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Association between Interleukin-6 rs1800795 Polymorphism and Serum Interleukin-6 Levels and Full-Term Neonatal Sepsis

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Abstract

Objective Cytokines are involved in the pathogenesis of sepsis. Association between IL-6 rs1800795 G/C polymorphism and the risks of sepsis is controversial. The aim of this study was to investigate the association of IL-6 rs1800795 G/C gene polymorphism with full-term neonatal sepsis and to determine its effect on the serum IL6 levels in these infants by a prospective study.

Methods The study included 200 full-term neonates from January 2019 to December 2020: 100 with sepsis (sepsis group), 47 with culture proven sepsis, and 53 with clinical sepsis, and 100 without infection (control group). The concentrations of IL-6 in serum were determined using enzyme-linked immunosorbent assay (ELISA). The polymorphisms of IL-6 rs1800795 G/C were analyzed to compare the genotypic and allelic frequencies in the groups by using the first-generation sequencing (Sanger sequencing). The association was studied between IL-6 rs1800795 G/C polymorphisms and serum IL-6 levels, and neonatal sepsis. The relationships between IL-6 rs1800795-G/C polymorphisms and sepsis and serum IL-6 levels were separately analyzed by logistic regression and analysis of variance.

Results There were no significant differences in genotypic frequencies and allelic frequencies of IL-6 rs1800795(G/C) in the groups ($p > 0.05$). There were no relations between IL-6 rs1800795G/C polymorphisms and sepsis and serum IL-6 levels by statistical analysis ($p > 0.05$).

Conclusion IL-6rs1800795G/C may not be genetic risk factors for full-term neonates; There was no association between serum IL-6 levels and IL-6 rs1800795G/C polymorphisms.

Keywords
- interleukin-6
- polymorphism
- sepsis
- full-term neonates

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Introduction

Sepsis is the major cause of neonatal morbidity and mortality in developing countries, and requires early diagnosis and treatment. An immature immune system and exposure to infective agents from the environment and their mothers are the risk factors that make neonates susceptible to sepsis.[1] A change in immune function is considered the crucial factor in the onset of sepsis.[2] During an infection, the host's immune system produces a series of substances, such as cytokines, in response to the infection or injury. IL-6 is a pro-inflammatory cytokine involved in the inflammatory response during the early stage of sepsis. IL-6 is used as a biomarker of sepsis. Elevated serum IL-6 levels are an early indicator of severe disease and higher mortality.[3,4] The possible association between the rs1800795 G/C polymorphism in the IL-6 promoter region and both risk of, and mortality from, sepsis has been extensively studied in adults and children.[5–7] However, reports rarely involve neonates, and studies including serum IL-6 levels are especially uncommon. Therefore, we adopted a prospective approach to measuring serum IL-6 levels and performed a case–control study on IL-6 rs1800795G/C polymorphism of neonatal sepsis. We used first-generation sequencing technology (Sanger sequencing) [8] to detect IL-6 rs1800795 polymorphism, so as to explore the association between IL-6 rs1800795 gene polymorphism and serum IL-6 levels, and between IL-6 rs1800795 gene polymorphism and the susceptibility to neonatal sepsis.

Methods

We selected 100 full-term neonates (gestational age $\geq$37 weeks and $<$42 weeks) hospitalized in our department from January 2019 to December 2020. All of them were of the Han ethnicity and had clinical signs and symptoms of sepsis, including 47 cases with positive blood cultures, and 53 cases with negative blood cultures. In detail, the diagnostic criteria were[9]: newborns with clinical manifestation of sepsis (temperature instability, increased oxygen requirement, respiratory distress, cyanosis, poor perfusion, hypotension, hypotonia, lethargy, seizures, abdominal distension, and vomiting) and (1) culture-positive in blood, cerebrospinal fluid, or other normally sterile body fluid or (2) culture-negative but meeting any of the following conditions: (a) more than two non-specific laboratory tests suggestive of infection; (b) cerebrospinal fluid examination consistent with purulent meningitis; (c) pathogenic DNA detected in blood. Another 100 non-infected full-term neonates hospitalized during the same period were selected as the control group, diagnoses in this group included cases of swallowing syndrome, non-infectious diarrhea, and mild non-hemolytic jaundice. There were no infection-related risk factors before or during delivery, and there were no symptoms of clinical infection or abnormalities in indicators of infection in laboratory testing. Exclusion criteria were presence of concomitant severe congenital malformations or inherited metabolic disorders. The study was approved by the hospital ethics committee (2020-03-042-K01), and all parents of participating children signed an informed consent form.

