Tohoku J. Exp. Med., 2019, **247**, 259-264

Association between Tumor Necrosis Factor-*α* Promoter -308 G/A Polymorphism and Early Onset Sepsis in Preterm Infants

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Early-onset neonatal sepsis (EOS) is diagnosed during the first 7 days of neonatal life and is the major cause of morbidity and mortality among preterm infants. Genetic predisposition may have an impact on EOS susceptibility and outcome. The aim of our study was to explore the association between *TNF-α -308 G/A* or *IL-6 -174 G/C* gene polymorphism and the susceptibility and outcome of EOS in preterm infants. The study included 471 preterm infants: 282 with EOS (151 with culture proven sepsis and 131 with clinical sepsis) and 189 without infection (control group). *TNF-α -308 G/A* and *IL-6 -174 G/C* were genotyped using Real-time RCR method. We observed significantly higher frequency of A allele of *TNF-α -308 G/A* polymorphism in blood culture proven EOS ($p = 0.017$) or clinical EOS ($p = 0.025$) compared with the control group. Logistic regression confirmed significant association between *TNF-α -308 GA+AA* genotypes and development of culture proven EOS ($B = –0.718$, $p = 0.013$) or clinical EOS ($B = –0.602$, $p = 0.027$). No significant differences in *IL6 -174G/C* alleles or genotypes distribution have been observed between culture proven EOS group, clinical EOS group and the control group. An association between *TNF-α -308 G/A* or *IL-6 -174 G/C* genotypes and EOS lethal outcome was not observed ($p = 0.652$ and $p = 0.384$, respectively). According to our analysis of large cohort of preterm infants with clearly defined EOS groups, the *TNF-α -308 A* allele may be a risk factor for the EOS occurrence.

Keywords: early onset sepsis; gene polymorphism; interleukin 6; preterm infants; tumor necrosis factor alpha
Tohoku J. Exp. Med., 2019 April, **247** (4), 259-264.

Introduction

Neonatal sepsis is caused by invasion of microbial pathogens on the normally sterile tissue of infants and the consequent neonatal inflammatory response (Lever and Mackenzie 2007; Shane et al. 2017). The incidence of sepsis in neonates with appropriate birth weight is 0.1% but it increases to up to 50% as a birth weight and gestational age decrease (Rønnestad et al. 2005a, b; Shane and Stoll 2014). Despite a significant improvement in quality of intensive neonatal care, neonatal sepsis is the major cause of morbidity and mortality among preterm infants.

Early onset sepsis (EOS) is diagnosed during the first 7 days of neonatal life and is caused by maternal-fetal pathogens transmission through placenta or through ascending vaginal route (Mukhopadhyay and Puopolo 2012). Besides maternal factors, virulence of infecting organism and neonatal host predisposition are also recognized as risk factors for neonatal sepsis (Mukhopadhyay and Puopolo 2012).

Numerous studies have provided evidence that a genetic base may have an impact on host immunity and susceptibility and outcome of sepsis (Bellamy and Hill 1998; Cooke and Hill 2001; Waterer and Wunderink 2003; Sutherland and Walley 2009). Preterm infants have unbalanced immune response to pathogens because of the immaturity of immune system. Inflammatory response to bacterial sepsis of a neonate is mediated by cytokines through a cascade that starts with the activation of macrophages. Activated macrophages then release tumor necrosis factor alpha (TNF-*α*) and interleukin 6 (Weirich et al. 1998; Levy et al. 2006).

TNF-*α*, which is an initiator of the inflammatory response, increases in the early phase of neonatal sepsis (Meem et al. 2011). The most extensively studied polymorphism in the *TNF-α* gene is located 308 bp upstream of the transcriptional start site (rs1800629). Presence of *TNF-α -308A* allele is associated with enhanced *TNF-α* transcrip-

Received January 17, 2019; revised and accepted April 8, 2019. Published online April 19, 2019; doi: 10.1620/tjem.247.259.
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tion (Kroeger et al. 1997) and higher production of TNF-α (Louis et al. 1998) in adults, but there are opposing data in neonatal patients (Allam et al. 2015). Recent data provide inconsistent results about the role of *TNF -308 G/A* polymorphism in neonatal sepsis. Namely, there are results that support association *TNF-α -308 G* allele with EOS in neonates (Allam et al. 2015), but there are studies that deny association between this polymorphism and culture proven neonatal sepsis (Härtel et al. 2011; Srinivasan et al. 2017).

