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Original Article

Correlation between *TLR4* genetic polymorphisms and susceptibility to neonatal early Crohn disease

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Abstract: Aims: The present study was aimed at exploring the relationship of the polymorphisms of rs12377632, rs1927907 and rs2149356 in *TLR4* gene with the susceptibility to early Crohn disease in neonate. Methods: The polymorphisms of rs12377632, rs1927907 and rs2149356 were tested in 100 neonatal patients with early Crohn disease and 100 healthy controls through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The linkage disequilibrium (LD) and haplotype analysis were performed with Haploview software. The chi-square test was employed to analyze the differences of genotype, allele and haplotype frequencies between cases and controls. The control group was matched with the case group by age and gender. Odds ratios (ORs) with 95% confidence intervals (CIs) were utilized to express the relative risk of neonatal early Crohn disease. Results: The frequencies of TT, TT and TT genotypes at rs12377632, rs1927907 and rs21479356 polymorphisms respectively were significantly higher in neonatal patients with early Crohn disease than in controls (P=0.031; P=0.024; P=0.042), and the ratios of T, T and T alleles expressed the same outcome between two groups (OR=1.744, 95% CI=1.159-2.623, P=0.010; OR=1.926, 95% CI=1.221-3.038, P=0.006; OR=1.695, 95% CI=1.121-2.565, P=0.016). The haplotype analysis and linkage disequilibrium test of alleles of three polymorphisms in *TLR4* gene showed that T-T-T haplotype was common in cases (P=0.004), indicating T-T-T might be the susceptible haplotype to neonatal early Crohn disease. Conclusion: The genetic polymorphisms of *TLR4* gene rs12377632, rs1927907 and rs2149356 may increase the risk of neonatal early Crohn disease.

Keywords: *TLR4*, polymorphisms, neonatal early Crohn disease

Introduction

Crohn disease, a common inflammatory bowel disease, is a chronic intestinal inflammatory, occurring on susceptible individuals who affected by environmental factors. Its morbidity shows a rising trend year by year [1]. Crohn disease can invade any part of digestive tract and express various clinical symptoms, so the diagnosis for it is quite difficult, and the misdiagnosis rate is impressively high, even up more than 60% before operation [2, 3]. At the moment, Crohn disease is considered to be related to autoimmune, intestinal bacteria and mycoplasma infection, and to be induced or exacerbated by other factors such as genetic and mental factors [4, 5]. In recent years, with the development of method for disease gene research, multiple susceptible loci in genes are discovered, like Toll-like receptor 4 (*TLR4*). Firstly reported by Poltorak, *TLR4* gene plays a vital role in determining the individual responsiveness to endotoxin, and its function will affect the human sensibility to bacterial infection [6, 7]. The polymorphisms of *TLR4* gene currently is one of the hot research areas in the field of infection and immune, but the study focus is restricted on the coding region [8-11].

In the present study, the association of *TLR4* gene with neonatal early Crohn disease was also examined based on three polymorphisms of rs12377632, rs1927907 and rs2149356.

Materials and methods

Objects of the study

100 neonatal patients with early Crohn disease diagnosed in the gastroenterology department of Qilu hospital were recruited in this case-control study as the case group, including 65 males and 35 females. 100 healthy controls con-

Table 1. Primer sequences

Polymorphism	Up-/down-primer		Length
rs12377632	Forward	5'-TATTTGGCTTTCTGTTCCTT-3	234 bp
	Reverse	5'-AACAGAAAGCCAAATACCAT-3'	
rs1927907	Forward	5'-TTATGTTCTAAATTTCAGTT-3'	183 bp
	Reverse	5'-CTACCTTAATAGAGTACTTG-3'	
rs2149356	Forward	5'-TTCCACAAAACTCGCTCCTA-3'	272 bp
	Reverse	5'-AGGTGATAGGAGCGAGTTTT-3'	

tained 69 males and 31 females. The clinical diagnosis of neonatal early Crohn disease was following the diagnosis criteria in the standard recommends for inflammatory bowel disease by Chinese Society of Gastroenterology in 2001 and the diagnostic points recommended by World Health Organization (WHO), and the final confirmation was determined by pathology [12]. All patients were unrelated and experienced no radiotherapy or chemotherapy before blood collection. The controls were healthy people from the physical examination center of the hospital during the same period, and their physical examination demonstrated no digestive disease history. They were randomly selected to pair with cases at the ratio of 1:1 with same ethnicity, gender and the place and duration of residence as well as an age difference less than 5 years. The essential information of age, gender, ethnicity and native place had no significant differences between two groups through statistical test (P>0.05). All participants were not related by blood. The present study obtained the approval from the ethics committee of Qilu hospital and the written informed consensus from every subject parents. Sample collection was conducted in accordance with the national ethics criterion for human genome research.

