg cells [7], and plays important roles in modulating cellular growth, differentiation, immunoregulation and extracellular matrix formation [8,9]. In addition, TGF-β1 is present in semen and expressed in several pregnancy-associated tissues. Moreover, in the placenta, both mRNA and protein of TGF-β1 are detected throughout gestation. It is suggested that TGF-β1 has an important role in the physiology of normal pregnancy [8], and may contribute to the regulation of maternal immune responses against the fetal allograft, and thereby prevent immunological rejection of the fetus [10,11]. Several studies demonstrated a positive correlation between the circulating concentration of TGF-β1 and high blood pressure (BP) in humans [12–14]. It was found that in PE the plasma level of TGF-β1 is significantly increased, which may be a risk factor for the preeclamptic [15,16]. Several single nucleotide polymorphisms (SNPs) in gene coding for TGF-β1 have been detected and certain alleles are suggested to be associated with the elevated serum levels of TGF-β1 both in vitro and in vivo [17–19]. For instance, polymorphism in the signal sequence at position 74G→C, which changes codon 25 (Arg→Pro), has been associated with variations in the levels of TGF-β1 production [20]. Previously, it was reported that 74G→C polymorphism was associated with a lower systolic pressure and a lower frequency of a history of hypertension [21]. Moreover, T→C transition at nucleotide 29 of TGF-β1 gene, which results in a Leu→Pro substitution at amino acid position 10 of the signal peptide, was reported to potentially affect the secretion of TGF-β1 [22]. So the aim of this study is to investigate whether there is an association between coding regions of TGF-β1 polymorphisms and serum level of this cytokine with PE.

Material and method

Subjects

The study group consisted of 123 women (aged 17–38 years, mean 28 years) with preeclampsia and no history of kidney disorders and hypertension before pregnancy. Patients with anatomical, hormonal, chromosomal, infections and autoimmune disorders were excluded from this study. All subjects attended the Department of Obstetrics and Gynecology of Shahid Beheshti University of Medical Sciences Tehran–Iran. The diagnosis of preeclampsia was made by a gynecologist after clinical and paraclinical examinations of cases. From 123 selected PE women; 102 suffered moderate (mild) and 21 suffered severe preeclampsia, patients with blood pressure (BP) of more than 140/90 after 20 weeks of pregnancy or proteinuria of more than 300 mg in 24 h urine collected were classified as preeclampsia. The severe form of preeclampsia was defined on the basis of symptoms of BP more than 160/110, proteinuria >300 mg in 24 h urine, with headache, oliguria, visual disturbances, upper abdominal pain, high creatinine and liver enzymes and thrombocytopenia. The control group consisted of 120 ethnically matched women; 100 pregnant of whom were in the third trimester of pregnancy and 20 non-pregnant women (aged 16–50 years, mean 35) with normal blood pressure (<140/90) and no proteinuria. The non-pregnant women had at least one previous pregnancy without PE. The gestational age of PE patients and control pregnant women were 32–37 and 30–35 weeks, respectively. Women that entered into this study did not have any intra uterine growth restriction (IUGR) or systemic disorders. None of the patients had received any medication before blood sampling. Twenty PE patients and six control women had a family history of preeclampsia. All case and control individuals in this study were non-smokers. Controls were healthy women with no histories of essential hypertension, chronic renal disease, diabetes, platelet disorders, or autoimmune conditions. The Ethics Committee at research General Hospital approved the use of the clinical information and the collection of samples for research purposes. Written informed consent was obtained from all enrolled subjects.

DNA extraction and TGF β1 genotyping

Peripheral venous blood was collected in EDTA-coated tubes. DNA was extracted from peripheral blood lymphocytes by using a standard salting out extraction method [23]. Genotyping of codon 10 was performed by the amplification refractory mutation system (ARMS) method [24]. The 241 bp fragment containing the polymorphic site in codon 10 of *TGF*-β1 was amplified using PCR primers (Alfa, Canada) as follows: the generic primer (sense), 5′-TCCGTGGGATACTGAGACAC-3′; the *C* allele specific primer (antisense), 5′-GCAGCGGTAGCAGCAGCG-3′; the *T* allele specific primer (antisense), AGCAGCGGTAGCAGCAGCA-3′; forward internal control primer was (P53F), 5′-TGCCC TGTGCAGCTGTGGGTTGATT-3′; and the reverse internal control primer was (P53R), 5′-GCCCCAGCTGCTCACCA TCGCTATC-3′. The internal control primers were used to amplify a segment of p53 gene located at chromosome 17p13.1 to check for successful PCR amplification [24]. The Genotyping of the codon 25 and codon 263 polymorphisms of TGF-β1 was performed by RFLP analysis. For position 74G→C (Arg 25 Pro), forward primer sequence: 5′-GCTACCGCTGCTGTGGCTACT, and the reverse primer sequence: 5′-ACGCGGGTGACCTCCTTG. For position 788C→T (Thr 263 Ile), forward primer sequence was: 5′-AAGCAGGGTTCACTACCGGC, and the reverse primer sequence was: 5′-AGGCCTCCATCCAGGCTACA. PCR was