Interleukin 6 (IL-6) plays an important role in the acute phase response to microbial invasion (Borden and Chin 1994), in the systemic inflammation, in the pathogenesis of sepsis and is associated with increased severe sepsis risk and mortality (Uusitalo-Seppälä et al. 2011; Tschaikowsky et al. 2011; Miguel-Bayarri et al. 2012; Palmiere and Augsburger 2014). The polymorphism *-174 G/C* resides upstream of the transcriptional start site, affects transcriptional activity of the *IL-6* gene and is associated with elevated IL-6 level in stimulated neonatal cells, but not in adult ones (Kilpinen et al. 2001).

Analysis of the polymorphisms in genes for proinflammatory cytokines *TNF-α* and *IL-6* in neonatal sepsis may allow for the development of new diagnostic markers and improve diagnosis of sepsis, and reveal predictors of patient outcomes. This is extremely important in premature neonates who are at the highest risk of developing severe bacterial infections (Abu-Maziad et al. 2010). Inconclusive results for premature infants from previous studies may be due to differences in study design, ethnical origin and small cohort size. Our study included homogenous, large cohort with clearly defined EOS groups.

The aim of our study was to evaluate the relationships between susceptibility and outcome of early onset sepsis and *TNF-α -308 G/A* or *IL-6 -174 G/C* gene polymorphism in Serbian preterm infants.

Materials and Methods

Study population

Our study enrolled 471 preterm infants (gestational age < 37 weeks) who were admitted to the Institute of Neonatology in Belgrade, Serbia, from January 2012 to September 2018. The study protocol was approved by the Ethics Committee Institute of the Neonatology in Belgrade and the Ethics Committee of the Faculty of Medicine, University of Belgrade. In all cases, the infants' parents were informed and their written consent was obtained.

Study design

Neonates with sepsis are diagnosed during the first 7 days of neonatal life and classified as EOS. Premature neonates with EOS (n = 282) were divided into the group with blood culture proven sepsis (n = 151) and the group with symptoms of clinical sepsis but negative blood culture (n = 131). The control group consisted of 189 preterm neonates with negative blood culture and with no symptoms of clinical sepsis during hospitalization.

Clinical sepsis was defined as the presence of at least three out of six clinical and three out of nine laboratory criteria, according to the protocol of the Institute of Neonatology (http://www.neonatologija.rs/files/rad16-KON-2.pdf) based on modified Töllner and Rodwell criteria (Töllner 1982; Rodwell et al. 1988). The clinical symptoms of sepsis are 1) apnea, dyspnea, tachypnea, cyanosis; 2) tachycardia, hypotension, bradycardia; 3) lethargy, hypotonia, irritability, convulsions; 4) poor perfusion, arterial hypotension; 5) feeding intolerance or abdominal distension; and 6) hepatosplenomegaly, jaundice or skin and subcutaneous lesions.

The laboratory criteria for detecting sepsis according to the hematological scoring system (HSS) of Rodwell et al. (1988) are: 1) a total white blood cell (WBC) count ≤ 5,000/μl or ≥ 25,000/μl; 2) a total polymorphonuclear leukocytes (PMN) count < 1,800/μl or > 5,400/μl, or no mature PMN seen; 3) increased immature PMN count from normal value 600/μl; 4) an immature to total neutrophil ratio (I/T) of ≥ 0.2; 5) an immature to mature neutrophil ratio (I/M) of ≥ 0.3; 6) presence of degenerative morphologic changes in PMN; 7) a platelet count ≤ 150,000/μl; 8) C-reactive protein (CRP) levels greater than 5 mg/L; and 9) acidosis as characterized by a base excess (BE) of 0.210 mmol/L.

For all neonates, the data about gender, gestational age, weight on birth (BMW), Apgar score determined in the 5th minute (AS5'), type of delivery and multiple pregnancies were collected.

Possible occurrence of intracranial hemorrhage (ICH) in patients was analyzed by ultrasound of the central nervous system (Papile et al. 1978). Respiratory distress syndrome (RDS) was confirmed by assessment of Chest X-rays (Bomsel 1970).

