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• *BRIEF REPORTS* •

Association of polymorphism of tumor necrosis factor-alpha gene promoter region with outcome of hepatitis B virus infection

Hong-Quan Li, Zhuo Li, Ying Liu, Jun-Hong Li, Jian-Qun Dong, Ji-Rong Gao, Chun-Yan Gou, Hui Li

Hong-Quan Li, Hui Li, Ying Liu, Jian-Qun Dong, Department of Epidemiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences; School of Basic Medicine, Peking Union Medical College, Beijing 100005, China
Zhuo Li, Jun-Hong Li, Ji-Rong Gao, Chun-Yan Gou, Institute of Hepatitis Research, Beijing You'an Hospital, Capital Medical University, Beijing 100005, China
Supported by the Beijing Municipal Government Commission for Science and Technology, No. H020920020590
Correspondence to: Hui Li, Department of Epidemiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences; School of Basic Medicine, Peking Union Medical College, 5 Dongdan Santiao, Beijing 100005, China. lihui99360@sohu.com
Telephone: +86-10-65296971 Fax: +86-10-65225752
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Abstract

AIM: To determine whether -238G/A and -857C/T polymorphisms of tumor necrosis factor-alpha (TNF-α), gene promoter and hepatitis B (HB) viral genotypes were associated with outcomes of HBV infection.

METHODS: A total of 244 HBV self-limited infected subjects, 208 asymptomatic carriers, and 443 chronic HB patients were recruited to conduct a case-control study. TNF-α -238G/A and -857C/T gene promoter polymorphisms were examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and HBV genotypes were examined by nested PCR.

RESULTS: The positive rate of HBV DNA in asymptomatic carrier group and chronic HB group was 46.6% and 49.9%, respectively. HBV genotype proportion among the asymptomatic carriers was 2.1% for genotype A, 25.8% for genotype B, 68.0% for genotype C, and 4.1% for genotype B+C mixed infection, and 0.9% for genotype A, 21.7% for genotype B, 71.5% for genotype C, 5.9% for genotype B+C mixed infection in chronic HB group. There was no significant difference in genotype distribution between the asymptomatic carrier group and chronic HB group ($\chi^2 = 1.66$, $P = 0.647$). The frequency of -238GG genotype in self-limited group was 95.1%, significantly higher than 90.7% in chronic HB group and 89.0% in asymptomatic carrier group ($P = 0.041$ and $P = 0.016$, respectively).The frequency of TNF-α-857 CC in chronic HB group was 79.7%, significantly higher than 64.4% in asymptomatic carrier group and 70.9% in self-limited group ($P<0.001$ and $P = 0.023$, respectively). A multiple logistic regression analysis revealed that TNF-α-238GA and -857CC were independently associated with chronic HB after gender and age were adjusted.

CONCLUSION: TNF-α promoter variants are likely to play a substantial role in the outcome of HBV infection.

Key words: Hepatitis B; TNF-α gene; Single nucleotide polymorphism; Genotype; Haplotype

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INTRODUCTION

It is estimated that HBV has infected more than 350 million people worldwide. HBV infection causes various clinical outcomes in patients. Ninety to ninety-five percent of adults infected with HBV can eliminate the virus and only 5-10% of them become chronic HBV carriers; 20-30% of chronic HBV carriers develop chronic hepatitis B (HB) and 5% of them develop liver cirrhosis and hepatocellular carcinoma in a long term of disease course. HB has become one of the severe public health problems.

The outcome of HBV infection may be associated with host genetic factors. Cytokines play an important role in defense against viral infection, indirectly through determination of the predominant pattern of the host response, and directly through inhibition of viral replication[1]. TNF-α is one of the important cytokines involved in noncytotoxic antiviral mechanism, and participates in the viral clearance and the host immune response to HBV[2]. It was found that individuals with acute HB have higher TNF-α plasma levels than controls[3]. The capacity for the cytokine production in individuals largely depends on promoter genetic polymorphisms[4]. At present, few studies corresponding to these results are reported in China.

The outcome of HBV infection is also associated with the genetic variability of virus, which influences the expression of viral antigens. In recent years, HBV genotypes A, B, C, and D have been reported in China, and genotypes B and C are the predominant genotypes[5].

In this case-control study, we analyzed TNF-α -238G/A and -857C/T promoter genetic polymorphisms among self-limited group, asymptomatic carrier group and chronic HB group, and HBV genotypes in asymptomatic carrier group and chronic HB group to elucidate whether host genetic and viral factors were associated with the outcome of HBV infection.

