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Research on the correlation between the fibrinogen β and attack of pediatric pneumonia

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Abstract. – OBJECTIVE: To investigate the correlation of the gene polymorphism of β-148C/T of fibrinogen with the expression of fibrinogen and the attack of pediatric pneumonia.

PATIENTS AND METHODS: We employed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to detect the gene polymorphism of beta-fibrinogen gene-148C/T (β-148C/T). The expression level of fibrinogen in plasma was measured using enzyme-linked immunosorbent assay (ELISA), and the expression level of fibrinogen β protein was determined using Western-blot method.

RESULTS: Compared with the healthy control group, the expression level of fibrinogen β was significantly higher in patients with pneumonia. Additionally, the frequency of CC genotype, as well as the allele of C, in the pneumonia group were significantly higher than that in the control group. Meanwhile, the frequency of TT genotype and the allele of T were remarkably lower in patients with pneumonia compared to those in the control group. No significant difference was found in comparison with the CT genotype frequency between the two groups. Compared with the patients with TT genotypes, expressions of fibrinogen, IL-6 and CRP were significantly higher in the patients with the CC and CT genotypes. However, the odds ratio (OR) of pediatric pneumonia patients with TT genotype was 0.21, OR of pediatric pneumonia patients with CT genotype was 0.77 and OR of pediatric pneumonia patients with CC genotype was 12.73. The OR of patients with T allele was 1.85 and OR of patients with C allele was 5.15.

CONCLUSIONS: We concluded that β-148C/T gene polymorphism of fibrinogen was correlated with the susceptibility of pediatric pneumonia, suggesting that it may be a genetic risk factor, and fibrinogen β-148C/T gene may be involved in the onset of pediatric pneumonia through affecting the concentration of fibrinogen β in plasma.

Key Words:
Pediatric, Pneumonia, Fibrinogen, Gene polymorphism.

Introduction

Pneumonia, as one of the most frequent respiratory system diseases in children, is attracting the attention of researchers due to its typical anatomical characteristics and complication. Variable condition of disease may result in difficulty in clinical treatment[1]. Epidemiologic studies revealed that the patients with pediatric pneumonia have ranked the first in the number of in-patients of pediatric department, particularly the neonates who have the highest prevalence of pneumonia, which has become a leading cause of death in neonates in some regions in China[2]. Currently, the main clinical treatment methods for pediatric pneumonia basically include the use of anti-infection medications, but long-term administration of Western drugs may increase the risk of drug resistance; due to the side effects associated with anti-infection drugs, pediatric patients with pneumonia carry the risk of organ damage and immunologic disorders[3]. Previous studies demonstrated that the pathogens and hereditary factors are closely associated with the attack of pediatric pneumonia, but the specific molecular mechanism remains unclear.

Fibrinogen, encoded by 3 genes mapped on the human chromosome 4, contains 3 peptide chains, i.e. α, β and γ with 610, 451 and 410 amino acid residues, respectively[4]. Fibrinogen can be transformed into the fibrin under the effect of thrombin to coagulate the blood for hemostasis[5]. Besides, fibrinogen also plays important roles in various reactions in human body, such as inflammatory reactions and tissue injuries[6]. Polymorphism of fibrinogen gene is a key genetic factor contributing to the increase of concentration of fibrinogen in plasma[7]. At present, researchers have identified at least 10 polymorphism loci on the cluster of fibrinogen β. A prior study[8] showed that the poly-

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morphism of -148C/T in the promoter region is closely correlated with the reaction fragment of interleukin-6 (IL-6), which affects the concentration of fibrinogen in plasma. However, we did not find any study reporting the susceptibility of pediatric pneumonia. Research on the healthy males and females indicated that the β-C148T polymorphism of fibrinogen is directly associated with the increased concentration of fibrinogen in plasma[9]. Thus, we studied the β−148C/T genetic polymorphism of fibrinogen and the concentrations of fibrinogen in plasma, in 84 pediatric pneumonia patients and 45 healthy children. We investigated the correlations of β−148C/T genetic polymorphism of fibrinogen with the attack of pediatric pneumonia and the concentration of fibrinogen in plasma.

