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BRIEF ARTICLE

Relationship between interleukin-6 polymorphism and susceptibility to chronic hepatitis B virus infection

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Abstract

AIM: To identify the relationship between tag single nucleotide polymorphisms (tag SNPs) of interleukin-6 (*IL-6*) gene and susceptibility to chronic hepatitis B virus (HBV) infection in a Han Chinese population.

METHODS: We performed a case-control study of 501 Chinese patients with chronic HBV infection and 301 self-limiting HBV-infected individuals as controls. Genomic DNA was isolated from the whole blood of all subjects using phenol/chloroform with MaXtract high-density tubes. Tag SNPs were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. Four tag SNPs in *IL-6* (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) were genotyped by the Multiplex SnaPshot technique. The genotype and allele frequencies were calculated and analyzed.

RESULTS: Five haplotypes were involved in the analysis, with frequencies higher than 0.03. One of the haplotypes, TTAA, was significantly different between the two groups. Overall haplotype *P* values were: ATAA, *P* = 0.605, OR (95%CI) = 1.056 (0.860-1.297); TGAG, *P* = 0.385, OR (95%CI) = 1.179 (0.813-1.709); TGGG, *P* = 0.549, OR (95%CI) = 1.087 (0.827-1.429); TTAA, *P* = 0.004, OR (95%CI) = 0.655 (0.491-0.873); TTAG, *P* = 0.266, OR (95%CI) = 1.272 (0.832-1.944). However, the four SNPs showed no significant genotype/allele associations with susceptibility to chronic HBV infection. Overall allele *P* values were: rs17147230, *P* = 0.696, OR (95%CI) = 1.041 (0.850-1.276); rs2066992, *P* = 0.460, OR (95%CI) = 1.090 (0.868-1.369); rs2069837, *P* = 0.898, OR (95%CI) = 0.983 (0.759-1.274); rs2069852, *P* = 0.165, OR (95%CI) = 0.859 (0.693-1.064). Overall genotype *P* values were: rs17147230, *P* = 0.625; rs2066992, *P* = 0.500; rs2069837, *P* = 0.853; and rs2069852, *P* = 0.380.

CONCLUSION: The four tag SNPs of *IL-6* gene may be associated with susceptibility to chronic HBV infection in the Han Chinese population.

Key words: Chronic hepatitis B virus infection; Interleukin-6; Single nucleotide polymorphism; Genetic susceptibility; Haplotype

Core tip: This study included a large number of subjects with a single ethnic background (Chinese). This would further add to the statistical power of the analysis and identify more single nucleotide polymorphisms. We selected self-limiting hepatitis B virus-infected subjects, but not unexposed subjects as controls, therefore, our results may be more reliable than other studies that

recruited blood donors as controls. In addition, we included only antiviral-naive subjects.

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INTRODUCTION

The outcome of hepatitis B virus (HBV) infection is mainly influenced by the virus, immune response and genetic diversity[1-3]. Many studies strongly support that host genetic variety plays an important role in determining the outcome of HBV infection[4,5]. Host genetics such as single nucleotide polymorphisms (SNPs) of a variety of genes have been implicated in the diversity of HBV clinical course[6-9]. Recent studies have shown that cytokine genetic polymorphisms are associated with the development of chronic HBV infection and progression of the infection[10].

Interleukin-6 (IL-6) is a pleiotropic cytokine with a pivotal function in regulation of the biological responses of several target cells including hepatocytes[11]. Kuo *et al*[12] found that IL-6 was able to effectively suppress HBV replication and prevent the accumulation of HBV covalently closed circular DNA (cccDNA) in a human hepatoma cell line. Cussigh *et al*[13] found that *IL-6* promoter polymorphisms influence the development of chronic hepatitis C virus infection. Another study suggested that *IL-6* gene polymorphisms may be associated with the outcome of allogeneic hematopoietic stem cell transplantation, particularly in patients transplanted from a related donor[14]. However, the exact mechanism involving *IL-6* in the outcome of HBV infection is unknown. The present study investigated the association between the tag SNPs of the *IL-6* gene and genetic susceptibility to chronic HBV infection.

