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Association study of *FOXP3* gene and the risk of 0020 pre-eclampsia

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ABSTRACT

Pre-eclampsia (PE) is a multifactorial pregnancy disorder, with serious consequences for both the mother and the fetus. Despite intense studies, the pathophysiology of PE remains enigmatic. Previous studies suggested that Treg dysfunction is involved in the pathogenesis of PE. We hypothesized that functional variants of the *FOXP3* gene might be associated with PE via dysregulation of Treg cells. Of the 276 subjects, we genotyped three variants of *FOXP3* by PCR-RFLP and Tetra ARMS-PCR methods. The genotypic frequencies of rs2232365 were found to be protective from the development of PE under codominant [odds ratio (OR) 0.49, 95 percent confidence interval (CI) 0.28–0.87, *p-value* = 0.043], dominant [odds ratio (OR) 0.54, 95 percent confidence interval (CI) 0.32–0.94, *p-value* = 0.027] and over dominant [odds ratio (OR) 0.57, 95 percent confidence interval (CI) 0.35–0.92, *p-value* = 0.02] models. Moreover, the rs3761548 conferred a risk of PE in recessive model [odds ratio (OR) 2.05, 95 percent confidence interval (CI) 1.08–3.88, *p-value* = 0.025]. However, no mutation was detected in *FOXP3* exon2 in any of the studied samples. Based on our results, thought that *FOXP3* variants may be an important contributor for the progression of PE in Iranian women.

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Association; genotype; *FOXP3*; pre-eclampsia; variants

Introduction

Pre-eclampsia (PE) is a pregnancy complication characterized by the onset of high blood pressure and often a remarkable amount of protein in the urine that develops after 20 weeks of pregnancy (1). In severe forms there may be red blood cell breakdown, a low blood platelet count, impaired liver function, kidney dysfunction, swelling, shortness of breath due to the fluid in the lungs, or visual disturbances. If left untreated, it may progress to eclampsia (2,3). PE complicates ranges from 2–7% of pregnancies and is one of prominent causes of maternal and perinatal morbidity and mortality (4). It has been proposed that PE correlated with deficient trophoblast invasion of maternal spiral arteries, impaired placental perfusion, and widespread endothelial cell dysfunction (5). Development of preeclampsia has a multifactorial nature and is influenced by different factors such as fetal/paternal genetic and environmental risk factors (6). Despite intensive research efforts, the etiology and pathogenesis of preeclampsia still remains unclear. Emerging document propose that an excessive maternal inflammatory response to cytokine mediated endothelial damage during pregnancy and it plays an important role in PE pathogenesis (7).

Regulatory T cells (Tregs) have an important role in immune response and hold peripheral tolerance against antigens, including auto immunogens and allergens by the generation of anti-inflammatory cytokines such as IL-10 and TGF-β (8,9). The forkhead/winged helix transcription factor (FOXP3) gene, mapped to chromosome Xp11-23, is a critical gene for the function and development of Treg cells (10,11). Deficiency of the *FOXP3* gene impairs the suppressive function of Treg cells and Th1 type immunity is predominant in PE patients with decreased expression of FOXP3 mRNA but the mechanism involved is still unclear (12,13). It has been reported that there is an association between *FOXP3* variants with several disorder such as pre-eclampsia (14), systemic lupus erythematosus (SLE) (15), autoimmune thyroid diseases (AITDs) (16) and acute coronary syndrome (ACS) (17). In the present study, we evaluated the role of two functional promoter polymorphisms of *FOXP3* gene, rs3761548A/C and rs2232365A/G, and a deletion mutation in exon-2 of *FOXP3* in pre-eclampsia patients with Iranian origin.

Material and methods

Subjects

In a case-control study, 133 women with preeclampsia and 143 healthy normotensive controls were included (Table 1). Informed consent was taken from all subjects with acknowledgment of their awareness of the theme of the study. Case and control groups shared the same socio-demographic characteristics and ethnic background. Preeclampsia was defined as including systolic blood pressure ≥140 or diastolic blood

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Table 1. Demographic and clinical characteristics of pre-eclampsia patients(133) and controls(143).

