Clinical Research

Toll-Like Receptor Polymorphism Associated with Neonatal Sepsis in Term Babies

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ABSTRACT

Objective: Previous studies have revealed that severity and variability in the spectrum comprising sepsis are associated with toll-like receptor (TLR) polymorphism. The purpose of this study was to investigate the association between neonatal sepsis and TLR polymorphism.
Material and Method: In this cross-sectional study, 40 newborns with sepsis and 27 healthy newborns in department of neonatal intensive care unit, were evaluated. Single nucleotide polymorphisms (SNPs) of TLR2 and TLR4 were investigated in peripheral blood of both term newborns with sepsis before treatment and healthy age- and weight-matched control newborns.
Results: Twenty-four (60%) newborns with sepsis had blood culture positivity. Nine SNPs were determined in these two genes in seven patients. In addition, TLR2 gene variants; Pro631His; C>A variants and TLR4 gene variants; Asp299Gln; A>G, Thr399Ile; and C>T variants were associated with neonatal sepsis (p =0.016). TLR2 and TLR4 are important candidate genes regulating the immune response in sepsis pathways.
Conclusion: The findings of this study can assist with the identification of the mechanism involved in sepsis and host susceptibility to sepsis in term newborns.

Keywords: *Newborn, Sepsis, Toll-Like Receptors, Single Nucleotide Polymorphism.*

ÖZET

Term Bebeklerde Toll-Like Reseptör Polimorfizminin Neonatal Sepsisle İlişkisi
Amaç: Önceki çalışmalarda sepsis spektrumunun şiddet ve değişkenliğinin Toll-like reseptör (TLR) polimorfizmiyle ilişkili olduğu gösterilmiştir. Bu çalışmanın amacı yenidoğan sepsisi ile TLR polimorfizmi arasındaki ilişkiyi saptamaktır.
Gereç ve Yöntem: Bu kesitsel çalışmada, hastanemizde 40 sepsisi olan ve 27 sağlıklı olan yenidoğan değerlendirildi. Sepsisi olan term bebeklerin tedavi öncesindeki ve yaş ve vücut ağırlığı eşleştirilmiş sağlıklı kontrol bebeklerin TLR2 ve TLR4 tek nükleotid polimorfizmleri (SNP) incelenmiştir.
Bulgular: Yirmi dört (%60) yenidoğanın kan kültürü pozitifti. Yedi hastada bu iki genin 9 SNP'si saptandı. Ayrıca, TLR2 gen varyantlarından; Pro631His; C>A varyantları ve TLR4 gen varyantlarından; Asp299Gln; A>G, Thr399Ile; ve C>T varyantlarının neonatal sepsisle ilişkili olduğu görülmüştür (p =0.016). TLR2 ve TLR4 sepsis yolaklarındaki immün yanıtı düzenleyen önemli aday genlerdir.
Sonuç: Bu çalışmanın bulguları, term yenidoğanlarda sepsis mekanizmasını ve konağın sepsise karşı duyarlılığını tanımlamada yardımcı olabilir.

Anahtar Sözcükler: *Yenidoğan, Sepsis, Toll-Like Reseptörleri, Tek Nükleotid Polimorfizmi.*

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Neonatal septicemia is a major worldwide health problem. During the neonatal period, infections are frequent and important causes of morbidity and mortality, in addition to other reasons (1, 2). In the United States of America, the incidence of early sepsis is 3.5-8.9, and that of late sepsis 6 in 1000 live term births (3, 4). In Turkey, the incidence of neonatal sepsis is 5.4% in term babies, with a mortality rate of 15-50% (4). Predisposing factors for neonatal sepsis include maternal and environmental exposures, immune status, and inflammatory responses. These interacting factors can be influenced by gene functions or expressions which may result in variability and significant clinical implications among individuals (5).

