

Common variants of the TLR9 gene influence the clinical course of HBV infection

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Abstract. Hepatitis B virus (HBV) infection leads to the development of liver inflammation, causing morbidity and mortality. Multiple factors influence HBV progression, including genetic factors. Toll-like receptor (TLR)9 plays a key role in innate immunity, and mutations in the genes encoding this receptor have been associated with liver damage progression. Our study aimed to investigate one-tag single nucleotide polymorphisms (rs187084) representing the majority of common variations in TLR9 in a population-based study of Chinese patients. A total of 209 Chinese patients with HBV infection (130 with chronic hepatitis and 79 with liver cirrhosis) and 193 healthy individuals were studied. Our results showed that the frequencies of the C/C genotype and C allele were statistically higher in patients with HBV-related liver cirrhosis than in the healthy controls (26.6 vs. 15.5%; OR=1.97, 95% CI 1.05-3.71, $\chi^2=4.483$, P=0.034/43.1 vs. 37.8%; OR=1.49, 95% CI 1.02-2.16, $\chi^2=4.323$, P=0.038). No significant differences in the frequencies of alleles or genotypes were found between patients with chronic hepatitis B and the control subjects. In conclusion, this study is the first to show that small effects within TLR9 contribute towards the development of HBV, supporting the hypothesis that little is currently known regarding the contribution of genetic factors to HBV.

Introduction

HBV infection leads to chronic liver inflammation in the majority of patients. A substantial proportion of patients develop fibrosis or cirrhosis, causing HBV-related morbidity and mortality. Multiple factors have an impact on the progression of chronic hepatitis B to cirrhosis, and its complications include gender, age, metabolic factors and alcohol consumption

(1). In addition, genetic factors influence the progression of fibrosis. Angiotensin II, the main peptide of the renin-angiotensin system, is involved in hepatic fibrosis through hepatic stellate cell activation (2,3), while polymorphisms in the promoter region of the angiotensinogen gene have been shown to be associated with liver cirrhosis in patients with chronic hepatitis B (4). Cirrhosis results from the defective repair of liver damage resulting from inflammation caused by the effector cells of the immune system. Cytokine interferon (IFN)- α is a key mediator at the interface between innate and adaptive immunity. It is mainly produced by plasmacytoid dendritic cells (pDCs) after the engagement of toll-like receptors (TLRs) (5,6). TLR9 is promising as an immune mediator candidate in HBV infection since it is expressed in pDCs, binds cytidine-phosphate-guanosine (CpG) DNA motifs that are present in viruses, and stimulates the secretion of interferon- α (5,6) when activated. There is evidence to support a role for TLR9 in HBV infection, in that a clinical study demonstrated an antiviral effect of the TLR9 agonist CpG ODN, which was able to significantly inhibit HBV replication *in vitro* (7). The low expression of TLR9 mRNA was also recently detected in the peripheral blood mononuclear cells of chronic hepatitis B patients (8). TLR9 may therefore be a potential candidate gene contributing to HBV development or influencing the clinical course of the disease. Despite a plausible role for TLR9 in the development of HBV infection, to our knowledge no previous study has been conducted to investigate whether a common variation in the TLR9 gene is involved in HBV aetiology. We aimed to examine this issue by selecting tag single nucleotide polymorphisms (SNPs) that predicted at least 90% of the entire common variation in this gene. We then assessed the association between these SNPs and HBV infection by means of a population-based study of Chinese patients.

Materials and methods

Study population. Two hundred and nine HBV-infected patients were enrolled at Ruijin Hospital, Shanghai Jiaotong University School of Medicine. Patients were positive for HBsAg but negative for anti-HCV and -HIV. As a control group, 193 healthy blood donors were analysed. None of the subjects had a history of alcohol or drug abuse. Patients did not receive antiviral or immunosuppressive therapy prior to or during the course of the study. The HBV-infected patients were categorised into two groups according to diagnosis

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Table I. Clinical patient characteristics.

