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Correlation of Toll-like Receptor Single Nucleotide Polymorphisms with Susceptibility to Infectious ARDS in Children

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Abstract

Purpose: Acute respiratory distress syndrome (ARDS) is a major contributing factor to the death in child intensive care units. Toll-like receptor (TLR) single nucleotide polymorphisms (SNPs) participate in several diseases. This study intends to analyze the correlation between TLR2 and TLR4 SNPs and the susceptibility to infectious ARDS in children.

Materials and Method: 32 patients with infectious ARDS diagnosed in our hospital were collected and 35 healthy children were collected as the control group followed by analysis of TLR2 and TLR4 mRNA expression in PBMCs by real time PCR. The SNaPshot method was used to detect TLR2 (rs5743708, rs3804099) and TLR4 genes (rs10759932, rs4986790) genotypes. Their correlation with disease susceptibility was analyzed.

Results: TLR2 and TLR4 level was significantly upregulated in PBMCs of children with infectious ARDS compared to control. The genotypes of TLR2 gene rs5743708 and TLR4 gene rs4986790 were positively correlated with BMI (P <0.05) but not with age and gender. There was no significant difference in the frequency composition ratios of rs3804099 and rs10759932 genotypes, which was not related to ARDS. TLR2 gene rs5743708 and TLR4 gene rs4986790 genotype distribution showed a significant difference between two groups (P <0.05) and they were related to ARDS, and their mutant alleles increased the risk of ARDS (OR 1.92, 95% CI1 .27-5.91; OR 1.88, 95% CI 1.32-6.76) (P <0.05).

Conclusions: TLR2 and TLR4 level is upregulated in children with infectious ARDS. TLR2 rs5743708 and TLR4rs4986790 may be ARDS susceptible genes.

Key words: infectious ARDS; TLR2; TLR4; gene; polymorphism; SNP.

Introduction

Acute respiratory distress syndrome (ARDS) is caused by intrapulmonary and / or extrapulmonary causes. The injury causes damage to the alveoli and capillaries. The pathological features are noncardiogenic pulmonary edema and inflammation. Refractory hypoxemia is a clinical syndrome which can ultimately lead to acute respiratory failure [1, 2]. The incidence of ARDS in children is lower than that of in adults. Although the proportion is small, treatment is difficult and the mortality rate is high and it is an important cause of the death in child intensive care units [3, 4]. The definition of ARDS in children is to exclude perinatal-related lung diseases, including meconium aspiration syndrome, acquired pneumonia in preterm birth, and other perinatal lung injuries. PaO2 / FiO2 or SaO2 / FiO2 are still used for non-invasive full-face ventilation, and the oxygenation index (OI) is utilized for invasive ventilation or oxygen saturation index (OSI) [5-7]. Infectious ARDS is the most common type of ARDS in children [8, 9]. The diagnosis of infectious ARDS in children is not difficult, but the treatment is challenging. The current treatment approach is mainly to control the risk factors for ARDS, correct hypoxia through respiratory support, improve oxygen delivery, and achieve the goal of controlling ARDS [10, 11]. Therefore, exploring the pathogenesis of childhood infectious ARDS can help find effective treatment targets for this disease.

Innate immune response is a hereditary and innate natural immune defense system, which constitutes the body's non-specific anti-infective immunity against the invasion of pathogenic organisms [12, 13]. Toll-like receptors (TLRs), the innate immune recognition receptors, are an important part of molecules that recognize pathogenic patterns and are involved in the activation and regulation of innate and adaptive

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immune responses [14, 15]. Toll-like receptor 2 (TLR2) and TLR4 are members of the Toll-like receptor family and play cirical roles in antibacterial and antiviral immunity [16, 17]. In recent years, the single nucleotide polymorphism (SNP) of TLR gene has been demonstrated to be closely associated with several inflammatory diseases [18]. This study is to analyze the correlation between TLR2 and TLR4 single nucleotide polymorphisms and susceptibility to childhood infectious ARDS.

Materials and methods

Research Object

32 patients with infectious ARDS were treated in pediatric intensive care unit (PICU) from June 2018 to June 2019. Diagnosis of infectious ARDS in children occurs from neonatal period to adolescence; the cause of infection was diagnosed; PaO2 / FiO2 or SaO2 / FiO2 was used for non-invasive ventilation of the mask, and oxygenation index (OI) or oxygen saturation index (OSI) was utilized for invasive ventilation. There were 17 males and 15 females, aged 2-6 years (average: 3.2 ± 0.5). Exclusion criteria: perinatal-associated lung diseases including meconium aspiration syndrome, preterm birth pneumonia and other perinatal lung injuries, infants with congenital malformations such as alveolar capillary dysplasia and congenital diaphragmatic hernia, thyroid dysfunction, autoimmune diseases, etc. Another 35 children who received a physical examination in our hospital at the same time were selected as the control group, including 18 males and 17 females, aged 1-5 years (average: 3.5 ± 0.7). There was no statistical difference in general clinical conditions such as gender and age between the two groups of patients, and they were comparable. In this study, the family members of patients signed the informed consent. This study was approved by the Medical Ethics Committee of our hospital.