It was previously shown that both neutrophil function and unique cytokine responses were impaired in newborn infants, and in particular extremely preterm neonates (5-7). Sepsis comprised of highly complex interactions between invading pathogens, the innate and adaptive immune systems of the host, and multiple downstream events leading to organ dysfunction and death. Circulating cellular mediators alter cellular signaling, and hence, appropriate mechanisms involving immune regulation, tissue repair, and cellular stress

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responses are damaged (5, 8). By means of pattern recognition receptors (PRRs), the innate immune system has the capability to recognize conserved microbial structures, also known as pathogen-associated molecular patterns (PAMPs) (9). PAMPs are recognized via cell surface receptors as the typical initial response to bacterial infection (10-12). Toll-like receptors (TLRs) are one class of PRRs that sense bacterial, viral or fungal molecular structures or nucleic acids and induce systemic inflammation (9). TLR family members, which are type 1 transmembrane proteins expressed by macrophages and dendritic cells, are vital regulators of both innate and adaptive immune responses (10-13). Ten types of TLR have to date been identified (14). While TLR-2 appears to be mostly involved in the recognition of Gram-positive bacteria, TLR-4 takes a role in the recognition of Gram-negative bacteria (10-12, 15). The importance of host genetic factors which contribute significantly to interindividual variation in susceptibility to infections has been shown by studies of twins. One or more candidate genes have been suggested to be involved in the pathogenesis of sepsis. Abu-Maziad et al. (5) has shown that gene-environment interactions result in certain patients having heightened susceptibility to neonatal sepsis.

Numerous studies have investigated the relationship between TLR mutations, polymorphism, the immune system and infections in adults and children (5, 16-22). The purpose of this study was to investigate the relationship between polymorphism related to TLR2 and TLR4 and neonatal sepsis in term newborns.

MATERIAL AND METHOD

This prospective case-control study was performed in neonatal intensive care units (NICUs) at the Erzurum Regional Training and Research Hospital, Turkey, from February to December, 2014. Following ethics committee approval, written consent was obtained from all families, and newborns with gestational age ≥37 weeks and diagnosis of sepsis were included. Neonatal sepsis was defined as the presence of two or more of the following clinical features:

1. Respiratory compromise, including tachypnea, increased apnea, severe apnea, increased ventilation, or desaturation,
2. Cardiovascular compromise or hypotension,
3. Metabolic changes, including hypothermia, feeding intolerance, glucose instability, or metabolic acidosis,
4. Neurological changes, including lethargy, hypotonia, or decreased activity.

In addition, the following previously validated hematological criteria were also used as indicators of sepsis:

1. An absolute neutrophil count (ANC) of <7,500 or >14,500 cells/mm^3,
2. An immature/total neutrophil ratio > 0.20, and
3. A platelet count of <150,000 cells/mm^3.

Infants with positive cultures were diagnosed with confirmed sepsis, while those with negative culture results but a positive clinical septic picture with two or more of the hematological and two or more of the clinical features detailed above were categorized as suspected sepsis (1, 5).

Exclusion criteria consisted of the presence of major congenital abnormalities, serious congenital heart diseases, or congenital metabolic diseases.

Sixty-seven infants were enrolled in this study. The patient group included 40 babies with diagnosis of sepsis. Twenty-seven randomly selected age- and weight-matched babies, with no medical problems and gestational age over 37 weeks, brought in for routine controls, were enrolled in the control group. Data including demographic characteristics, antenatal, prenatal and postnatal history, physical examination findings (including vital signs), routine blood tests, culture results, and treatment of each patient at baseline and during follow-up were recorded.

Concurrently with routine investigation including complete blood count, C-reactive protein, serum biochemical tests, and arterial blood gases before the start of treatment, blood samples for TCRs analysis were collected and stored in non-additive EDTA-containing vacutainer tubes at -80° C until analyis.

Genomic DNA was extracted using a MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany). We examined frequencies of single-nucleotide polymorphisms (SNPs) in TLRs and focused on two cosegregating SNPs, Pro631His (rs5743704) and Arg753Gln (rs5743708), within the gene encoding TLR2, and two cosegregating SNPs Asp299Gln (rs4986790) and Thr399Ile (rs4986791) within the gene encoding TLR4. Genotyping was carried out using LightSNiP typing assay (TIB-MolBiol, Berlin, Germany) by analyzing the melting curves with the LightCycler 480 II system (Roche Applied Science, Mannheim, Germany). Samples were set up in a total volume of 20 ml, containing 5 ml of DNA solution, 2 ml of FastStart DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany), 2 ml of LightSNiP typing assay (TIB-MolBiol, Berlin, Germany), 1.6 ml of 25 mM $MgCl_2$, and 9.4 ml of distilled H_2O. The cycling conditions were initial denaturation at 95° C for 10 min, followed by 45 cycles of denaturation at 95° C for 10 sec, annealing at 60° C for 10 s, and extension at 72° C for 15 sec. Fluorescence was monitored at the end of each annealing phase at 60° C. After completion of the PCR, a melting curve of the amplification products was plotted by denaturation at 95° C for 20 sec. The sample was held at 40° C for 10 sec, and then slowly heated to 80° C with a ramp rate of 0.1° C/sec and continuous fluorescence acquisition. The four polymorphisms were determined using a commercially available LightSNiP assay according to the manufacturer's instructions.