Sampling and analyses

Genomic DNA from buccal swabs taken from patients was extracted using the conventional phenol: chloroform-based method. All molecular genetic analyses were conducted at the Institute of Human Genetics, Faculty of Medicine University of Belgrade.

In our study, we analyzed the single nucleotide polymorphisms of *TNF-α* (*-308 G/A*, rs1800629) and *IL-6* (*-174 G/C*, rs1800795) genes. Genotyping was performed with custom TaqMan® SNP Genotyping Assays and TaqMan® Genotyping Master Mix (Applied Biosystems, Foster City, CA) in 10 μL volumes. Genotype analyses were carried out in Applied Biosystems' 7500 Real-Time PCR System (Applied Biosystems).

Statistical analysis

Mean values between groups of neonatal patients were compared by ANOVA or Kruskal-Wallis test, depending on variable distribution. Differences in genotype and allele frequencies between the group with blood culture proven sepsis, the one with clinical sepsis and the control group were analyzed by Chi-square test and Fisher exact test. Association between analyzed polymorphisms and sepsis occurrence or outcome in preterm infants with sepsis was explored by logistic regression analysis after the adjustment for BMW, gestational age, Apgar score and culture proven sepsis. Statistical analyses were performed using SPSS statistical package, version 16.0 (SPSS Inc, Chicago, IL, USA).

Results

We analyzed 471 premature infants. EOS groups included 151 (32.1%) preterm infants with culture proven sepsis and 131 (27.8%) with clinical sepsis. The control group included 189 (40.1%) preterm infants who had no signs of sepsis during hospitalization and did not require any antimicrobial treatment. The clinical data of the ana-

lyzed infants are shown in Table 1.

Gestational age, weight mass at birth and Apgar score were statistically significantly lower in the culture proven EOS group and the clinical EOS group than in the control group. In the culture proven EOS group and the clinical EOS group frequencies of intracranial hemorrhage and death were statistically significantly higher, while caesarean section delivery was significantly lower than in the control group (Table 1).

In preterm infants with positive blood culture, 106 (70.2%) cases of sepsis were caused by Gram-negative bacteria and 45 (29.8%) by Gram-positive bacteria.

Both analyzed polymorphisms in premature infants were in Hardy-Weinberg equilibrium (Table 2). We observed statistically significant differences in *TNF-α -308G/A* polymorphism alleles frequencies between blood culture proven EOS (p = 0.017) or clinical EOS (p = 0.025) and the control group of premature infants (Table 2). Also, a statistically significant difference was observed in *TNF-α -308G/A* genotypes distribution between the culture proven EOS group and the control group by dominant model (GG vs. GA/AA) (p = 0.011; Risk ratio 1.40, 95% CI 1.047-1.869; Odds ratio 1.98, 95% CI 1.163-3.356). A similar trend was observed between the clinical EOS group and the control group by dominant model (p = 0.057; Risk ratio 1.27, 95% CI 0.968-1.676; Odds ratio 1.71, 95% CI 0.981-2.992). The frequency of A allele *TNF-α -308G/A* polymorphism is significantly higher in the culture proven EOS group and the clinical EOS group than in the control group (Table 2). After the adjustment for BMW, gestational age, AS5' and type of delivery, logistic regression confirmed statistically significant association between *TNF-α -308G/A* genotypes and development of culture proven EOS (B = –0.718, p = 0.013) or clinical EOS (B = –0.602, p = 0.027) in premature infants.

Genotype and allele frequencies of *IL-6* gene *-174G/C* polymorphism among the culture proven EOS group, the clinical EOS group and the control group did not show significant differences (Table 2). Also, genotype frequencies among the culture proven EOS group, the clinical EOS group and the control group by recessive (GG/GC vs. CC) model did not show significant differences.

TNF-α -308G/A genotypes distribution in infants who did/did not die because of sepsis were: AA 1.2%, GA 23.5%, GG 75.3% and AA 2.5%, GA 25.2%, GG 72.3% respectively (p = 0.652). *IL-6 -174G/C* genotypes distribution in infants who did/did not die because of sepsis were: CC 10.8%, GC 42.8%, GG 46.4% and CC 14.3%, GC 47.1%, GG 38.7% respectively (p = 0.384). In the EOS group *TNF-α -308G/A* and *IL-6 -174G/C* genotypes were not predictors of lethal EOS outcome.

