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CD14-*159* and -*260* Gene Polymorphisms Are Associated with HBV-Related Cirrhotic Injury in Chinese Han Patients

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Abstract

The present study aimed to investigate the association between TLR4 mutations (*Asp299Gly* and *Thr399Ile*) and CD14 polymorphisms (base pair -*159* and -*260*) with HBV-related cirrhosis in Chinese Han patients. By use of a polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) analysis technique, we genotyped Toll-like receptor 4 (TLR4) Asp299Gly and *Thr399Ile* and CD14-*159* and -*260* polymorphisms in 110 HBV-related cirrhotic patients and 110 healthy controls from the Chinese Han population. We found significant differences in the genotypes and allele frequencies of CD14-*159* (but not -*260*) between healthy controls and liver cirrhotic patients, and both the CD14-*159* and -*260* genotypes were significantly different among Child-Pugh grades in cirrhotic patients. No TLR4 Asp299Gly and *Thr399Ile* mutations were detected in any cirrhotic patients or healthy controls in the Chinese Han population. These findings indicated that the polymorphisms of CD14, but not TLR4 *Asp299Gly* and *Thr399Ile* mutations, may be an important genetic factor for HBV-related cirrhotic injury in the Chinese Han population.

Key Words: CD14, Toll-like receptor 4, liver cirrhosis, polymorphism

Introduction

Pattern-recognition receptors (PRRs) are specific for discrete determinants on microorganisms. The best-study PRRs are Toll-like receptors (TLRs) involving in the recognition of pathogens by the innate immune system. TLRs can be secreted, can be expressed on the cell surface, or can be residents in intracellular compartments (23, 31). These receptors recognize and bind conserved patterns on pathogens such as lipopolysaccharide (LPS), peptidoglycan, lipopeptides, bacterial DNA and flagellin, and they are responsible for initiating acute inflammatory responses against invading pathogens (19, 21). TLRs signaling leads to the activation of phagocytosis and direct killing of the pathogens and also affects the initiation of adaptive

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immune response through co-stimulatory molecule expression on dendritic cells. Thus, TLRs-mediated recognition creates a link between innate and adaptive immune system. The CD14 receptor of monocytes is an important mediator for the activation of monocytes and macrophages by Gram-negative bacterial endotoxin (4). Enzymatic cleavage of membrane-associated CD14 releases a soluble CD14 (sCD14) fragment, which can bind circulating endotoxin, thereby reducing biological activity. Membrane-bound CD-14 enhances endotoxin-initiated signal transduction, which is mediated through the TLR4. The recognition of LPS through TLR4/CD14 activates monocytes and macrophages to produce cytokines such as tumor necrosis factor alpha (TNF-α), interleukin -1(IL-1) and IL-6, which, in turn, serve as endogenous mediators of inflammation.

Recently, base-pair changes in the genes for both CD14 and TLR4 have been described in humans (4). The first is an A/G substitution at nucleotide 896 from the start codon of the TLR4 gene, which results in an aspartic acid -to-glycine substitution at position 299 of the amino acid sequence (*Asp299Gly*). A cosegregating point mutation that results in a threonine-to-isoleucine substitution at position 399 of the amino acid sequence (*Thr399Ile*) was also identified. These mutations are associated with functional changes, as demonstrated by decreased airway responsiveness after LPS stimulation (24), increased risk of pancreatic necrotic infection in acute pancreatitis (5) and the risk of development of distal gastric cancer (6). So far, two forms of CD14 gene polymorphisms have been identified. The first form involves a C-to-T transition at bp-*260* (*260 C/T*). Hubacek *et al*. (10) showed higher incidence of T allele in position 260 in Czech patients who survived myocardial infarction (MI) and also more pronounced expression of CD14 membrane receptor (mCD14) on monocytes in patients with TT genotype compared to CT or CC genotypes. Due to the discrepancy of results between different ethnic groups, our interest focused on another polymorphism of the CD14 receptor gene involving a C-to-T transition at bp-*159* (*159C/T*) (11, 15, 25).