The Statistical Packages for the Social Sciences (SPSS) version 20 (SPSS, Inc., Chicago, IL) was used for statistical analyses. For descriptive statistics, mean± standard deviation for normally distributed and median (minimum-maximum) for non-normally distributed

variables were used. In addition to descriptive methods, the independent-samples T-test was used to assess changes between variables with normal distribution. The Mann Whitney U test was used to assess changes between variables with non-normal distribution. Yates continuity correction (Yates-corrected chi square) test was used to compare qualitative data. Spearman's correlation analysis was used to evaluate correlation between variables. Significance was set at $p < 0.05$.

RESULTS

Forty term infants with sepsis and 27 healthy control infants were included in this study. Demographic and clinical characteristics are presented in table 1.

Table 1. *Demographic and clinical characteristics of the patients and control newborns.*

Variables	Patients (n =40)	Controls (n =27)	p value
Gestational age, week, mean ± SD	39,4 ± 1.4	39.1 ± 1.2	**0.395**
Gender			
Female, n (%)	19 (47.5)	15 (55.5)	**0.493**
Male, n (%)	21 (52.5)	12(44.5)	**0.560**
Birthweight, grams, mean ± SD	3203.1±726.7	3285.5 ±514.6	**0.533**
Type of delivery			
Cesarean section, n (%)	24 (60)	16 (40)	**0.695**
Vaginal delivery, n (%)	16 (59.3)	11 (40.7)	**0.645**
Clinical chorioamnionitis, n (%)	7 (17.5%)	0	**0.001***
Rupture of membranes, n (%)	12 (15)	0	**<0.0001***
Duration of rupture of membranes, hours, mean ± SD	60 ± 21.4	0	**<0.0001***
White blood cell, number/mm^3, mean ± SD	20,008.7 ± 10,322.8	11,170 ± 3,448.4	**<0.0001***
CRP levels, mg/dl, median (minimum-maximum)	8.6 (0.25 -45.6)	0.4 (0.2- 0.9)	**<0.0001***
Duration of antibiotic treatment, days, mean ± SD	12 ± 3.8	NA	-
Sepsis (Positive cultures), n (%)	24 (60)	NA	-
Suspected sepsis, n (%)	16 (40)	NA	-
SNEPs, n (%)	7 (17.5)	0	**<0.0001***
Death, n (%)	1 (2.5)	NA	-

**statistically significant; SNEPs, single nucleotide polymorphisms; CRP, C-reactive protein; NA, not applicable.*

The mean gestational ages of the patients and control subjects were 39.4±1.4 and 39.1±1.2 weeks, respectively. Nineteen (47.5%) of the patients and 15 (55.5%) of the controls were female. The mean birth weights of the patients and controls were 3203.1±726.7 and 3285.5±514.6 g, respectively. There was no statistically significant difference between the patients and controls in terms of gestational age, sex, or birth weight. Twenty-four (60%) of the patients and 16 (59.3%) of the controls were born by cesarean section (C/S). There was no statistically significant difference between the groups in terms of mode of delivery. Mothers of 12 (30%) patients experienced premature membrane rupture (PMR), the mean duration of which was 60±21.1 days. Mothers of 7 (17.5%) patients had clinical chorioamnionitis. Presence of PMR and clinical chorioamnionitis and duration of rupture of membranes were significantly associated with sepsis. Patients' white blood cell counts and CRP values were significantly higher than those of the control infants (for both $p < 0.0001$). One patient died due to sepsis-related complications of sepsis during treatment. Single nucleotide polymorphisms were present in 7 (17.5%) patients, but in none of the controls ($p < 0.0001$).

Twenty-four (60%) patients had blood culture positivity (Table 2).

Table 2. *The pathogens recovered in blood cultures of 40 newborns with sepsis.*

Pathogen	Number (%)
Gram positive infections	
Coagulase negative Staphylococcus	5 (12.5)
Enterococcus species	2 (5)
Gram negative infections	
Klebsiella pneumoniae	8 (20)
Escherichia coli	4 (10)
Pseudomonas species	3 (7.5)
Acinetobacter baumanii	1 (2.5)
Fungal infections	
Candida albicans	1 (2.5)
Total of culture positivity	24 (60)
No growth	16 (40)

Gram-negative organisms were more frequent than Gram-positives, and *Klebsiella pneumonia* was the most frequently isolated microorganism in blood cultures.