Characteristics	NC (n=193)	CHB (n=130)	LC (n=79)
Sex (M/F)	120/73	105/65	65/14
Age (years)	42	44	50
ALT (n.v. 10-60 IU/l) ^a	24 (19-37)	405 (16-1369)	160 (14-1262)
AST (n.v. 10-42 IU/l) ^a	21 (14-38)	192 (16-845)	158 (25-1607)
TB (n.v. 3.4-24.0 μ mol/l) ^a	17 (4.6-20)	84 (5.3-511)	150 (10.3-650)
DB (n.v. 0-6.8 μ mol/l) ^a	3.2 (1.4-4.8)	45 (1.3-266)	75 (2.6-315)
PT (n.v. 13 \pm 3 sec)	11 (10-13) ^d	13 (11-15) ^e	21 (12-36) ^b

^aMedian (inter-quartile range). P<0.01 for comparison with all other groups; ^b vs. ^c, P<0.01; ^b vs. ^d, P<0.01. n.v., Normal values. NC, normal control; CHB, chronic hepatitis B; LC, liver cirrhosis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IU, international units; TB, total bilirubin; DB, direct bilirubin; PT, prothrombin time.

(Table I). The groups were well-characterised, with patients exhibiting symptoms such as chronic hepatitis B (n=130) and liver cirrhosis (n=79). Classification of the groups was based on chronic viral hepatitis B-fatigue, anorexia, jaundice, hepatomegaly, splenomegaly, hyperbilirubinemia, increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and positivity for HBsAg for >6 months. Patients with liver cirrhosis had a history of HBV infection. At the time of the study, the symptoms observed were: anorexia, nausea, vomiting, malaise, weight loss, abdominal distress, jaundice, oedema, cutaneous arterial 'spider' angiomas, palma erythrema, ascites, shrunken liver, splenomegaly, mild or pronounced hyperbilirubinemia, elevated levels of serum ALT and AST and decreased serum albumin levels, along with increased serum globulins, an albumin/globulin (A/G) rate <1, and positivity for HBsAg. For the healthy controls, blood was collected from 193 blood donors. These individuals were confirmed to be negative for HBsAg, anti-HCV and -HIV by routine serology. Oral and written informed consent from the participating subjects was obtained prior to obtaining patient peripheral blood samples.

Detection of hepatitis B virus antigens and antibodies. Serum HBsAg, HBeAg, anti-HBs, anti-HBe and anti-HBc were assayed by ELISA kits (BioKit, Spain), according to the manufacturer's instructions.

Selection of single nucleotide polymorphisms. For SNP selection, Haploview software (<http://www.broad.mit.edu/mpg/haploview>) was used to conduct linkage disequilibrium using Hapmap phase genotype data for chromosomal region 3: 52,230,137-52,235,218 (chronic hepatitis B database, Hapmap release 21a, January 2007). The amplicon of interest is a 5.082-kb region encompassing the domain ~3 kb upstream and 3 kb downstream of TLR9. TagSNP selection was performed by running the tagger program implemented in Haploview. The criteria for r^2 were set at >0.8, meaning that any marker that was not eventually selected as a tagging marker was already strongly correlated with at least one of the tagging markers with r^2 >0.8. One-tag SNP (rs187084) within TLR9 was examined in 209 patients, including 130 with chronic HB and 79 with HBV-related liver cirrhosis, as well as in 193 healthy controls.

Genotype analysis. Genomic DNA was extracted from peripheral blood mononuclear cells using a commercially available kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymorphisms of T-1486C (rs187084) in the TLR9 gene were detected by PCR restriction fragment length polymorphism analysis. A 356 base-pair fragment containing the polymorphic site was amplified using TLR9-specific primers (forward 5'-CATTTCATTCAGCCTTCACTCA-3'; reverse 5'-TGACATGGGAGCAGAGACATA-3'). PCR was performed using a mixture containing 1.5 mM MgCl₂, 10 mM dNTP, 50 ng genomic DNA, 20 μ mol of each primer and 2.5 U Taq DNA polymerase (Sangon, Shanghai, P.R. China) at a volume of 50 μ l. Amplification was performed for 30 cycles with preheating at 95°C for 3 min, followed by denaturation at 94°C for 30 sec, annealing at 58°C for 40 sec and extension at 72°C for 45 sec. The PCR product was incubated with the restriction enzyme Afl for 2 h at 37°C, and the digestion products were resolved on a 3% agarose gel stained with ethidium bromide.