There are little attempts to reveal an association between gene polymorphisms of TLR4 and CD14 with liver cirrhosis. In the present study, we investigated whether CD14-*159* and -*260* polymorphisms and TLR4 *Asp299Gly* and *Thr399Ile* polymorphisms are associated with HBV-related cirrhosis in Chinese Han patients.

Materials and Methods

Study Subjects

In total, 110 patients (Child-Pugh A, n = 24; Child-Pugh B, n = 28; Child-Pugh C, n = 58) with HBV-related cirrhosis from Ruijin Hospital affiliated with Shanghai Jiaotong University, School of Medicine and affiliated Hospitals of Shaoxing University, School of Medicine were studied. Among the patients, there were 65 males and 45 females. The mean age was 43.46 ±13.27 years, ranging from 18 to 65 years. One hundred and ten age- and sex-matched healthy individuals (HBS-Ab positive) from the medical examination population were recruited as a control group. The study protocol was approved by the Ethics Committees of Shanghai Jiaotong University, School of Medicine and Shaoxing University, School of Medicine. All patients and healthy controls gave their written informed consent to participate in the study. Diagnosis of HBV-related cirrhosis was confirmed either by pathology and histology or clinic and ultrasound data if biopsy was not available. Further exclusion criteria were the presence of primary biliary cirrhosis, HCV-related cirrhosis and alcoholic cirrhosis. None of the patients and healthy individuals suffered from other factors that may influence circulating endotoxin and/or immunomodulatory drug use within the previous 6 weeks.

DNA Isolation

Genomic DNA was extracted from 5 ml EDTA-anticoagulated peripheral blood with the ReadyAmp genomic DNA purification system (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. After extraction, the DNA concentration was measured photometrically and the DNA was diluted to a concentration of 5 ng/μl.

Detectioon of TLR4 and CD14 Gene Polymorphisms

TLR4 Asp299Gly and Thr399Ile polymorphisms. Genotyping for the TLR4 *Asp299Gly* and TLR4 *Thr399Ile* polymorphisms was performed using PCR-RFLP as previously described (17). Specifically, primers for the TLR4 *Asp299Gly* (249 bp) allele were (P1: 5'-gat tag cat act tag act act acc tcc atg-3', P2: 5'-gat caa ctt ctg aaa aag cat tcc cac-3'). Primers for the TLR4 *Thr399Ile* (406bp) allele were (P1: 5'-ggt tgc tgt tct caa agg att ttg gga gaa-3', P2: 5'-acc tga aga ctg gag agt gag tta aat gct-3'). PCR was performed in a total volume of 25 μl: 20 ng DNA, 1 × buffer (MBI Fermentas, Vilnius, Lithuania), 0.2 mM each dNTP, 0.4 μM primer, 2 mM $MgCl_2$, 1U Taq DNA Polymerase (MBI Fermentas, Vilnius, Lithuania). Amplification was carried out on an Applied PCR Biosystems PE7500 (Foster City, CA, USA) and the cycling conditions were 95°C for 2 min, 35 cycles of 95°C for 50 s, 56 °C (TLR4 *Asp299Gly*) or 60°C (TLR4 *Thr399Ile*) for 50 s, 72°C for 50 s, and a final extension at 72°C for 5 min.

The PCR products (20 μl) were digested with *NcoI* (*Asp299Gly*) or *HinfI* (*Thr399Ile*), respectively.

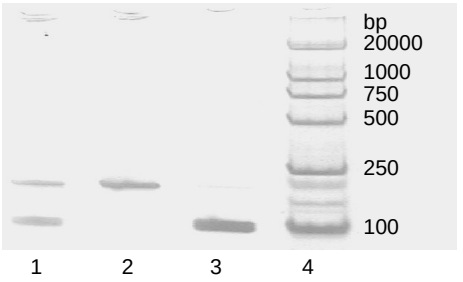

Fig. 1. 3% agarose gel electrophoresis of CD14-*159* PCR-RFLP products. Lane 1: C/T heterozygotes; Lane 2: C/C hrmozygotes; Lanes 3: T/T homozygotes; Line 4: DL2000 DNA Marker.