SNP was not detected in any of the 27 control children in this study. SNPs and their related genes determined in the patients are summarized in table 3.

Table 3. *Single nucleotide polymorphisms and their related genes.*

Cases	Gene	Chromosome	Single gene nucleotide polymorphism	TLs	Major/minor allele	Pathogen in culture
1	TL2	4q33.1	Pro631His Heterozygote	TL1 (C>A)	C/A	*Klebsiella pneumonia*
2	TL4	9q33.1	Thr399Ile Heterozygote	TL4 (C>T)	C/T	*Klebsiella pneumonia*
3	TL4	9q33.1	Asp299Gln Heterozygote	TL3 (A>G)	A/G	*Escherichia coli*
	TL4	9q33.1	Thr399Ile Homozygote	TL4 (C>T)	T/T	
4	TL4	9q33.1	Thr399Ile Heterozygote	TL4 (C>T)	C/T	*Escherichia coli*
5	TL4	9q33.1	Asp299Gln Heterozygote	TL3 (A>G)	A/G	Coagulase negative *Staphylococcus*
	TL4	9q33.1	Thr399Ile Heterozygote	TL4 (C>T)	C/T	
6	TL4	9q33.1	Thr399Ile Heterozygote	TL4 (C>T)	C/T	*Pseudomonas* species
7	TL4	9q33.1	Asp299Gln Heterozygote	TL3 (A>G)	A/G	*Candida albicans*

SNP, single nucleotide polymorphism.

In this study, 9 SNPs were determined in 2 genes. TLR2 gene variant, Pro631His; C>A variants and TLR4 gene variants; Asp299Gln; A>G and Thr399Ile; C>T variants were associated with neonatal sepsis in 7 of the 40 patients (p =0.016). Pro631 His Heterozygot Genotype C/A SNP was detected in TLR2 in 1 (2.5%) patient, and Thr399Ile Heterozygote Genotype C/A SNPs in TLR4 SNPs in 3 (7.5%) patients. In 2 (5%) patients, two SNPs were found together. In one of these patients, Asp299Gln Heterozygote Genotype A/G and Thr399Ile Homozygote Genotyp T/T SNPs were detected, while the other patient had Asp299Gln Heterozygote Genotype A/G and Thr399Ile Heterozygote Genotyp C/T SNPs. In 1 (2.5%) patient, Asp299Gln Heterozygote Genotype A/G SNP was detected only in TLR4. There was a positive correlation between blood culture positivity with Gram-negative microorganisms and TLR4 SNPs (r =0.485, p =0.003).

DISCUSSION

Despite the significant burden caused by sepsis, studies investigating the genetic association in newborn infants are still limited in comparison to those involving adults and older children (9, 17-21, 23, 24). This is one of the rare studies demonstrating the relationship between polymorphism related to TLR2 and TLR4 and neonatal sepsis in term newborns. Pathogen-associated molecular mechanisms play a major role in innate immune responses. The relevant reports mainly concern premature infants (5, 18, 22). Each type of TLR elicits specific cellular responses to pathogens using different intracellular adapter proteins (25). TLR-4 has been reported to be mostly involved in Gram-negative bacterial infections (10-12, 15). Similarly, in this study, a positive correlation was identified between Gram-negative sepsis and TLR4 SNPs in term newborns. Three Asp299Gln and five Thr399Ile SNPs in the TLR4 gene were present in these patients.