MATERIALS AND METHODS

Patients

Eight hundred and two Han Chinese with HBV infection were enrolled in this study. Following recruitment, the subjects gave informed consent for genetic analysis. No abnormalities were observed in these subjects based on physical examination, chest radiography, electrocardiogram, urinalysis and routine laboratory blood testing. Liver, renal, endocrine and cardiovascular disorders were excluded. Five hundred and one were chronic HBV infected patients (221 males and 280 females). The remaining 301 were HBV natural clearance individuals and served as the control group (143 females and 158 males). The average age was 44.2 years for HBV chronic carriers and 44.9 years for controls. All patients with chronic HBV infection fulfilled the diagnostic criteria: positive for hepatitis B surface antigen (HBsAg) for a period of at least 6 mo, serum HBV DNA level > 1000 copies/mL, and elevated alanine aminotransferase or aspartate aminotransferase (> 40 IU/mL). The clinical criteria for self-limiting HBV infected patients were: positive for hepatitis B surface antibody and hepatitis B core antibody, but negative for HBsAg, and no history of HBV vaccination. Controls were age- and sex-matched subjects ($P > 0.1$). All cases and controls were followed for more than 6 mo. None of the patients had received anti-HBV therapy.

Isolation of DNA from whole blood

Genomic DNA was isolated from the whole blood of all subjects using phenol/chloroform with MaXtract high-density tubes. Genomic DNA was extracted from the peripheral blood leukocyte pellet using a DNA extraction kit (Yuan Ping-Hao Biotechnology Co., Ltd. Tianjin, China), according to the manufacturer's instructions. The DNA samples were stored at -80 ℃ at a concentration of 100 ng/μL.

Tag SNP selection

We selected SNPs on the basis of the following principal criteria: tag SNPs were identified using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The criteria for tag SNPs were $r^2 > 0.8$, minor allele frequency (MAF) > 0.1, functional relevance and importance, and SNPs significantly associated with diseases in previous studies. Four tag SNPs in the *IL-6* gene (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) were selected, which captured 100% of common SNPs (MAF > 0.1) in the HapMap Chinese database at $r^2 > 0.8$.

Genotyping

The four SNPs in *IL-6* were genotyped using the Multiplex SNaPshot technique. The primers and probes were designed by Primer 5.0 software and were rs17147230 (5' to 3'): forward primer: AAAAGGGCAAGGAAGGGAGGTA, reverse primer: CACGA GTCATTTG AGCCATCTTTG, and extension primer: TTTTTTTTTTTTTTTTTTTTTGAGTT CAGTGTCATCAGCAGAAACT; rs2066992 (5' to 3'), forward primer: CTTCCTGCTGGAACATTC-TATGGC, reverse primer: TTTCTGCCAGTGC CTCTTTGC, and extension primer: TTTTTTTTTTT TTTTTTTTTTTTTTTTT TCCTAAAACAAACAC-CACTAGAGGG. rs2069837 (5' to 3'), forward primer: CTTCCTGCT GGAACATTCTATGGC, reverse primer: TTTCTGCCAGTGC CTCTTTGC, and extension primer: TTTTTTTAAATTTGTTTTGAAGATTAG ACACAATATTTAT. rs2069852 (5' to 3'): forward primer: CGTCATTTAACCCCAGCACTTG, reverse primer: GGATTTTCTACATCAT CCCTCAGTTCC, and extension primer: TTTTTT TTTTGCACTTG-CACA CTCCTTTCTG. The polymerase chain reaction

(PCR) amplification conditions were: a 15-μL final volume containing 10 × 1.5 μL buffer, 0.3 μL dNTPs (10 mmol/L), 0.9 μL $MgCl_2$ (25 mmol/L), 0.1 μL Taq DNA polymerase (TAKARA Biotechnology Co. Ltd., Dalian, China), 0.5 μL each primer (10 pmol/L), and 1 μL DNA template (20 mg/L). Conditions for the multiplex PCR reaction using Touch-down PCR response procedures included initial denaturation at 95 ℃ for 15 min, denaturation at 94 ℃ for 40 s, annealing at 63 ℃ for 1 min, and recursive-descent at 0.5 ℃, followed by extension at 72 ℃ for 1.5 min, for a total of 15 cycles. This was followed by 25 cycles of denaturation at 94 ℃ for 40 s, annealing at 56 ℃ for 40 s, and extension at 72 ℃ for 1.5 min, with a final extension at 72 ℃ for 8 min. Amplified samples were stored at 4 ℃. After amplification, 1.5 μL PCR product was examined on an agarose gel to test for successful amplification.