Patients and Methods

Patients

Between November 2014 and March 2016, 84 pediatric pneumonia patients who were admitted in the Respiratory Department of Xuzhou Children's Hospital were enrolled in this study. There were 49 males and 35 females aging from 18 days to 11 years. Meanwhile, 45 children without any history of pneumonia were selected as the control group. In the control group, there were 22 males and 23 females aging from 27 days to 12 years. There was no relationship among the subjects. In this study, patients with pediatric pneumonia were taken as pneumonia group and healthy subjects as control group. Diagnosis of pediatric pneumonia was carried out according to previous criteria[10]. This research was approved by the Ethics Committee of Xuzhou Children's Hospital.

Methods

Major Reagents

The sequences of primers used for detecting the polymorphism were as follows: upstream primer of β-148C/T of fibrinogen: 5'-CCTAACTTCCCATCATTTTGTCAATTAA-3'; downstream primer: 5'- TGTCGTTGACA CCTTGGGACTTAACTAG-3' (synthesized by Nanjing GenSpcript Biology Technology Co., Ltd, Nanjing, Jiangsu, China). The recognition sequence of restriction enzyme for Hind III was AAGCTT, and the restriction enzyme of Hind III was bought from TaKaRa (Dalian, China). GenElute™ blood genome DNA extraction kit was bought from Sigma-Aldrich (St. Louis, MO, USA). Amplification enzyme for PCR and the relevant reagents were purchased from the TransBionovo Co., Ltd (Beijing, China). Agarose gel extraction kit was purchased from Beijing Kangwei Century Biotech Co., Ltd (Beijing, China). ELISA kit was purchased from Wuhan Boster Bioengineering Co., Ltd (Wuhan, Hubei, China). Antibody for fibrinogen β, β-actin and the relevant secondary antibody were all bought from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Equipment

Polymerase Chain Reaction (PCR) instrument was purchased from ABI (Foster City, CA, USA). Horizontal gel box system, vertical gel box system and membrane-transferring system were bought from Beijing Liuyi Biotechnology Co., Ltd (Beijing, China). NanoDrop 2000 ultramicro-spectrophotometer and multi-functional microplate reader were bought from the Thermo Fisher Technology Co., Ltd (Waltham, MA, USA).

Blood Sample Collection and DNA Extraction

In the collected samples of peripheral venous blood (1 mL), 100 μL 2% EDTA was added for anti-coagulation. Samples were stored at -20 °C. The genomic DNA was extracted using (the) phenol-chloroform method; concentration and purification of extracted DNA were assayed using spectrophotometer; the extracted DNA was preserved at -20 °C for later PCR experiment.

Detection of Gene Polymorphism

Using the DNA extracted from the peripheral venous blood as the template, we analyzed the β-148C/T gene polymorphism of fibrinogen in both groups using PCR-RFLP. The PCR reaction system was set as follows: initial degeneration for 4 min at 94 °C, degeneration at 94 °C for 30 s, annealing at 54 °C for 40 s, and extension for 45 s at 65 °C. This cycle was repeated for 25 times followed by the final extension at 65°C for 10 min. Gel electrophoresis was performed for the PCR product, and the target fragment was extracted for purification after the gel was cut. The extracted DNA was digested using Hind III restriction enzyme. 1.5% agarose gel was used for horizontal electrophoresis for 30 min. Thereafter, the gel was analyzed using UV gel imaging system. The molecular weight of relevant fragment was read for analyzing the genotypes[11].