Discussion

Pathophysiology of sepsis is a complex process which includes interaction of microorganisms with the innate and adaptive host immune system. Fragile immature skin and immature mucosal barriers as well as delayed maturation of the specific humoral and cellular immune response and complete physiological immaturity are some of the key reasons why sepsis is the main cause of morbidity and mortality of preterm infants (Kamalakannan 2018). The most relevant risk factors associated with EOS are low gestational age and low birth weight and Apgar Score ≤ 6 at 5 min (Simonsen et al. 2014). Our study confirmed these risk factors for development of EOS. As expected, vaginal delivery is significantly more frequent in our septic group infants because of the exposure of infants to microorganisms in birth canal (Plano 2010). Gram-negative bacteria are the

Table 1. Comparison of clinical data of preterm neonates with and without sepsis.

Clinical characteristics	Culture proven EOS	Clinical EOS	Control group	p values
GA weeks*	27.8 ± 3.2	28.5 ± 3.4	30.2 ± 3.4	0.000
BWM grams*	1,018.8 ± 410.4	1,161.8 ± 510.3	1,363.7 ± 460.0	0.000
Male	85 (56.3)	74 (56.5)	97 (51.3)	> 0.5
AS 5'*	3.7 ± 2.3	3.9 ± 2.3	5.4 ± 2.4	0.000
C-section delivery**	66 (43.7)	57 (43.5)	113 (59.8)	0.003
Multiple births**	39 (25.8)	36 (27.5)	64 (33.9)	> 0.5
RDS**	145 (96.0)	122 (93.1)	176 (93.1)	> 0.5
ICH**	30 (19.9)	30 (22.9)	16 (8.5)	0.001
Death**	98 (64.9)	67 (51.5)	63 (33.7)	0.000

*mean value ± SD; **No (%).

GA, gestational age; BWM, weight on birth; AS 5', apgar score at 5th minute; RDS, respiratory distress syndrome; ICH, intracranial hemorrhage.

Table 2. Analysis of genotypes and alleles distribution of *TNF-α -308G/A* and *IL-6 -174* polymorphisms in the study group.

Polymorphism	Genotype	Culture proven EOS n (%)	Clinical EOS n (%)	Control group n (%)	*p value
TNF-α -308G/A	GG	110 (72.8)	99 (75.6)	159 (84.1)	0.010
	GA	40 (26.5)	28 (21.4)	30 (15.9)	
	AA	1 (0.8)	4(3.0)	0 (0)	
TNF-α -308G/A	GG	110 (72.8)	99 (75.6)	159 (84.1)	0.031
	GA+AA	41 (27.2)	32 (24.4)	30 (15.9)	
Allele %	G	86.1	86.3	92.1	0.035
	A	13.9	13.7	7.9	
IL-6 -174G/C	GG	65 (43.0)	57 (43.5)	84 (44.4)	0.878
	GC	66 (43.7)	61 (46.6)	80 (42.3)	
	CC	20 (13.2)	13 (9.9)	25 (13.2)	
IL-6 -174G/C	GG+GC	131 (86.8)	118 (90.1)	164 (86.8)	0.619
	CC	20 (13.2)	13 (9.9)	25 (13.2)	
Allele %	G	64.9	66.8	65.6	0.887
	C	35.1	33.2	34.4	

*Chi-square test or Fisher exact test.

most commonly isolated pathogens in positive blood cultures of our septic infants, and those findings are in concordance with previous studies (Plano 2010; Cortese et al. 2016).

TNF-*α* and IL-6 are primary proinflammatory cytokines that mediate the initial response of the innate immune system to infection and have an important role in the pathogenesis of sepsis. Previous studies have shown that septic neonates had elevated levels of circulating TNF-*α* and IL-6 cytokines in plasma when compared with healthy infants (Kurt et al. 2007; Allam et al. 2015). These cytokines were classified as biomarkers of early phase of EOS (Meem et al. 2011).