Main instruments and reagents

RNA extraction and reverse transcription kit were from American ABI Company. DNA extraction kit was purchased from Qiagen, USA. The electrophoresis apparatus was purchased from Beijing Liuyi Instrument Factory. The AU680 automatic biochemical analyzer was purchased from Beckman Coulter, Germany. The DNA amplification instrument was purchased from PE Gene Amp PCR System 2400.

General data collection and specimen collection of the research object

The height, weight and body mass index (BMI) were recorded. The fasting venous blood PBMCs were isolated and DNA was extracted from each group.

Real time PCR

PBMCs were obtained by density gradient centrifugation for RNA extraction followed by cDNA synthesis according to the kit instructions. The primers were designed by Primer Premier 6.0 and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table 1). Real-time PCR reaction conditions: 55 ° C for 1 min, the cycle was 92 ° C 30 S, 58 ° C 45 S, 72 ° C 35 S, and a total of 35 cycles were performed. Data was collected using the PCR reactor software and GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn, and the semi-quantitative analysis was carried out using the 2-ΔCt method.

SNaPshot method to detect TLR2 and TLR4 gene polymorphisms

DNA was extracted using a whole blood DNA extraction kit and its quality was measured by a spectrophotometer through detecting the absorption value at 260 nm and 280 nm, and OD260 / OD280 = 1.7-1.9 was defined as a high quality of DNA. The primers were designed by Primer6.0 based and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table 2). The total reaction system was 6 μL, including 2 μL of PCR products, 1 μL of SNaPshot Mix reagent, 1 μL of each of the extension upstream and downstream primers, and 2 μL of water. Reaction conditions: 96 °C 1 min; 96 °C 10 s, 52 °C 5 s, 60 °C 30 s, a total of 30 cycles. The SNP site was determined corresponding to the PCR product and GeneMaper 3.0 software was applied for analysis.

Statistical method

SPSS 19.0 software was applied for analyzing data which were displayed as mean ± standard deviation and assessed by t test for measurement data. The chi-square test was for comparison of allele frequencies and genotype frequencies and determined whether the gene distribution conformed to Hardy-Weinberg's law genotype and frequency comparison of alleles. The correlation between TLR2 SNPs and TLR4 SNPs genotypes and infectious ARDS in children was analyzed and odds ratios (OR) and 95% confidence intervals (CI) were calculated. P <0.05 indicates a difference.

Results

Changes of TLR2 level in infectious ARDS

TLR2 mRNA level in PBMCs of children with infectious ARDS was significantly upregulated compared to control (P <0.05) (Figure 1).

Changes of TLR4 level in infectious ARDS

The expression of TLR4 mRNA in PBMCs of children with infectious ARDS was significantly elevated in comparison to control (P <0.05) (Figure 2).

Correlation between genotype frequency and susceptibility in children with infectious ARDS

The genotype distribution of the TLR2 (rs5743708, rs3804099) and TLR4 genes (rs10759932, rs4986790) in control group and ARDS group was conformed to the Hardy-Weinberg equilibrium law (points X2 = 1.267; 0.489; 0.661; 0.586; P> 0.05); the homozygous and heterozygous mutations of rs5743708 are CC and TC, respectively; the homozygous and heterozygous mutations of rs3804099 are CC and GC; The heterozygotes are AA and GA, respectively; the mutant homozygotes and heterozygotes of rs4986790 are CC and GC, respectively. After the homozygous and heterozygous mutations of TLR2 SNPs and TLR4 SNPs were combined, compared to control, the frequency composition ratio of the TLR2 gene rs3804099 and TLR4 gene rs10759932 was not statistically significant. However, the frequency composition ratio of TLR2 gene rs5743708 and TLR4 gene rs4986790 showed a significant difference (P <0.05) (Table 3).

Correlation analysis between TLR2 and TLR4 SNPs and clinical parameters in children with infectious ARDS

The relationship of TLR2 and TLR4 SNPs with the clinical parameters in children with infectious ARDS was further analyzed. The TLR2 gene rs5743708 and TLR4 gene rs4986790 genotypes were positively correlated with BMI (P <0.05) and not with age and gender. In addition, the TLR2 gene rs5743708 and TLR4 gene rs4986790 genotypes were not related (Table 4).

