



CLINICAL ARTICLE

Heterozygosity in *CTLA-4* gene and severe preeclampsia

A. Samsami Dehaghani^a, M. Doroudchi^{b,c,*}, T. Kalantari^c,
A.M. Pezeshki^c, A. Ghaderi^{b,c}

^aDepartment of Obstetrics/Gynecology, Shiraz University of Medical Sciences, Shiraz, Iran

^bDepartment of Immunology, Shiraz University of Medical Sciences, Shiraz, Iran

^cShiraz Institute for Cancer Research, Shiraz University of Medical Sciences, Shiraz, Iran

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KEYWORDS

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Abstract

Objective: One of the major complications of pregnancy, preeclampsia makes pregnancy termination inevitable in most cases. Similarities exist between the mechanisms that maintain normal pregnancy, allograft transplants, and, it is postulated, peripheral self-tolerance. In addition, the critical role of the cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) molecule in maintaining self-tolerance has been established. Therefore, the frequency of *CTLA-4* A49G polymorphism was investigated in severe preeclampsia. **Patients and Methods:** Genomic DNA extracted from mononuclear cells of the peripheral blood of 36 pregnant women with severe preeclampsia and 151 healthy women was analyzed. A49G polymorphism in position 49 of exon-1 of the *CTLA-4* gene was studied by the polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) method. **Results:** The frequency of the GG genotype was 2 (5.6%) in patients and 19 (12.6%) in controls, while the frequency of the AA genotype was 4 (11.1%) and 60 (39.7%). Interestingly, the frequency of the AG genotype was significantly higher in preeclamptic than in healthy women from the general population (83.3% vs. 47.7%; $P=0.0005$). **Conclusion:** These data suggest that heterozygosity in the *CTLA-4* A49G allele might be a predisposing factor for severe preeclampsia. Whether the observed association results from linkage imbalance with other loci on chromosome 2 or other polymorphisms of the *CTLA-4* gene or

* Corresponding author. Department of Immunology, Shiraz University of Medical Sciences, P.O. Box: 71345-3119, Shiraz, Iran. Tel.: +98 711 2303687; fax: +98 711 2304952.

E-mail address: mdoroud@sums.ac.ir (M. Doroudchi).

even from a preferential transfer and/or expression of one allele from a heterozygous mother to the fetus will be the subject of future investigations.

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1. Introduction

One of the major complications of pregnancy, preeclampsia causes maternal and fetal morbidity and mortality [1]. The preeclamptic syndrome is defined by hypertension and proteinuria or hypertension accompanied by headache, visual disturbances, abdominal pain, rapid weight gain and/or abnormal laboratory tests [2]. Despite extensive research, the etiology of the disease is still unknown. However, one of the likely hypotheses for the pathogenesis of the disease is the poor adaptation of the maternal immune system to the fetal allograft [3,4]. In this regard, it has been proposed that a Th₁/Th₂ cell disequilibrium occurs because of an abnormal activation of a Th₁-type immune response during a preeclamptic pregnancy followed by the breakdown of peripheral tolerance to the foreign antigens of the fetus [5,6]. Moreover, a familial tendency for this syndrome suggests that genetic elements are predisposing factors [7,8].

Considering the similarities between the maintenance of a normal pregnancy and that of an allograft transplant, it has been suggested that mechanisms involved in the tolerance of the fetus as allograft are also similar to the mechanisms involved in peripheral self-tolerance [9]. Recent studies have shown the critical role of CD4⁺CD25⁺ regulatory T cells in the maintenance of self-tolerance via constitutive expression of the CTLA-4 molecule [10]. These regulatory T cells exert a dominant down-regulatory role in the activation and proliferation of T cells. It is suggested that CD4⁺CD25⁺ T cells are specific for self-antigens in natural self-tolerance or are specific for alloantigens in transplant tolerance [11]. In this regard, it has been shown that CTLA-4 A49G polymorphism is an independent risk factor associated with acute allograft rejection after liver transplant [12]. The expression of the CTLA-4 gene in the placental fibroblasts and the preferential transfer of CTLA-4 alleles to the fetus during a successful pregnancy have raised the possibility of involvement of the CTLA-4 molecule in the maintenance of tolerance at the maternal–fetal interface [9,13]. The CTLA-4 gene is placed on chromosome 2, a candidate chromosome to carry maternal susceptibility loci