DNA extraction

5 mL peripheral venous blood from each participant was performed anticoagulant with ethylene diamine tetraacetic acid (EDTA) and reserved in -80°C refrigerator. DNA was extracted with phenol chloroform method and stored at -20°C for later.

Polymerase chain reaction (PCR) amplification

PCR test primers for SNPs were all designed by Primer5.0 primer design program and synthesized by Shanghai Genecore Biotechnologies Co., Ltd.. Upstream and downstream primer sequences are listed in **Table 1**. *TLR4* genetic polymorphisms were analyzed with PCR-restriction fragment length polymorphism (PCR-RFLP) technique. PCR were performed in a total volume of 25 μl containing 4 μl template DNA, each 1.0 μl of forward and reverse primers, 1 μl Taq DNA polymerase, 2.5 μl 10× Buffer, 1.0 μl 4×dNTPs and the rest volume of sterile water. The PCR cycle conditions consisted of an initial degeneration step at 94°C for 3 min, followed by 35 cycles of degeneration at 94°C for 45 s, annealing at 51°C for 45 s and extension at 72°C for 45 s, and at last a final extension at 72°C for 5 min. PCR products were digested with MspI enzyme and determined the genotypes of all genetic variations by agarose gel electrophoresis.

Statistical analyses

χ^2 test was conducted using PASW Statistics 18.0 software, and utilized to compare the differences of genotypes and alleles distribution at every SNP between cases and controls (P<0.05 represents statistically significant difference). Linkage disequilibrium (LD) and haplotype analysis were performed with haploview software. PLINK1.07 software was employed to detect whether the distribution in cases deviated from Hardy-Weinberg equilibrium (HWE) (statistically significant difference with P>0.05). Odds ratios (ORs) and 95% confidence intervals (CIs) expressed the relative risk of neonatal early Crohn disease.

Results

General characteristics of the subjects

The distribution of *TLR4* gene rs12377632, rs 1927907 and rs2149356 polymorphisms was corresponded to HWE in 100 controls. Meanwhile, the HWE test in controls showed that the goodness of fit to the law in each site was fine (P>0.05), indicating that controls were in equilibrium state and had good representativeness.

Correlation analysis between alleles and genotypes of TLR4 and neonatal early Crohn disease risk

The genotypes distribution of *TLR4* gene rs12377632, rs1927907 and rs2149356 polymorphisms, shown in **Table 2**. Results manifested that TT, TT and TT genotypes in these

Table 2. Distribution of every genotype and allele at rs12377632, rs1927907 and rs2149356 polymorphisms

genotype/allelotype	Cases (n=100) (%)	Controls (n=100) (%)	χ^2	*P* value	OR (95% CI)
rs12377632					
CC	35 (35.0)	51 (51.0)	-	-	1
CT	41 (41.0)	35 (35.0)	2.844	0.115	1.707 (0.915-3.184)
TT	24 (24.0)	14 (14.0)	5.330	0.031	2.499 (1.137-5.488)
C	111 (55.5)	137 (68.5)	-	-	1
T	89 (44.5)	63 (31.5)	7.173	0.010	1.744 (1.159-2.623)
rs1927907					
CC	52 (52.0)	67 (67.0)	-	-	1
CT	31 (31.0)	26 (26.0)	1.767	0.200	1.536 (0.814-2.898)
TT	17 (17.0)	7 (7.0)	5.890	0.024	3.129 (1.208-8.106)
C	135 (67.5)	160 (80.0)	-	-	1
T	65 (32.5)	40 (20.0)	8.071	0.006	1.926 (1.221-3.038)
rs2149356					
GG	38 (38.0)	53 (53.0)	-	-	1
GT	41 (41.0)	35 (35.0)	2.468	0.123	1.634 (0.884-3.019)
TT	21 (21.0)	12 (12.0)	4.648	0.042	2.441 (1.072-5.556)
G	117 (58.5)	141 (70.5)	-	-	1
T	83 (41.5)	59 (29.5)	6.289	0.016	1.695 (1.121-2.565)

Table 3. Linkage disequilibrium and haplotype analysis of alleles at rs12377632, rs1927907 and rs2149356 polymorphisms

Haplotype site1-site2-site3	Cases (2n=200)	Controls (2n=200)	χ^2	*P* value	OR (95% CI)
C-C-G	111	137	-	-	1
T-T-T	65	40	8.675	0.004	2.006 (1.258-3198)
T-C-T	18	19	0.197	0.724	1.169 (0.586-2.335)
T-C-G	6	4	0.901	0.519	1.851 (0.510-6.723)

three polymorphisms respectively were more frequent in cases than in healthy controls. The differences between case and control groups were statistical significant, indicating that the genotypes increased the risk of early Crohn disease in neonate (OR=2.499, 95% CI=1.137-5.488; OR=3.129, 95% CI=1.208-8.106; OR=2.441, 95% CI=1.072-5.556). Moreover, the frequencies of rare alleles T, T and T at rs12377632, rs1927907 and rs2149356 polymorphisms were remarkably higher in cases than in controls, showing that they were closely related to the incidence of neonatal early Crohn disease (OR=1.744, 95% CI=1.159-2.623; OR=1.926, 95% CI=1.221-3.038; OR=1.695, 95% CI=1.121-2.565).

