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Original Article

Toll-like receptors 2 and 9 gene polymorphisms in severe sepsis and septic shock: a single center study in the pediatric intensive care unit

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Abstract: Background: Toll-like receptors (TLRs) are associated with innate and adaptive immunity. In study, the aim was to investigate the possible relation between genetic polymorphisms of the TLR genes 2 and 9 in pediatric patients with severe sepsis and septic shock. Patients and methods: Fifty-nine pediatric patients were admitted with severe sepsis/septic shock in the pediatric intensive care unit, and 59 healthy controls were investigated for the frequency of TLR2-1350 T/C (rs3804100) and TLR9-1237 T/C (rs5743836) gene polymorphisms. Results: The frequency of TLR2 1350T/C polymorphic genotype in patients was 13.6% for the TC genotypes and 1.7% for the CC genotypes. There was no significant difference between cases and controls in TLR2 1350T/C (p 0.308). The frequency of TLR9 1237T/C polymorphic genotypes was 57.6% for the genotype TC and 1.7% for the genotype CC. The frequency of TLR9 1237T/C genotype was significantly higher in patients than in controls (p 0.006), with two-fold increased risk of severe sepsis/septic shock (relative risk = 1.8, 95% confidence interval = 1.2-2.6) and male sex predisposition (p 0.014). Conclusion: TLR9-1237T/C polymorphism is a risk factor for progression of infection to severe sepsis in pediatric patients.

Keywords: TLR2, TLR9, gene polymorphism, septic shock, sepsis, critical illness

Introduction

Sepsis is the most common cause of pediatric deaths worldwide [1]. TLRs play a critical role in innate and adaptive immunity. At least 10 TLRs have been identified in humans: TLR1, TLR2, TLR4, TLR5, and TLR6 work on the plasma membrane, while TLR3, TLR7, TLR8, and TLR9 are located intracellular. In sepsis, bacterial-induced toll-like receptors (TLR) signaling may be elaborated in cytokine production. Bacterial endotoxins bind to TLR, initiating a cascade of inflammatory cytokines, hence massive cytokine production [2-5]. TLR2 has a major effect in defense against gram-positive bacteria. A tendency to *Staphylococcus aureus* sepsis by TLR-2 Arg753Gln polymorphism has been reported in animal studies [6-8].

In addition to being crucial for immunity against viral, fungal, mycobacterial and *Helicobacter pylori* infections, TLR9 plays a role in autoimmune diseases. In some animal studies, blockage of TLR9 increased the survival of septic mice by decreasing the inflammatory response [9, 10]. There is a relation between sepsis and decreased serum TLR-9 levels polymorphisms due to sepsis-associated immunosuppression [11]. (Atalan N et al, 2016). This study explored the possible association between TLR2 (13-50T/C-rs3804100) and TLR9 (1237T/C-rs57-43836) pleomorphism and severe sepsis in pediatrics admitted into Cairo University Intensive Care Unit.

Material and methods

Patients

This cross-sectional study was conducted over a 1-year period in the Pediatric Intensive Care Unit (PICU) of Cairo University Pediatric Hospital,

Egypt. All pediatric patients with severe sepsis admitted to PICU were included in this study after informed consent was obtained from the parent/guardian. The study was conducted according to the Declaration of Helsinki. Sepsis was defined and classified as per the 2005 International Consensus Conference [12]. Critical patients who died within 24 hours of admission were excluded. Patients with chronic underlying diseases were also excluded.

Methods

All patients underwent full history taking and clinical examination. The reason for PICU admission was recorded. The pediatric risk of mortality (PRISM III) score, the Pediatric Logistic Organ Dysfunction (PELOD) score and inotrope score were calculated for all patients. The results of blood cultures, length of hospital stay and outcome (survival and death) were reported.