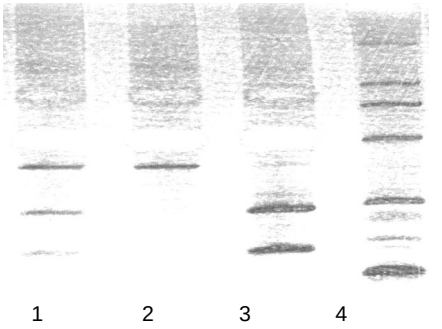

Fig. 2 3% agarose gel electrophoresis of CD14-*260* PCR-RFLP products. Lane 1: C/T heterozygotes; Lane 2: T/T hrmozygotes; Lanes 3: C/C homozygotes; Line 4: DL2000 DNA Marker.

When 249 bp PCR product was digested with *NcoI*, fragments of 223 bp and 26 bp were obtained in the variant allele. The wild-type allele showed a loss of one *NcoI* cleavage site, resulting in the presence of fragments 249 bp in length. The 406 bp PCR product was digested with the restriction enzyme *HinfI*, into the fragments of 337 and 29 bp in length in the presence of the variant allele. The wild-type allele showed a loss of one *HinfI* cleavage site, resulting in the presence of fragments 406 bp in length. All products were separated on a 3% agarose gel and stained with ethidium bromide. After running electrophoresis at 100 V for 30 min, the gels were visualized on a UV light box (MiniBis DNR BioImaging Systems, Jerusalem, Israel).

CD14 -159 and -260 polymorphisms. Genotyping for -*159* and -*260* of the CD14 gene was performed using the method described by Hubacek *et al*. (10). In brief, the promoter of the CD14 receptor gene was amplified by the primers CD14 -*159* (P1: 5'-ctt agg ctc ccg agt caa ca-3', P2: 5'-cct ctg tga acc ctg atc ac-3', 208 bp) and CD14 -*260* (P1: 5'-cta agg cac tga gga tca tcc-3', P2: 5'-cat ggt cga taa gtc ttc cg-3', 417 bp). A 25 μl PCR amplification mixture containing 50 pmol of each primer, 2.5 μl of 10 × buffer, 1.5 μl of 25 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 1 μl of template DNA, 1 μl of Ampli Taq DNA polymerase, and 17 μl ddH_2O, was run in a Perkin-Elmer thermalcycler. After an initial denaturation at 95°C for 5 min, followed by 35 cycles at 92 for 40 s, at 62°C (CD14 -*159*) or 58°C (CD14 -*260*) for 35 s, and at 72°C for 50 s. The final extension step was prolonged to 5 min.

The 208 bp PCR product was digested with the restriction enzyme *AvaII*, into the fragments of 108 and 100 bp in length in the presence of the TT genotype. The CC genotype showed a loss of one *AvaII* cleavage site, resulting in the presence of fragments 208 bp in length. However, the 417 bp PCR product was digested with the restriction enzyme *Hae III*, into the fragments of 262 and 155 bp in length in the presence of the CC genotype. The TT genotype showed a loss of one *Hae III* cleavage site, resulting in the presence of fragments 417 bp in length. The resulting fragments were separated on a 3% agarose gel and stained with ethidium bromide. After running electrophoresis at 100V for 30 min, the gels were visualized on a UV light box (MiniBis DNR BioImaging Systems, Jerusalem, Israel).

Statistical Analysis

The data were analyzed using SPSS10.0 software. Genotypes and allele frequencies were calculated by direct counting. X^2 test was to compare variables distribution. The probability level of $P < 0.05$ was considered statistically significant.

Results

To determine if CD14 and TLR4 polymorphisms confer susceptibility in HBV-related cirrhotic patients, we assessed the genotypes in 110 HBV-related cirrhotic patients and 110 healthy controls. In our study, the functional gene polymorphisms of the TLR4 *Asp299Gly* and *T hr399Ile* alleles were not detected in all subjects. Because PCR products were sensitive to *Nco I (Asp299Gly)* and *Hinf I (Thr399Ile)* and digested at the -*299* and -*399* site into two fragments (223 bp and 26 bp, 337bp and 29 bp), they were all wild-type homozygotes. Therefore, a significant association was not observed between TLR4 *Asp299Gly* and *Thr399Ile* alleles and HBV-related cirrhosis and healthy controls in this Chinese Han population.

