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Cellular Physiology and Biochemistry
Cell Physiol Biochem 2015;37:1927-1933
DOI: 10.1159/000438553
Published online: November 17, 2015
Accepted: October 14, 2015
Open access

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1421-9778/15/0375-1927$39.50/0
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Original Paper

Role of Toll-Like Receptor 3 Gene Polymorphisms in Preeclampsia

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Key Words
TLR3 • Polymorphism • Preeclampsia • Chinese

Abstract
Background/Aims: Accumulating evidence suggests that an excessive maternal systemic inflammatory response to pregnancy with exaggerated activation of the innate immune system plays a critical role in the development of preeclampsia (PE). In this study, we investigated whether polymorphisms in the *Toll-like receptor 3* (*TLR3*) gene are associated with susceptibility to PE in the Chinese Han population. ***Methods:*** We recruited 987 PE patients and 1227 healthy pregnant women. Two polymorphisms (rs3775291 and rs3775296) located in *TLR3* were genotyped by TaqMan allelic discrimination real-time PCR. The association between the genotype or allele frequencies and PE was examined using chi-square tests. Clinical data were compared between cases and controls using Student's t test. ***Results****:* No significant difference was determined in the genetic distribution of rs3775291 and rs3775296 between cases and controls. There were also no significant differences in the genotype and allele frequencies of either SNP between healthy pregnant women and patients with late or early onset PE, or with mild or severe PE. ***Conclusion****:* Although this is the first study of the association between *TLR3* polymorphisms and preeclampsia, we found that *TLR3* polymorphisms are unlikely to play a significant role in the development of preeclampsia in the Chinese Han population.

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Introduction

Preeclampsia (PE) is a pregnancy-specific syndrome that usually occurs after 20 weeks gestation. It is determined by increased blood pressure accompanied by proteinuria. The incidence of preeclampsia ranges from 6% to 8% of all pregnancies worldwide [1]. As one of the leading causes of maternal and perinatal morbidity and mortality, PE is a severe threat

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to maternal and infant health. Although numerous studies on the etiology and pathogenesis of PE have been reported, such as inadequate trophoblast invasion [2, 3], placental and endothelial dysfunction [4], immune maladaptation and exaggerated systemic inflammatory response [5, 6], the exact causes of PE remain unclear. Previous studies have also indicated an excessive maternal systemic inflammatory response to pregnancy with exaggerated activation of the innate immune system playing a critical role in the development of PE [7, 8].

Toll-like receptor 3 (TLR-3), a member of the toll-like receptor family, recognizes specific pathogen-associated molecular signatures, including dsRNA of viral origin or from dying cells, to activate the innate immune system, which in turn leads to inflammatory responses [9]. Expressed predominantly by innate immune cells, including dendritic cells, macrophages, natural killer cells and mast cells, TLR3 potently activates interferon regulatory factor 3 (IRF3) and nuclear factor-KappaB (NF-κB), resulting in the release of IFN-β and other pro-inflammatory cytokines, such as tumor-necrosis factor (TNF) and interleukin-1 (IL-1) [10-12]. Additionally, activation of TLR3 may induce placental miR-210 via HIF-1 and NF-κB p50, leading to decreased levels of STAT6 and IL-4, which results in increased levels of pro-inflammatory cytokines [13].

PE displays multifactoral inheritance and both environmental risk factors and genetic components influence the development of PE. Although various candidate genes, such as *IFN-γ*, *IL-1* and *TNF-α* [14-16], have been shown to have associations with PE, the exact genes involved remain controversial. As important functional variants, *TLR3* rs3775296 (in the 5′-UTR) and rs3775291 (in exon 4) polymorphisms affect the expression and cell surface localization of TLR3 and, thereby, influence NF-κB cascade induction [17]. Moreover, polymorphisms in *TLR3* have associations with a variety of autoimmune disorders and inflammatory diseases, including systemic lupus erythematosus, sero-negative rheumatoid arthritis (RA), tick-borne encephalitis and multiple types of cancer [18-21]. However, the association between *TLR3* polymorphisms and PE has not been investigated. As TLR3 plays a crucial role in the innate immune system, which is excessively activated in PE, we hypothesized that *TLR3* genetic variants may affect the susceptibility and clinical symptoms of preeclampsia. In this study, our aim was to investigate whether *TLR3* polymorphisms are associated with PE in the Chinese Han population.