Genotyping

All patients and controls underwent genotyping for TLR2 and 9 polymorphisms. Whole blood samples were collected in sterile vacutainers containing K3EDTA to prevent blood clotting (BD, Becton, Dickinson Company, USA) in the patients with severe sepsis or septic shock. For quality control, genotyping was repeated with respect to case/control status from 59 normal healthy individuals coming to the outpatient clinic. Genotyping of TLR2 (1350T/C, rs3804-100) and TLR9 (1237T/C, rs5743836) was done. Genomic DNA extraction from peripheral blood leucocytes was performed using Gene JET. Whole Blood Genomic DNA purification Mini kit (Fermentas Life Sciences, Canada). TLR2-1350 T/C and TLR9-1237 T/C genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. All PCR was performed in a total volume of 25 µl containing 150 ng genomic DNA, 2X Dream TaqGreen PCR Master Mix, 25 pM of each forward reverse primers (Fermentas, Lithuania). The PCR products were visualized by 3% agarose gel electrophoresis under UV light.

Genotyping of TLR2-1350 T/C (rs3804100) was performed as per Takahashi et al. 2011 [13]. The primer set used was forward: 5'TCATTTGGCATCATTGGAAA-3' reverse: 5'GAGTTGCGGCAAATTCAAAG-3'. The thermocycler program conducted was an initial denaturation at 0°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and a final extension step at 72°C for 10 minutes. The generated amplicon was a 251 bp fragment, which was digested by MwoI enzyme (Fermentas-Lithuania). The wild type allele (T allele) produced a single b of 251 bp, while the polymorphic allele (C allele) produced 2 bs of 167 bp 84 bp. For TLR-9 1237T/C (rs5743836) genotyping, the primer set used was forward: ATGGGAGCAGAGACATAATGGA-3' reverse: 5'-CTGCTTGCAGTTGACTGTGT-3. The thermocycler program conducted was an initial denaturation at 95°C for 5 min, followed by 36 cycles of denaturation at 94°C for 40 seconds, annealing at 62°C for 40 seconds, extension at 72°C for 1 minute. A final extension step was performed at 72°C for 10 minutes as per Liu et al. 2012 [14]. This generated a 135 bp fragment. The amplified material was digested by BstNI enzyme (Fermentas-Lithuania). The wild type allele (T allele) showed two bs of 108 bp 27 bp, while the polymorphic allele (C allele) showed 3 bs of 60, 48 27 bp. Results of genotyping were interpreted blindly by 2 different observers.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 21.0 for Windows (SPSS Inc., Armonk, NY, USA). The values of variables with normal distribution are presented as mean and standard deviation, while values of variables without normal distribution are expressed as median (interquartile range [IQR]). According to the type of distribution, parametric or non-parametric for the comparisons between groups were used for Student's t test or the Mann-Whitney test, respectively. A comparison of multiple variables between groups was performed using one-way ANOVA algorithm with the post hoc Tukey test. The association between categorical variables was analyzed using the test χ^2 or Fischer's exact test. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Fifty-nine patients with severe sepsis were included in this study in addition to fifty-nine

Table 1. Characteristics of the study population

Variable	Frequency
Age in months	
Median (IQR)	8 (5)
Min-Max	1-144
PRISM III score	
Median (IQR)	20 (17)
Min-Max	4-47
PELOD score	
Median (IQR)	12 (11)
Min-Max	1-43
Etiology	
Pneumonia; N (%)	32 (54)
Gastroenteritis; N (%)	20 (34)
Meningitis; N (%)	8 (5)
Abdominal Surgery; N (%)	2 (3.4)
Duration of stay in days	
Median (IQR)	13 (19)
Min-Max	3-90
Isolated Organisms; N (%)	
Pseudomonasaeruginosa	7 (12)
Staphylococcus aureus	6 (10)
Klebsiella species	5 (8.5)