SNaPshot reaction

The purified PCR product, each concentration of 0.2 μmol/L SNaPshot primer mixtures, and SNaPshot fluorescent mixtures (containing Taq DNA polymerase and different fluorescently labeled ddNTP, TAKARA Biotechnology Co. Ltd., Dalian, China) consisted of a PCR system. SNaPshot response procedures were: initial denaturation at 96 ℃ for 10 s; denaturation at 96 ℃ for 10 s, annealing at 53 ℃ for 5 s, extension at 60 ℃ for 30 s, for a total of 25 cycles, and finally extension at 60 ℃ for 30 s. Amplified samples were stored at 4 ℃. SNaPshot PCR products using SAP purification, in 10 μL SNaPshot PCR product with 1 U SAP or 1 U CIP, were mixed and incubated at 37 ℃ for 1 h, and 75 ℃ for 15 min to inactivate the enzyme. The samples can be stored at 4 ℃ for 24 h or -20 ℃ permanently.

DNA sequencing

The SNaPshot product was diluted 20-fold. In a total volume of 10 μL, we mixed 8.6 μL HiDiFormamide (high-purity formamide), 0.9 μL GeneScan-120 LIZ Size Standard, and 0.5 μL SNaPshot purification product. Samples were incubated at 95 ℃ for 5 min, chilled quickly for 4 min, and then loaded on an ABI 3730XL Genetic Analyzer (Applied Biosystems, CA, United States) for capillary electrophoresis, running GeneMapper 4.0 software for analysis of the experimental results.

Statistical analysis

Allele and genotype frequencies were obtained by direct counting, and the χ^2 test was used to compare allele and genotype distributions. The quality of the genotype data was assessed by Hardy-Weinberg equilibrium in the case and control samples using Fisher's exact test ($P > 0.05$). OR and 95%CI were calculated according to Woolf's method.

RESULTS

We investigated the distribution of the four SNPs in 501 Chinese HBV-infected patients (cases) and 301 self-limiting HBV-infected patients (controls). All genotypes of the *IL-6* polymorphisms were in Hardy-Weinberg equilibrium in both the cases and controls.

The genotype frequencies and allele distributions of the *IL-6* polymorphisms in each subgroup of HBV-infected patients are summarized in Table 1. The genotype frequencies for AA, AT, and TT of *IL-6* rs17147230 were 30.9%, 48.1%, and 21.0% in case samples, and 28.2%, 51.5%, and 20.3% in control samples, respectively, without significant differences between cases and controls ($P = 0.625$). The genotype frequencies for GG, GT and TT of *IL-6* rs2066992 were 9.0%, 37.9%, and 53.1% in case samples, and 6.6%, 39.2%, and 54.2% in control samples, respectively, without significant differences between cases and controls ($P = 0.510$). The genotype frequencies for AA, AG, and GG of *IL-6* rs2069837 were 66.1%, 30.1%, and 3.8% in case samples, and 67.1%, 28.6%, and 4.3% in control samples, and no significant differences were noted ($P = 0.853$). The genotype frequencies for AA, AG and GG of *IL-6* rs2069852 were 41.5%, 445.7%, and 12.8% in case samples, and 45.8%, 43.9%, and 10.3% in control samples, and no significant differences were noted ($P = 0.380$). In addition, no statistically significant differences were found when the allele frequencies of SNPs rs17147230, rs2066992, rs2069837 and rs2069852 were compared between patients with chronic HBV infection and controls. Overall allele *P* values were: rs17147230, $P = 0.696$, OR (95%CI) = 1.041 (0.850-1.276); rs2066992, $P = 0.460$, OR (95%CI) = 1.090 (0.868-1.369); rs2069837, $P = 0.898$, OR (95%CI) = 0.983 (0.759-1.274); rs2069852, $P = 0.165$, OR (95%CI) = 0.859 (0.693-1.064).