Materials and Methods

Participants

We collected 987 patients with PE and 1227 healthy pregnant women in their third trimester from The Affiliated Hospital of Qingdao University, The Zaozhuang Maternal and Child Care Service Centre, The Liaocheng People's Hospital. All women were ethnically Chinese Han and healthy controls were enrolled by random selection. The control groups were composed of normal pregnant women, matching the cases according age (maternal age > 26 years). The mean ages of PE patients and controls were 30.02 ± 5.89 years and 30.05 ± 5.24 years, respectively. Exclusion criteria of the controls were multifetal gestation, macrosomia, premature rupture of membrane, placenta previa, poly- or oligo-hydramnios, threatened abortion, diabetes mellitus, hypertension and autoimmune disease.

Preeclampsia was defined as the development of hypertension (≥ 140 mm Hg systolic blood pressure or ≥ 90 mm Hg diastolic blood pressure on two or more occasions at least 6 h apart) and proteinuria (≥ 300 mg/24 h, ≥ 30 mg/dL, ≥1+ dipstick proteinuria) that occurred after 20 weeks of gestation in a woman who previously had normal blood pressure [1, 6, 22]. Severe preeclampsia was diagnosed if any of the following criteria was present: blood pressure ≥160/110 mm Hg, or proteinuria ≥ 5 g/24 h, or the presence of multiorgan involvement, such as visual or cerebral disturbance, abnormal liver enzymes, oliguria, pain in the epigastric area or right upper quadrant, pulmonary edema or thrombocytopenia (platelet count < 100×10^9/L) [1, 6, 20]. Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation. The study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University and all the participants gave written informed consent.

Genetic studies

Total genomic DNA was extracted from 300 μl peripheral blood taken for routine laboratory investigations using a Qiagen DNA extraction kit. Two polymorphisms (rs3775291and rs3775296) located in TLR3 were genotyped by TaqMan allelic discrimination real-time PCR. The Taqman probes and primers were designed by Applied Biosystems, Life Technologies. For rs3775291, the primer sequences were: forward, 5′-ACT TGC TCA TTC TCC CTT ACA CATA-3′ and reverse, 5′-TCA ACC TAA CCA AGA ATA AAA TCTC-3′. For rs3775296, the forward primer was 5′-ACT TTT TAA TGT TTC TTT TCT ACAG-3′, and reverse primer was 5′-AGA ATC ATG AGA CAG ACT TTG CCTT-3′. The polymerase chain reaction (PCR) mixture contained 20×SNP Genotyping Assay 1.25 μl, 2×PCR Master Mix 12.5 μl, DNA and DNase-free water 11.25 μl in a total volume of 25 μl. The amplifications were carried out on a C1000TM thermal cycler system with the following conditions: 95°C for 3 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. The fluorescence was detected in real time during PCR thermal cycling. The discrimination of genotypes was conducted with BioRad CFX manager 3.0 software.

Statistical analysis

Statistical analyses were performed using the software package SPSS21.0. Goodness-of-fit χ^2 test was applied to check whether the observed genotype frequencies of the control group were in Hardy-Weinberg equilibrium. Clinical data were compared between cases and controls using Student's t test, and are presented as the mean ± standard error of the mean. Pearson's χ^2 test was performed to compare the allele and genotype frequencies between cases and controls (Fisher's exact test was used when expected values were below 5). For all statistical analyses, a p-value < 0.05 was considered statistically significant.

Results

Clinical characteristics

Clinical characteristics and pregnancy outcomes of cases and controls are presented in Table 1. As compared with controls, preeclampsia patients had earlier delivery (36.27 ± 3.09 weeks vs. 39.33 ± 1.30 weeks, p < 0.001), fewer abortions (0.66 ± 0.96 vs. 0.79 ± 0.99, P < 0.05), lower new-born birth weight (2622.44 ± 945.08 g vs. 3401.92 ± 373.12 g, P < 0.001) and higher blood pressure (P < 0.001).

