Toll-like Receptor 2 Polymorphism Is Associated With Preterm Birth

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ABSTRACT: Evidence is increasing for a role of polymorphisms in maternal or fetal innate immune response genes in preterm birth. Toll-like receptors (TLRs) are important receptors in the innate immunity. The genotype distribution of two TLR2 single nucleotide polymorphisms (SNPs) and one TLR4 SNP were determined among 524 neonates and associated with gestational age (GA). Genomic DNA was isolated from prospectively collected blood samples and polymorphisms in TLR2 (T-16934A, RS4696480 and Arg753Gln, RS5743708) and TLR4 (Thr399Ile, RS4986791) were determined using sequence specific primers by PCR. Allele frequencies of two TLR2 SNPs and one TLR4 SNP were analyzed according to prematurity. Analysis among 305 infants, after exclusion of infants born after multiple pregnancy or because of preeclampsia, revealed significantly shorter GAs for infants carrying two polymorphic TLR2 alleles (-16934TA/AA and 753ArgGln/GlnGln) compared with infants carrying one polymorphic and one wild-type allele or two wild-type alleles (median GA 30.6 wk *versus* 34.1–36.8 wk, respectively, $p < 0.02$). Carriage of two variant TLR2 alleles potentially leads to aberrant innate immune responses, which may have contributed to very preterm birth. (*Pediatr Res* 62: 474–476, 2007)

Preterm birth remains a major public health concern because of its associated perinatal morbidity and mortality (1). Evidence is increasing of an important role of polymorphisms in maternal or fetal immune response genes in preterm birth (2,3). Several single nucleotide polymorphisms (SNPs) in immune response genes, particularly in proinflammatory cytokine genes, have been investigated for their contribution to the etiology of preterm birth (2–5). The innate immune system's rapid recognition of pathogens is mediated through toll-like receptors (TLRs) and binding of a microbe to a TLR leads to activation of several inflammatory pathways (6–9). SNPs in TLR genes may affect TLR function, contributing to a modulated inflammatory response. Up-regulation of TLRs may be one of the mechanisms by which intrauterine inflammation signals parturition and triggers preterm labor (10). This is supported by the fact that the majority of pregnancies that end with very preterm birth are more likely to be complicated by chorioamnionitis and/or neonatal infection acquired by

vertical transmission from the mother (11,12). Moreover, an increased frequency of two TLR4 polymorphisms has been found in a population of preterm Finnish infants when compared with term control infants (5). The most important TLR4 ligand is LPS from Gram-negative microorganisms.

We hypothesize that polymorphisms in the TLR2 and TLR4 genes result in modulation of fetal inflammatory responses, which trigger preterm birth. To address this issue, we determined the genotype distribution of two common TLR2 (T-16934A, Arg753Gln) and two cosegregating TLR4 (Asp299Gly and Thr399Ile) SNPs among 524 infants admitted to the neonatal intensive care unit of our hospital and associated this with gestational age (GA).

METHODS

Patient population. In a prospective study, we separately stored a portion of the whole blood sample collected for routine laboratory analyses of 524 infants admitted to the neonatal intensive care unit of our tertiary care hospital (Wilhelmina Children's Hospital, University Medical Center, Utrecht, The Netherlands) between 2002 and 2004. Clinical data of the infants and their mothers were collected prospectively. Infants born preterm due to the condition of mothers with preeclampsia or multiple pregnancy were excluded because they are confounding. The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht. Informed parental consent was obtained from all participating subjects.

DNA isolation and SNP genotyping. Genomic DNA was isolated from whole blood using a Qiaamp DNA blood kit (Qiagen, Hilden, Germany).

Polymorphisms in TLR2 [T-16934A (RS4696480) and Arg753Gln (RS5743708)] and TLR4 [Thr399Ile (RS4986791)] were determined using sequence specific primers and polymerase chain reaction (SSP-PCR) technique (13). After SSP-PCR, samples were loaded on a 2% agarose gel containing ethidium bromide, electrophoresed for 15 min at 300 V, and visualized under ultraviolet light. The TLR4 Asp299Gly polymorphism (RS4986790) was determined using a TaqMan genotyping assay.

Statistical analyses. The median GA according to combinations of the TLR2 SNPs was compared using one-way analysis of variance (ANOVA). Data analyses were performed using SPSS version 12.0.1 for Windows (SPSS Inc., Chicago, IL). P values <0.05 were considered statistically significant.

RESULTS

The relevant clinical characteristics of the 524 infants are shown in Table 1. Because preeclampsia and multiple pregnancies are important risk factors for prematurity, we excluded infants born of mothers with these conditions. There-

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Abbreviations: GBS, group B streptococcus; SNP, single nucleotide polymorphism; TLR, toll-like receptor

Table 1. Clinical characteristics of all study subjects ($n = 524$) and of 305 infants, after exclusion of infants born in multiple pregnancies or after preeclampsia

Total no. of patients	All ($n = 524$)	Multiple birth and eclampsia excluded ($n = 305$)
Male/female, n	314	191
Birth weight, g, (95% CI)	1958.2 (1873.6–2042.8)	2324.6 (2207.3–2441.9)
Gestational age, wk (95% CI)	33.2 (32.8–33.6)	34.5 (33.9–35.0)
Term: ≥ 37 wk, n (%)	117 (22.3)	105 (34.4)
Moderately preterm: 32–37 wk, n (%)	143 (27.3)	81 (26.6)
Very preterm: < 32 wk, n (%)	264 (50.4)	119 (39.0)
Infants born from multiple pregnancies, n (%)	125 (23.9)	Excluded
Preeclampsia, n (%)	108 (20.6)	Excluded
Preterm premature rupture of membranes, n (%)	58 (11.1)	49 (16.1)
Early-onset sepsis, n (%)	11 (2.1)	9 (3.0)