TNF-*α* plays a prominent role in the acute inflammatory response through induction of release of numerous inflammatory mediators and through activation of immune and structural cells (El-Tahan et al. 2016). Genetic variations in *TNF-α* gene may affect circulatory levels of TNF-*α* (Sutherland and Walley 2009; Wynn and Wong 2010). Allele A in *TNF-α -308 G/A* polymorphism is associated with higher expression of *TNF-α* and increased susceptibility to sepsis and septic shock in adults (Mira et al. 1999). Our study showed association between *TNF-α -308 A* allele and the risk of culture proven and clinical EOS in preterm infants. Moreover, all analyzed preterm infants with *TNF-α AA* genotype developed EOS. Previous studies have shown that the AA genotype has affected TNF-*α* production, secretion, or function (El-Tahan et al. 2016). Abnormal TNF-*α* is implicated in the pathogenesis of sepsis (Zhang et al. 2017), and *TNF-α* gene expression is closely related to neonatal sepsis in very low birth weight infants (Cernada et al. 2014). In our study a significant association was found between *TNF-α -308 G/A* polymorphism and EOS (culture proven and clinical) under the dominant model (for GG vs. GA/AA). A strong association between the *TNF-α -308 G/A* polymorphism and EOS in premature infants is confirmed by logistic regression (covariates BMW, GA, AS5' and type of delivery).

A recently published meta-analysis with pooled data from 34 publications that included 12,284 subjects (only 4 studies included neonates) suggested that *TNF-α -308 G/A* polymorphism may contribute to risk of sepsis and septic shock under the dominant model (Wang et al. 2017). The results are consistent in part with the present study. Moreover, to the best of our knowledge, we are able to show for the first time that *TNF-α -308 A* allele is a risk factor for EOS in premature infants. Härtel and coworkers (2011) in two large cohorts of very low birth weight infants did not find an association between *TNF-α -308 G/A* polymorphism and neonatal sepsis. Similar findings were also

obtained by Srinivasan and coworkers (2017), but with the emphasis on the fact that GG genotype shows a trend towards an increased sepsis risk in infants with culture proven sepsis. A study from Saudi Arabia on newborn infants who were 40 ± 2 weeks of gestation has shown the association between GG genotype and G allele of *TNF-α -308 G/A* polymorphism with EOS (Allam et al. 2015). Inconclusive results for premature infants may be due to the differences in study design, ethnical origin and small cohort size, which could all be limiting factors. Hence, we have focused on a homogenous, large premature infant cohort with clearly defined EOS groups.

One of the first studies of the relationship between *TNF-α -308 G/A* polymorphism and sepsis in infants found strong association of GA/AA genotypes and mortality (Hedberg et al. 2004). Our study did not confirm the connection of *TNF-α -308 G/A* genotypes with EOS mortality.

IL-6 mediates early response to infection, it is a major inducer of the hepatic protein synthesis and it precedes the increasing C-reactive protein concentration. Because of this it is a good marker for EOS with a high degree of sensitivity and specificity (Machado et al. 2014). We did not find any association between genotype or allele distribution of *IL-6 -174 G/C* polymorphisms and culture proven and clinical EOS or EOS outcome. Numerous studies on pediatric and neonatal patients have found association of *IL-6 -174 G/C* polymorphism with sepsis, septic shock or sepsis outcome (Harding et al. 2003; Ahrens et al. 2004; Baier et al. 2006; Wynn et al. 2010; Jabandziev et al. 2014). However, in two large meta-analyses, the authors failed to detect any association of *IL-6 -174 G/C* polymorphism and neonatal sepsis **(**Chauhan and McGuire 2008; Machado et al. 2014), the results of which are in agreement with our study.

To our knowledge, this is the first study that has found the association of *TNF-α -308G/A* polymorphism and blood culture proven EOS or clinical EOS. Additionally, this is one of the largest cohorts of preterm neonatal patients analyzed for the association between *TNF-α -308 G/A* and *IL-6 -174 G/C* polymorphisms and EOS. Our results suggest that *TNF-α -308 GA and AA* genotypes are risk factors for EOS occurrence in Serbian population. *TNF-α -308 G/A* and *IL-6 -174 G/C* polymorphisms are not genetic risk factors for lethal EOS outcome.

Acknowledgments

This work was supported by the Serbian Ministry of Education, Science and Technological development (Grant 175091).

Conflict of Interest

The authors declare no conflict of interest.

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