CD14 gene polymorphisms at position -*159* and -*260* were found in both HBV-related cirrhotic patients and healthy controls (Fig. 1, Fig. 2). As shown in Table 1, of 110 patients with HBV-related cirrhosis, CD14-*159* CC genotype was found in 21.82%, CT genotype in 40.91%, and TT genotype in 37.27% of subjects, whereas in healthy controls it was found in 42.72%, 39.09% and 18.18% subjects, respectively

Table 1. Genotype and allele frequency of CD14-*159*(C/T) in cirrhotic patients and healthy controls

	Genotype, n (%)			Allele Frequency, n (%)	
	CC	CT	TT	C	T
Healthy controls (n = 110)	47 (42.72)	43 (39.09)	20 (18.18)	137 (62.27)	83 (37.73)
Liver cirrhosis (n = 110)	24 (21.82)	45 (40.91)	41 (37.27)	93 (42.27)	127 (57.73)
Child A (n = 24)	11 (45.83)	9 (37.50)	4 (16.67)	31 (64.58)	17 (35.42)
Child B (n = 28)	6 (21.43)	13 (46.43)	9 (32.14)	25 (44.64)	31 (55.36)
Child C (n = 58)	7 (12.07)	23 (39.66)	28 (48.27)	37 (31.90)	79 (68.10)

Healthy controls *versus* liver cirrhosis: genotype (CC *vs.* CT *vs.* TT: $x^2 = 13.91$, $P < 0.01$), allele frequency (C *vs.* T: $x^2 = 17.64$, $P < 0.01$). Genotype among Child-Pugh grades in cirrhosis (CC *vs.* CT *vs.* TT = $x^2 = 14.97$, $P < 0.01$).

Table 2. Genotype and allele frequency of CD14-*260*(C/T) in cirrhotic patients and healthy controls

	Genotype, n (%)			Allele Frequency, n (%)	
	CC	CT	TT	C	T
Healthy controls (n = 110)	40 (36.36)	58 (52.73)	12 (10.91)	138 (62.73)	82 (37.27)
Liver cirrhosis (n = 110)	44 (40.00)	52 (47.27)	14 (12.73)	140 (63.64)	80 (36.36)
Child A (n = 24)	11 (45.83)	5 (20.83)	8 (33.34)	27 (56.25)	21 (43.75)
Child B (n = 28)	6 (21.43)	19 (67.86)	3 (10.71)	31 (55.36)	25 (44.64)
Child C (n = 58)	27 (46.55)	28 (48.28)	3 (5.17)	82 (70.69)	34 (29.31)

Healthy controls *versus* liver cirrhosis: genotype (CC *vs.* CT *vs.* TT: $x^2 = 0.67$, $P < 0.22$), allele frequency (C *vs.* T: $x^2 = 0.04$, $P < 0.84$). Genotype among Child-Pugh grades in cirrhosis (CC *vs.* CT *vs.* TT = $x^2 = 20.02$, $P < 0.01$).

(healthy controls *vs.* cirrhosis: $x^2 = 13.91$, $P < 0.01$). Allele frequencies for the CD14-159 polymorphism were also found to be significant between the cirrhotic patients and healthy controls ($x^2 = 17.64$, $P < 0.01$). Compared to controls, the CD14-*260* genotype and allele frequencies were found to be not significantly different in the cirrhotic patients (genotype: $x^2 = 0.67$, $P = 0.22$; allele frequencies: $x^2 = 0.04$, $P = 0.84$) (Table 2). However, when compared among Child-Pugh grades in cirrhotic patients, both CD14-*159* and -*260* genotypes were significantly different.

Discussion

In the clinical setting, several studies have shown significant, although relatively modest, increases in circulating endotoxin levels in patients with cirrhosis (2, 18, 26). Many factors promote endotoxemia in this setting, including increased translocation of endotoxin from the gut lumen and a reduction in hepatic clearance capacity (27). LPS-induced IL-1 production is mediated by CD14-dependent mechanisms in the presence of plasma, whereas in its absence, both CD14-dependent and CD14-independent pathways are involved. The LPS-LBP complex attaches to CD14, that in turn activates TLR4 and other Toll-like receptors, which act as transmembrane transducers of LPS-induced cellular signaling to produce cytokines. Therefore, the TLR4 and CD14 polymorphisms may be a genetic factor responsible for individual differences in the expression of TLR4 and CD14 and the inflammatory response for luminal bacterial infections.