The Measurement of Fibrinogen β, IL-6 and CRP Levels in Plasma

The plasma concentration of fibrinogen was assayed using ELISA. The assay was carried out according to the instruction provided by the of kit. Plasma and standard samples were added to the 96-well plate (100 μL/well), which was later sealed using film. Samples were incubated in a thermostat incubator at 37 °C for 90 min. Diluted solution of antibody (100 μL/well) was added for incubation in the thermostat incubator at 37 °C for 60 min. Subsequently, 100 μL enzyme-binding working liquid was added to each well, and samples were incubated in the thermostat incubator at 37 °C for 30 min. After 96-well plate was washed 4 times, OD_{450nm} value was assayed using multi-functional microplate reader.

Western Blot

Plasma samples, after being mixed with the protein loading buffer, was heated at 100 °C for 5 min, and were then subjected to SDS-PAGE. Gel were then transferred to polyvinylidene difluoride (PVDF) membrane which was then blocked for 30 min using 5% skimmed milk containing tris Tris-buffered saline +0.05% Tween (TBST). Samples were then incubated with primary antibody in the antibody hybrid bag at 4 °C overnight. After the membrane was washed using TBST for 3 times (10 min/time), it was incubated with the second antibody marked by HRP at room temperature for 1 h followed by washing (3 times and 15 min/time). The membrane was stained using Electro-Chemi-Luminescence (ECL) kit for color development. Fixation and exposure using X-ray were consecutively performed in a dark room. Results were quantitatively analyzed using Image J software.

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Experiment data were presented as mean ± standard deviation (mean ± SD). Student *t*- test was performed for intergroup comparison. Chi-square test was carried out for the intergroup comparison of the measurement data. $^{*}p<0.05$, $^{**}p<0.01$ or $^{***}p<0.001$ suggested that the difference was statistically significant.

Results

Expression of Fibrinogen β in plasma

For pediatric pneumonia patients, the level of fibrinogen β in the plasma was (4.34±0.52) mg/L, and the level of fibrinogen β in the control group was (3.07±0.44) mg/L. This showed that the expression of fibrinogen β was significantly higher in pediatric pneumonia children. The difference was statistically significant ($^{*}p<0.05$, Figure 1A). The immunoblotting results revealed that the fibrinogen β level was significantly elevated in the plasma of pediatric pneumonia children ($^{*}p<0.01$, Figure 1B).

Frequency of β-148C/T and Allele of Fibrinogen

The gene polymorphism of β-148C/T of fibrinogen was presented as C/T, in which C alleles contained the recognition sequence of Hind III restriction enzyme, and, thus, could be cut into two fragments, i.e. 97bp and 265bp. When the fragment of 148C was mutated into T, the digested fragment of T allele, due to the deficiency of Hind III specific restriction enzyme cutting site, was 362bp. PCR results showed a band at 362 bp

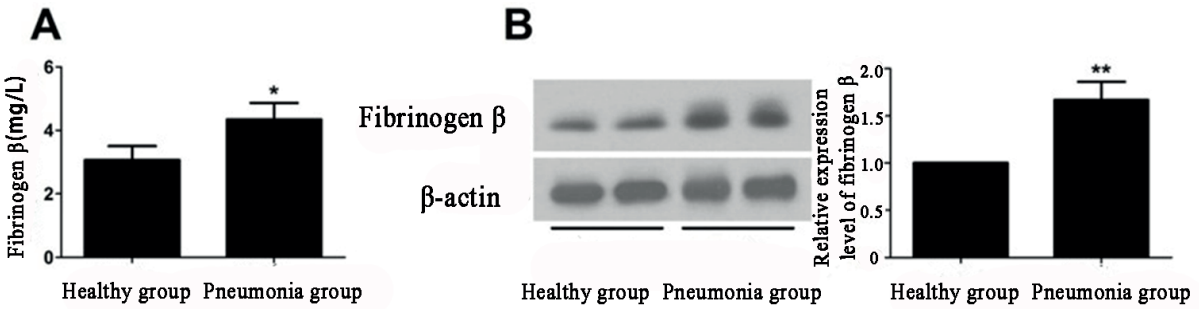

Figure 1. The expression levels of fibrinogen β in the healthy group and the pneumonia group.

