RESEARCH PAPER

Association of *TLR4* and *TNF-α* Gene Polymorphisms and TLR4 mRNA Levels in Preterm Birth in a Northern Indian Population

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Objective: To assess the association of *TLR4* (rs4986790 and rs4986791) and *TNF-α* (rs1800629) genes polymorphisms and TLR4mRNA levels with preterm birth. **Methods:** Hospital-based case-control study on women of Caucasoid morphological subtype ethnicity in Northern India. Inclusion criteria for cases: women aged between 18-40 years with preterm birth (<37 weeks gestation), and for controls: women who delivered a term neonate consecutive to an enrolled case. Three polymorphisms *TLR4* (Asp299 Gly, Thr399 Ill) and *TNF-á* (-308G/A) and TLR4 mRNA levels were compared between cases and controls. ***Results*:** From 2012-2015, 559 cases and 559 controls were recruited. TLR4 mRNA levels were found to be higher (*P*<0.001) in cases [(0.7 (0.04)] than in controls [(0.5 (0.04)]. No association was found between *TLR4* Asp299 Gly, *TLR4* Thr399 Ill and *TNF-α* (-308G/A) with preterm birth. ***Conclusion*:** Increased TLR4 mRNA levels seem to be associated with preterm birth, and can be investigated further as a potential biomarker for identifying women at risk.

Keywords: *Biomarker, Inflammation, Pregnancy complications*, *Pematurity.*

Preterm births contribute significantly to neonatal mortality and childhood morbidity in developing countries [1]. Globally, the incidence of preterm birth is highest in India at 23.6% [2]. There is a need to find etiological causes of preterm birth, including its genetic associates and biomarkers of inflammation. *TLR-4* and *TNF-α* are the strong candidate genes of inflammatory pathway. *TLR*-4 has leucine rich repeats or pattern recognition receptors that help to identify the molecular pattern of several pathogens which initiates the activation of cascade of inflammatory pathway [2]. The objective of this study was to assess the association of the SNP at the rs4986790 and rs4986791 of the *TLR4* gene and rs361525 of *TNF-α* gene and the expression analysis of *TLR-4* gene with preterm birth.

METHODS

This was a case control study conducted in two hospitals in Lucknow (India): King George's Medical University and Ram Manohar Lohia Hospital. Ethical clearance was obtained from Institutional committees of the respective hospitals. Cases were mothers (age 18-40 years) of live preterm (<37 weeks) neonates, while controls were eligible mothers who delivered a neonate at or after 37 weeks of gestation, consecutive to an enrolled case. Mothers with known clinically diagnosable causes of preterm birth, twin delivery, congenital abnormalities or complications in pregnancy were excluded. Clinical, anthropometry and demographic data were extracted from hospital records. The peripheral blood was collected and genomic DNA was extracted from the mothers after obtaining their consent.

Both the polymorphisms of *TLR4*, SNP rs4986790 (A+896 G) and rs4986791 (C+1196T) are located in the coding region which further regulate the transcription of the gene. These polymorphic sites cause the exchange of an amino acid on alteration with the rare allele: an aspartic acid in exchange for glycine at position 299 *i.e. Asp299Gly* and threonine for an isoleucine at position 399*: Thr399Ill* [2]. Alternatively, *TNF-α* (rs361525) SNP is located on the promoter region of the gene and is reported to be associated with elevated expression of the gene [2]. The details of primers, PCR condition, restriction enzymes, and their products are given in ***Web Table* I**. Total RNA was extracted the peripheral blood with the help of QIAGEN kit. All the reverse transcription was carried out by Fermentas reverse transcriptase kit using oligo dT priming.

Real-time PCR was performed with 1 µL cDNA, Syber green universal PCR mix, and 20X primer (Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems).The 18S rRNA gene was used as an endogenous control. Results were evaluated using pfaffa method: the delta-delta Ct method, where delta Ct was calculated as

(TLR4Ct - 18sRNA Ct), and the relative quantity of TLR4 mRNA expression was calculated by the delta–delta Ct as 2^{-}[(case delta Ct) " (control sample delta Ct)] [3].

Statistical analysis: All the analyses was carried out by SPSS (version 22.0). The genotypic and expression analysis was done blinded toward the patient's status. For categorical variables chi-square test was used. Student's t-test was used to determine the association of preterm birth with other factors. We calculated genotypic distribution by Pearson's $\chi 2$ test taking 95% of confidence interval (C.I.) in consideration. $P \leq 0.05$ was considered significant.

RESULTS

A total of 559 cases and 559 controls were recruited. ***Table* I** contains the demographic and anthropometric details of neonates and mothers. ***Table* II** depicts genotypic and allelic frequency of *TLR4* Asp 299 Gly, Thr 399IIl and *TNF-α* (-308G/A) in association with preterm birth. The prevalence of genotype *TLR4* Asp 299 Gly, Thr 399IIl did not differ significantly in cases and controls. Increased risk of preterm birth was found with AA genotype of TNF-α (OR 1.5; CI 1.02, 2.20; *P*=0.03; *i.e.* AA *Vs* GA+GG). TLR4 mRNA expression (***Fig* 1**) was found to be higher (*P*<0.001) in cases [0.7 (0.04)] than in controls [0.5 (0.04)].