MATERIALS AND METHODS

Subjects

From November 2002 to March 2004, 244 self-limited HBV-infected subjects, 208 asymptomatic carriers, and 443 chronic HB patients were recruited at Beijing You'an Hospital. The diagnostic criteria used for three groups of subjects were determined, according to the protection and treatment protocol for viral hepatitis amended by Infectious Disease Branch and Liver Disease Branch of Chinese Medical Association in September 2000, and the diagnostic criteria and treatment principle of viral HB published by National Technical Supervision Bureau and Ministry of Public Health in January 1996 (GB15990-1995). All subjects enrolled in this study provided the informed consent. All subjects were Chinese Han people and not infected with other viral hepatitis.

Serological test

ELISA was used for detection of serum HBsAg, anti-HBs, and anti-HBc (IMX, Abbott Diagnostics, North Chicago, IL, USA).

Measurement of TNF-α gene promoter polymorphism

Genomic DNA was extracted from peripheral blood leukocytes collected in EDTA by standard phenol-chloroform extraction. Allelic polymorphisms in the TNF-α gene promoter at positions -238G/A[6] and -857C/T[7,8] were amplified into 152- and 131-bp fragments by PCR with the primers (for 152-bp fragment, sense 5'AGAAGACC-CCCCTCGGAACC3' and antis-ense 5'ATCTGGAG-GAAGCGGTAGTG3'; for 131-bp fragment, sense 5'AAGTCGAGTATGGGGACCCCCCGTTAA3' and anti-sense 5'CCCCAGTGTGTGGCCATATCTTCTT3'). Amplifications of the -238 and -857 fragments were performed in a volume of 25 μL containing 50 ng of genomic DNA, 20 pmol/L of each primer, 200 μmol/L dNTP, 1.5 mmol/L $MgCl_2$, buffer and 1 U *Taq* polymerase (Shanghai Biocolor). PCR conditions of -238 were as follows: pre-denaturation at 94 ℃ for 2 min, 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, extension at 72 ℃ for 30 s, and a single final extension at 72 ℃ for 10 min in Perkin Elmer thermocycler (2700, Applied Biosystems, Foster City, CA, USA). For amplification of -857 polymorphism, annealing temperature was at 64 ℃ and other thermal cycling parameters were as the same as those for amplification of -238.

The PCR products of -238 and -857 were digested with *Msp*I and *Hinc*II restriction enzymes respectively. TNF-α -238 allele 1 was identified by 20- and 132-bp fragments and allele 2 by a single 152-bp fragment. TNF-α -857 allele 1 was identified by 25- and 106-bp fragments and allele 2 by a single 131-bp fragment.

HBV genotyping

Sequence data for the amplified region of HBV DNA were aligned from GenBank. Alignment of the sequences was performed with Vector NTI suite and Tree View software.

According to the comparisons of HBV DNA sequences and other papers[9,10], outer primers and inner primers specific for HBV genotypes are summarized in Table 1.

HBV genotypes were determined by PCR. Nested PCR primers amplified HBV DNA with two stages (Table 1). The first-stage amplification was performed in a volume of 30 μL containing 5 μL of genomic DNA, 0.1 mol/L of primers Ps1 and Ps2, 200 μmol/L dNTP, 1.5 mmol/L $MgCl_2$, buffer and 1 U *Taq* polymerase (Shanghai Biocolor). The procedure of amplification was as follows: pre-denaturation at 94 ℃ for 3 min, 35 cycles of denaturation at 94 ℃ for 40 s, annealing at 55 ℃ for 40 s, extension at 72 ℃ for 45 s. Two microliters of PCR products were required in the second-stage amplification, which involved two groups. The primers contained 0.1 μmol/L B2, 0.08 μmol/L BA1R, BB1R, and BC1R in group A. In group B, the primers contained 0.1 μmol/L B2R, 0.08 μmol/L BD1, BE1, and BF1. Thermal cycling parameters involved 94 ℃ for 3 min, 20 cycles of 94 ℃ for 40 s, 58 ℃ for 40 s, and 72 ℃ for 40 s, and 20 cycles of 94 ℃ for 40 s, 60 ℃ for 40 s, and 72 ℃ for 40 s. PCR products of the amplification were illuminated using ultraviolet rays, after electrophoresis on 6% acrylamide gel stained by EB.

Table 1 Nested PCR primer sequences for HBV genotype

Primer	sequence
Outer primers	
Ps1	5′TCACCATATTCTTGGGAACAAGA3′
Ps2	5′CGAACCACTGAACAAATGGC3′
Inner primers	
Group A	
B2	5′GGCTCCAGTTCCGGAACAGT3′
BA1R	5′CTCGCGGAGATTGACGAGATG3′
BB1R	5′CAGGTTGGTGAGTGACTGGAG3′
BC1R	5′GGTCCTAGGAATCCTGATGTTG3′
Group B	
BD1	5′GCCAACAAGGTAGGAGCT3′
BE1	5′CACCAGAAATCCAGATTGGGACCA3′
BF1	5′GCTACGGTCCAGGGTTACCA3′
B2R	5′GGAGGCGGATVTGCTGGCAA3′

Statistical analysis

Comparisons were performed using one-way ANOVA and χ^2 test. SAS version 6.12 software package was used to analyze data. All P values were two-tailed, and $P<0.05$ was considered statistically significant. Haplotypic analysis was performed using EH2.0 program.