Analysis of TLR4 gene haplotype frequencies

LD analysis was performed on the alleles of *TLR4* gene rs12377632, rs1927907 and rs2149356 polymorphisms using Haplo View software (**Table 3**). The results showed that there were 4 haplotypes formed by three polymorphisms (rs12377632, rs1927907 and rs2149356) in *TLR4* gene. The correlation analysis of 4 haplotypes with neonatal early Crohn disease risk demonstrated that T-T-T haplotypes showed significant differences between cases and controls ($P<0.05$), might increase the risk of neonatal early Crohn disease (OR=2.006, 95% CI=1.258-3.198).

Discussion

Crohn disease is a chronic inflammatory disease with the main expression of gastrointestinal lesions. Its pathogenesis currently is attributed to the activation of intestinal immune and non-immune systems in susceptible individuals caused by the combination effects of environmental factors and intestinal floras [13-23]. In

other word, Crohn disease is induced as a result of the tissue damages produced by local inflammation after the amplification of immune responses and inflammation processes amid the continuity of antigen stimulation and immune dysfunction.

As the first immunologic barrier against pathogenic microorganism invading, innate immune system maintains the intestinal homeostasis through identifying pathogenic microorganisms with recognition receptors, such as Toll-like receptors. Toll-like receptors (TLRs) is one of the important pattern recognition receptors in innate immune system. As yet, 13 members (TLR1~TLR13) of TLRs family has been identified in mammals, of which 10 are functional ones. TLRs can be generally divided into two kinds: one kind is located on the cell membrane surfaces, including TLR1, 2, 4, 5, 6 and 10; and the other locates in cells, containing TLR3,7,8 and 9 [24]. Different members in TLRs family mediate various biological effects [25]. Numerous studies have shown that TLRs signal pathways are closely associated with multiple dysimmunity-relevant diseases, and that *TLR4* plays an important role in maintaining intestinal homeostasis through regulating intestinal inflammation and resisting foreign pathogen invasion [26-28]. But the research about the correlation of *TLR4* polymorphisms and neonatal early Crohn disease risk was rare.

In this case-control study, the correlation between 3 polymorphic variations in *TLR4* gene and the risk of neonatal early Crohn disease was analyzed. HWE test in controls revealed that the control group had representativeness ($P>0.05$). The calculation demonstrated that the TT, TT and TT genotype frequencies of *TLR4* rs12377632, rs1927907 and rs2149356 polymorphisms were substantially higher in neonatal patients with early Crohn disease than in controls. Results indicated that T, T and T allele frequencies were also higher in the former than in the later, suggesting the genotypes and alleles had a close relationship with the pathogenesis of early Crohn disease in neonate. Besides, the TT, TT and TT genotypes at rs12377632, rs1927907 and rs2149356 polymorphisms respectively all increased the risk of neonatal early Crohn disease (OR=2.499, 95% CI=1.137-5.488; OR=3.129, 95% CI= 1.208-8.106; OR=2.441, 95% CI=1.072-5.556). Additionally, there were significant differences of rare alleles T, T and T of rs12377632, rs1927907 and rs2149356 polymorphisms between two experimental groups ($P<0.05$), so the alleles increased the morbidity of neonatal early Crohn disease (OR=1.744, 95% CI=1.159-2.623; OR=1.926, 95% CI=1.221-3.038; OR=1.695, 95% CI=1.121-2.565).

The alleles of *TLR4* gene three polymorphisms (rs12377632, rs1927907 and rs2149356) were performed haplotype analysis and disequilibrium test in the present study. The results showed that T-T-T haplotype was common in cases ($P=0.004$), indicating that T-T-T might be the susceptible haplotype to neonatal early Crohn disease. Combined with the above results, as susceptible haplotype T-T-T contains susceptible alleles T, T and T, further evidence has been given to prove that T, T and T at rs12377632, rs1927907 and rs2149356 polymorphisms might be the susceptible alleles to neonatal early Crohn disease.

As for Crohn disease, doctors should be familiar with its diagnostic criteria, and early perform corresponding examination when facing cases with long disease courses. Thus, they could exclude the possibility or make a definite diagnosis of Crohn disease, and avoid the situations of misdiagnosis or missed diagnosis, improving the life quality of patients.

Disclosure of conflict of interest

None.

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