Basic data collected for all neonates included gestational age, day age, gender, birth weight, and mode of delivery.

Routine blood tests, including C-reactive protein measurement, and a blood culture test were performed on all neonates in both groups upon admission to the hospital. Enzyme-linked immunosorbent assay (ELISA) kits (provided by Wuhan Elabscience Biotechnology Co., Ltd.) were used to measure the serum interleukin-6 (IL-6) levels of the neonates. Ethylenediaminetetraacetic acid anticoagulant tubes were used to collect 2 mL of venous blood from each child, which was stored in a refrigerator at −80°C. Magnetic bead DNA extraction kits (provided by Chongqing Mygenostics Gene Technology Co., Ltd.) were used to extract genomic DNA from whole blood, agarose gel electrophoresis was used to analyze the degree of DNA degradation and determine any RNA contamination, and a Nanodrop 2000 was used to measure the purity of the DNA. DNA samples with an OD260/OD280 ratio of 1.8 to 2.0 and a concentration greater than 50 ng/μL were used to build a library.

PrimerZ was used to design and synthesize primers for the 200 bp upstream and downstream sequences of IL-6 rs1800795. IL-6 rs1800795 upstream primer: 5′- AGACATGCCAAAGTGCTGAG-3′; downstream primer: 5′- CCTGGAGGGGAGATAGAGCT-3′.

The total volume of the PCR amplification system was 20 μL: 1 μL of DNA templates (50 ng/μL); 10 μL of Extender PCR-to-Gel Master Mix (2 $\times$); 2 μL of PCR Primer mix; diluted to 20 μL with ddH2O. Amplification conditions: pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 67°C for 30 seconds, extension at 72°C for 1 minute, 14 cycles in total; denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 1 minute, 30 cycles in total; re-extension at 72°C for 7 minutes, cooling at 4°C. The PCR purification was completed in a Beckman automated workstation. The product was purified by the magnetic bead method and the purified product was assayed and analyzed for amplification using agarose gel electrophoresis.

The purified PCR product was diluted by 1:3 to 1:6 to 8 ng/μL, and the total volume of the sequencing system was 10 μL: 1 μL of purified and diluted PCR product, 1 μL of primers (1 μM upstream or downstream), 8 μL of 10-fold BigDye (2.5x) dilution. Amplification conditions: pre-denaturation at 96°C for 1 minute, denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes, 25 cycles in total, cooling at 4°C. The PCR product was purified with a mixture of alcohol and sodium acetate at a ratio of 25:1. After the PCR product was purified, 10 μL of Hi-Di (highly deionized) formamide was added for sequencing.

The purified PCR product for sequencing was added to the BigDye reagent (contains four fluorescently labeled dideoxynucleotide triphosphates [ddNTPs], four dNTPs, DNA polymerase, magnesium ions, and pH buffer) to initiate a polymerization reaction. The mixture was filtered to remove ddNTPs and other impurities, leaving only DNA fragments of

