Original Article

Toll-Like Receptor 4 Gene Polymorphisms and Preeclampsia: Lack of Association in a Caucasian Population

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Preeclampsia is a multifactorial disorder with genetic and environmental components. As Toll-like receptor 4 (TLR4) has an essential role in innate immune response, which is exaggeratedly activated in preeclampsia, our aim was to investigate whether two single nucleotide polymorphisms (SNPs) of the TLR4 gene—Asp299Gly (A896G) and Thr399Ile (C1196T)—are associated with preeclampsia in a Caucasian population from Hungary. In a case-control study, we analyzed blood samples from 180 preeclamptic patients and 172 normotensive, healthy pregnant women with the polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method. The linkage disequilibrium (LD) profile of the TLR4 gene was investigated and tag SNPs were identified using data from the International HapMap Project. There were no significant differences in the genotype and allele frequencies of Asp299Gly and Thr399Ile polymorphisms between the two study groups. Additionally, no significant difference was found in the distribution of the estimated haplotypes created by the two polymorphisms between the preeclamptic and the control group. Furthermore, no significant differences were detected in the genotype, allele and haplotype frequencies of Asp299Gly and Thr399Ile TLR4 SNPs between patients with mild and severe preeclampsia, between patients with late and early onset of the disease, or between preeclamptic patients with and without fetal growth restriction. In conclusion, we did not find an association between TLR4 Asp299Gly and Thr399Ile gene polymorphisms and preeclampsia. As the Thr399Ile polymorphism is a highly informative tag SNP of the TLR4 gene, our results suggest that variations in this genomic region are not associated with preeclampsia. Nevertheless, further studies are required with determination of fetal TLR4 genotypes to explore the role of TLR4 gene polymorphisms in the risk of preeclampsia, especially in ethnically different populations. (*Hypertens Res* 2008; 31: 859–864)

***Key Words*: Toll-like receptor, gene, polymorphism, preeclampsia, innate immune response**

Introduction

Preeclampsia remains a major, worldwide problem in obstetric practice, and is one of the leading causes of maternal and perinatal morbidity and mortality (*1*). Despite intensive research efforts, the etiology and pathogenesis of preeclampsia are not fully understood. Increasing evidence suggests that an excessive maternal systemic inflammatory response to pregnancy with exaggerated activation of the innate immune

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system plays a crucial role in the pathogenesis of the disease (*2*, *3*). It has also been shown that the development of preeclampsia is influenced by both genetic and environmental risk factors, suggesting its multifactorial inheritance (*4–9*).

Toll-like receptor 4 (TLR4) belongs to the family of pattern recognition receptors (PRRs), which recognize specific pathogen-associated molecular patterns (PAMPs) (*10*, *11*). TLR4 is expressed by many cell types, predominantly by innate immune cells such as monocytes/macrophages, granulocytes and dendritic cells (*12*). Toll-like receptors are transmembrane proteins with an extracellular domain characterized by leucine-rich repeats (LRRs) and an intracellular domain homologous to that of the human interleukin-1 receptor (Toll/interleukin-1 receptor [TIR] domain) (*11*). TLR4 is the receptor for lipopolysaccharide, which is the outer membrane component of Gram-negative bacteria (*11*). However, TLR4 might also recognize endogenous ligands, the so-called danger signals released by damaged cells, including heat shock proteins (*13*). Following ligation, TLR4 signals through the adapter molecule myeloid differentiation protein 88 (MyD88), leading to the release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α *via* the transcription factor nuclear factor (NF)-κB. However, a second, MyD88-independent pathway also exists, which leads to the induction of type 1 interferons *via* interferon regulatory factor 3 (IRF3) (*11*).

The gene encoding TLR4 is located on the long arm of chromosome 9 (9q32–q33) (*14*). Several polymorphisms have been described within the TLR4 gene, of which the Asp299Gly and Thr399Ile single nucleotide polymorphisms (SNPs) have been most extensively investigated in candidate gene association studies. The Asp299Gly SNP corresponds to an adenine-to-guanine transition at nucleotide 896 (A896G) leading to an aspartic acid-to-glycine amino acid substitution at position 299, whereas the Thr399Ile SNP is a cytosine-to-thymine transition at nucleotide 1196 (C1196T) that results in an amino acid change of threonine to isoleucine at position 399 (*15*).