for preeclampsia [14,15]. The gene carries several polymorphisms that have been shown to be of clinical importance. It has been shown that thymine at position –318 and adenine at position 49 in exon-1 are associated with higher expression of the CTLA-4 protein and mRNA [16]. In addition, stretches of AT repeats in the 3'-untranslated region of the CTLA-4 gene has been suggested to affect mRNA stability and fetal survival in humans [9,17].

In the present study, the frequency of CTLA-4 A49G polymorphism in women with severe preeclampsia was compared with the frequency of the polymorphism in healthy Iranian women.

2. Subjects and methods

2.1. Subjects

From April 2001 to March 2003, 73 women were diagnosed with preeclampsia in the teaching hospitals of Shiraz University of Medical Sciences after their 20th week of pregnancy. Early diagnosis was made based on elevated blood pressure (>140/90 mmHg) in at least three successive measurements every 15 min and proteinuria (dipstick reading >1+ per 24 h). None of the women had a history of hypertension before pregnancy. Of these women, 36 who had a systolic blood pressure of 160 mm Hg or higher and a diastolic blood pressure of 110 mmHg or higher in addition to a proteinuria of 1+ or greater were entered in the study. After delivery, their blood pressure was monitored, and in none of them, stable hypertension was observed. The control group consisted of 151 healthy pregnant women who had normal blood pressure readings in at least three successive measurements followed by another measurement after delivery. In addition, women in the control group had no personal or familial history of autoimmune diseases and/or malignancy. Demographic data including age, admission date, parity, gestational age, type of delivery, birth weight, blood group and the results of platelet level measurement and liver function tests were recorded from the patients' files.

2.2. DNA preparation and *CTLA-4* gene amplification

Peripheral blood samples of 10 mL were collected by venous puncture, and genomic DNA was extracted by the salting out method, as described by Miller et al. [18].

Polymerase chain reaction (PCR) was used to amplify the desired fragment of *CTLA-4* gene using the primers described by Donner et al. [19] and the method described elsewhere [20].

2.3. Single-strand conformation polymorphism analysis

One microliter of PCR product was subjected to polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis according to the previously described method [20]. Bands were visualized after silver staining of gel (Fig. 1), as described in Ref. [20].

2.4. Statistical analysis

The results were analyzed with a Chi square (χ^2) test and the Spearman correlation coefficient using EPI Info 2000 software (public domain application). Software package SPSS, version 10 (SPSS, Chicago, Ill) was used for the analysis of correlation between different parameters. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed vs. expected genotype frequencies in each group. Multiple

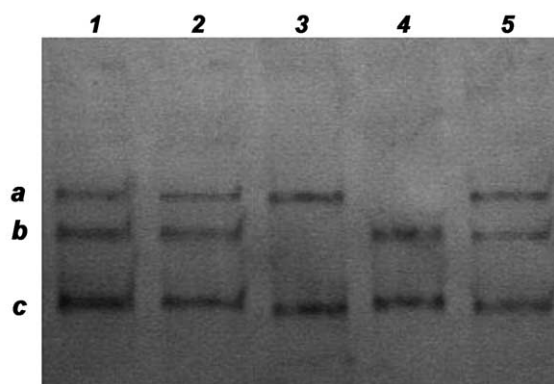


Figure 1 Silver-stained polyacrylamide gel of *CTLA-4* PCR–SSCP fragments. Band ‘a’ shows a ‘‘G’’, band ‘b’ indicates an ‘‘A’’ at position 49 of exon-1, whereas band ‘c’ is detected in all samples. Individuals in lanes 1, 2 and 5 are heterozygous A/G (Thr/Ala), individual in lane 3 is homozygous GG (Ala), and individual in lane 4 is homozygous AA (Thr).