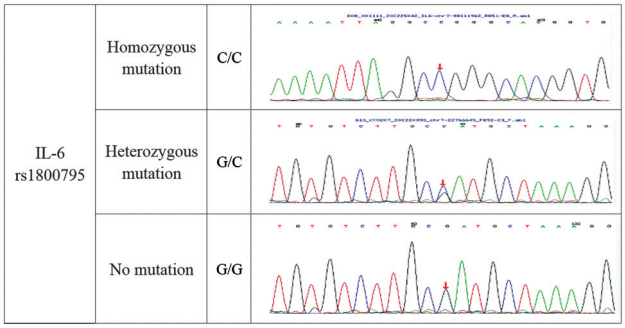

Fig. 1 The first-generation sequencing diagram of IL-6 rs1800795-G/C. IL-6, interleukin-6.

different lengths, and then sequencing was performed using an ABI 3130XL sequencer by capillary electrophoresis.

"Mutation Surveyor" software was used to analyze the reference sequence as well as the original data. The first-generation sequencing diagram of IL-6 rs1800795G/C was seen in ►**Fig 1**.

Statistical analysis was performed on the data using SPSS22.0 and Prism7.0 statistical software. The genotype distribution of IL-6 rs1800795 in both groups was tested using the principle of Hardy-Weinberg equilibrium. Measurement data conforming to the normal distribution are expressed as mean ± standard deviation ($x \pm s$) and subject to an independent-samples *t*-test. Enumeration data are expressed as a percentage (%), and the Chi-square test was used for comparison between groups. If 1≤ theoretical frequency <5, the continuity-corrected Chi-square test was used. ANOVA was used to analyze the association between IL-6 gene polymorphism (G/C) and serum IL-6 levels; logistic regression analysis was used to investigate the association between genotype and sepsis. p <0.05 indicates that the difference was statistically significant.

Results

There was no statistically significant difference in gestational age, post-gestational age, birth weight, gender, or mode of delivery between the two groups (p >0.05) (►**Table 1**).

The genotype distribution of IL-6 rs1800795 in the control sepsis groups conformed to the principle of Hardy-Weinberg equilibrium ($\chi^2 = 0.201, 0.312, p > 0.05$). There was no significant difference in the distribution of genotypic and allelic frequencies of IL-6 rs1800795 between the two groups (p> 0.05). On comparison of the serum IL-6 levels between the two groups, those of the sepsis group were significantly higher than those of the control group (p <0.05) (►**Table 2**).

Based on the Neonatal Critical Illness Score (NCIS)[10] neonates in the sepsis group were divided into critically ill and non-critically ill groups, and their serum IL-6 levels were compared. IL-6 levels of the critically ill group were significantly higher than those of the non-critically ill group(p

Table 1 Comparison of clinical data between the two groups

Group	Cases	Gestational ($\chi \pm s$, w)	Age ($\chi \pm s$, *d*)	Birth weight ($\chi \pm s$, g)	Gender (%cases)		Mode of delivery (%cases)	
					Male	Female	Vaginal delivery	Cesarean delivery
Control group	100	38.9 ± 0.1	11.2 ± 1.1	3,130 ± 390	49 (49)	51 (51)	76 (76)	24 (24)
Sepsis group	100	39.2 ± 0.3	12.4 ± 1.2	3,206 ± 448	52 (52)	48 (48)	68 (68)	32 (32)
t/χ^2		1.451	0.674	2.152	0.182		0.991	
p		0.150	0.502	0.051	0.855		0.321	

Table 2 Genotype and allele distribution of IL-6 rs1800795 and serum IL-6 levels in the two groups

Genotype	Sepsis group	Control group	χ^2/t	p
	n = 100 (%)	n = 100(%)		
GG	65 (65)	52 (52)	4.891	0.087
GC	23 (23)	33 (33)		
CC	12 (12)	15 (15)		
Allele				
G	153 (76.5)	137 (68.5)	2.127	0.093
C	47 (23.5)	63 (31.5)		
IL-6(pg/mL)	46.56 ± 8.45	8.78 ± 2.47	4.091	<0.0001

Table 3 Comparison of genotypes and serum IL-6 levels between critically ill and non-critically ill neonates in the sepsis group ($\chi \pm s$)

