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ORIGINAL ARTICLE

Immunoregulatory gene polymorphisms in women with preeclampsia

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The costimulatory molecules CD28, cytotoxic T-lymphocyte antigen-4 (CTLA-4) (cytotoxic T-lymphocyte-associated antigen-4) and inducible costimulator (ICOS) are believed to have a critical modulatory role in the immune response. However, few studies have been performed on the role of these immune regulatory molecules and their polymorphisms in women with preeclampsia (PE). The aim of our study was to evaluate the *CTLA4* (+49 A/G) (rs 231775), *CD28* (+17 T/C) (rs 3116496) and *ICOS* (−1564 T/C) (rs 4675378) gene polymorphisms in Brazilian women with PE. This case–control study included 130 patients with PE and 261 control women without any obstetric or systemic disorders. Genomic DNA was extracted from peripheral blood, and the polymorphism genotyping was performed by digesting the PCR products with the restriction endonucleases *Bbv*I (*CTLA-4*), *Afe*I (*CD28*) and *Alu*I (*ICOS*). Data were analyzed by χ^2 or Fisher's exact test; a *P*-value of <0.05 was considered as significant. There were significant differences in the *ICOS* genotype and allelic frequencies between the PE and control groups ($P=0.01$ and $P=0.01$, respectively). We found a significantly lower frequency of the *ICOS (−1564)* T allele in women with mild PE compared with the controls. There were no differences in the *CTLA-4* (+49 A/G) and *CD28* (+17 T/C) genotypes and allelic frequencies between the PE patients and controls. Our data suggest that PE is associated with *ICOS*, but is not associated with the *CTLA-4* or *CD28* gene polymorphisms.
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INTRODUCTION

Preeclampsia (PE) affects approximately one in every 10 pregnancies and is an important cause of maternal and perinatal adverse outcomes. In Latin America, hypertensive disorders are the most common cause of maternal death.[1] Despite its clinical relevance and frequency, the etiology and pathogenic mechanisms of PE remain unclear, possibly because PE is a multi-factorial disease with several different risk factors. Additionally, this condition can present in various clinical forms, ranging from mild PE occurring late in pregnancy to severe forms that may present early in the second trimester, which, in fact, could have different etiopathogenic pathways.[2–5]

Many investigations suggest that PE is an immune-mediated disorder.[6,7] However, there is a paucity of studies that involve immunoregulatory molecules and their polymorphisms in women with PE.

The expression of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) molecule on regulatory T cells is considered to be critical for the maintenance of tolerance at the maternal–fetal interface.[8,9] Together with CTLA-4, the costimulatory molecule CD28 and the inducible costimulator (*ICOS*) gene are important regulators of the immune system, and their corresponding genes are located on the chromosome 2q33.[10–12]

The single-nucleotide polymorphisms of these T-cell regulatory genes have been associated with autoimmune diseases[13,14] and with acute allograft rejections after liver transplants.[15] According to current theories, PE can also be considered a type of immune disorder.[3,16] Until now, only two studies have analyzed the *CTLA-4* gene polymorphism in women with PE. The first study involved a small group of Iranian patients[17] and suggested that heterozygosis could be a predisposing factor for severe PE. However, Jääskeläinen *et al.*[18] analyzed the same polymorphism in a Finnish population and reported that the *G* allele appeared to be the risk allele for PE. To the best of our knowledge, there have been no previous studies assessing the association between PE and the *CD28* or *ICOS* genetic polymorphisms.

Although the exact mechanisms are not yet completely understood, there seems to be a clear genetic predisposition to PE,[16,19] and chromosome 2 appears to be implicated in this tendency. Coincidentally, the *ICOS*, *CD28* and *CTLA-4* genes are located on this chromosome.[10,11]

The aim of our study was to evaluate the *CTLA4* (+49 A/G) (rs 231775), *CD28* (+17 T/C) (rs 3116496) and *ICOS* (-1564 T/C) (rs 4675378) gene polymorphisms in Brazilian women with PE.