RESULTS

Characteristics of subjects

The average age of chronic HB patients, asymptomatic carriers, and self-limited subjects was 33.37±12.67, 31.2±10.78, and 34.68±11.34 years, respectively. A significant difference was found in age of the three groups ($F = 4.985$, df = 2, $P = 0.008$).

The ratio of male to female patients (354/89) in the chronic HB group was significantly higher than those (136/108 and 113/95) of the self-limited group and the asymptomatic carrier group ($P<0.01$).

Association of HBV genotypes with outcomes of HBV infection

The positive rate of HBV DNA in the asymptomatic carrier group and the chronic HB group was 46.6% and 49.9%, respectively (Table 2).

The proportions of subjects with HBV genotypesA, B, C and mixed genotype B+C among the asymptomatic carriers were 2.1%, 25.8%, 68.0% and 4.1%. Among the chronic HB patients, the proportion of HBV genotypes was 0.9% for genotype A, 21.7% for genotype B, 71.5% for genotype C, 5.9% for mixed genotypes B+C. The difference in genotype distribution between the asymptomatic carrier group and chronic HB group was statistically significant ($\chi^2 = 1.66$, $P = 0.647$) (Table3).

Table 2 Positive rate of HBV DNA in asymptomatic carrier group and chronic HB group

Group	*n*	HBV DNA positive (%)
Carrier	208	97 (46.6)
Chronic HB	443	221 (49.9)

Table 3 Proportions of HBV genotype in asymptomatic carrier group and chronic HB group

Group	*n*	A	B	C	B+C
Carrier group	97	2 (2.1)	25 (25.8)	66 (68.0)	4 (4.1)
Chronic HB group	221	2 (0.9)	48 (21.7)	158 (71.5)	13 (5.9)

$\chi^2 = 1.66$, df = 3, $P = 0.647$.

Association of TNF-α -238G/A and -857C/T genotypes with outcomes of HBV infection

The frequency of -238GG genotype in the self-limited group was 95.1%, significantly higher than 90.7% in the chronic HB group and 89.0% in the asymptomatic carrier group ($P = 0.041$ and $P = 0.016$), respectively. No significant difference was found between chronic HB and carrier groups ($P = 0.496$). The frequency of -238G allele in the self-limited group was 97.5%, significantly lower than that in the other two groups ($\chi^2 = 3.99$, $P = 0.046$). There was no significant difference in Hardy-Weinberg among the three groups ($\chi^2 = 0.155$, $P = 0.69$, Table 4).

The frequency of TNF-α -857CC in chronic HB group was 79.7%, significantly higher than 64.4% of carrier group and 70.9% of self-limited group ($P<0.001$ and $P = 0.023$), respectively. The frequency of -857C allele in chronic HB group was 87.6%, significantly higher than those of the two groups ($\chi^2 = 17.953$, $P<0.001$). There is no significant difference in Hardy-Weinberg among the three groups ($\chi^2 = 3.54$, $P = 0.059$, Table 5).

The results of haplotype analysis are summarized in Table 6. The frequency of haplotype -238G/-857 C in asymptomatic carrier group was lower than that in the self-limited group ($P = 0.035$) and that of carrier group ($P = 0.04$). Frequency of haplotype -238G/-857T in carrier group was higher than that of chronic HB group ($P = 0.030$, Table 6).

Multivariate logistic regression analysis

Multivariate unconditional logistic regression model was

Table 4 TNF-238 polymorphism and allele frequencies in chronic HB, asymptomatic carrier and self-limited groups

Group	*n*	Genotype (%)					Alleles (%)[1]	
		GG	GA	AA	χ^2	*P*	G	A
Chronic HB group	433	402 (90.7)	41 (9.3)	0 (0.00)			845 (95.4)	41 (4.6)
Carrier group	208	187 (89.0)	23 (11.0)	0 (0.00)	0.464 (to patient)	0.496 (to patient)	397 (94.5)	23 (5.5)
Self-limited group	244	232 (95.1)	12 (4.9)	0 (0.00)	4.157 (to patient)	0.041 (to patient)	476 (97.5)	12 (2.5)
					5.776 (to carrier)	0.016 (to carrier)		

[1]$\chi^2 = 3.99$, $P = 0.046$. Hardy–Weinberg $\chi^2 = 0.155$, $P = 0.69$.