Haplotype analysis

We also estimated the *IL-6* haplotype frequencies and evaluated the association among these variants and HBV infection. We observed five haplotype combinations, but found no significant association in the distribution of the haplotype frequencies between cases and controls ($P > 0.05$) except "TTAA" where the protective haplotype was associated with lower disease susceptibility. The haplotype "TTAA" was observed to be significantly associated with control subjects compared with patients [$P < 0.05$, OR (95%CI) 0.655 (0.491-0.837)]. Haplotype frequencies lower than 0.03 were ignored in the analysis (Table 2).

DISCUSSION

Several cytokine polymorphisms are associated with the natural history of HBV infection. Zhang *et al*[15] proved that persistent HBV infection susceptibility is associated with the gene polymorphism *IL-10* -1082 GA in the Chinese population and that clearance of HBV is associated with the gene polymorphism *IL-10* -592 CA in the Chinese population. *IL-10* -1082 G/G and *IL-12β* -10993 C/G are associated with early, spontaneous HBeAg seroconversion[16]. Another study indicated that the -148C,

Table 1 Genotype and allele distributions of interleukin-6 tag single nucleotide polymorphisms in patients with chronic hepatitis B virus infection and those with self- limiting hepatitis B virus infection *n* (%)

IL-6 SNP site	Chronic HBV infection	Self-limiting HBV infection	*P* value	OR (95%CI)
rs17147230	*n* = 501	*n* = 301		
AA	155 (0.309)	85 (0.282)		1.0
AT	241 (0.481)	155 (0.515)	0.348	0.853 (0.611-1.189)
TT	105 (0.210)	61 (0.203)	0.784	0.944 (0.625-1.425)
A	551 (0.550)	325 (0.540)	0.696	1.041 (0.850-1.276)
T	451 (0.450)	277 (0.460)		
rs2066992				
GG	45 (0.090)	20 (0.066)		1.0
GT	190 (0.379)	118 (0.392)	0.252	0.716 (0.403-1.271)
TT	266 (0.531)	163 (0.542)	0.261	0.725 (0.414-1.272)
G	280 (0.279)	158 (0.262)	0.460	1.090 (0.868-1.369)
T	722 (0.721)	444 (0.738)		
rs2069837				
AA	331 (0.661)	202 (0.671)		1.0
AG	151 (0.301)	86 (0.286)	0.670	1.072 (0.780-1.472)
GG	19 (0.038)	13 (0.043)	0.758	0.892 (0.431-1.845)
A	813 (0.811)	490 (0.814)	0.898	0.983 (0.759-1.274)
G	189 (0.189)	112 (0.186)		
rs2069852				
AA	208 (0.415)	138 (0.458)		1.0
AG	229 (0.457)	132 (0.439)	0.364	1.151 (0.850-1.559)
GG	64 (0.128)	31 (0.103)	0.198	1.370 (0.848-2.213)
A	645 (0.644)	408 (0.678)	0.165	0.859 (0.693-1.064)
G	357 (0.356)	194 (0.322)		

IL-6: Interleukin-6; HBV: Hepatitis B virus; SNP: Single nucleotide polymorphism.

Table 2 Distribution of haplotypes of interleukin-6 tag single nucleotide polymorphisms in patients with chronic hepatitis B virus infection and those with self-limiting hepatitis B virus infection

Haplotypes	Frequency (cases)	Frequency (controls)	χ^2	*P* value	OR (95%CI)
ATAA	526.10 (0.525)	306.81 (0.510)	0.267	0.6051	1.056 (0.860-1.297)
TGAG	89.34 (0.089)	45.98 (0.076)	0.753	0.3854	1.179 (0.813-1.709)
TGGG	172.46 (0.172)	96.25 (0.160)	0.358	0.5494	1.087 (0.827-1.429)
TTAA	117.81 (0.118)	101.19 (0.168)	8.380	0.0038	0.655 (0.491-0.873)
TTAG	70.30 (0.070)	33.8 (0.056)	1.237	0.2660	1.272 (0.832-1.944)

+8925G and +13925C alleles of the *IL-18* gene are likely associated with HBV clearance in a Korean population[17]. However, Lee *et al*[18] found that the polymorphisms near the *IL-28B* gene, rs8099917T>G, rs12979860C>T and rs12980275A>G, are not significantly associated with the natural course of chronic HBV infection.

IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine. Hösel *et al*[19] found that IL-6 ensures early control of the virus, limiting activation of the adaptive immune response and preventing death of HBV-infected hepatocytes. IL-6 may play an extremely important role in determining liver progression[20]. Polymorphism of the *IL-6* promoter -572 loci may be associated with HCC occurrence in men[21]. Fabris *et al*[22] found that fewer patients aged < 50 years who carried one of the IL-6 high producer (G/G or G/C) genotypes experienced HBsAg loss in comparison with patients aged > 50 years and/or carriers of the IL-6 low producer C/C genotype. This indicates that possessing an IL-6 low producer phenotype may provide some advantage to older patients with chronic HBV infection. This is consistent with other findings which showed that *IL-6* -174 G>C polymorphism may play a role in the clinical evolution of HBV infection at least in European countries where a higher prevalence of the C allele was detected in comparison with patients from the Far East[23]. However, Park *et al*[24] found that variants in the *IL-6* gene are not associated with subsequent HBV outcomes, although *IL-6* has been found to be functionally significant in other diseases[25-27].

To identify the relationship between the SNPs of *IL-6* gene and genetic susceptibility to chronic hepatitis B virus infection in the Han Chinese population, we selected four SNPs in *IL-6* (*rs1714230*, *rs2066992*, *rs2069837* and *rs2069852*) using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The four tag SNPs captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at r^2 > 0.8. We analyzed the associations of four SNP alleles with chronic HBV infection compared with self-limiting HBV infection. The results indicated that tag

SNPs of *IL-6* may be a prognostic factor for chronic hepatitis B infection. There are several advantages of this study which strengthen it compared with previous similar studies, and increase the reliability of our results. First, our study included a large number of subjects with a single ethnic background (Chinese). This would further add to the statistical power of the analysis and identify more SNPs. Second, we selected self-limiting HBV-infected subjects, but not unexposed subjects, as controls, therefore, our results may be more reliable than other studies that recruited blood donors as controls. Third, we included only antiviral-naive subjects. In contrast, previous similar studies which investigated the natural course of chronic HBV infection did not set any limitation regarding antiviral treatment.

In conclusion, *IL-6* is a functional gene which plays a relevant role in the pathogenesis of HBV. Our study demonstrates that the tag SNPs of *IL-6* may be related to genetic susceptibility to chronic HBV infection in Chinese patients. Further genetic studies are needed to examine other SNPs in *IL-6* and their possible association with disease progression in chronic HBV infection.

COMMENTS

Background

Persistent hepatitis B virus (HBV) infection is considered a multifactorial and polygenic disorder with viral, environmental, and genetic components, as well as contributions from HBV genomic variability, host age, gender, concurrent infection with the hepatitis C virus, hepatitis D virus, and human immune deficiency virus. Interleukin-6 (IL-6) plays an important role in the response of the innate immune system to viral infection. *IL-6* polymorphisms affect induction of IL-6 expression.

Research frontiers

This study is the first to investigate the association between four tag single nucleotide polymorphisms (tag SNPs) (*rs17147230A/T*, *rs2066992G/T*, *rs2069837A/G* and *rs2069852A/G*) of *IL-6* and genetic susceptibility to chronic HBV infection in Chinese patients using the Multiplex SNaPshot technique.

Innovations and breakthroughs

The four tag SNPs of *IL-6* may be related to genetic susceptibility to chronic HBV infection.

Applications

Based on the results of this study, further genetic studies are needed to examine the roles of other *IL-6* SNPs and their association with disease progression in chronic HBV infection.

Terminology

IL-6 is a pleiotropic cytokine with a pivotal function in regulation of the biological responses of several target cells including hepatocytes. It acts as both a pro-inflammatory and anti-inflammatory cytokine.

Peer review

The manuscript analyzed the association between polymorphisms of *IL-6* and clinical outcomes of HBV infection. Four SNPs of *IL-6* were genotyped using Multiplex SNaPshot technique and compared between chronic HBV infection and self-limiting HBV infection patients. The overall data showed that SNPs of *IL-6* may affect the outcome of HBV infection. The data may have a significant clinical implication.

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