Statistical analysis. Distribution of alleles and genotypes among the studied groups was analysed by the χ^2 test for 2x2 or 2x3 tables. Statistical significance was defined as P<0.05. Deviation from the Hardy-Weinberg equilibrium was tested using the Pearson χ^2 test statistic (SPSS software version 11.5, SPSS Inc., Chicago, IL, USA).

Results

Clinical symptoms and liver parameters of hepatitis B virus-infected patients. In total, 209 HBV-infected individuals with well-characterised clinical profiles, including different forms of hepatic disease (chronic hepatitis and liver cirrhosis), were enrolled. Clinical details and the results of biochemical analysis from the different patient groups at the time of the study are shown in Table I.

In accordance with other studies, we observed that prothrombin time was significantly longer in patients with liver cirrhosis than in other groups (P<0.01). The AST/ALT ratio increased significantly from chronic hepatitis B to liver cirrhosis (P<0.01) patients, indicating a positive correlation with liver function impairment and progressively severe pathological processes.

Table II. Genotype distributions and allele frequencies of TLR9 polymorphisms in patients with chronic hepatitis B (CHB) and normal controls (NC).

	NC No. (%)	CHB No. (%)	χ^2 -value	OR (95% CI)	P-value
Allele					
T	240 (62.2)	152 (58.5)	0.898	1.17 (0.85-1.61)	0.343
C	146 (37.8)	108 (41.5)			
Total	386 (100)	260 (100)			
Genotype					
TT	77 (39.9)	48 (36.9)	1.076	TT+TC vs. CC	0.3
TC	86 (44.6)	56 (43.1)		1.36 (0.76-2.43)	
CC	30 (15.5)	26 (20.0)			
Total	193 (100)	130 (100)			

Table III. Genotype distributions and allele frequencies of TLR9 polymorphisms in patients with liver cirrhosis (LC) and normal controls (NC).

	NC No. (%)	CHB No. (%)	χ^2 -value	OR (95% CI)	P-value
Allele					
T	240 (62.2)	83 (56.9)	4.323	1.49 (1.02-2.16)	0.038
C	146 (37.8)	75 (43.1)			
Total	386 (100)	158 (100)			
Genotype					
TT	77 (39.9)	25 (31.6)	4.483	TT+TC vs. CC	0.034
TC	86 (44.6)	33 (41.8)		1.97 (1.05-3.71)	
CC	30 (15.5)	21 (26.6)			
Total	193 (100)	79 (100)			

Association between the TLR9 polymorphism and hepatitis B virus infection. Genotype frequencies of patients and control subjects were compatible with the Hardy-Weinberg law. Frequencies of the C/C genotype and C allele were higher in patients with HBV-related liver cirrhosis than in the healthy controls (26.6 vs. 15.5%; OR=1.97, 95% CI 1.05-3.71, $\chi^2=4.483$, P=0.034/43.1 vs. 37.8%; OR=1.49, 95% CI 1.02-2.16, $\chi^2=4.323$, P=0.038) (Table III). No significant differences in the frequencies of alleles or genotypes were found between the chronic hepatitis B patients and the control subjects (Table II).

Association between the TLR9 polymorphism and gender. Regulation of innate immune response is gender-dependent, 43-45 in the present study. A comparative study of the TLR9 polymorphism in males and females was also conducted, but no statistical differences were found in the comparison of the frequencies of alleles or genotypes with gender composition.