Genotype	Sepsis group		χ^2/t	p
	Critically ill	Non-critically ill		
	$n = 42$ (%)	$n = 58$ (%)		
GG	24 (57.2)	42 (72.4)	3.499	0.174
GC	13 (30.9)	9 (15.5)		
CC	5 (11.9)	7 (12.1)		
Allele				
G	61 (72.6)	93 (80.2)	1.253	0.210
C	23 (27.4)	23 (19.8)		
IL-6 (pg/mL)	91.58 ± 16.36	15.19 ± 2.42	5.508	<0.0001

Table 4 Association between IL-6 rs1800795G/c genotypes and serum IL-6 levels

	GG n (%)	GC n (%)	CC n (%)	F	p
Sepsis group	66 (66)	22 (22)	12 (12)		
IL-6 (pg/mL)	43.9 ± 8.1	60.9 ± 19.7	19.9 ± 7.1	2.451	0.095

Table 5 Univariate logistic regression analysis of neonatal sepsis

Variable	β	SE	Waldχ^2	OR	95% CI	p
rs1800795(GG)	0.296	0.212	1.947	1.7	0.887–2.039	0.163
rs1800795(CC)	0.239	0.215	1.268	1.4	0.748–1.345	0.260

<0.05), although the distributions of genotypic and allelic frequencies of IL-6 rs1800795 in the two groups were not significantly different ($p > 0.05$) (►**Table 3**).

There was no statistically significant association between the IL-6 rs1800795 G/C genotypes and serum IL-6 levels of neonates in the sepsis group ($p > 0.05$) (►**Table 4**). IL-6 rs1800795 genotypes GG and CC were not those susceptible to neonatal sepsis (►**Table 5**).

Discussion

Sepsis is characterized by the body's systemic inflammatory response to microbial invasion. Neonates are a special group with an immature immune system and susceptible to infectious diseases. Although antibiotic use and clinical supportive treatments have seen significant improvement, the mortality of neonatal sepsis is still very high, approaching 20%, especially for low birth weight infants.[11] Identifying and managing neonatal sepsis have become an important issue in the NICU. Therefore, it is necessary to find a predictive marker that can identify patients at high risk for developing sepsis and assist with early intervention in these patients to prevent the occurrence of sepsis.

Cytokines play a vital role in regulating the host's immune response, and changes in cytokine levels have been proven to be involved in the development of sepsis.[12] Studies have shown that genetic variation among cytokines, especially single nucleotide polymorphisms, may affect the risk of sepsis.[5,13] IL-6 gene is responsible for the regulation of the transcriptional activity during inflammation reaction. IL-6 is an important inflammatory cytokine produced by leukocytes, endothelial cells, and fibroblasts, playing an important role in the immune response and regulation of the inflammatory response.[14] High IL-6 levels have been proven to be associated with an increased risk of severe sepsis and increased mortality.[5,15] The IL-6 gene is located on chromosome 7p21, 5 kb in length, and consists of four introns and five exons. Several polymorphisms have been found in the IL-6 promoter region. rs1800795 G/C is located in the exon region, is responsible for the regulation of transcriptional activity during inflammation and regulates the expression of the IL-6 gene. This study found that the frequencies of genotypes GG, GC, and CC of IL-6 gene rs1800795 G/C polymorphism in the sepsis group were 65, 23, and 12%, respectively, and the allele frequencies were 76.5 and 23.5%. The frequencies of the genotypes in the control group were 52, 33, 15%, respectively, and the allele frequencies were 68.5 and 31.5%. The current study showed that there is no statistically significant difference between IL-6 rs1800795 polymorphisms, indicating that the IL-6 rs1800795 G/C