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performed in a 10 μl volume containing 300 ng of genomic DNA as template. The following restriction enzymes (Fermentas Lithuania) were used: *BglI* for digestion of PCR products contain position74G→C (Arg 25 Pro), and *BseGI* for PCR product contain position 788C→T (Thr 263 Ile).

Measurement of TGF-β1 protein level

Peripheral venous blood was obtained from the patients and control women that were in the same gestational age (third trimester). The venous blood were collected into tube and centrifuged at 1600*g* for 10 min then plasma isolated and stored at −80 °C until assay. The biologically active TGF-β1 concentration was determined using a solid-phase TGF-β1-specific sandwich ELISA (Bender med system, USA) as described by the manufacturer's protocol. Prior to analysis plasma were transiently acidified to activate latent TGF-β1, and then tested at a 1:12 dilution. Plasma optical density was measured at 450 nm by using ELISA reader (Anthos 2020 Version 1.8).

Statistical analysis

Frequencies of each polymorphic site were calculated by the allele counting method. Differences in the genotype and allele frequencies between patients and controls were tested by χ^2 analysis. Differences in serum TGF-β1 concentration between the PE patients and normal control group were determined by Student's *t*-test. Serum TGF-β1 levels in PE patients and control groups in relation to different genotypes were analyzed by an ANOVA test. $P < 0.05$ was considered statistically significant. Haplotype estimation and differences in the haplotype frequencies between PE cases and control groups were analyzed by Arlequin software version 3.11 (http://anthro.unige.ch/arlequin).

Results

The distribution of observed genotypes was not significantly different from the expected distribution, according to Hardy–Weinberg equilibrium in both the PE and control women groups.

ARMS-PCR [position 29T→C (Leu 10 Pro)]

Genotyping at position 29T→C (Leu 10 Pro) of TGF-β1 gene was studied by ARMS-PCR among 123 cases of pre-eclampsia and 120 normal subjects. Results indicated that 45 (36.6%) of PE cases, and 39 (32.5%) of normal subjects were homozygote TT at this position. Moreover, 50 (40.6%) of PE cases and 46 (38.3%) of normal controls were heterozygote TC. Furthermore, 28 (22.8%) of PE cases and 35 (29.2%) of normal subjects were homozygote CC (Table 1). There was no statistically significant difference in genotype and allele frequency distributions between PE women and normal controls at this position ($P = 0.5$).

PCR-RFLP [position 74G→C (Arg 25 Pro)]

Results of genotyping at position 74G→C (Arg 25 Pro) revealed that 100 (81.3%) of PE women, and 96 (80%) of the normal subjects were homozygote GG. In addition, 18 (14.6%) of PE cases and 22 (18.4%) of controls were heterozygote GC. Moreover, 5 (4.1%) of PE cases and 2 (1.6%) of controls were homozygote CC (Table 1). Statistical analysis indicated that there was no significant difference between PE and control women ($P = 0.3$).

PCR-RFLP [position 788C→T (Thr 263 Ile)]

The change at position 788C→T (Thr 263 Ile) indicated that 114 (92.6%) of PE cases and 100 (83.3%) of normal

Table 1
Distribution of *TGF*-β1 genotypes and allele frequencies in pre-eclampsia and controls.

TGF-β1 genotype and allele	Pre-eclampsia ($n = 123$)	Controls ($n = 120$) (pregnant and non-pregnant)	*P*-value*
Genotype: 29T→C(Leu10Pro)			
TT	45(36.6%)	39(32.5%)	0.5
TC	50(40.6%)	46(38.3%)	
CC	28(22.8%)	35(29.2%)	
Allele frequencies			
T	0.57	0.5	0.2
C	0.43	0.49	
Genotype: 74G→C(Arg25Pro)			
GG	100(81.3%)	96(80%)	0.3
GC	18(14.6%)	22(18.4%)	
CC	5(4.1%)	2(1.6%)	
Allele frequencies			
G	0.89	0.91	0.6
C	0.11	0.09	
Genotype: 788C→T(Thr263Ile)			
CC	114(92.6%)	100(83.3%)	0.02
CT	9(7.4%)	20(16.7%)	
TT	0(0%)	0(0%)	
Allele frequencies			
C	0.97	0.91	0.06
T	0.03	0.09	

* Statistical test: χ^2.