DISCUSSION

In this study, increased expression of TLR4 mRNA levels was reported in cases as compared with controls.

TABLE I DETAILS OF THE NEONATES AND MOTHERS ENROLLED

Characteristics	*Cases (n=559)*	*Controls (n=559)*
Neonatal details		
Birthweight (kg)*	1985 (473.7)	2768.6 (423.9)
Gestational age (wks)*	33.9 (1.9)	38.2 (1.0)
Gestational age (wks)#	34 (27-36)	38(37-42)
Gestation ≤34 wk, *n* (%)	119 (21.2%)	0
Male sex, n (%)	318 (56.9)	314 (56.2)
Head Circumference (cm)*	30.9 (1.7)	33 (1.3)
Length (cm)*	43.2 (3.0)	46.6 (3.7)
Maternal details		
Age (y)*	25.8 (4.2)	25.7 (3.7)
Weight (kg)*‡	52.9 (7.4)	56.3 (6.8)
Gravida <3, *n* (%)	390 (69.7%)	420 (75.1%)
Parity <3, *n* (%)	424 (75.8%)	443 (79.2%)

**mean (SD); #Median (Range);‡P<0.001.*

TABLE II GENOTYPIC FREQUENCY OF *TLR4* ASP 299GLY, THR 399 ILE AND TNF-α POLYMORPHISMS

Gene	*Cases, n (%)*	*Controls, n (%)*	*P value*
AA	495 (88.5%)	497 (89%)	0.81
AG	64 (11.9%)	61 (11%)	
CC	515 (92%)	531 (95%)	0.053
CT	44 (8%)	28 (9%)	
GG	307 (55%)	318 (57%)	
GA	180 (32%)	190 (34%)	0.2
AA	72 (13%)	51 (9%)	0.1
Dominant Model			
GG	307 (55%)	318 (57%)	0.5
GA+AA	252 (45%)	241 (43%)	
Overdominant Model			
GA	180 (32%)	191 (34%)	0.5
GG+AA	379 (68%)	368 (66%)	
Recessive Model			
AA	72 (13%)	50 (9%)	0.03
GG+GA	487 (87%)	509 (91%)	

However, no association was found between TLR4 SNP rs4986790 (A+896 G) and rs4986791 (C+1196T) and TNF-α SNP rs361525 with preterm birth.

Our findings are supported by results from another study [4] that reported increased expression of TLR4 in patients with preterm labour [4]. Our study also concurs with the findings of few other studies [5-8] which observed TLR4 as a contributing factor in inflammation. Patni, *et al*. [9] reported no difference of TLR 4 expression in term and preterm placenta.

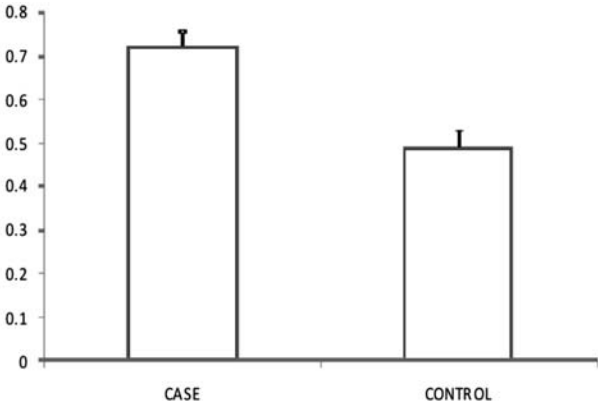

FIG.1 *Quantitative Real time-PCR analysis of TLR-4 gene expression which showed 2.5 fold increase in cases as compared to controls; the y axis of 2(–ΔCt) represents the relative gene expression of TLR-4.*

WHAT THIS STUDY ADDS?

- Increased TLR4 mRNA levels in mother may be associated with the risk of preterm birth.

A study conducted in Netherland by Krediet, *et al*. [10] found no association of *TLR4* (Thr 399 Ill) polymorphism with the gestational age. While Lorenz, *et al*. [11] reported that *TLR4* T allele was higher in singleton preterm neonates as compared to multiple preterm neonates and term neonates. Elovitz, *et al*. [12] reported that *TLR4 G* allele *(*Asp 299 Gly) populates are more at risk of premature rupture of membrane before or at 33 weeks. In case of TNF-α (-308G/A), no association was found in our study; similar results are seen in few earlier studies [13-15]. A study conducted on maternal-fetal genotype interface showed that mother carrying *TNF-α*-308GA genotype and fetus with the carrier of *TNF-α*-308GG genotype are at risk of preterm birth [16]. Speer, *et al*. [17] also supported the higher incidence of inflammation associated preterm birth in fetus with *TNF-a*-308GG genotype.

We conclude that the increased level of TLR4 in preterm birth supports its role in causation of preterm birth.

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