As TLR4 has an essential role in innate immune response (*10*), which is exaggeratedly activated in preeclampsia (*3*), our aim was to investigate whether the Asp299Gly and Thr399Ile single nucleotide polymorphisms of the TLR4 gene are associated with preeclampsia in a Caucasian population from Hungary. We hypothesized that the two investigated polymorphisms decrease the risk of preeclampsia by acting as loss-of-function variants of the TLR4 gene. To our knowledge, this is the first report on TLR4 gene polymorphisms in preeclampsia.

Methods

Study Patients

Our study was designed as a retrospective study. The study consisted of 180 preeclamptic patients and 172 normotensive (systolic blood pressure [SBP]<140 mmHg and diastolic blood pressure [DBP]<90 mmHg) healthy pregnant women with uncomplicated pregnancies. The study participants were enrolled in the Department of Obstetrics and Gynecology at the Kútvölgyi Clinical Center and in the 1st Department of Obstetrics and Gynecology at Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease and renal disease.

Preeclampsia was defined by increased blood pressure (≥140 mmHg SBP or ≥90 mmHg DBP on two or more occasions at least 6 h apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24 h) (*16*). Blood pressure returned to normal by 12 weeks postpartum in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: SBP≥160 mmHg or DBP≥110 mmHg, or proteinuria≥5 g/24 h (*17*). Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation. Fetal growth restriction was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender (*18*).

The study protocol was approved by the Regional, Institutional Committee of Medical Ethics at Semmelweis University, and written informed consent was obtained from each patient.

Biological Samples and Genotyping

Peripheral blood samples taken for routine laboratory investigations were used for genotyping. Total genomic DNA was extracted from the buffy coat of EDTA blood samples by the method of Miller *et al*. (*19*). The prevalence of TLR4 A896G (Asp299Gly) and C1196T (Thr399Ile) genotypes was examined (*20*). The polymerase chain reaction (PCR) mixture contained 1 μL 10× PCR buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L dNTP mix, 1 μmol/L of both primers, 1 U recombinant HotStar Taq DNA Polymerase (QIAGEN Diagnostics GmbH, Hamburg, Germany) and 100 ng genomic DNA in a total reaction volume of 10 μL. Primers for TLR4 A896G SNP were F5′-GATTAGCATACTTAGACTACTACCTCCATG (forward) and R5′-GATCAACTTCTGAAAAAGCATTCC CAC (reverse). Primers for TLR4 C1196T SNP were F5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA (forward) and R5′-CCTGAAGACTGGAGAGTGAGTTAA ATGCT (reverse). The PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 30 s, ending with a final extension at 72°C for 10 min and cooling to 10°C in an AB 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). The PCR products were digested with *NcoI* and *HinfI* restriction enzymes (Fermentas International Inc., Burlington, Canada) at 37°C overnight, producing fragments of 249 base pairs (bp)

Table 1. Clinical Characteristics of Preeclamptic Patients and Normotensive, Healthy Pregnant Women

Characteristics	Preeclamptic patients (n=180)	Controls (n=172)	Statistical significance (p)
Age (years)	28.5±5.6	29.5±4.8	>0.05 (n.s.)
Prepregnancy BMI (kg/m^2)	24.2±4.5	22.1±3.9	<0.001
Smokers (n (%))	15 (8.3)	10 (5.8)	>0.05 (n.s.)
Primiparas (n (%))	130 (72.2)	96 (55.8)	<0.05
Blood pressure, systolic (mmHg)	168±18	112±12	<0.001
Blood pressure, diastolic (mmHg)	105±11	71±9	<0.001
Gestational age at delivery (weeks)	34.5±3.9	39.4±1.4	<0.001
Fetal birth weight (g)	2,081±944	3,444±448	<0.001
Fetal growth restriction (cases) (n (%))	61 (33.9)	0 (0.0)	<0.001

Data are presented as mean±SD for continuous variables and as number (%) for categorical variables. n.s., not significant.