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METHODS

Subjects

This case–control study comprised pregnant women who were referred to the obstetric ward of the Sao Paulo Hospital (Sao Paulo Federal University) or admitted to the Dr Mário de Moraes Altenfelder Municipal Teaching Hospital (both facilities located in Sao Paulo, Brazil) between 2007 and 2009. The study group included 130 women diagnosed with PE according to the criteria proposed by the 'National High Blood Pressure Education Program'.[20] According to these recommended guidelines, PE was defined as the presence of systolic blood pressure $\geqslant$140 mm Hg and diastolic blood pressure $\geqslant$90 mm Hg after 20 weeks of gestation in a previously normotensive woman, accompanied by proteinuria (urinary excretion of $\geqslant$0.3 g protein in a 24-h specimen). Women with blood pressure $<$160/110 mm Hg (systolic/diastolic), a normal platelet count, normal liver enzyme values and no maternal symptoms were classified as having mild PE; all others were classified as cases of severe PE.

The exclusion criteria consisted of the presence of following: a multiple gestation, fetal death, autoimmune diseases, diabetes, uterine malformation, *in vitro* fertilization treatment, placental abruption, infection and cancer or any other systemic disease, including pre-existing hypertension.

The control group included 261 pregnant women matched by race to the study group. Control women had to be normotensive during the index pregnancy and must have had a history of at least two previous normal pregnancies without any maternal or fetal disorders.

The study protocol was approved by the Ethics Committees of both aforementioned hospitals, and written informed consent was obtained from all participants.

Upon admission, 10 ml of peripheral blood was collected from each participant in tubes containing ethylenediamine tetraacetic acid (BD Diagnostics, Franklin Lakes, NJ, USA). The tubes were immediately centrifuged to obtain the buffy coat layer containing the polymorphonuclear cells, and the DNA was extracted by the dodecyl trimethyl ammonium bromide/cetyl trimethyl ammonium bromide method.[21]

Genotyping of polymorphisms

The sequences of the primers and cycling conditions, as well as the genotyping methods have been previously described.[12] The *CTLA-4* (rs231775), *CD28* (rs3116496) and *ICOS* (rs4675378) gene polymorphisms were genotyped by the restriction fragment length polymorphism method.[12] Briefly, the *CTLA-4*, *CD28* and *ICOS* PCR products were digested with the *Bbv*I, *Afe*I and *Alu*I restriction endonucleases (New England Biolabs, Beverly, MA, USA), respectively.

The *CTLA-4* gene polymorphism genotyping resulted in fragment sizes of 464 bp (A) or 239 and 255 bp (G). The *CD28* gene polymorphism resulted in sizes of 149 bp (C) or 125 and 24 bp (T). Finally, the *ICOS* gene polymorphism genotyping resulted in fragment sizes of 385 bp (T) or 339 and 289 bp (C).

All PCR products were visualized by electrophoresis on an agarose gel that was stained with ethidium bromide.

Statistical analysis

Two-tailed, pooled Student's *t*-tests were used to analyze continuous variables.

The sample size estimates were based on the *CTLA4* (+49 A/G) gene polymorphism (rs 231775) frequency, according to the Hap Map Project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap27_B36/) data, which defined an allele risk of 39% in the European Caucasian population. Applying the allele risk data in the Power and Sample Size Program Version 2.1.30[22] and considering an 80% power and a two-tailed α value of 0.05, a sample size of 108 patients per group was determined to be sufficient to detect an association between the alleles and PE.

The Hardy–Weinberg equilibrium tests were performed by calculating the expected frequencies of each genotype and comparing them with the observed values. The single allelic and single genotype frequencies (obtained by direct count) were analyzed by the Fisher's exact or χ^2-tests, with the level of significance set at 0.05 and the Bonferroni correction for multiple testing. The odds ratios and 95% confidence intervals were calculated. A statistical analysis was performed with standard software (SPSS for the Social Science, v13.1 for Windows).