Table 1 Genotypic frequencies of *CTLA-4* gene A/G polymorphism in preeclamptic patients and control group

	Patients	Control group	Healthy blood donors*
GG	2 (5.6%)	19 (12.6%)	44 (9.6%)
AG	30 (83.3%)	72 (47.7%) ^a	227 (49.6%) ^b
AA	4 (11.1%)	60 (39.7%)	187 (40.8%)
Total	36 (100%)	151 (100%)	458 (100%)

^a $P=0.0005$.

^b $P=0.0004$.

* The population included 178 women and 280 men.

comparisons using a χ^2 test were performed according to the Fleiss method [21].

3. Results

Table 1 shows the frequency of each genotype in the preeclamptic and healthy women. As shown, there was a statistically significant difference between the frequencies of *CTLA-4* genotypes in the two groups. In this regard, the frequency of the heterozygous AG genotype was higher in preeclamptic women than in controls ($P=0.0005$). Multiple comparisons using a χ^2 test revealed that the increase in AG genotype was significant only in comparison with the AA genotype in both the control group ($P<0.001$) and the general population ($P<0.001$). Of 72 chromosomes studied in pregnant women with severe preeclampsia, the frequencies of the A and G alleles were 38 (52.8%) and 34 (47.2%), respectively, whereas in the controls, of 302 chromosomes, the frequencies of the A and G alleles in the controls were 192 (63.6%) and 110 (36.4%). In this respect, there was an increase in the frequency of the G allele in the preeclamptic group. In addition, a significant deviation from the Hardy–Weinberg equilibrium was observed in the preeclamptic group ($P<0.01$) but not in the control group ($P>0.5$).

Table 2 illustrates the characteristics of the patients. There was no significant association in the preeclamptic group regarding these variables.

4. Discussion

Cytotoxic T lymphocyte antigen is an immune attenuator molecule, which exerts a regulatory function and results in the suppression of T-cell activation [22,23]. The molecule may also have a critical role in T-cell differentiation and Th₁/Th₂ equilibrium [24]. Constitutive expression of the *CTLA-4* molecule on a specific lineage of regulatory

Table 2 The Characteristics of preeclamptic patients

Patient No.	Age	GA [§]	UP	BP	BG	PT *1000	LFT	Delivery*	BW	Genotype
1	20	31	3+	160/110	AB+	117	N	C/s	1200	AG
2	20	33	3+	210/120	A+	300	N	C/s	1300	AG
3	30	26	4+	170/110	B+	120	ND	C/s	ND	AG
4	32	34	3+	160/110	A+	142	N	C/s	1950	AG
5	39	40	ND	180/110	A+	173	N	C/s	3900	AG
6	35	28	4+	160/110	B-	169	N	C/s	750	AA
7	30	27	3+	160/110	O+	45	ABN [‡]	C/s	900	AG
8	20	35	3+	170/115	AB+	87	N	C/s	1850	AG
9	30	24	2+	160/110	O+	213	N	C/s	600	AG
10	18	42	4+	170/120	A+	325	N	C/s	3700	AG
11	29	39	3+	180/110	A+	218	ND	NVD	1700	AG
12	44	39	3+	160/120	AB-	150	N	C/s	1800	AG
13	30	38	4+	160/120	A+	266	N	NVD	2500	AG
14	38	36	2+	170/120	A+	383	ND	C/s	5500	AG
15	30	36	4+	170/120	B+	110	N	C/s	2700	GG
16	30	36	ND	190/130	O-	166	N	C/s	3600	AA
17	30	33	2+	180/110	O+	235	ABN	C/s	1550	AG
18	20	33	2+	160/110	O+	218	ABN	NVD	2300	AG
19	27	37	ND	170/110	O+	181	N	NVD	2500	AG
20	19	24	4+	170/115	O+	187	N	C/s	1700	AA
21	30	36	2+	160/115	A-	320	N	C/s	4300	AG
22	16	36	3+	165/110	AB+	112	ND	NVD	2250	AG
23	18	34	4+	165/115	A+	27	ABN	C/s	1950	AG
24	24	37	2+	160/110	A+	79	N	C/s	1550	AG
25	25	37	2+	160/110	B+	53	ABN	NVD	1900	AG
26	29	34	2+	170/110	B+	190	ABN	NVD	1600	AG
27	25	35	3+	180/110	A+	245	ABN	NVD	2600	AG
28	19	35	3+	160/110	O+	208	ABN	NVD	2200	AG
29	19	28	4+	170/120	B+	106	N	C/s	2400	AG
30	24	37	3+	160/110	B+	60	ND	NVD	ND	AG
31	37	32	3+	160/110	B+	300	N	C/s	1250	AG
32	20	27	2+	160/110	A-	200	N	C/s	730	AG
33	22	36	3+	180/110	B+	196	N	C/s	1575	AA
34	36	35	2+	170/110	A+	230	N	C/s	1960	AG
35	23	32	ND	170/120	B+	250	N	NVD	1450	GG
36	19	42	2+	170/110	O+	227	N	C/s	3600	AG