Discussion

It is well known that HBV elimination by the infected host is attributable to a coordinated innate and adaptive humoral and

cell-mediated immune response. TLR genes encode a family of transmembrane proteins that play an essential role in the activation and regulation of innate and adaptive immunity through their recognition of specific molecular patterns of pathogens (6). SNPs of other TLRs influence the efficacy of immune response in bacterial, viral and parasite infections. TLR2 binds lipoproteins, and its polymorphisms have been implicated in *Borrelia burgdorferi* infection as well as in tuberculosis and leprosy (9,10). TLR4 binds lipopolysaccharide, and its polymorphisms influence the course of disease in meningococcal infection (11), candidiasis (12), gram-negative bacteremia (13-15) and respiratory syncytial virus (16). Polymorphisms of the gene of flagellin-binding TLR5 are associated with susceptibility to Legionnaires' disease (17). TLR7 binds single-stranded RNA, and its polymorphisms have been implicated in HCV infection (18). Although TLR7 stimulation shows antiviral effects in HBV infection (18), TLR7 binding has only been demonstrated for RNA viruses (6), and the binding of HBV to TLR7 is unlikely. TLR9 is engaged by unmethylated CpG-rich DNA, common in bacteria and also prevalent in the genomes of DNA viruses, then triggers a cascade of events that lead to innate and adaptive immune response activation. It has been demonstrated

that the ligands for TLR9 can inhibit HBV replication in the livers of HBV transgenic mice (19), suggesting that TLR9 plays a role in regulating HBV. In our previous study, we used CpG as a tool to establish a model of innate immune response to viral molecules, and our data suggested that IFN- α secretion decreased significantly in patients with HBV infection compared to healthy controls. This was compatible with results from other studies (20,21), but these prior data did not account for potential genetic mechanisms underlying the response to the CpG motif. Recognition of these motifs requires TLR9, which induces cell signaling pathways including MAPKs and NF- κ B (5). In other viral infectious diseases, such as HIV, TLR9 polymorphisms have been found to be associated with the rapid progression of HIV-1 infection (22). For these reasons, we have studied whether such SNPs play a role in susceptibility to HBV.

Mutations in the coding region of several human genes that profoundly affect the protein sequence and influence the course of hepatitis B have been described (23-25). As well, mutations in the promoter region of various genes influence the severity of HBV infection (26). In the present study, we found the TLR9 T-1486C polymorphism to be associated with HBV-related liver cirrhosis. Although the mechanism by which this variant causes variability in the response to TLR9 activation is poorly understood, a role for an endogenous antiviral immune mediator such as TLR9 in liver damage progression seems plausible. Watanabe *et al* indicated that apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via TLR9 (27). In HCV infection, Huang *et al* demonstrated a significant difference between TLR9 gene expression in HCV cirrhosis compared to healthy liver (28). Innate immune responses linked to TLR7, whose activation stimulates the secretion of IFN- α , and TLR7 polymorphisms have been shown to be associated with inflammation and fibrosis in chronic HCV infection (18). The innate immune response generates soluble growth factors, cytokines, chemokines and extracellular proteinases, which contribute to early neoplastic development and potentiate tumorigenesis (29). TLRs are important in the innate response to pathogens. TLR9 is detected in CpG-responsive but not unresponsive monocytes (30). Altered responsiveness to viral stimuli on hepatocytes or to immune cells that infiltrate the liver may reduce the amount of damage caused by an antiviral immune response. Consequently, fibrosis and cirrhosis progression would also be delayed. TLR9 activation in the course of HBV infection is therefore a double-edged sword: activation is probably warranted in the setting of acute infection to clear the virus, but is responsible for liver scarring in the setting of chronic disease.

The regulation of innate immune response is gender-dependent. This may explain why men are more prone to infections (31), while women are more likely to suffer from autoimmune disease (32). Innate immune responses linked to TLR4 have been shown to differ between men and women, rendering men more susceptible to sepsis or endotoxin shock (33,34). A recent study observed gender differences in IFN- α production by pDCs in response to TLR7 stimulation (35). TLR7 SNPs have been found to offer protection against advanced inflammation and fibrosis in male patients with chronic HCV infection (18). In the present study, we aimed to

determine the correlation between the TLR9 polymorphism and gender. However, no associations between this SNP and gender were found.

In summary, we present the first analysis of TLR9 SNPs in patients with chronic HBV infection. Our data suggest that the TLR9 T-1486C polymorphism is associated with the occurrence of liver cirrhosis. Knowledge of this polymorphism may have predictive significance in patients with chronic HBV, allowing physicians to use more aggressive therapy in patients with a high risk of disease progression.

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