Table 1 Characteristics of women included in the study

Variable	*Preeclampsia* n=*130*	*Control* n=*261*	P-*value*
Age[a]	25.79 ± 6.85	30.72 ± 9.29	<0.0001
Number of pregnancies[a]	1.9 ± 1.49	2.51 ± 1.38	0.0003
Race[b]			
White	73 (56%)	174 (67%)	0.09
Mulatto	36 (28%)	60 (23%)	
Black	21 (16%)	27 (10%)	
Gestational age at collection[a]	33.18 ± 4.98	31.93 ± 5.05	0.14

[a]Data presented as mean ± s.e.m. and analyzed by Student's *t*-test.
[b]Data analyzed by χ^2-test.

RESULTS

The clinical characteristics of participants are presented on Table 1.

All single-nucleotide polymorphisms in the PE and control groups were in Hardy–Weinberg equilibrium. The genotype and allele frequencies are shown in Table 2. The genotype and allelic frequencies of the controls and of the patients with mild and severe PE are presented in Table 3.

There were significant differences in *ICOS* (−1564 T/C) genotype (TT *vs.* TC *vs.* CC—corrected P=0.03, χ^2 test) and allelic frequencies (corrected P=0.03, χ^2 test) between PE and controls. We also found a significantly lower frequency of the T allele in the mild PE patients compared with the controls (corrected P=0.03). However, there were no statistically significant differences in the genotype and allelic frequencies between the mild and severe PE groups.

There were no differences in the *CTLA-4* (+49 A/G) and *CD28* (+49 A/G) genotypes and allelic frequencies between the PE patients and controls. We detected a significant difference in the *CTLA4* +49 genotype frequencies between the mild and severe PE groups (P=0.03, χ^2-test), although this was not confirmed after the Bonferroni correction (corrected P=0.12). Additionally, we did not identify any association between the *CTLA-4* and *CD28* gene polymorphisms when comparing the controls *vs.* the patients with mild or severe PE.

DISCUSSION

Our results suggest an association between the *ICOS* gene polymorphism and PE. However, we did not detect any relationship between the *CTLA-4* and *CD28* gene polymorphisms and PE.

The genotype frequencies were in Hardy–Weinberg equilibrium in both the patients and controls. Bearing in mind the importance of ethnical matching in disease association studies, our groups were matched by race.[23–26] In addition, the genotype frequencies were similar to those previously reported for the Brazilian population.[12]

Most association studies between pregnancy and immunoregulatory molecules have focused mainly on *CTLA-4*.[17,18,27] The T regulatory (Treg) cells that express the activation marker CTLA-4 are critical for the maintenance of maternal tolerance to fetal antigens.[8,9] Additionally, a decreased ratio of CTLA-4(+)/CD28(+) expression, both in peripheral blood and in deciduas, seems to be associated with pregnancy loss.[28] Several studies have shown that ICOS has a pivotal role in T-cell activation and in Th1/Th2 differentiation.[29] Despite this, the role of ICOS has not been previously investigated in pregnancy, a condition that is clearly influenced by these processes. To the best of our knowledge, this is the first study to simultaneously evaluate these three immunoregulatory molecule gene polymorphisms in pregnancy.

We observed a significantly lower frequency of the *ICOS* −1564 T allele and of the TT genotype in the women with PE compared with

Table 2 Genotype and allele frequencies of *CTLA-4, CD28* and *ICOS* gene polymorphisms in women with preeclampsia and controls