Characteristics	Patients (mean ± SD)	Controls (mean ± SD)
Maternal age (years)	29.49 ± 6.09	28.10 ± 5.40
Marriage age (years)	21.86 ± 4.75	20.81 ± 4.51
Gestational age at delivery (weeks)	34.26 ± 4.96	39.13 ± 1.10
Gestational age at preeclampsia (weeks)	31.93 ± 5.68	-
Infant weight at birth	2655 ± 714	3254 ± 453
Delivery type		
Vaginal delivery	24	50
Caesarian section	109	93
Preeclampsia		
Mild	88	-
Severe	45	-

pressure ≥90 with evidence of proteinuria (1+ on urinalysis or ≥300 mg on a 24-hour urine collection). Severe pre-eclampsia was defined as a higher blood pressure ≥160 mmHg systolic or ≥110 mmHg diastolic on two occasions >6 h apart, and a proteinuria level >5 g/24 h or >3+ by dipstick testing on at least two separate occasions. The patients with gestational hypertension, history of hypertension, diabetes mellitus before/during pregnancy, autoimmune diseases, endocrine diseases and history of cancers were not included in the present study. The control group containing 134 women with at least one live birth was derived from volunteers undergoing routine annual gynecological examination. This group of women had no pregnancy complications such as spontaneous abortion, preterm labor, or preeclampsia. This study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (SBMU1.REC.1394.34).

Genotype determination

Genomic DNA was extracted from whole blood using MTU DNA extraction kit according to the manufacturer's instruction and stored at −20°C until genotyping. The quality of DNA was checked by electrophoresis on 1% agarose gel prepared in 0.5X TBE buffer. The rs3761548 polymorphism and Exon-2 deletion mutation were genotyped using PCR-RFLP method. Genotyping of rs2232365 was conducted by using the tetra-primer amplification refractory mutation system-polymerase chain reaction (Tetra- ARMS PCR). The PCR reactions were carried out in a final volume of 20 µl containing: 1 µl of 100–300 ng DNA, 0.1–0.5 mM of each primer, 10 µl of Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark) and 8 µl of ddH2O. The protocol for the PCR was as follows: 95°C for 5 min and 35 cycles of denaturing at 94°C for 30s, annealing (61°C for rs2232365, 58°C for rs3761548 and 59°C for potential Exon-2 deletion) for 35s, and extension at 72°C for 40s and a single final extension at 72°C for 5 min. The PCR products of rs3761548 and Exon-2 deletion underwent a RFLP reaction with restriction enzyme *Pst1* and *BfaI* (Thermo Scientific) according to the manufacturer's instructions, respectively, and were incubated at 37°C for 6 hours (Table 2). The amplified PCR and digested PCR products were run on a 2 and 3% agarose gel electrophoresis. To ensure that the results were repeatable, a 5 percent specimen of the subjects in the patient group and the control group were genotyped twice, and the reproducibility was 100 percent.

Statistical analysis

To examine whether the genotype frequencies were in Hardy–Weinberg equilibrium, goodness of fit $\chi 2$-test was used. $\chi 2$-test was applied for the analysis of genotypic and allelic distributions. Odds ratio and 95% confidence intervals were computed to evaluate the relative risk. These analyses were performed by using SNPStats online program (http://bioinfo.iconcologia.net/SNPstats) and MedCalc statistical software (https://www.medcalc.org/calc/odds_ratio.php). p value of <0.05 was considered statistically significant.

Results

Characteristics of preeclampsia cases and controls are summarized in Table 1. Genotyping of the three *FOXP3* analyzed variants (rs3761548A/C, rs2232365A/G and potential deletion mutation in exon-2) was successful for all tested samples and the resulting allelic and genotype distributions are shown in Tables 3 and 4. The genotype frequencies in the control subjects were in agreement with the Hardy-Weinberg equilibrium and supporting absence of confounding population stratification in the tested samples.