Genetic analysis

The genetic distribution of rs3775291 and rs3775296 in cases and controls are displayed in Table 2. The control group was in Hardy–Weinberg equilibrium for both SNPs (for rs3775291, χ^2 = 1.650, P = 0.198; for rs3775296, χ^2 = 0.842, P = 0.359). There were no significant differences in the genotype and allele frequencies of rs3775291 and rs3775296 between cases and controls (for rs3775291, χ^2 = 2.120, P = 0.346 by genotype; χ^2 = 0.374, P = 0.541, OR = 1.043, 95% CI 0.911-1.195 by allele. For rs3775296, χ^2 = 2.225, P = 0.329 by genotype; χ^2 = 2.26, P = 0.133, OR = 0.9, 95% CI 0.789-1.033 by allele).

To further examine the association between rs3775291 and rs3775296 and PE, the distribution of the two SNPs in preeclamptic subgroups was also investigated. There were no

Table 1. Clinical characteristics

	Cases	Controls	*t*	P-value
Age(years)	30.02±5.89	30.05±5.24	0.104	0.918
Gestational age(weeks)	35.44±3.62	39.05±1.49	30.537	P<0.001
Gestational age at delivery (weeks)	36.27±3.09	39.33±1.30	29.298	P<0.001
Number of abortion	0.66±0.96	0.79±0.99	2.979	0.003
Birth weight of offspring (g)	2622.44±945.08	3401.92±373.12	24.55	P<0.001
Systolic blood pressure (mmHg)	154.12±23.22	114.09±9.95	-53.623	P<0.001
Diastolic blood pressure (mmHg)	100.65±16.02	73.33±7.72	-51.771	P<0.001

Table 2. The genotypic and allelic frequencies of rs3775291 and rs3775296 in cases and controls

	Cases	Controls	X^2	P-value	OR(95%CI)
rs3775291					
Genotypes					
AA	84	85	2.120	0.346	
AG	342	443			
GG	563	699			
Alleles					
A	510	613	0.374	0.541	1.043(0.911-1.195)
G	1468	1841			
rs3775296					
Genotypes					
TT	59	87	2.225	0.329	
GT	351	456			
GG	579	684			
Alleles					
T	469	630	2.260	0.133	0.9(0.789-1.033)
G	1509	1824			

Table 3. The comparison of genotype distributions and allelic frequencies between early/late-onset PE and control groups

		rs3775291					rs3775296				
Group	N	AA	AG	GG	A	G	GG	GT	TT	G	T
Early-onset PE	289	17	94	178	128	450	171	100	18	442	136
Control	1227	85	443	699	613	1841	684	456	87	1824	630
χ^2		2.090			2.035		1.156			1.138	
P-value		0.352			0.154		0.561			0.286	
OR					0.854					1.123	
95%CI					0.688-1.061					0.908-1.388	
Late-onset PE	700	65	244	391	374	1026	414	249	37	1077	323
Control	1227	85	443	699	613	1841	684	456	87	1824	630
χ^2		3.476			1.408		3.468			3.24	
P-value		0.176			0.235		0.177			0.072	
OR					0.913					0.868	
95%CI					0.787-1.061					0.744-1.013	

significant differences in the genotype and allele frequencies of either SNP between controls and patients with late and early onset of the disease (shown in Table 3). We also did not find any difference in the genotype and allele frequencies of rs3775291 and rs3775296 between controls and patients with mild and severe PE (displayed in Table 4).

Discussion

Accumulating evidence has shown that an excessive maternal systemic inflammatory response to pregnancy with exaggerated activation of the innate immune system results in endothelial cell damage, which subsequently leads to the signs and symptoms of PE. TLR3 plays a key role in mediating innate immune responses, as well as in regulating adaptive immunity. TLR3 recognizes dsRNA of viral origin and from endogenous dsRNA that is released from dying cells. This initiates specific signaling pathways, including those leading to the activation of the transcription factors NF-κB and IRF3. NF-κB mediates the production of several proinflammatory cytokines, whereas IRF3 regulates the expression of beta interferon (IFN-β), which contributes to the excessive inflammatory response. Moreover, TLR activation may trigger TLR autoamplification feed-forward loops involving circulating