Table 2. Allele frequencies of the TLR2-16934 (RS4696480), TLR2 2285 (RS5743708), and TLR4 8851 (RS4986791) SNPs in the total study group and in subgroups

	TLR2		TLR4
	-16934 A/T (RS4696480)	2258 G/A (RS5743708)	8851 C/T (RS4986791)
All ($n = 524$; %)	517/531 (49.3/50.7)	1019/29 (97.2/2.8)	992/56 (94.7/5.3)
Preterm premature rupture of membranes ($n = 58$; %)	57/59 (49.2/50.8)	112/4 (96.6/3.4)	110/6 (94.8/5.2)
Preeclampsia ($n = 108$; %)	109/107 (50.5/49.5)	205/11 (94.9/5.1)	204/12 (94.4/5.6)
Born in multiple pregnancy ($n = 125$; %)	121/129 (48.4/51.6)	241/9 (96.4/3.6)	233/17 (93.2/6.8)
Preeclampsia or multiple pregnancies excluded ($n = 305$; %)	298/312 (48.9/51.1)	596/14 (97.7/2.3)	581/29 (95.2/4.8)

fore, the statistical analysis for a possible association between TLR polymorphisms and prematurity was performed in 305 of the 524 infants.

The allele frequencies of the examined TLR2 and TLR4 SNPs were in Hardy-Weinberg equilibrium (Table 2). Because the TLR4 Asp299Gly and Thr399Ile TLR4 SNP were totally cosegregating, we analyzed one SNP (Thr399Ile).

The median GA of infants was similar across the individual TLR2 and TLR4 genotypes. However, comparing the median GA of infants being wild type on both TLR2 positions, infants carrying one polymorphic allele and infants carrying two polymorphic alleles showed that the infants with a double polymorphism in the TLR2 allele had more than 3 wk shorter GA

(median GA 30.6 wk in the latter group *versus* 34.1–36.8 wk in the other groups; $p < 0.02$) (Fig. 1). The polymorphism in TLR4 did not contribute to GA.

DISCUSSION

The present study showed a significant association between the carriage of two TLR2 polymorphic alleles and very preterm birth. Carriage of only one TLR2 polymorphic allele did not influence the duration of gestation.

A study in a Finnish population by Lorenz *et al.* (5) found a trend toward a higher frequency of TLR2 polymorphisms (TLR Arg753Gln) in preterm infants compared with term infants, but this association was not significant. TLR2 predominantly responds to Gram-positive bacteria, such as group B streptococci (GBS), the most frequent causative microorganism for neonatal early-onset sepsis. However, we were not able to establish an association between TLR2 mutations and GBS sepsis, probably due to the very low incidence of culture-proven early-onset GBS sepsis (14). Elovitz and colleagues (10) studied the involvement of TLRs in preterm parturition using a mouse model with localized intrauterine inflammation and found that lipopolysaccharide stimulates both TLR4 and TLR2 mRNA expression in the uterus, cervix, and placenta, resulting in an exaggerated host response to microorganisms colonizing the vagina, such as GBS. However, whether the enhanced maternal host response through activated TLRs, or, alternatively but not mutually exclusively, a modulated fetal host response through polymorphisms in TLRs is prevalent cannot be concluded yet. Studies among larger cohorts are needed to confirm the hypothesis that fetal TLR2 polymorphisms contribute to the development of Gram-positive bacterial infections in the perinatal period.

The TLR2 SNPs used in this study are not tagging SNPs for this gene but were chosen because of the functional conse-

Figure 1. Median GA according to polymorphisms in the TLR2 gene. Infants born in multiple pregnancy or preeclampsia were excluded. Data are grouped according to -16934 and 2258 genotype: -16934 TT 2258 GG, $n = 79$; -16934 AT/AA 2258 GG, $n = 212$; -16934 TT 2258 GA/AA, $n = 7$; -16934 AT/AA 2258 GA/AA, $n = 7$. Median values are indicated by a bar, the boxes represent the 25th–75th percentile and the whiskers minimum and maximum; p values as calculated by one-way ANOVA are indicated in the figure. * $p = 0.016$; ** $p = 0.009$; † $p = 0.003$.

quences. The TLR2 gene does, however, reside in a large block of LD on chromosome 4.

An important issue for the interpretation of our data is the varying distribution of innate immunity genes among populations of different ethnic origin (15). However, although we were not informed on the exact ancestral origin of our study population, the vast majority of our neonatal intensive care unit population is white, and therefore ethnicity was not considered a confounding factor.

Associations between polymorphisms in other maternal genes and preterm birth have been described: SNPs in tumor necrosis factor, interleukin (IL)-1 β receptor antagonist, IL-4, matrix metalloproteinase 9, β_2 -adrenergic receptor, vascular endothelial growth factor, and factor V Leiden are all associated with preterm delivery (4). Therefore, both maternal and fetal polymorphisms in inflammatory pathway genes should be investigated for their impact on preterm birth.

In conclusion, our study suggests that carriage of two variant TLR2 alleles potentially leads to an aberrant fetal innate immune response, contributing to very preterm birth. Additional studies involving polymorphisms of both maternal and fetal innate immunity genes may further substantiate the importance of these genes as risk factors for intrauterine complications and preterm birth.

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