Molecular Human Reproduction Vol.14, No.9 pp. 555–559, 2008
Advance Access publication on August 22, 2008 doi:10.1093/molehr/gan049

Toll receptor 4 Asp299Gly polymorphism and its association with preterm birth and premature rupture of membranes in a South American population

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Preterm birth (PTB) is a worldwide health problem and remains the leading cause of perinatal morbidity and mortality. Systemic and local intrauterine infections have been implicated in the pathogenesis of preterm labor and delivery. Common pathways between PTB, premature rupture of ovular membranes (PROM) and altered molecular routes of inflammation have been proposed. There is evidence to support a genetic component in these conditions. Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is thought to play a key role in eliciting an inflammatory response. LPS is recognized by proteins of the innate immune system, including Toll-like receptor 4 (TLR4). Individuals from some European countries carrying the variant alleles resulting in an amino acid substitution (Asp299Gly) are at increased risk of Gram-negative infections and premature birth. The objective of this study was to determine if preterm newborns have different allele frequency of the Asp299Gly TLR4 variant from healthy term neonates in Uruguay. The impact of PROM was also examined. There was an increase in the risk for fetuses carrying the Asp299Gly substitution in TLR4 of being severely premature (<33 weeks) and to present PROM at the same time.

Keywords: TLR4 polymorphism; preterm birth; premature rupture of membranes; genetic association; South American population

Introduction

Preterm birth (PTB), defined as the delivery of a fetus that has not completed 37 weeks of gestation, is a worldwide health problem. Preterm delivery remains the leading cause of perinatal morbidity and mortality (Esplin, 2006). Prematurity results in a 40-fold increase in neonatal mortality (Rush *et al*., 1976; Goldenberg and Rouse, 1998; Goldenberg *et al*., 1998). Among surviving infants, PTB is implicated in approximately half of all pediatric neurodevelopmental disabilities, including cerebral palsy (Goldenberg and Rouse, 1998), long-term morbidity and high healthcare costs (Ananth and Vintzileos, 2006). Unfortunately, the rate of PTB continues to rise according to recent incidence reports, and is currently 12–13% in the USA (Goldenberg *et al*., 2001; Ananth *et al*., 2006; Ananth, 2007). In South America, more than 10% of newborns are preterm, and the incidence is between 8% and 10% in Uruguay (http://www.msp.gub.uy/subcategoria_4_1_1.html). The rising incidence of PTB is linked to an increase of iatrogenic termination of pregnancy associated with severe pre-eclampsia, intrauterine growth restriction, severe maternal health conditions and others. However, an increase of spontaneous preterm delivery has also been reported (Ananth *et al*., 2006).

Despite the impact of spontaneous PTB on current obstetric practice, our understanding of this complication is still not clear. Spontaneous PTB probably has different underlying causes (Norwitz *et al*., 1999). Recognized etiologies of PTB such as maternal and fetal stress, inflammation, infection and hemorrhage or placental abruption ultimately converge in a final common pathway that leads to organized uterine contractions, rupture of membranes and cervical changes followed by preterm delivery (Lettieri *et al*., 1993). Systemic and local intrauterine infections have been implicated in the pathogenesis of preterm labor and delivery (Romero *et al*., 1987, 1988; Romero and Mazor, 1988). Indeed, either local or systemic exposure to microbial products leads to PTB in several animal models (Elovitz and Mrinalini, 2004). Intrauterine infection has been implicated in the etiology of preterm premature rupture of ovular membranes (PPROM), which accounts for 30–40% of all preterm deliveries (Parry and Strauss, 1998). Common pathways between PTB, PPROM and altered molecular routes of inflammation have been proposed. This common pathway could occur without even the presence of chorioamnionitis or clinical infection (Romero *et al*., 2006).