Two common mutations in the human TLR4 gene, *Asp299Gly* and *Thr399Ile*, have been observed to occur at a frequency of 6 to 10% in Caucasian populations (20). Individuals heterozygous for these, typically cosegregating, mutations have been shown to have reduced airway responsiveness to inhaled *Escherichia coli* LPS (1). Furthermore, primary airway epithelial cells of individuals heterozygous for these mutations were shown to be incapable of producing IL-1 in response to LPS challenge, whereas this response was intact in cells extracted from wild-type individuals (3, 22). In our study, the functional polymorphisms of TLR4 *Asp299Gly* and *Thr399Ile* alleles were not detected in all subjects. Like Guo *et al*. (8) in the colorectal cancer study, we could not find any association between TLR4 *Asp299Gly* and *Thr399Ile* alleles and HBV-related cirrhosis. However, it is worth noting that we first demonstrated that CD14 promoter -159 and -260 gene polymorphisms were associated with HBV-related cirrhotic injury in Chinese Han population.

Polymorphisms within the CD14 gene may alter the inflammatory response. CD14 -260 C /T polymorphism is located at one of the binding sites for the specificity protein transcription factors, which

are involved in regulation of the CD14 transcription and level of expression, and affects the CD14 density on the surface of monocytes in T/T homozygotes. Moreover, this polymorphism also affected the concentrations of sCD14 produced mainly by two sources, including cleavage of the receptor from monocytes and direct secretion. A recent study showed that circulating monocytes in T/T homozygotes had an increased production of interleukin-6 in response to LPS (14). The problem of the CD14 -159 and-260 gene polymorphisms and its association with the presence of coronary atherosclerosis (15, 25), asthmatic (11), periodontitis (9, 13), Crohn's disease (12), ulcerative colitis (21), *Helicobacter pylori*-related gastric malignancies (29) and liver enzyme levels (7) was investigated in a few previous studies in various ethnic groups. However, no data for the liver cirrhosis have been published so far.

The major finding of the present study is the association between the CD14 gene polymorphisms and liver cirrhosis. Our data indicated that CD14-159 CT/TT genotype frequency in cirrhotic patients is higher in comparison with healthy controls, but CD14-*260* genotypes have no significance between HBV-related cirrhosis and healthy controls. This suggested that CD14-159 CT/TT appeared to influence the HBV-infectious result. Meanwhile, in this report we demonstrated that both CD14-*159* and CD14-*260* genotype expressions were different among Child-Pugh grades in cirrhotic patients. The data implicated that CD14-*159* and -*260* gene polymorphisms might confer a genetic predisposition to HBV-related advancing cirrhosis. We hypothesized that the mutation of CD14-*159* and -*260* might influence the immunity by interfering with the signal and promoting inflammatory cytokines release, which would imply a higher risk of HBV-related cirrhotic injury.

We compared our findings with those from other investigations on the prevalence of the CD14-*159* genotypes in Germany with coronary angiography (TT 22%, CC 28%, CT 50%)(16) and Polish with myocardial infraction (CC 43.8%, CT 52.6%, TT 3.6%)(28), and that of the CD14-260 genotype in a Dutch Caucasian population (TT 23.7%)(20) and Czech patients (TT 19. 2%)(24). Our study and Yamazaki *et al*. (30)'s report indicated that the distribution of CD-159 genotype was quite different between Asian and Western populations.

In conclusion, we found no TLR4 *Asp299Gly* and *Thr399Ile* polymorphism in cirrhotic patients or healthy controls. This finding indicates the rarity of *Asp299Gly* and *Thr399Ile* polymorphisms of TLR4 among Chinese Han population. On the other hand, we found that the CD14-159 and -260 allele are significantly associated with HBV-related cirrhotic injury in this Chinese Han population. These data might implicate that CD14 is an important genetic factor for HBV-related cirrhotic injury in the Chinese Han population.

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