polymorphism had no significant association with the risk of sepsis in full-term neonates. Varljen et al[16] found no association between the genotypes or alleles of IL-6 rs1800795 G/C polymorphism and early-onset sepsis in premature infants, in agreement with the results of this study regarding full-term neonatal sepsis. The results of a meta-analysis by some researchers[17,18] showed that IL-6 rs1800795 G/C polymorphism was not associated with the risk or mortality of sepsis in any age or ethnic group. Allam et al[19] found that the IL-6 rs1800795 G allele was associated with early-onset neonatal sepsis in Saudi Arabia. Mao et al[6,7] believed that the IL-6 rs1800795 C allele was a risk factor for pneumonia-induced sepsis. The results of a meta-analysis by Hu et al[14] showed that IL-6 rs1800795 G/C polymorphism might be a risk factor for susceptibility to sepsis in Africans and Asians. The results of a meta-analysis by Ferdosian, et al[20] showed that there was no significant association between IL-6 rs1800795G/C polymorphism and the risk of sepsis in children. However, a subgroup analysis found that among Caucasians and Africans, the risk of sepsis increased in children. In this study, neonates in the sepsis group were divided into a critically ill and non-critically ill group based on the NCIS. The respective frequencies of genotypes GG, GC and CC in the critically ill group were 57.2, 30.9, and 11.9%, and 72.4, 15.5, and 12.1% in the non-critically ill group. There was no statistically significant difference ($p > 0.05$), inconsistent with the study.[5]

The serum IL-6 levels of the sepsis group were significantly higher than those of the control group, and the levels of the critically ill group were also higher than those in the non-critically ill group, but this study did not find an association between serum IL-6 levels and IL-6 rs1800795 G/C polymorphism. Lorente, et al[5] found an association between IL-6 rs1800795 G/C polymorphism and serum IL-6 levels in patients with severe sepsis. Patients with genotype CC had lower serum IL-6 levels, indicating a comparatively lower inflammatory response, and lower severity of sepsis and risk of death. Zidan, et al[21] found that in children with community-acquired pneumonia (CAP), IL-6rs1800795 genotype GG and allele G polymorphisms, were significantly associated with CAP susceptibility, and the GG genotype and G allele had a protective effect on severe sepsis, acute respiratory failure, and hospital mortality. The serum IL-6 levels of these children were significantly increased, while the GG genotype exhibited no association with serum IL-6 levels. There are conflicting reports about the role of IL-6 polymorphism in infectious diseases. The inflammatory response, especially production of IL-6, depends largely on the pathogens and the route of infection.[22,23] The pathogenesis of sepsis is complex, involving pathogenic bacteria, environmental exposure, host immune status, severity of infection, and interaction of various factors. At the same time, there is significant variation in genetic polymorphism among different regions, ages, populations, and races.

Our findings showed that the serum IL-6 levels were significantly higher in the sepsis group than those in the control group. The serum IL-6 levels in the critically ill group were also higher than those in the non-critically ill group, but showed no association between serum IL-6 levels and IL-6 rs1800795 G/C polymorphism. The study demonstrated IL-6 rs1800795 G/C polymorphism might not be a genetic risk factor for sepsis in full-term neonates and there was no association between IL-6 rs1800795 G/C polymorphism and outcome of sepsis.

Our study has certain limitations. First, we detected only one genetic polymorphism of IL-6.The sample size was relatively small, which may have influenced the analysis of IL-6 gene polymorphism. Second, our aim was to determine whether there was an association between the polymorphism and sepsis risk of full-term newborn, and not to analyze the association between the polymorphism and the appearance of sepsis. Third, we did not report data on treatments and treatment response over time. Fourth, being a single-center study is inevitably a limitation. Sepsis is a complex systemic inflammatory response process, which involves multiple cytokines. Anti-inflammatory mediators such as interleukin-10 will be further investigated to identify genetic risk factors related to the outcome of sepsis in our next study. Further researches regarding non-Han ethnic minorities might help to understand the association between IL-6 rs1800795 G/C polymorphism and neonatal sepsis.

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Conflict of Interest
None declared.

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