The documented increased risks in patients with a personal or family history of PTB and the ethnic disparities in the incidence of PTB suggest a genetic component in this condition (Varner and Esplin, 2005). In this way, preterm delivery appears to be a complex mechanism determined by both genetics and environmental factors.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is thought to play a key role in eliciting an inflammatory response including the activation of the immune cells and the release of enzymes involved in remodeling of the extracellular matrix leading to PPROM and PTB (Parry and Strauss, 1998). LPS is recognized by proteins of the innate immune system, including

Toll-like receptor 4 (TLR4) (Medzhitov *et al*., 1997). Hyporesponse to an LPS challenge has been associated with a TLR4 variant resulting in an amino acid substitution (Asp299Gly) lying between leucine-rich repeats (Arbour *et al*., 2000).

The biological relevance of this TLR4 single nucleotide polymorphisms (SNPs) has been widely investigated; individuals carrying the variant alleles are at increased risk of Gram-negative infections (Agnese *et al*., 2002; Lorenz *et al*., 2002b), and premature birth (Lorenz *et al*., 2002a) but not for PPROM in African Americans (Ferrand *et al*., 2002a). The same variant protects individuals from atherosclerosis (Kiechl *et al*., 2002; Ameziane *et al*., 2003).

In this research, we first determined the allele frequency of the Asp299Gly TLR4 gene polymorphism in term Uruguayan neonates. Then, we examined TLR4 genotypes in a cohort of preterm neonates. We considered PROM as an inflammatory co-factor of PTB and studied the impact of carrying this polymorphism in newborns that had PROM.

The discovery of genetic factors involved on PTB may lead to medical breakthroughs and reduction therefore in spontaneous PTB, neonatal morbidity and mortality. Understanding causes of PTB is especially important in underdeveloped countries. Complications of PTB are associated with increase of medical expenses, representing billions of dollars of direct costs and unrealized potential each year in developed countries (Esplin, 2006) and unaffordable for poor countries.

Materials and Methods

Subjects

Subjects in this study were offspring of women receiving obstetrical care at the Pereira Rossel Hospital, Montevideo, Uruguay. The study protocol was approved by the School of Medicine Ethics Committee of the Republic University, Uruguay. Subjects were recruited sequentially between May 2003 and May 2008. Informed written consent was obtained from mothers prior to collection of biological material, including specimens for extraction of DNA.

Cases ($n = 226$) were neonates from pregnancies complicated by spontaneous PTB. Two subgroups were distinguished; neonates born between 33 and 36 weeks were considered as moderate PTB ($n = 118$) and neonates of gestational age (GA) <33 weeks were included in a group of severe PTB ($n = 108$). Control subjects ($n = 250$) were neonates delivered at term. Subjects with PROM were detected in all categories. Patients with multiple gestations and fetal anomalies were excluded.

Analysis of TLR4 alleles

DNA was extracted either from umbilical cords or from newborn cheek swabs by conventional methods (Ferrand *et al*., 2002b). To detect *TLR4* 896 A>G non-synonymous polymorphism, we used the PCR amplification strategy previously described (Lorenz *et al*., 2001; Ferrand *et al*., 2002a), employing mismatch primers designed to detect the wild-type and variant *TLR4* alleles based on the presence of a restriction site in the variant allele. The following primers were used: forward 5′-GATTAGCATACTTAGACTACTACCTCCATG-3′ and reverse 5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′. The underlined base in the forward primer indicates the nucleotide altered to create an *Nco*I restriction site in the presence of the polymorphism. PCR was carried out using PureTaq™-Ready-To-Go™ PCR beads (Amersham Biosciences). After initial denaturation at 94°C for 5 min, PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension for 30 s at 72°C, after which a 20 μl sample was digested with *Nco*I (New England Biolabs) and fractionated on a 3% agarose gel.

Statistical analysis

Cases and controls were compared on demographic factors using ANOVA followed by the Tukey test. Comparisons of proportions of allele frequencies and the Hardy–Weinberg equilibrium test were performed using the χ^2 test or Fisher's exact test (Guo and Thompson, 1992).