controls. Thirty-one patients (52.5%) were males. The ages of the patients ranged from 1 to 144 months, median (IQR) was 8 (5) months. Thirty-two patients (54%) had pneumonia, 20 (33.9%) had gastroenteritis, and 5 (8%) had meningitis as a diagnosis on admission; while 2 patients (3.4%) were admitted to PICU after they underwent abdominal surgery for intestinal perforation. The PRISM III score ranged from 4 to 47, median (IQR) was 20 (17). The PELOD score ranged from 1 to 43, median (IQR) was 12 (11). The duration of stay of the patients ranged from 3 to 90 days, median (IQR) was 13 (19) days. Thirty-three patients (55.9%) died and 26 (44.1%) were discharged. Seven patients (12%) had *Pseudomonas aeruginosa*, 6 (10%) had *Klebsiella* species, and 5 (8.5%) had *Staphylococcus aureus* infections (**Table 1**). All 59 critical pediatric patients with severe sepsis were assessed for the presence of TLR2 (1350T/C, rs3804100) and TLR9 (1237T/C, rs5743836) polymorphisms. TLR2 polymorphic variants (T/C and CC) were detected in 9 (15.3%) out of 59 patients; 8 patients (13.6%) were heterozygous (T/C), while 1 (1.7%) was homozygous (CC). There was no significant difference between cases and controls in the distribution of TLR2 1350T/C genotypes; *p* value was 0.308. TLR9 polymorphic variants (T/C and CC) were detected in 34 (57.9%) and 1 (1.75%) out of 59 patients, respectively. The frequency of TLR9 1237T/C genotype was significantly higher in patients than in controls; *p* value was 0.006 and conferred almost two-fold increase of deterioration of sepsis to critical illness (relative risk = 1.8, 95% confidence interval = 1.2-2.6) (**Table 2**). None of the patients had both polymorphisms of TLR2 and 9. Twenty-three males (74.2%) vs. 12 females (42.85%) carried the polymorphic allele TLR9-1237 TC or CC. There was a significant difference between males and females in TLR9 1237TC and CC where the *p* value was 0.014. Two males (6.5%) and 7 females (25%) carried the polymorphic allele TLR2 1350TC or CC. There was no significant difference between males and females in TLR2 1350TC/CC where the *p* value was 0.48 (**Table 3**). The wild type TT of TLR2 was associated with longer median duration of stay: 16 days in TT genotype vs. 10 days in the heterogeneous group TC/CC, with *p* value approaching statistical significance (0.063). There was no association between need for mechanical ventilation, PRISM III, PELOD or inotrope score and TLR 2 or 9 gene polymorphism. Out of the 33 patients who died, 7 (21%) were carriers of the wild type TLR 2-1350 TC/CC, and 19 (58%) were carriers of the TLR 9-1237 TC/CC. There

Table 2. Comparison between patients with severe sepsis and healthy controls regarding genetic polymorphisms in TLR2 and 9

	TLR2		TLR9	
	Cases (N=59)	Controls (N=59)	Cases (N=59)	Controls (n=59)
TT; N (%)	50 (85)	46 (78)	24 (41)	41 (69.4)
TC; N (%)	8 (13.6)	13 (22)	34 (58)	18 (30.5)
CC; N (%)	1 (1.7)	0	1 (1.7)	0
P value	0.308		0.006	

Table 3. Sex distribution of TLR 2 and 9 genetic polymorphisms in patients with severe sepsis

	TLR2		TLR9	
	TT	TC & CC	TT	TC & CC
Male; N (%)	29 (93)	2 (6.5)	8 (25.8)	23 (74)
Female; N (%)	21 (75)	7 (25)	16 (57)	12 (43)
P value	0.48		0.014	