[§] GA—gestational age, UP—urine protein, BP—blood pressure, BG—blood group, PT—platelet count, LFT—liver function test, BW—birth weight.

* C/s—cesarean section, NVD—normal vaginal delivery.

[‡] ABN—abnormal, N—normal, ND—not determined.

T cells has been suggested to be one of the main factors in peripheral self-tolerance, which can be mimicked in the maintenance of tolerance to allogenic antigens [11]. From an immunological point of view, the fetus is an allograft bearing allogenic antigens. Therefore, the maintenance of this allograft is in part related to the mechanisms and molecules involved in naturally programmed self-tolerance.

In this study, the exon-1 A49G polymorphism of the *CTLA-4* gene that corresponds to an amino acid change (threonine to alanine) in the leader peptide of the expressed protein was investigated in 36 women with severe preeclampsia. The genotype and allele frequencies were compared with those of 151 healthy ethnic-matched women. Interestingly, the results indicated that the frequency of heterozygosity was highly increased, and the fre-

quency of the AA homozygous genotype was decreased among preeclamptic women. This was contrary to the slight difference between the frequencies of AA and AG genotypes found in 458 healthy blood donors (Doroudchi et al., unpublished data). A comparison of genotype frequencies between preeclamptic women and healthy blood donors revealed a highly significant difference ($P=0.00042$; Table 1).

In a previous study on idiopathic recurrent spontaneous miscarriage, Tsai et al. [9] showed that there was an association between the transmission of longer repeats of (AT)_n alleles of the *CTLA-4* gene in heterozygous pregnant women and miscarriage. They also suggested that only maternal alleles of the *CTLA-4* gene might be expressed in the fetus. Inasmuch as there is a linkage disequilibrium between the G allele in exon-1 and

the common long (AT)_n allele (i.e., 106 bp allele), the slight increase in the frequency of the G allele in our patients is noteworthy. However, the increased susceptibility of heterozygous women to preeclampsia cannot simply be ascribed to either of A or G alleles. There is a possibility that the interaction between the A and G alleles may result in a new functional or expressional pattern of the gene, or that the two allelic products may become activated in different phases of disease development, as suggested by others [25].

Tsai et al. [9] observed a preferential transmission of maternal *CTLA-4* alleles to children contrary to the random transmission of paternal alleles. In their study, the short (AT)_n allele was transmitted from heterozygous mothers to 74% of liveborn children compared with the 47% transmission of the allele from heterozygous fathers. Although our study cannot address this question, there might be a preferential transfer of one of A/G alleles from heterozygous mothers to their fetuses, or there might be a difference in the expression of paternal and maternal *CTLA-4* alleles in the fetus. It is also possible that the observed effect of heterozygosity can solely be related to the linkage disequilibrium to other closed loci on chromosome 2 or even to other polymorphisms of the *CTLA-4* gene. However, it is worth mentioning that a significant increase in A/G heterozygosity has previously been reported in female patients with rheumatoid arthritis and multiple sclerosis [25,26]. There are also several reports on the other genes regulating the immune system for which heterozygosity confers susceptibility to common diseases [27,28].

In conclusion, this study points to the *CTLA-4* gene as a susceptibility locus for preeclampsia in Iranian women, and it should be confirmed in other ethnic groups.

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