(A allele) or 226+23 bp (G allele) and of 406 bp (C allele) or 377+29 bp (T allele), respectively. The cleavage products were electrophoresed on 2% agarose gel and stained with ethidium bromide. The restriction fragments were detected by visual inspection on an ultraviolet table.

Statistical Analysis

The normality of continuous variables was assessed using the Shapiro-Wilk's W-test. As the continuous variables were normally distributed, parametric statistical methods were used. To compare continuous variables between the preeclamptic and control groups, the t-test was applied. The Fisher exact and Pearson χ^2 tests were performed to compare categorical variables between groups. Multivariate logistic regression analysis was carried out with adjustment for maternal age, prepregnancy body mass index (BMI), primiparity and smoking status.

An exact test using a Markov chain was applied to check whether the observed genotype frequencies were in Hardy-Weinberg equilibrium, which is more suitable for a small sample size than χ^2 analysis. Linkage disequilibrium (LD) was determined with a permutation test using the Expectation-Maximization (EM) algorithm. Our data were genotypic data with an unknown gametic phase. Therefore, we performed haplotype estimation with a pseudo-Bayesian approach (Excoffier-Laval-Balding [ELB] algorithm). The LD profile of the TLR4 gene was investigated and tag SNPs were identified using data from the International HapMap Project (available free online at http://www.hapmap.org) (*21*, *22*).

Statistical analyses were carried out using the software package STATISTICA (version 7.1; StatSoft Inc., Tulsa, USA) and the Statistical Package for the Social Sciences (version 13.0 for Windows; SPSS Inc., Chicago, USA). For population genetic data analysis, the Arlequin software package (version 3.0; CMPG, University of Berne, Switzerland) was applied. For all statistical analyses, values of $p<0.05$ were considered statistically significant.

Data are presented as the means±SD for continuous variables and as percentages for categorical variables.

Results

Patient Characteristics

The clinical characteristics of the study participants are described in Table 1. There were no statistically significant differences in maternal age or the percentage of smokers between the two study groups. The prepregnancy BMI and the frequency of primiparas were significantly higher in the preeclamptic group compared to the control group. However, there was no significant difference in the percentage of prepregnancy obesity (prepregnancy BMI≥30 kg/m^2) between the two groups (data not shown). The SBP and DBP values were significantly higher, whereas the gestational age at delivery and the fetal birth weight were significantly lower in the preeclamptic group than in the control group. Fetal growth restriction was absent in control subjects, whereas the frequency of this condition was 33.9% in the preeclamptic group.

TLR4 Genotypes

The genotype and allele frequencies of the Asp299Gly (A896G) and Thr399Ile (C1196T) TLR4 gene polymorphisms in the preeclamptic and control groups are displayed in Table 2. The Asp299Gly and Thr399Ile TLR4 genotype distributions met the requirements of Hardy-Weinberg equilibrium both in the preeclamptic and the control groups. There were no significant differences in the genotype and allele frequencies of Asp299Gly and Thr399Ile polymorphisms between the two study groups. In addition, the risk of homozygous and heterozygous mutant allele carriers of either polymorphism for preeclampsia did not differ significantly from that of homozygous wild-type allele carriers, even after adjustment for maternal age, prepregnancy BMI, primiparity and smoking status in multiple logistic regression analyses

Table 2. Genotype and Allele Frequencies of the Asp299Gly (A896G) and Thr399Ile (C1196T) Toll-Like Receptor 4 Gene Polymorphisms in Preeclamptic Patients Compared to Normotensive, Healthy Pregnant Women

	Preeclamptic patients	Controls
Genotype frequencies (Asp299Gly) (*n* (%))		
Genotype	180 (100)	172 (100)
AA	165 (91.7)	150 (87.2)
AG	15 (8.3)	21 (12.2)
GG	0 (0.0)	1 (0.6)
$p>0.05$		
Allele frequencies (Asp299Gly) (*n* (%))		
A	345 (95.8)	321 (93.3)
G	15 (4.2)	23 (6.7)
$p>0.05$		
Genotype frequencies (Thr399Ile) (*n* (%))		
Genotype	180 (100)	172 (100)
CC	165 (91.7)	149 (86.6)
CT	15 (8.3)	22 (12.8)
TT	0 (0.0)	1 (0.6)
$p>0.05$		
Allele frequencies (Thr399Ile) (*n* (%))		
C	345 (95.8)	320 (93.0)
T	15 (4.2)	24 (7.0)
$p>0.05$		