Correlation analysis between TLR2 and TLR4 SNPs and the incidence of childhood infectious ARDS

Mutated alleles of TLR2 gene rs5743708 and TLR4 gene rs4986790 were associated with the development of childhood infectious ARDS, which increases the risk of childhood infectious ARDS ((OR 1.92, 95% CI 1.27-5.91; OR 1.88, 95% CI 1.32- 6.76), with a statistical difference (P <0.05), while the other two TLR2 rs3804099 and TLR4 SNP rs10759932 were not related to the onset of infectious ARDS in children and did not have statistical differences (Table 5).

Discussion

The pathogenesis of infectious ARDS in children is complicated and it is believed to be related to the deficiency of immune function, especially the developmental disorder of innate immune function. At the same time, it is accompanied by pathogenic inflammatory factors, which leads to the reduction of immune function, eventually leading to multiple organs functional failure and even death [19, 20]. TLRs are important receptors during innate immune defense. TLRs recognize and respond to a large number of different PAMPs in invading microorganisms through pattern recognition receptors (PRRs), and protect them from virus and bacterial invasion. The functional changes of immune system in ARDS caused by TLRs gene regulation may be a key risk factor for the development of ARDS [21, 22].

Single nucleotide diversity (SNP) is a single-base mutation analysis at the genomic level, including deletions, insertions, or substitutions, which results in single-base mutation frequencies greater than 1%. Some SNP mutations can cause changes of gene transcription and post-transcriptional translation and the amount and function of expressed proteins might be different [23]. With the deepening study of genomics, 90% of human genetic information is confirmed to be caused by gene SNP, which determines the human susceptibility and tolerance to disease or stress and can affect the occurrence and development of human non-hereditary diseases [24]. TLRs have so far identified at least 10 family members. Among them, TLR2 mainly recognizes and binds to the components of G + bacteria, including lipoproteins, peptidoglycans, glycoproteins and teichoic acid. TLR2 (rs5743708, rs3804099) is a common genetic mutation site and is related to the occurrence and development of various diseases [25]. TLR4 is the main receptor of endotoxin lipopolysaccharide and a type I transmembrane signal transduction receptor protein [26]. TLR4 regulates innate immune response and can be expressed to varying degrees in Kupffer cells, hepatocytes, adipocytes, and sinusoidal endothelial cells, and thus participates in the occurrence and development of infectious ARDS in children [27]. The interaction between TLR4 and endotoxin leads to the release of inflammatory mediators by ARDS. Genetic mutations in the coding genes cause individuals to have different susceptibility to infection and disease. TLR4 gene SNPs are associated with disease susceptibility. Different TLR4 gene SNPs (rs10759932, rs4986790) cause imbalances of pro-

inflammatory and anti-inflammatory factors [28]. This study confirms that TLR2 and TLR4 expressions were upregulated in children with infectious ARDS. The genotypes of TLR2 gene rs5743708 and TLR4 gene rs4986790 were positively correlated with BMI ($P < 0.05$) without association with age and gender. There was also no significant difference in the frequency composition ratios of rs3804099 and rs10759932 genotypes, which was not related to ARDS. The TLR2 gene rs5743708 and TLR4 gene rs4986790 genotype distributions were statistically different between two groups and they were associated with ARDS, and their mutant alleles increase the risk of ARDS. The results suggest that TLR2 rs5743708 and TLR4rs4986790 can be used as a biomarker for clinical analysis of the incidence and prognosis of children with infectious ARDS. However, with limited number of patients in our study, which is a main limitation, more patients sample is required to confirm the association of the polymorphism of TLR2 and TLR4 with infectious ARDS in the future.

Disclosure of conflict of interest

None

Conclusion

TLR2 and TLR4 level is elevated in children with infectious ARDS. TLR2 rs5743708 and TLR4rs4986790 may be ARDS susceptible genes and they might be used as a biomarker for the diagnosis of infectious ARDS.