Table I. Distribution of genotypes and allele frequencies of fibrinogen β-148C/T gene.

Group	Cases	Genotype TT	CT	CC	Allele T	C
Pneumonia group	84	19 (22.1)	25 (30%)	40 (47.9%)	63 (37.5%)	105 (62.5%)
Control group	45	26 (57.9%)	16 (34.5%)	3 (8.6%)	68 (75.6%)	22 (24.4%)
X^2 value			25.44		33.96	
p			< 0.001		< 0.001	

in the TT genotype, 2 bands (265bp and 97bp) in the CC genotype and 3 bands (362bp, 265bp and 97bp) in the CT genotype (Figure 2).

The frequency distribution of β-148C/T genotype and allele of fibrinogen is shown in Table I. Compared with the control group, the frequency of CC genotype as well as C allele in the pneumonia group was significantly higher, while the frequency of TT genotype and T allele in pneumonia group was significantly lower. However, no significant differences were found in the comparison of the variations of CT genotype frequency between the two groups. These data revealed that there were significant differences in the comparison of 3 genotypes and 2 alleles between the pneumonia group and control group, in which the χ^2 values were respectively 25.44 and 33.96 with statistically significant differences.

The Concentrations of Fibrinogen β, IL-6 and CRP in Plasma of Patients with Different β-148C/T Genotypes of Fibrinogen

To assay the expression level of fibrinogen in plasma, the pediatric pneumonia patients were divided into three subgroups, i.e. TT, CC and TC. Compared with TT genotype, the expression levels of fibrinogen β, IL-6 and CRP in the patients with CC and CT genotypes were significantly higher. However, there was no statistically significant differences between the expression levels of fibrinogen β, IL-6 and CRP in pediatric pneumonia patients with CC and CT genotypes (Figure 3).

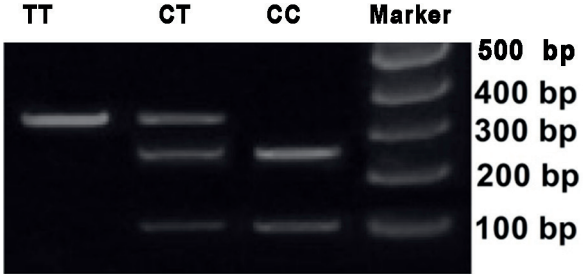

Figure 2. Gene polymorphism of fibrinogen β-148C/T by PCR-RFLP.

The Correlation Between the Fibrinogen β-148C/T Genotype and the Attack of Pediatric Pneumonia

The distribution of fibrinogen β-148C/T genotype and the allele in pediatric pneumonia patients is shown in Table II. To evaluate the correlation between the fibrinogen β-148C/T genotype and the attack of pediatric pneumonia, in this study we used odds ratio (OR) to demonstrate the risk of pediatric pneumonia. The OR of TT genotype was 0.21 (19 × 19/65 × 26), the OR of CT genotype was 0.77 (25 × 29/59 × 16). The OR in CC genotype was 12.73 (40 × 42/44 × 3). The OR of T allele was 1.85 (63 × 68/105 × 22), and the OR of C allele was 5.15 (105 × 68/63 × 22). These results suggested that the CC genotype and C allele remarkably increased the risk of pediatric pneumonia.

Discussion

Pediatric pneumonia is a common pulmonary inflammation disease in infants and children[12]. Once progressed into the severe condition, it may lead to the cardiac failure. Fibrinogen plays an important role in the pathologic courses of pneumonia, cardiovascular diseases, immunologic diseases and cancer[13,14]. IL-6 level is increased significantly in the bronchi-alveolar lavage fluid in the child patients with refractory pneumonia, suggesting the critical role of IL-6 in the immunologic process of children patients with refractory pneumonia[15]. IL-6 can also exert its functions in the inflammatory reactions through upregulating the expression of fibrinogen gene. -148 locus, close to the IL-6 reaction fragment, is of great significance for activating the transcription of fibrinogen gene[16]. β-C148T polymorphism of fibrinogen is a major regulatory factor[16]. Schmidt et al[17] confirmed that the β-148TT