Table 4. The comparison of genotype distributions and allelic frequencies between mild/severe PE and control groups

		rs3775291					rs3775296				
Group	N	A	AG	GG	A	G	GG	GT	TT	G	T
Mild PE	215	21	75	119	117	313	127	78	10	332	98
Control	1227	85	443	699	613	1841	684	456	87	1824	630
χ^2		2.168			0.962		2.016			1.610	
P-value		0.338			0.327		0.365			0.204	
OR					1.123					1.170	
95%CI					0.891-1.415					0.918-1.492	
Severe PE	774	63	266	445	392	1156	457	270	47	1184	364
Control	1298	85	443	699	613	1841	684	456	87	1824	630
χ^2		1.370			0.060		2.320			2.368	
P-value		0.504			0.807		0.313			0.124	
OR					1.018					1.123	
95%CI					0.880-1.179					0.969-1.303	

leukocytes. The interaction between the maternal endothelium and activated peripheral blood leukocytes [23] would further reduce, or cross, the threshold for the development of clinical preeclampsia [24]. Several studies have shown that TLR3 activation during pregnancy causes preeclampsia-like symptoms indicating that TLR3 plays a key role in the development of PE [25-27].

Our investigation into the effects of two SNPs in *TLR3* on the risk of PE in a Chinese Han population failed to detect an association between genotypes or rs3775291 and rs3775296 allele frequencies and PE. Furthermore, no difference was found in the genotype and allele frequencies of the two SNPs between controls and patients with mild and severe PE or with late and early onset of the disease. To our knowledge, this is the first report of the association between *TLR3* and susceptibility to PE. This study used relatively large samples, which indicated a low false negative rate; therefore, our results suggest that variants in this genomic region are not associated with preeclampsia. Similar to this conclusion, *TLR2* and *TLR4* have also been reported to have no association with PE, even though they may play an important role in placental development [28, 29].

rs3775291, located in the protein-coding sequence, results in an amino acid substitution, which may reduce or abolish TLR3 activity [17]. Previous studies have shown this SNP to be associated with several inflammatory diseases. Magdalena et al. identified rs3775291 to be significantly associated with RA and suggested a significant association of sero-negative RA with the A allele and disease activity after subgroup analysis [19]. Andrey et al. have reported the frequencies of the rs3775291 G allele, and G/G homozygotes were significantly more numerous among patients with tick-borne encephalitis (TBE) in the Russian population, compared with the control group, especially among patients with severe TBE [30]. However, contrasting results were also observed. Hsin-Yi et al. observed that the rs3775291 has no significant genotype and allele association between osteoarthritis cases and healthy controls in a Chinese Han population [31]. In this study, we did not find an association in the genotype and allele frequencies of rs3775291 between PE patients and healthy pregnant women.

Hsin-Yi et al. have shown that rs3775296, located in the 5′-UTR of *TLR3*, is associated with elevated *TLR3* expression and susceptibility to knee osteoarthritis in a Chinese Han population [31]. Moreover, the rs3775296-T allele was associated with photosensitivity and anemia of system lupus erythematosus in females in a Taiwanese population [32]. However, in the present study, we failed to observe an association between rs3775296 and PE.

In conclusion, no significant difference was found between rs3775291 and rs3775296 in *TLR3* and PE in our study. However, there were several limitations that should not be ignored. First, although the sample size of our study was large enough to draw statistically significant conclusions, the Han ethnicity does not represent the entire Chinese population because ethnicity and regional variation play crucial roles. Second, Over 136 SNPs in *TLR3* have been identified in the human population, however, only two SNPs were investigated

in our study. Also, complex interactions among genetic and environmental factors cannot be ignored. While PE is generally considered to be a multifactorial disease, environmental interactions may play a role in the pathogenesis of PE. Many environmental factors, including exposure to chemicals, pre-pregnancy weight, height, diet, smoking, and alcohol use may be related to the risk of PE. A limitation of our study is that we did not analyze the interaction and co-morbidity among environmental risk factors, polymorphisms of *TLR3* and PE. Therefore, studies with more SNPs and multiple populations are needed to further explore the association between *TLR3* polymorphisms and PE.

Acknowledgements

We thank all subjects for their participation. This work was supported by the National Natural Science Foundation of China (81371499 and 30971586).

Disclosure Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us.

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