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controls were CC homozygous. Moreover, 9 (7.4%) of PE cases and 20 (16.7%) of normal subjects were CT heterozygous. None of the individuals in the two studied groups were TT homozygous (Table 1). The genotype distribution at this position was a statistically significant difference in genotype distribution between PE cases and control subjects ($P = 0.02$).

Comparison of haplotype frequencies between PE and control women

Six haplotypes were constructed at the three polymorphic sites among PE and control subjects (Table 2). Among them, two most frequent in both study groups were "TGC" and "CGC". Statistical analysis showed no significant difference in their frequency distributions between the two study groups ($P = 0.8$).

Measurement of serum TGF-β1 concentration

The biologically active TGF-β1 protein concentration was determined using a solid-phase TGF-β1-specific sandwich ELISA. A TGF-β1 standard curve was constructed using 200, 100, 50, 25, and 12.5 ng/ml of recombinant human TGF-β1 protein. Mean serum levels of TGF-β1 in PE patients were 62.73 ng/ml, compared with 47.01 and 40.68 ng/ml in pregnant and non-pregnant control women, respectively. As summarized in Table 3, the mean serum TGF-β1 level was significantly higher in preeclamptic patients in comparison with both of the two control groups ($P = 0.0001$; Table 3). The serum TGF-β1 levels in relation to different genotypes in PE, pregnant control and non-pregnant control women are summarized in Table 3.

Table 2
Comparison of haplotype frequency distributions between pre-eclampsia and controls.

Haplotype	Pre-eclampsia ($n = 123$)	Controls ($n = 120$)	*P*-value
TGC	0.45	0.45	
CGC	0.39	0.36	0.8
TCC	0.08	0.06	
CCC	0.02	0.05	

Table 3
Serum levels of TGF-β1 in whole pre-eclampsia and control groups and in relation to different genotypes.

		Serum TGF-β1 (ng/ml)	*P*-value*
Preeclampsia ($n = 123$)		62.73	
Pregnant control ($n = 100$)		47.01	0.0001
Non-pregnant control ($n = 20$)		40.6	0.0001

SNP position	Genotype	Pre-eclampsia ($n = 123$)	Pregnant ($n = 100$)	Non-pregnant ($n = 20$)	
788	CC	70.59(114)	51.57(72)	44.41(11)	0.146
	CT	54.88(9)	42.46(28)	35.97(9)	0.176
	TT	0(0)	0(0)	0(0)	
P-value		0.524	0.532	0.548	
74	GC	62.11(18)	46.08(23)	45.51(3)	0.131
	GG	74.18(100)	50.57(76)	31.17(16)	0.121
	CC	51.81(5)	44.22(1)	43.46(1)	0.217
P-value		0.542	0.627	0.519	
29	TC	51.64(50)	45.08(43)	44.53(5)	0.134
	TT	71.90(45)	50.47(33)	39.97(8)	0.142
	CC	64.85(28)	45.42(24)	35.54(7)	0.232
P-value		0.521	0.614	0.517	

* Statistical test: ANOVA.