The association between TLR4 genotypes and PROM related to GA was examined by logistic regression analysis. The presence of differential effects of TLR4 genotypes on the risk of PROM was explored by the inclusion of interaction or conditional terms. The analysis was performed with the Epi Info 2000 software (Center for Disease Control and Prevention, Atlanta, GA, USA). Probability values of 0.05 or less were considered significant.

Results

To determine if there was an association between PTB and the hyporesponsive TLR4 allele, we performed a case–control study in which we genotyped fetal DNA from cases (PTB $<$ 37 weeks) and controls. Since severe PTB showed particular susceptibility to inflammation, two groups of PTB were distinguished: moderate and severe newborns. The demographic characteristics of our study population are presented in Table I. Mothers were similar in age, parity and previous reproductive history. As expected, there were significant differences in birthweight of newborns and percentage of PROM.

Regarding the TLR4 polymorphism, we found that all carriers of the Asp299Gly allele were heterozygotes.

The TLR4 Asp299Gly allele frequency in the Uruguayan population shows no strong differences from other studies (Table II).

There was no significant difference in the frequencies of the TLR4 polymorphism among cases and controls (28 carriers in a total of 250 newborns of 37 weeks or more versus 25 carriers in 226 PTB; odds ratio 0.99; 95% confidential interval 0.557–1.748).

Regarding the combination of both effects, GA and PROM, allele frequency of the Asp299Gly variant was higher only in the severe PTB group with PROM versus without PROM. This difference was statistically significant (Table III).

The presence of TLR4 polymorphism was not associated with GA. When we analyzed PROM related to GA conditional on TLR4, a significant association in the severe preterm group was found (Table IV).

Table I. Clinical and demographic characteristics.

	≥37 weeks	33–36 weeks	<33 weeks
n	250	118	108
Maternal age (years)[a]	24 ± 0.4	24 ± 1	25 ± 1
Parity (% of nulliparous mothers)	26	33	29
Birthweight (g)[a]	3184 ± 30	2226 ± 32*	1414 ± 41*◇
Poor obstetric history (%)[b]	8	15	15
PROM (%)	20	40*	38*

[a]Values are expressed as mean ± SEM.
[b]Previous preterm birth, low birthweight, stillbirth or recurrent miscarriage.
*$P < 0.05$ versus term newborn.
◇$P < 0.05$ versus preterm 33–36 weeks.

Table II. TLR 4 Asp299Gly allele frequencies in different populations.

	Asp299Gly	*N*
Finland (Lorenz *et al*., 2002a)	0.083	351
USA (Arbour *et al*., 2000)	0.066	155
Mexico (Garza-Gonzalez *et al*., 2007)	0.077	259
China (Gao *et al*., 2007)	0.056	80
Portugal (Carvalho *et al*., 2007)	0.055	388
Africa (Mockenhaupt *et al*., 2006))	0.095	290
African American (Ferrand *et al*., 2002a)	0.066	218
Chile (Santos *et al*., 2006)	0.046	227
Spain (Santin *et al*., 2006)	0.08	269
Uruguay (present study)	0.05	250

Table III. TLR4 Asp299Gly allele frequencies in neonates delivered with or without PROM.

	Without PROM[a]		PROM[a]		*P*-value[a]
	Asp299Gly	*N*	Asp299Gly	*N*	
≥37 weeks	0.050	200	0.080	50	0.89
33–36 weeks	0.029	70	0.042	48	1.00
<33 weeks	0.045	67	0.134*	41	0.935

[a]Hardy–Weinberg test results for each GA group.
*$P < 0.05$ versus without PROM same GA.

Logistic regression analysis showed evidence of an association between PROM and GA as it has been already described in Table I (Table IV).

The observed genotype distributions did not deviate from the Hardy–Weinberg equilibrium in any of the GA groups (Table III).

Discussion

We have detected an increase of the risk for fetuses carrying the Asp299Gly substitution in TLR4 of being severely premature (<33 weeks) and to present PROM at the same time. Heterozygous individuals showing an increased risk of presenting both effects represents a new finding regarding the action of TLR4.