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Tables and Figure legends

Table 1. **Primer sequences.**

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	GAAGCTGAAGGTCGGAGTCA	GGAAGATGGTGATGGGATT
TLR2	GTACGATGGAAGTACAG	GTGACTATTGGCGCCTACTA
TLR4	GTGGAAGTTGAACGAATG	CCTGGCTTGAGTAGATAACA

Table 2. **PCR primers for TLR2 and TLR4 gene SNPs.**

SNPs	Primers	5'-3'
rs5743708	Forward	GCTGGAAGTATAGACTCGATGG
	Reverse	GAGGTTATCAGTGTAGCTGTGA
rs3804099	Forward	GGCCTCTTTCACCATCACAG
	Reverse	AGATGGCCACAGCAAAAAGT
rs10759932	Forward	GGTTGCTGGTGATTTTTCTCAAATG
	Reverse	ACCTGAGTGAAGACTGGAGAGT
rs4986790	Forward	TACTGGCCGGACGCTTTGAGA
	Reverse	CCAGCACCATTCATACTTGCTC

Table 3. **Correlation between genotype frequency of each point and susceptibility of children with infectious ARDS (%).**

Group	rs3804099			rs10759932			rs4986790			rs5743708		
	GG	GC	CC	TT	TC	CC	CC	GC	GG	TT	TC	CC
Control	23.7	31.1	45.2	28.8	37.7	24.5	64.9	28.0	7.1	58.8	37.7	3.5
ARDS	34.3	33.5	32.2	37.6	31.6	31.8	64.1	34.9	1.0	27.1	57.2	15.7
P	0.057			0.065			0.031			0.022		

Table 4. **Correlation analysis between TLR2 and TLR4 SNPs and clinical parameters in children with infectious ARDS.**

		Age	Gender	BMI
TLR2	rs5743708	0.198	0.147	0.941*
	rs3804099	0.147	0.341	0.229
TLR4	rs10759932	0.236	0.219	0.175
	rs4986790	0.119	0.232	0.671*

Compared with the control group, * P<0.05.

Table 5. **Correlation analysis between TLR2 and TLR4 SNPs and the incidence of childhood infectious ARDS.**

	SNPs	OR	95%CI
TLR2	rs5743708	1.92	1.27-5.91
	rs3804099	1.31	1.21-2.92
TLR4	rs4986790	1.88	1.32-6.76
	rs10759932	0.52	1.22-2.18

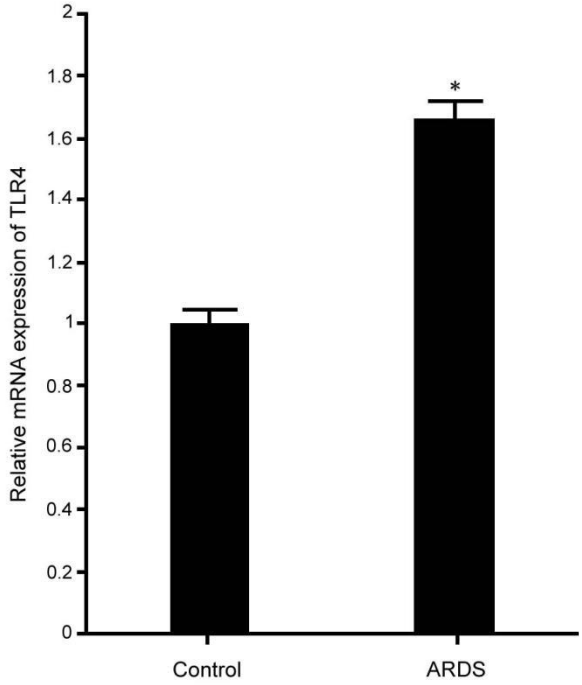

Figure 1. **TLR2 mRNA expression changes in PBMCs of children with infectious ARDS. Compared with the control group, * P<0.05.**

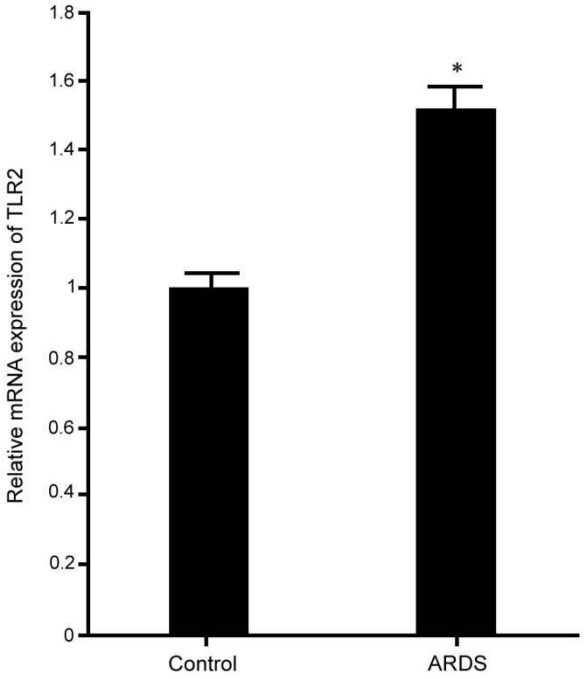

Figure 2. **TLR4 mRNA expression changes in PBMCs of children with infectious ARDS. Compared with the control group, * P<0.05.**