	Preeclampsia	*Control*	*OR (95% CI)*	P-*value*	*Corrected* P-*value*[a]
CTLA-4 (+49)					
Genotypes[b]	*n*=125	*n*=158			
AA	58 (46.4%)	76 (48.1%)		0.62	—
AG	47 (37.6%)	63 (39.9%)			
GG	20 (16%)	19 (12%)			
GG×(AG+AA)[c]			1.39 (0.70–2.74)	0.38	—
Alleles[c]	*n*=250	*n*=316			
A	163 (65.2%)	215 (68%)	1.13 (0.79–1.61)	0.52	—
G	87 (34.8%)	101 (32%)			
CD28 (+17)					
Genotypes[b]	*n*=125	*n*=171			
TT	84 (67.2%)	125 (73.1%)		0.38	—
TC	34 (27.2%)	41 (24%)			
CC	7 (5.6%)	5 (2.9%)			
TT×(TC+CC)[c]			0.63 (0.38–1.06)	0.08	—
Alleles[c]	*n*=250	*n*=342			
T	202 (80.8%)	291 (85.1%)	0.73 (0.47–1.13)	0.18	—
C	48 (19.2%)	51 (14.9%)			
ICOS (−1564)					
Genotypes[b]	*n*=124	*n*=167			
TT	52 (41.9%)	99 (59.3%)		0.01	0.03
TC	60 (48.4%)	54 (32.3%)			
CC	12 (9.7%)	14 (8.4%)			
TT×(TC+CC)[c]			0.49 (0.30–0.79)	0.004	0.012
Alleles[c]	*n*=248	*n*=334			
T	164 (66.1%)	252 (75.4%)	0.63 (0.44–0.91)	0.01	0.03
C	84 (33.9%)	82 (24.6%)			

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Bonferroni correction.
[b]χ^2-test.
[c]Fischer's exact test.

the controls. Similar results were reported in patients with type 1 diabetes.[30] In a study involving Finnish and Estonian participants, Douroudis *et al.*[30] detected a marginally significant decrease in frequency of the CTIC154_1 T allele in the group of type 1 diabetics. Thus, this gene polymorphism might be related to the immune/inflammation processes. Although little is known about the functional role of this polymorphism, other investigators have reported that variations in noncoding regions may influence the mRNA of the *ICOS* gene and subsequently affect its expression.[29,31]

Jääskeläinen *et al.*[18] did not detect an association between the *CTLA-4 +49* genotype and PE, but did detect an increased frequency of the *G* allele in Finnish PE patients. In contrast, an increased frequency of the GA genotype was reported among Iranian women with severe PE.[17] Our data also showed an increased frequency of the GA genotype in Brazilian women with severe PE; however, this difference was not confirmed after the Bonferroni correction. These controversial findings could be attributed to the sample size, statistical analysis and differences in the polymorphism distribution among races.

The CTLA-4 expression seems to be critical for the suppressive effects of Treg cells.[32] Although none of the three studies evaluated the expression of this molecule, differences in this polymorphism seem to modify the CTLA-4 expression and compromise the activity of the Treg cells.[8,33,34]

Further studies are needed to validate the biological significance of the *ICOS* (−1564 T/C) gene polymorphism identified in the present study. Although the number of participants was not large, ours is the first study to investigate the association between PE and this specific genetic marker. These results need to be replicated in larger groups of women and in more ethnically diverse populations.

The genes of the costimulatory molecules *CD28*, *CTLA-4* and *ICOS* are situated in the same region on chromosome 2q33.[11,12,31] As previously reported by other investigators, this chromosome seems to be implicated in a woman's predisposition to PE.[10] In addition to being located closely together on the same chromosome, these three molecules seem to be implicated in the Th1—Th2 balance through Treg activation.[8,29] Previous studies have reported that women with PE have altered Treg cell numbers and/or function.[8,35] Due to these common characteristics, we can speculate that these three genes could be involved in the susceptibility to the same disease. On the basis of this assumption, we investigated the possible existence of associations between different combinations of these three genetic polymorphisms and PE but detected none (data not shown).

The role of the genes of these costimulatory molecules needs to be further investigated in healthy pregnant women and in those with PE. Specifically, studies assessing the expression of these molecules need to be conducted, including investigations on the relationship between maternal and fetal genotype interactions.

In conclusion, our data suggest an association between PE and the *ICOS* gene polymorphism, but do not suggest an association between PE and the *CD28* and *CTLA-4* gene polymorphisms.