The relationship between TLR polymorphism and sepsis has previously been examined in adult studies (9, 16, 17). TLR4 gene (Asp299Gly) polymorphism has been evaluated in adult patients with brucella, and the G allele frequency of TLR4 has been reported to be significantly higher in adult patients with brucella than in control subjects (17). Biebl et al. (16) determined no association between TLR4 Asp299Gly SNP and invasive meningococcal disease in adult patients. In contrast, Van Well et al. (21) examined TLR SNPs in a wide range of pediatric patients with meningococcal meningitis (MM). In that study, the carriage state of mutant TLR4 +896 GG predisposed to susceptibility to MM. The combined carriage state of TLR2 +2477 and TLR4 +896 mutants was also strongly associated with MM. A carrier trait of TLR4 +896 mutants was gain strongly associated with susceptibility to development of MM. In their large cohort study, Kumpf et al. (9) reported that higher TLR4 polymorphism was assocated with a greater risk of severe sepsis and pneumonia after surgery. In one pediatric study, TLR4 polymorphism was associated with asymptomatic bacteriuria and an increased risk for renal scarring (26). TLR genes, especially changes in TLR2 and TLR4 genes, have also been potentially implicated in the development and various complications of toxoplasmosis during pregnancy (27). One previous study suggested that TLR4 Asp299Gly polymorphism resulted in an increased risk of repeated *M. catarrhalis* colonization (28). Agbeko et al. (23) reported that a low platelet count was more prevalent in TLR4 variant allele carriers than wild type gene carriers, but no association was determined with the incidence of systemic inflammatory response syndrome (SIRS) in the pediatric intensive care unit. Similarly, TLR4 299/399 genotype polymorphism had no prominent effect on disease severity in Dengue virus-infected children (24). In addition, no association has been reported between TLR2 and TLR4 SNPs and susceptibility and severity of *Plasmodium falciparum* infection in children (28).

Lorenz et al. (19) reported an association between Asp299Gly and Thr399Ile polymorphism in TLR4 and premature birth in a study of premature infants aged less than 35 weeks and their mothers in a Finnish population. The frequencies of 299Gly allele and Asp/Gly or Gly/Gly genotype carrier status in premature singleton infants were higher than those in term singleton infants or in premature multiples. On the basis of their findings, they reported that an allelic variation in the TLR4 receptor was associated with increased risk of premature birth (18). Szebeni et al. (22) observed no relationship between very low birth weight premature infants with necrotizing enterocolitis and healthy infants in terms of TLR4 SNP carriage. One study of 40

infants showed that the presence of a TLR4 single nucleotide polymorphism is associated with increased risk of Gram-negative bacterial infections. Interestingly, the presence of a TLR5 variant was associated with an increased circulating white blood cell count (29). In one recent study, 598 patients treated in the pediatric intensive care unit for various reasons were analyzed for SNPs. The results demonstrated that SNP variants are associated with sepsis outcomes (30). A prospective cohort study involving 28 healthy newborns and 128 newborns with prenatal chorioamnionitis at term showed that the variant T allele for the common SNP rs352140 in the gene encoding TLR9, which recognizes bacterial DNA, was associated with increased placental inflammation (31).

TLR2 is predominantly responsible for recognizing Gram-positive cell wall structures (11, 12). Studies have hypothesized that mutations in TLR2 may be associated with a diminished response to Gram-positive lipoproteins and may place individuals at higher risk of Gram-positive infections. Lorenz et al. (19) demonstrated a link between polymorphism and severe staphylococcal infections (11, 12, 19). Also it was reported that children with Arg753Gln TLR2 polymorphism may predispose to fatal staphylococcal infections (32). In addition, significant correlation has been found between TLR2 polymorphisms and congenital cytomegalic virus infection in children (33). Abu-Maziad et al. (5) observed no association between the SNPs of TLR2 and sepsis in premature infants with sepsis aged less than 37 weeks. In our study, pro631His SNPs in TLR2 was positive in only 1 (2.5%) patient. Coagulase negative staphylococcus grew in blood culture from this patient.

Although this is one of the main studies to focus on the genetic association of neonatal sepsis in term babies, the small sample size involved is a major limitation that should be taken into consideration when interpreting the results. A multicenter study or a study with a larger population would produce more certain and generalizable results. Because sepsis consists of a multifactorial cascade, the findings of this study are important in terms of the genetic association in sepsis. This study will go some way to answering the question of why clinical response in some babies with sepsis is different to that of others. More detailed gene / SNP studies in larger groups with more patients are now needed. Identification of other relevant genetic risk factors possibly affecting the immune system can help pave the way to a better understanding of the pathophysiology and suggest new avenues for treatment and prevention of neonatal sepsis. Other possible contributing genetic or environmental factors, such as choice of antibiotic, resistance patterns, or immunological investigations should be studied together with TLR polymorphism.

Conclusion

This study is an analysis of important genes potentially involved in regulatory pathways in the immune response important for sepsis in the neonatal population. Our data demonstrated an association between TLR2 (Pro631His; C>A) and TLR4 (Asp299Gln; A>G, Thr399Ile; C>T) polymorphisms and sepsis. This study sheds new light on the mechanism involved in sepsis and host susceptibility to sepsis in term newborns.

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