(for GG and AG genotype carriers *vs*. AA genotype carriers: odds ratio [OR]=0.62, 95% confidence interval [CI]=0.31–1.24, $p=0.18$, adjusted OR=0.60, adjusted 95% CI=0.27–1.32, adjusted $p=0.20$; for TT and CT genotype carriers *vs*. CC genotype carriers: OR=0.59, 95% CI=0.30–1.17, $p=0.13$, adjusted OR=0.58, adjusted 95% CI=0.27–1.28, adjusted $p=0.18$).

We observed a strong LD between the Asp299Gly and Thr399Ile TLR4 gene polymorphisms. As shown in Table 3, no significant difference was found in the distribution of the estimated haplotypes created by the two polymorphisms between the preeclamptic and the control group. Furthermore, there was no significant difference in the risk of preeclampsia between G-T haplotype carriers (homozygous and heterozygous) and non-carriers, even after adjustment for confounding variables in multivariate logistic regression analysis (G-T haplotype carriers *vs*. non-carriers: OR=0.65, 95% CI=0.32–1.31, $p=0.23$, adjusted OR: 0.65, adjusted 95% CI=0.29–1.44, adjusted $p=0.29$).

We also investigated the occurrence of the two single nucleotide polymorphisms in preeclamptic subgroups. However, no significant differences were detected in the genotype, allele and haplotype frequencies of Asp299Gly and Thr399Ile TLR4 SNPs between patients with mild and severe preeclampsia, between patients with late and early onset of the disease, or between preeclamptic patients with and without fetal growth restriction (data not shown).

Table 3. Occurrence of the Estimated Haplotypes Created by the Toll-Like Receptor 4 Asp299Gly (A896G) and Thr399Ile (C1196T) Gene Polymorphisms in Preeclamptic Patients and Normotensive, Healthy Pregnant Women

Haplotype	Preeclamptic patients *n*=360 (100%)	Controls *n*=344 (100%)
A-C (*n* (%))	345 (95.8)	319 (92.7)
A-T (*n* (%))	0 (0.0)	2 (0.6)
G-C (*n* (%))	0 (0.0)	1 (0.3)
G-T (*n* (%))	15 (4.2)	22 (6.4)
$p>0.05$		

n, the number of chromosomes examined.

In the *in silico* analysis, the Thr399Ile polymorphism (rs4986791) was found to be a highly informative tag SNP of the TLR4 gene. In the CEU population (Utah residents with ancestry from northern and western Europe), it was in perfect LD ($D'=1$ and $r^2=1$) with the following 5 SNPs of the TLR4 gene: rs10818073 (intronic), rs7864330 (intronic), rs12344353 (intronic), rs4986790 (missense, Asp299Gly) and rs10983756 (intronic).

Discussion

In the present examination of the effects of two single nucleotide polymorphisms of the TLR4 gene on the risk of preeclampsia in a Caucasian population from Hungary, we did not observe an association between the TLR4 Asp299Gly (A896G) and Thr399Ile (C1196T) alleles, genotypes and haplotypes and preeclampsia. As the Thr399Ile polymorphism is a highly informative tag SNP of the TLR4 gene, which captures 5 additional SNPs spread throughout the whole TLR4 gene, our results suggest that variations in this genomic region are not associated with preeclampsia.

The Asp299Gly and Thr399Ile single nucleotide polymorphisms of the TLR4 gene, which affect the extracellular domain of the receptor and might cause decreased ligand recognition, have been investigated in several genetic association studies. They were found to be associated with a blunted response to inhaled lipopolysaccharide in humans. Overexpression studies indicated that the Asp299Gly polymorphism might have a greater functional impact than the Thr399Ile polymorphism (*15*). However, the two polymorphisms frequently cosegregate. The Asp299Gly SNP in the absense of a cosegregating mutation at residue 399 has been found exclusively in patients with Gram-negative septic shock as compared to healthy blood donors (*23*). In another study, pregnant women carrying the mutant allele of the Asp299Gly polymorphism were found to have ten-fold higher vaginal levels of *Gardnerella vaginalis* and anaerobic Gram-negative rods than homozygous wild-type allele carriers (*24*). The Asp299Gly SNP has also been related to premature birth (*25*). Finally, both polymorphisms have been associated with an increased

risk of severe respiratory syncytial virus bronchiolitis in previously healthy infants (*26*).