Table 3 Genotype and allele frequencies of *CTLA-4, CD28* and *ICOS* gene polymorphisms in women with mild and severe PE and controls

	Mild PE	*Severe PE*	*Control*	*OR (95%CI)*	P-*value*	*Corrected* P-*value*[a]
CTLA-4 (+49)						
Genotypes[b]	*n*=47	*n*=76	*n*=158			
AA	25 (53.2%)	33 (43.4%)	76 (48.1%)			
AG	11 (23.4%)	34 (44.7%)	63 (39.9%)		0.03[c]	0.09[c]
					0.05[d]	0.15[d]
					0.77[e]	—
GG	11 (23.4%)	9 (11.8%)	19 (12%)			
GG×(AG+AA)[f]				2.27 (0.86–5.99)[c]	0.13[c]	—
				2.23 (0.97–5.11)[d]	0.06[d]	—
				0.98 (0.42–2.28)[e]	1.0[e]	—
Alleles[f]	*n*=94	*n*=152	*n*=316			
A	61 (64.9%)	100 (65.8%)	215 (68%)			
G	33 (35.1)	52 (34.2%)	101 (32%)	0.96 (0.56–1.65)[c]	0.89[c]	—
				0.86 (0.53–1.41)[d]	0.62[d]	—
				0.90 (0.59–1.36)[e]	0.67[e]	—
CD28 (+17)						
Genotypes[b]	*n*=46	*n*=79	*n*=171			
TT	34 (73.9%)	50 (63.3%)	125 (73.1%)			
TC	9 (19.6%)	25 (31.6%)	41 (24%)		0.33[c]	—
					0.45[d]	—
					0.27[e]	—
CC	3 (6.5%)	4 (5.1%)	5 (2.9%)			
TT×(TC+CC)[f]				1.64 (0.73–3.66)[c]	0.24[c]	—
				1.04 (0.49–2.18)[d]	1.0[d]	—
				0.63 (0.35–1.12)[e]	0.14[e]	—
Alleles[f]	*n*=92	*n*=158	*n*=342			
T	77 (83.7%)	125 (79.1%)	291 (85.1%)			
C	15 (16.3%)	33 (20.9%)	51 (14.9%)	1.35 (0.69–2.66)[c]	0.40[c]	—
				0.89 (0.48–1.68)[d]	0.74[d]	—
				0.66 (0.40–1.07)[e]	0.12[e]	—
ICOS (−1564)						
Genotypes[b]	*n*=47	*n*=77	*n*=167			
TT	16 (34%)	36 (46.8%)	99 (59.3%)			
TC	27 (57.4%)	33 (42.9%)	54 (32.3%)		0.28[c]	—
					0.005[d]	0.02[d]
					0.19[e]	—
CC	4 (8.5%)	8 (10.4%)	14 (8.4%)			
TT×(TC+CC)[f]				0.58 (0.27–1.24)[c]	0.19[c]	—
				0.35 (0.18–0.69)[d]	0.002[d]	0.006[d]
				0.60 (0.35–1.03)[e]	0.07[e]	—
Alleles[f]	*n*=94	*n*=154	*n*=334			
T	59 (62.8%)	105 (68.2%)	252 (75.4%)			
C	35 (37.2%)	49 (31.8%)	82 (24.6%)	0.78 (0.45–1.34)[c]	0.40[c]	—
				0.54 (0.33–0.89)[d]	0.02[d]	0.06[d]
				0.69 (0.45–1.06)[e]	0.1[e]	—

Abbreviations: CI, confidence interval; OR, odds ratio; PE, preeclampsia.
[a]Bonferroni correction.
[b]χ^2-test.
The *P*-values were determined between the following: [c]women with mild preeclampsia and severe preeclampsia; [d]women with mild preeclampsia and controls; and [e]women with severe preeclampsia and controls.
[f]Fischer's exact test.

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