Analyzing of rs3761548 revealed significance association between AA genotype with increasing preeclampsia risk in Recessive model [odds ratio (OR) 2.05, 95 percent confidence interval (CI) 1.08–3.88, *p-value* = 0.025 Table 3], while significant association were not observed in other models and in the allele frequency (*p-value*> 0.05). The genotypic frequencies of rs2232365 polymorphism differ significantly under codominant [odds ratio (OR) 0.49, 95 percent confidence interval (CI) 0.28–0.87, *p-value* = 0.043], dominant [odds

Table 2. The primer sequences and conditions for genotyping of *FOXP3* variants.

Marker & method	Allele & enzyme	Primers	PCR product Tm/°C
rs3761548 PCR-RFLP	A/C *Pst1*	Forward: 5′ GCCCTTGTCTACTCCACGCCTCT 3′ Reverse: 5′ CAGCCTTCGCCAATACAGAGCC 3′	487bp, 329 bp + 158 bp 58 ◦C
rs2232365 Tetra-ARMS PCR	Outer	Forward: 5′ ACAGGGAAAGAGGAGAAGGAGTGGGCAT 3′ Reverse: 5′ GGATTGGGTGCAAAAGTGCAGGTGTAGA 3′	314bp 61 ◦C
	Inner A/G	Forward: 5′ CTACAGGCCCCAGCTCAAGAGACCACA 3′ Reverse: 5′GCATGGCAAGTGACAGAGAGGAGGAGATAC 3′	204 & 166bp 61 ◦C
Exon-2 deletion mutation	*Bfal*	Forward: 5′ TAGCATCTACCATGTGGGCTT 3′ Reverse: 5′ TGAGGTGTTACCAGGTGGGA 3′	451bp 335bp + 116bp 60°C

Table 3. Genotype and allele frequencies of rs3761548 in patients with PE and normal controls (n = 276).

Model	Allele/genotype	Status = case	Status = control	OR (95% CI)	*p-Value*
	C	163 (61)	157 (55)	1	
	A	103 (39)	129 (45)	1.30 (0.92–1.82)	0.12
Codominant	C/C	47 (35.3%)	47 (32.9%)	1.00	0.077
	A/C	69 (51.9%)	63 (44.1%)	0.91 (0.54–1.55)	
	A/A	17 (12.8%)	33 (23.1%)	1.94 (0.95–3.95)	
Dominant	C/C	47 (35.3%)	47 (32.9%)	1.00	0.67
	A/C-A/A	86 (64.7%)	96 (67.1%)	1.12 (0.68–1.84)	
Recessive	C/C-A/C	116 (87.2%)	110 (76.9%)	1.00	**0.025***
	A/A	17 (12.8%)	33 (23.1%)	**2.05 (1.08–3.88)**	
Overdominant	C/C-A/A	64 (48.1%)	80 (55.9%)	1.00	0.19
	A/C	69 (51.9%)	63 (44.1%)	0.73 (0.45–1.17)	

*$P \leq 0.05$ are considered statistically significant.

Table 4. Genotype and allele frequencies of rs2232365 in patients with PE and normal controls (n = 276).

Model	Allele/genotype	Status = case	Status = control	OR (95% CI)	*p-Value*
	G	136 (51)	160 (56)	1	
	A	130 (49)	126 (44)	0.82 (0.58–1.15)	0.25
Codominant	G/G	28 (21.1%)	47 (32.9%)	1.00	**0.043***
	A/G	80 (60.1%)	66 (46.1%)	**0.49 (0.28–0.87)**	
	A/A	25 (18.8%)	30 (21%)	0.71 (0.35–1.45)	
Dominant	G/G	28 (21.1%)	47 (32.9%)	1.00	**0.027***
	A/G-A/A	105 (79%)	96 (67.1%)	**0.54 (0.32–0.94)**	
Recessive	G/G-A/G	108 (81.2%)	113 (79%)	1.00	0.65
	A/A	25 (18.8%)	30 (21%)	1.15 (0.63–2.07)	
Overdominant	G/G-A/A	53 (39.9%)	77 (53.9%)	1.00	**0.02***
	A/G	80 (60.1%)	66 (46.1%)	**0.57 (0.35–0.92)**	

*$P \leq 0.05$ are considered statistically significant.

ratio (OR) 0.54, 95 percent confidence interval (CI) 0.32–0.94, *p-value* = 0.027] and over dominant [odds ratio (OR) 0.57, 95 percent confidence interval (CI) 0.35–0.92, *p-value* = 0.02] model between the case and control groups, nevertheless no association were observed in recessive model and in the allele frequency (*p-value*> 0.05). As for *Foxp3* deletion mutation in exon-2 shown no mutation was not found for heterozygous and not for homozygotes condition.