In a Finish population, Lorenz *et al*. (2002a) showed that preterm singleton infants had a significantly higher carrier rate for the TLR4 Asp299Gly variant compared with term singleton infants. These data suggest an association of this polymorphism and PTB of singleton newborns. In agreement with Lorenz *et al*., we found that the Asp299Gly allele frequencies in severe preterm infants presenting PROM were higher than in the term infants. However, there is no significant association between the TLR4 Asp299Gly polymorphism and the risk of preterm delivery in our population when this complication was considered independently of membrane rupture.

In a recent study of more than 500 newborns, Krediet *et al*. found a significantly higher frequency of the TLR2 polymorphism Arg753Gln in premature neonates of <30 weeks gestation. Carriage of the variant TLR2 alleles potentially leads to aberrant innate immune responses, which may have contributed to severe PTB (Krediet *et al*., 2007). Interestingly, a tendency toward the latter association was also found in the Finnish study, but this did not reach statistical significance. To date, these are the only studies that have looked at the relationship between TLR polymorphisms and PTB (Fleer and Krediet, 2007). The inflammatory response of placental tissues, considered to play a prominent role in the pathogenesis of premature labor and birth (Goldenberg and Rouse, 1998), could be most relevant in the severe preterm newborns. These findings suggest that newborns delivered before 33 weeks of GA should be further studied.

The lack of statistical differences between preterm and term newborns carrying the polymorphism in our study also suggests that other factors could be acting in conjunction to the toll receptor. Some of these genetic variations require the presence of certain environmental stimuli to have any clinical significance. Macones *et al*. (2004) described a gene–environment interaction between the tumor necrosing factor (TNF) alpha polymorphism and bacterial vaginosis. Women found to have both the TNF alpha polymorphism and bacterial vaginosis were at synergistically increased risk of PTB when compared with women with only the TNF alpha polymorphism or bacterial vaginosis alone. We did not check for bacterial vaginosis in all of our patients, so we cannot rule out the possibility of the influence of this factor.

The variations of the TLR4 gene may impact in different ways according to the genetic background of the population and that could explain the differences between the Finish and Uruguayan populations. Ancestral origin differences in PTB have been well documented in the USA. African American women present twice the rate of PTB compared with European American women, even when confounding social and economic variables are controlled (Schieve and Handler, 1996). However, regarding TLR4 Asp299Gly polymorphism, there is no difference between these populations with respect to PPROM (Ferrand *et al*., 2002a). The prevalence of the polymorphism seems to be similar in all the populations studied so far (Table II). The data were collected from different sources and it is difficult to establish an accurate worldwide distribution, but it seems that African and European populations have the highest frequencies. Interestingly, the studies that found a relevance of this TLR4 SNP were carried out in populations with a high frequency of TLR4 Asp299Gly polymorphism as in a Finnish study (Lorenz *et al*., 2002a).

The structure of the population can explain the differences found in our study. Uruguayan population has been described fundamentally as of European origin. However, more recently, genetic admixture analysis demonstrated a Native American and African contribution to the Uruguayan population of 10.4% and 5.6%, respectively (Sans *et al*., 1997; Hidalgo *et al*., 2005). The Uruguayan Asp299Gly observed frequency is less than that of Europeans or Africans and equal to the admixed Chilean population (Santos *et al*., 2006), which suggests the effect of admixture on this gene. The studies of the impact of different polymorphism on the inflammatory diseases in South American population have only recently started and sufficient data have yet to be collected and reported (Santos *et al*., 2006; Garza-Gonzalez *et al*., 2007). This is the first report concerning association between TLR4 and PTB or PPROM on a South American country.