Table 5 TNF-857 polymorphism and allele frequencies in chronic HB, asymptomatic carrier and self-limited groups

Group	*n*	Genotype (%)					Alleles (%)[1]	
		CC	CT	TT	χ^2	*P*	C	T
Chronic HB	433	345 (79.7)	69 (15.9)	19 (4.4)			759 (87.6)	107 (12.4)
Carrier	208	134 (64.4)	59 (28.4)	15 (7.2)	17.358 (to patient)	<0.001 (to patient)	327 (78.6)	89 (21.4)
Self-limited	244	173 (70.9)	60 (24.6)	11 (4.5)	7.710 (to patient)	0.023 (to patient)	406 (83.2)	82 (16.8)
					2.728 (to carrier)	0.079 (to carrier)		

[1]$\chi^2 = 17.953$, $P<0.001$. Hardy-Weinberg $\chi^2 = 3.54$, $P = 0.059$.

Table 6 Frequencies of TNF-238/857 haplotype among chronic HB, carrier and self-limited groups

Number	Haplotype	Self-limited group (%)	Carrier group (%)	Chronic HB group (%)	*P* (1 to 2)	*P* (1 to 3)	*P* (2 to 3)
1	GC	0.8115	0.7297	0.8278	0.035	0.618	0.040
2	AC	0.0205	0.0554	0.0478	0.052	0.069	0.708
3	GT	0.1639	0.2148	0.1246	0.154	0.156	0.030
4	AT	0.0041	0.0002	0.0004	0.367	0.184	0.487

Table 7 Multivariate logistic regression analysis

Variable	Self-limited group to chronic HB group			Carrier group to chronic HB group			Self-limited group to carrier group		
	χ^2	*P*	OR	χ^2	*P*	OR	χ^2	*P*	OR
Intercept	11.05	0.0009	–	8.86	0.0029	–	11.43	0.0007	–
Sex (male = 1, female = 0)	46.74	0.0001	3.02	34.32	0.0001	3.67	0.6694	0.4133	1.18
Age (yr)	4.06	0.044	0.984	4.22	0.0399	0.903	11.41	0.0007	0.97
-238GA (GA = 1, GG = 0)	4.82	0.036	1.4	4.09	0.0427	1.927	8.91	0.0028	3.19
-857CC (CC = 1, TT+CT = 0)	8.54	0.0035	1.59	9.14	0.0001	2.41	0.664	0.415	1.19

used to analyze the association of the outcome of HBV infection with sex, age, TNF-α promoter polymorphisms. The results showed that, after confounding effects of gender and age were adjusted, TNF-α -238GA and -857CC were independently associated with chronic HB compared to self-limited group and asymptomatic carrier group. TNF-α -238GA was associated with asymptomatic carrier compared to self-limited group (Table 7).

DISCUSSION

HBV has eight different genotypes (A-H) according to the homogeneity of the viral sequence[11]. HBV genotypes have distinct geographical distribution. The results of several studies[12,13] indicated that HBV genotypes B, C, and mixed genotype are prevalent in China, and genotypes B and C are the major genotypes. The results of our study are in agreement with the studies conducted in other areas of China[12,13].

Some studies[13] indicated that there is a significant difference in HBV genotype distribution in different places of China. It was reported that the proportion of genotype C in northern cities is higher than that of southern cities of China[13]. In addition, infection with mixed genotypes has also been found in China.

We also found that there was no statistically significant difference in genotype distribution between the asymptomatic carrier group and chronic HB group, But another study[14] reported that the proportion of genotype C in the chronic HB group was higher than in the asymptomatic carrier group. The results are similar to another study[14].

Our findings suggest that both genotypes TNF-α -238GA and -857CC are positively associated with chronic HB. The frequency of -238GA genotype in self-limited group was significantly lower than that in the chronic HB group and asymptomatic carrier group, which is consistent with that in Caucasian[6] and Korean[15] population. The frequency of TNF-α -857CC in the chronic HB group was significantly higher than that in the self-limited group, which is consistent with another study[2]. TNF-α can suppress expression and replication of HBV. *In vitro* recombinant TNF-α inhibits HBV replication through a post-translational mechanism that accelerates the degradation of HBV messenger RNA[16]. Furthermore, HBV core promoter element is sensitive to TNF-α[17].

The results of multiple logistic regression analysis indicate that TNF-α -238GA and -857CC are independently associated with chronic HB, after adjustment for sex and age. Males seem more likely to develop chronic HB. The mechanism underlying the association remains unclear, but viral replication is sensitive to sex hormones[18].

In conclusion, HBV genotypes may play an important role in the outcome of HBV infection, and TNF-α promoter genetic polymorphisms may play an important role in the development of HBV infection.

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