Discussion

Normal pregnancy requires maternal immunological tolerance to the semi allogeneic fetus (18). Tregs are thought to play an important role in immune suppression, and are involved in the pathogenesis of a variety of autoimmune disorders, including multiple sclerosis, lupus, type I diabetes, and rheumatoid arthritis (19). Tregs also participate in mediating human maternal tolerance to the fetus (20). In a recent study, Treg $^{CD4+CD25+FoxP3+}$ cells were diminished both in number and functional activity in PE patients compared with normal pregnancies (21). *FOXP3* mutations has been identified in patients with immune dysregulation polyendocrinopathy and enteropathy X-linked (IPEX) syndrome disorder (11). *FOXP3* expression now serves as the most specific marker of Tregs and it has been used to determine the proportion of Tregs in humans with a variety of disease states (22). Down-regulation of *FOXP3* in PE has been reported in previous studies and thus results in the decreased number of *FOXP3* regulatory T cells in preeclampsia, however, the mechanism involved in is still unclear (23,24). Therefore, we assumed that *FOXP3* variants might be involved in development of PE via quantitative and functional influences on T-regs$^{CD4+CD25+}$.

Our data showed that the rs3761548 and rs2232365 were significantly associated with pre-eclampsia, which suggests that *FOXP3* polymorphisms appear to affect the susceptibility to pre-eclampsia in Iranian population. Some previous studies implicated that rs3761548 and rs2232365 may contribute to the genetic susceptibility of several diseases. It has been suggested that the CC genotype for rs3761548 contribute to the risk of PE and acute coronary syndrome (ACS) (14,25). However, the recent study of Iranian patients with multiple sclerosis revealed that allelic and genotype frequencies of rs3761548 are not associated with this neurological disorder (26). In a previous study in Iranian patients with acute coronary syndrome (ACS), it was shown that the higher frequency of the C allele in the controls has a protective function. However, in the genotype level there was no significant association (17). Another study in a Han Chinese population revealed that the AA genotype in rs3761548 was associated with recurrent spontaneous abortions, suggesting patients with AA genotype may have fewer Treg cells and or weaker suppressive function thereby difficult to achieve fetal tolerance leading to early fetal loss. This is consistent with our findings identifying the AA genotype as a risk factor for pre-eclampsia (27).

In a previous study concerning the role of rs2232365 polymorphism in PE, no significant association was observed (28). Interestingly, we found that AG and AA+AG genotypes of rs2232365 polymorphism were protective for preeclampsia. These results are also consistent with the previous work showing that the A allele and AA genotype have a lower occurrence of unexplained recurrent spontaneous abortion

(27). In a previous study on rs2232365 A/G polymorphism, the G allele and GG+AG genotype were found to be a risk factor for vitiligo patients, which was consistent with our finding (29). In this study, deletion mutation in exon2 was not detected in any of the pre-eclampsia patients. However, in a study from south India the mentioned mutation was observed in 1.06% of PE patients in heterozygous condition. This mutation results in a truncated protein product of 108 amino acids compared with 431 residues in the impact protein (14).

Pre-eclampsia is a multifactorial disorder in which many genes and many risk factors may play role. It is also possible that *FOXP3* gene polymorphisms are involved in pre-eclampsia predisposition through an interaction with other genes. The exact mechanism by which these polymorphisms are involved in the pathogenesis of pre-eclampsia is still unclear. In conclusion we suggest that genetic variants in *FOXP3* gene may contribute to the pathogenesis of preeclampsia. Larger molecular epidemiological studies are needed to confirm these findings and to further elucidate the pathogenesis of this important clinical complication of pregnancy.

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Declaration of interest

The authors declare no conflict of interest.

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