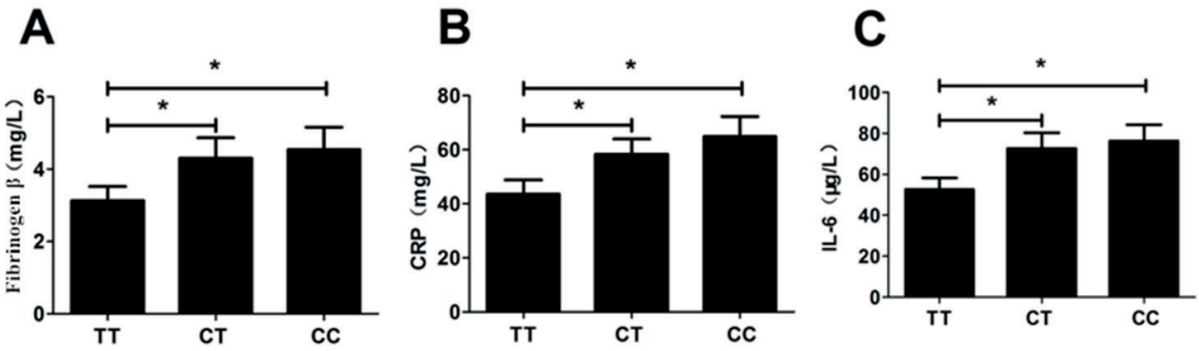

Figure 3. The levels of plasma fibrinogen, CRP and IL-6 in different fibrinogen β-148C/T genotypes.

Table II. Distribution of genotypes and allele of fibrinogen β-148C/T gene in children with pneumonia.

		Genotype			Allele	
Group	Cases	TT	CT	CC	T	C
Pneumonia group	84	19	25	40	63	105
Control group	45	26	16	3	68	22

genotype of fibrinogen can increase the risk of carotid atherosclerosis by 6.17 times. They also found that the β-148C/T polymorphism of fibrinogen is associated with the fraction of disease of carotid atherosclerosis, which is considered as a risk factor for cerebrovascular disease[17]. Passamonti et al[18] confirmed that the occurrence and the development of pneumonia in neonates are correlated with the β-455A/G polymorphism of fibrinogen. Nevertheless, the relationship between β-C148T polymorphism of fibrinogen and pediatric pneumonia remains unclear.

In this work, we discovered that the level of fibrinogen β in plasma in pediatric pneumonia patients was significantly elevated. A previous investigation[19] indicated that β-C148T gene polymorphism of fibrinogen could affect the level of fibrinogen in plasma. Our data suggested that the frequencies of CC genotype and C allele in pneumonia group were significantly higher than those in the control group, while the frequencies of TT genotype and T allele in pneumonia group were significantly lower than those in the control group. This showed that β-C148T gene polymorphism of fibrinogen is associated with the onset of pediatric pneumonia. It has been confirmed that the β-C148T gene polymorphism of fibrinogen can affect the concentration of fibrinogen in plasma in the healthy and smoking population with higher levels of IL-6 and CRP[20]. In this study, we found that the level of fibrinogen β in the pediatric pneumonia patients with CC and CT genotypes was higher, which further proved that β-148C/T gene polymorphism of fibrinogen is associated with the level of fibrinogen β in plasma.

Conclusions

We showed that β-148C/T gene polymorphism of fibrinogen is correlated with the susceptibility of pediatric pneumonia, suggesting that it may be a genetic risk factor. Fibrinogen β-148C/T gene may be involved in the onset of pediatric pneumonia through affecting the fibrinogen β concentration in plasma. Results obtained from the present work can be used for the research of pathogenesis of pediatric pneumonia, which can promote the development of clinical treatment methods with better efficacy.

Conflict of interest

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest about the work submitted.

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