Although these SNPs increase the risk of several infectious diseases, they seem to protect from atherosclerosis and related disorders (*27*, *28*). Mutant allele carriers of Asp299Gly SNP have a decreased risk of carotid atherosclerosis (*29*). Furthermore, the Asp299Gly polymorphism has been associated with a lower risk of acute coronary events (*30*, *31*). Interestingly, the Asp299Gly variant decreased the susceptibility to rheumatoid arthritis (*32*) and late-onset Alzheimer's disease (*33*). Moreover, both polymorphisms decreased the risk of diabetic neuropathy in patients with type 2 diabetes (*34*), as well as of acute allograft rejection after lung transplantation (*35*).

A generalized intravascular inflammatory reaction involving innate immune cells (monocytes and granulocytes) with production of proinflammatory cytokines leading to a Th1 bias, as well as the clotting and complement systems and acute phase proteins, seems to be the cause of the maternal syndrome of preeclampsia (*2*, *3*). The excessive innate immune activation results in inflammatory-mediated endothelial cell damage, which in turn leads to the signs and symptoms of the disease (*3*). TLR4 plays a central role in mediating innate immune responses, as well as in regulating adaptive immunity (*10*). Therefore, our null hypothesis was that Asp299Gly and Thr399Ile single nucleotide polymorphisms are associated with a decreased risk of preeclampsia by acting as loss-of-function variants of the TLR4 gene.

The reason for the lack of association between the TLR4 Asp299Gly and Thr399Ile SNPs and preeclampsia observed in this study remains unclear. It is possible that the investigated gene polymorphisms are not truly functional. Indeed, although associations have been reported between the two polymorphisms and various pathological conditions, several studies have also suggested a lack of such association (*36*–*38*). Additionally, transmission disequilibrium tests (TDT) should have been undertaken or multiple unlinked markers should have been used to exclude the possibility of spurious association caused by population stratification. The findings that the mutant allele variants do not influence the activation of monocytes or whole blood by lipopolysaccharide, also cast doubt on the possibility that these SNPs play a role in inflammatory disease susceptibility (*39*, *40*). Furthermore, the existence of an endogenous (non-microbial) TLR4 ligand is still controversial (*41*).

TLR4 is also expressed in the human placenta, and ligation of TLR4 with its specific ligand lipopolysaccharide induces cytokine production (*42*), as well as chemokine secretion by trophoblast cells with increased monocyte and neutrophil chemotaxis (*43*). TLR4 protein expression was found to be increased in interstitial trophoblasts at the placental bed of patients with preeclampsia. The authors hypothesized that danger signals (host or microbial in nature) at the feto-maternal interface, which are recognized by trophoblasts through TLR4, may play a key role in creating a local abnormal cytokine milieu leading to the development of preeclampsia (*44*). As trophoblast cells are fetal in origin, this finding raises the possibility that fetal instead of maternal TLR4 gene polymorphisms influence the risk of preeclampsia. Moreover, while TLR4 ligation resulted in the production of cytokines by trophoblast cells, TLR2 activation induced trophoblast apoptosis (*42*). Since preeclampsia is associated with an increase in placental apoptosis (*45*), the role of fetal TLR2 gene polymorphisms in the risk of preeclampsia also warrants investigation.

The limitations of this study are its case-control design and relatively small sample size. In light of the low population prevalence of the examined SNPs (*27*), our results should be confirmed in a study with a larger sample size.

In conclusion, we did not find an association between TLR4 Asp299Gly and Thr399Ile gene polymorphisms and preeclampsia in a Caucasian population from Hungary. Nevertheless, further studies are required with determination of fetal TLR4 genotypes to explore the role of TLR4 gene polymorphisms in the risk of preeclampsia, especially in ethnically disparate populations.

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