TLR4 Asp299Gly allele frequencies are similar between the studied populations and none of them has an allele frequency higher than 10% (Table II). This is consistent with the idea that a mildly negative selection pressure is not sufficient to eliminate the allele (Smirnova *et al*., 2001) and that could explain the frequencies found in the worldwide populations. Modifications on the effect of TLR4 produced by TLR4 Asp299Gly polymorphism are not so deleterious by themselves, which means that selective evolutionary process has not been strong enough to eliminate the variant. Recently, an SNP in the promoter of the SERPINH1 gene was found. This variation of the SERPINH1

Table IV. Logistic regression and ORs on TLR4 genotype and PROM of control samples versus 33–36 or <33 weeks.

	33–36 weeks		<33 weeks	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
TLR4	0.546 (0.180–1.655)	0.285	0.885 (0.340–2.306)	0.803
PROM	2.857 (1.719–4.750)	0.000	2.108 (1.215–3658)	0.008
Interaction	0.875 (0.162–4.735)	0.877	2.175(0.535–8.836)	0.278
PPROM conditional on TLR4	2.500 (0.500–12.511)	0.395	4.583 (1.263–16.635)	0.032

gene reduces promoter function in amnion fibroblast cells and is strongly associated with risk of PPROM, the leading identifiable cause of PTB. This SNP is enriched in individuals of African ancestry (Wang *et al*., 2006). Once again, ethnic origin and the environment interact in order to determine the distribution of allele variants.

Recent evidence suggests that TLR4 SNPs 299 and 399 haplotypes are geographically clustered and this could be related to the susceptibility to infection, especially malaria, because it has a high prevalence in sub-Saharan Africa. There should be a beneficial effect of Asp299Gly in malaria that seems to override the negative effect in Gram-negative infections. The data provide an understanding of how our innate immune system has been molded by infectious pressures (Ferwerda *et al*., 2007). The high prevalence of Gram-negative infectious diseases in pregnancies in South American countries and Hispanic populations (Conde-Gonzalez *et al*., 1987; Gonzalez Pedraza *et al*., 1995; Gunn *et al*., 1995; Narcio *et al*., 1996) makes especially relevant the studies of the role of genes and polymorphisms in the susceptibility to infections.

If a multitude of rare variants (rather than restricted number of common variants) underlie the genetic susceptibility to these traits, we need more powerful strategies than association studies such as we performed here. Admixture mapping or linkage disequilibrium mapping strategies would be options that would help to uncover the effect of uncommon alleles (Reich and Patterson, 2005).

Ferrand *et al*. (2002a) detected no risk for newborns carrying the Asp299Gly variant of having preterm PROM. Interestingly, we have been able to detect an increased risk of PROM in fetuses carrying the Asp299Gly substitution only in severe PTB, a condition that was not considered separately in their report. Their research was carried out in an African American population so ethnic variations could explain different predispositions to inflammatory pathologies, underlying again the importance of population studies. Further investigations should be done where PROM and ethnics differences would be closely considered.

Our findings underline a role for genetic variation in LPS responsiveness in determining the risk of obstetric complications that leads to PTB only in the group of severe preterm. However, the interaction between LPS and TLR4 also relies on a range of chaperone molecules including LPS-binding protein (LBP), CD14 and MD2. Therefore, either maternal or fetal variation in any of these molecules may influence gestational outcome. Additional studies involving other polymorphisms of such genes as CD14, LBP and MD-2 may further substantiate the importance of host innate immunity genes as risk factors for pregnancy complications leading to premature birth and severe PTB (Hirschfeld *et al*., 2007).

Hence, our data show an association between TLR4 polymorphisms and the risk of PTB and PROM in the group of severe preterm suggesting differences of susceptibility to the inflammatory process between preterm neonates. However, in order to better understand the role of TLR4 on these obstetrical complications, novel strategies need to be developed to further uncover the effects of environmental factors, other genes and genetic backgrounds.

Funding

Funding for this work was provided by Fogarty International Center 'National Institutes of Health' grant RO1TW006223.

Acknowledgement

We acknowledge Robert O'Neill, English Language Centre, Hove, UK, for his careful review of the manuscript language and Maria de los Angeles Michel, for her assistance with medical records.

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Submitted on December 27, 2007; resubmitted on August 8, 2008; accepted on August 12, 2008