Table 4. Comparison of TT genotype versus CC & TC in TLR2 & TLR 9 polymorphism

	TLR2			TLR9		
	TT (N=50)	TC & CC (N=9)	*P* value	TT (N=23)	TC & CC (N=36)	*P* value
Etiology:						
Pneumonia; N (%)	26 (52)	6 (66)	0.676	11 (47)	21 (58)	0.20
Gastroenteritis; N (%)	19 (38)	1 (11)		9 (39)	11 (30)	
Meningitis; N (%)	3 (6)	2 (22)		3 (13)	2 (5)	
Abdominal surgery; N (%)	2 (4)	0		1 (4)	1 (2)	
Mechanical ventilation; N (%)	44 (88)	9 (100)		21 (91)	32 (89)	
PRISM III						
Median	20	22	0.619	20	22	0.75
Min-Max	(6-42)	(4-47)		(4-40)	(6-47)	
PELOD						
Median	12	13	0.165	12	13	0.194
Min-Max	(1-41)	(1-43)		(1-41)	(10-43)	
Inotrope score						
Median	25	20	0.748	20	27	0.560
Min-Max	(10-240)	(10-70)		(10-70)	(10-240)	
Duration of stay in days						
Median	16	10	0.063	10	17	0.93
Min-Max	(3-47)	(3-90)		(93-47)	(3-90)	
Death; N (%)	26 (79)	7 (21)	0.152	14 (42)	19 (58)	0.758

was no statistically significant association between survival and either TLR 2-1350 TC/CC or TLR 9-1237 TC/CC (P 0.152 and 0.75, respectively) (**Table 4**).

Discussion

Sepsis (the systemic inflammatory response syndrome) remains the primary cause of death in intensive care units despite the advances in antibiotics [15]. The mortality rate of sepsis in children is higher than 50% [16]. Genetic differences between individuals determine the susceptibility to infections, the clinical manifestations and the mortality risk [17].

Our results show that the TLR9-1237T/C, rs5743836 is a risk for severe sepsis in pediatric critical care patients. Males had a more pronounced allele frequency TLR9-1237T/C than females. Apparently, females were protected from severe sepsis and septic shock by their wild allele. Zinkernagel et al. 2012 reported that TLR9 plays an important role in host defense against group A *Streptococcus* (GAS) infections. In addition, they reported that stimulation of TLR9 improves macrophage killing of this leading human pathogen. In contrast, Hu et al. 2011 demonstrated a protective role of TLR9 inhibition against the dysregulated inflammatory response and tissue injury in sepsis in mice animal model [18, 10]. Trevelin et al. 2012 suggested that the poor outcome of sepsis and the spread of infection were due to reduced neutrophil migration which was associated with TLR9 activation in neutrophils and CXCR2 down-regulation in mice [19].

TLR9 polymorphism was described in many diseases. Vulnerability to allergic bronchopulmonary aspergillosis was associated with allele C on T-1237C (TLR9) [20]. The presence of the TLR9-1237 C variant allele generates a potential nuclear factor kappa B binding site which increases the transcriptional activity of TLR9 and raises cellular production of pro-inflammatory cytokines [21]. Whereas polymorphism of TLR9 (C-1237 T) TT genotype was associated with low parasitaemia in malaria as described in infected Ghanaian children [22]. Moreover, TLR9 polymorphisms provided protection against meningococcemia and decreased incidence of positive blood cultures in children carrying TLR9-1237 C allele and TLR9+2848 AA genotypes [23]. Chen et al. 2011 reported that TLR9 polymorphisms rs187084 and rs352162

might be a risk for the development of sepsis and multiple organ dysfunction in patients with major trauma [24]. The frequency of TLR9-1486C/C genotype was significantly higher in fatal Crimean-Congo hemorrhagic fever patients than in healthy controls [25]. Furthermore, TLR9 1237 TC was associated with malaria susceptibility and severity in Burundian children [26]. Additionally, conflicts exist about the role of TLR2 polymorphism in sepsis. The genetic variant in TLR-2 (rs3804100, T1350C) was suggested to protect the host from severe urinary tract infections [27]. Eleven meta-analyses related to pneumonia susceptibility and outcome suggested that TLR2 rs5743708 minor genotype appeared to be associated with CAP/Legionnaires' disease/pneumococcal disease. The IL-6 rs1800795-C allele was associated with severe sepsis/septic shock/severe systemic inflammatory response, while the IL-10 rs1800896-A allele protected against the deterioration to critical conditions [28].

In conclusion, TLR9 gene polymorphism-1237 T/C, rs5743836 was associated with progression into severe sepsis in our pediatric patients with male predilection.

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Disclosure of conflict of interest

None.

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