Discussion

Preeclampsia is pathophysiologically characterized by endothelial cell damage and dysfunction [25]. A hypothesis indicates that one or more factors released from the placenta may lead to endothelial activation [26]. In searching for those triggers, a possible pathway of endothelial cell activation by TGF-β1 has been described [27,28]. Another hypothesis proposed that endothelial dysfunction is a part of the intravascular inflammatory reaction and PE is actually an excessive maternal inflammatory response to pregnancy [29]. The balance between regulatory T cells (Tregs) and Th17 cells is thought to be important in the development of maternal systemic inflammation in PE patients [30,31]. Tregs can induce tolerance [32,33] and Th17 cells induce inflammation or rejection [34]. The prevalence of peripheral Tregs is decreased following PE [35,36] but that of Th17 cells is increased compared to healthy pregnant women [30,37]. Since TGF-β1 can induce the differentiation of Tregs [38] and inhibit that of Th17 cells [39] the increased Th17/Treg ratio in PE patients is possibly mediated by down regulation TGF-β1 signaling [30]. Thus, TGF-β1 becomes a candidate factor that is potentially relevant to the origin of PE. Several studies suggested that TGF-β1 may be involved in reproductive-related disorder, including preeclampsia, although data were controversial [40–42]. In this study we analyzed polymorphisms of the TGF-β1 coding region at 29T→C (Leu 10 Pro), 74G→C (Arg 25 Pro), and 788C→T (Thr 263 Ile) in 123 Iranian women with PE and 120 matched control individuals (100 pregnant and 20 non-pregnant) and we also measured serum levels of this cytokine in this study group. At position 29T→C TT and TC genotypes in cases and CC genotype in healthy controls were of high frequency. At position 74G→C GG and CC genotypes in PE cases and GC genotype in healthy subjects were of high frequency but the genotypes and allele frequency distribution of 29T→C and 74G→C SNPs showed no significant differences between PE and controls. Our results are in accordance with those of two recent studies by Daher et al. [43] and Stanczuk et al. [44] that show there is no association between these polymorphisms in PE and healthy pregnant controls in separate populations. In our study, at position 29T→C individual with TT genotype was high TGF-β1 serum level (Table 3), while other scientists reported that the amount of serum TGF-β1 is higher for CC genotype than TT genotype [45]. Kim and colleague found that the C allele was associated with an increased risk of preeclampsia in Korean patients [46]. Stanczuk and colleague reported that the homozygosity for the T allele could contribute to severe outcomes of preeclampsia [44]. However, De Lima and colleague reported similar results to our

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report that there was no apparent relationship between the codon +10 polymorphism and the risk of preeclampsia in the Brazilian population [47]. The reasons for these discrepancies are not clear; however, it may be based on ethnic and environmental factors. In addition, in agreement with previous findings the G allele of the 74G→C SNP was associated with high level production of TGF-β1 [20] and the CC genotype was lower of this cytokine (Table 3). At position 788C→T CC genotypes in cases and CT genotypes in control subjects were of high frequency and TT genotypes in both groups were of lower frequencies. In our investigation both genotypes and allele frequencies were significantly different between PE and control groups (pregnant and non-pregnant), ($P = 0.02$, Table 2). There are no pervious data on the 788C→T polymorphism in PE patients. Recently TGF-β1 gene polymorphisms have been studied in recurrent spontaneous abortion patients and no associations were found [48]. We have a hypothesis that this SNP 788C→T (Thr 263 Ile) by increasing the affinity of TGF-β1 to its receptor, promotes to enhance the formation of binding between TGF-β1 and its receptor, so in the same concentration of TGF-β1 more number of receptors will be activated. Therefore this SNP may be a risk factor for PE. Two analyzed haplotypes on the three SNPs showed no statistically significant differences between PE and control women. Two haplotypes TGC (45%) and CGC (39%) were the most frequent in the two groups of women, respectively (Table 3). Aguilar-Duran and colleague found no association between haplotypes at position −800G→A, −509C→T and 29T→C TGF-β1 gene with preeclampsia [49]. Moreover, results from Li and colleague support that the TGF-β1 869 T.C polymorphism was associated with the risk of PE [50]. Serum TGF-β1 levels in PE patients have been previously studied but the data are not conclusive. In agreement with other reports our results showed statistically significant differences in mean TGF-β1 serum level between PE and control groups [15,16,51–53] while in contrast to our findings, other scientists have been reported no significant differences in serum TGF-β1 between PE and control individuals [54]. This controversy may be attributed to the different racial and genetic background of the different cohorts, as well as the small sample size for PE patients in these studies. In Conclusion, the presence of G nucleotide at position 74G→C (Arg 25 Pro) of TGF-β1 is associated with elevated cytokine production, therefore the higher frequency of allele at position 74G→C in PE cases compared to control subjects may be considered as a genetic susceptibility factor for the development of PE. Therefore, polymorphisms of coding regions of TGF-β1 may play a role in PE susceptibility. Greater sample sizes and studies using a considerable number of informative ancestry markers are required to confirm these findings and also to identify populations, where TGF-β1 variants are mediating the casual pathway of Preeclampsia.

Ethics statement

The Ethics Committee at research General Hospital approved the use of the clinical information and the collection of samples for research purposes. Written informed consent was obtained from all enrolled subjects.

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Competing interests

The authors have declared that no competing interests exist.

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