Original Article

Single Nucleotide Polymorphisms rs2227284, rs2243283 and rs2243288 in the IL-4 Gene Show no Association with Susceptibility to Chronic Hepatitis B in a Chinese Han Population

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Objective To investigate the relationship between single nucleotide polymorphisms (SNPs) of the interleukin-4 (IL-4) gene and outcome of hepatitis B virus (HBV) infection in a Chinese Han population.

Methods Total of 501 patients with chronic hepatitis B virus (HBV) infection and 301 controls with self-limiting HBV infection were studied. Three tag SNPs in the IL-4 gene (rs2227284G/T, rs2243283C/G and rs2243288A/G) were genotyped by the Multiplex snapshot technique. The genotype and allele frequencies were calculated and analyzed.

Results The three SNPs showed no significant genotype/allele associations with chronic HBV infection. Overall allele *P* values were: rs2227284, *P* = 0.655, odds ratio (OR) [95% confidence interval (CI)] = 1.070 (0.793-1.445); rs2243283, *P* = 0.849, OR (95% CI) = 0.976 (0.758-1.257); rs2243288, *P* = 0.659, OR (95% CI) = 1.060 (0.818-1.375). Overall genotype *P* values were: rs2227284, *P* = 0.771; rs2243283, *P* = 0.571; rs2243288, *P* = 0.902. There were no statistically significant differences between patients with chronic HBV infection and controls. Haplotypes generated by these three SNPs also had no significant differences between the two groups.

Conclusions The three tag SNPs of IL-4 were not associated with the outcome of HBV infection in the Han Chinese population.

Key words: Hepatitis B virus; IL-4 gene; Polymorphism

Hepatitis B virus (HBV) infection is a worldwide health problem. Approximately 2 billion people worldwide have been infected with HBV, and more than 350 million are chronic carriers with increased risks of developing liver cirrhosis and hepatocellular carcinoma.¹ However, there are some cases that never evolve into any significant liver disease within the patient's natural lifespan.^{2,3} Variations outcome of HBV among individuals are influenced by several factors such as

environment, genetic, and immune components. Host immunological and genetic factors may influence the course of the disease.^{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

Interleukin-4 (IL-4) is a Th2 cytokine produced by Th2 cells, mast cells, basophils and NK cells. Like other cytokines, IL-4 can have a variable affect on a number of different target cells, including B cells, T cells, monocytes, endothelial cells and hepatocytes.¹⁰ It plays a key role in the modulation of the homeostasis of T lymphocyte subsets. Imbalance between these T lymphocyte subsets has been implicated in response to therapeutic vaccination in humans and susceptibility to human autoimmune diseases.^{11,12} What's more, IL-4 has the function of inhibiting the production of IFN- γ and modulating inflammatory responses by down regulating production of pro-inflammatory mediators

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Supported by Grants from the National Natural Science Foundation of China, No. 81072342; the National Pre-973 Program Projects, No. 2009CB526411

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and preventing inflammatory injury of the liver.¹³⁻¹⁵ Genetic mutations in the IL-4 gene may alter its expression and its downstream signaling, which may contribute to an individual's susceptibility to immune-related liver disease.¹⁶

This study attempted to use tag SNPs to investigate the relationship between three SNPs in IL-4 and chronic HBV infection susceptibility by comparing the results of chronic HBV infection cases to those of acute infection controls.

PATIENTS AND METHODS

Patients

The case-control group included 802 unrelated Han Chinese adults, who were outpatients and inpatients from the First and Second Hospitals Affiliated to Anhui Medical University. Among these patients, 501 chronic HBV infection patients (221 men and 280 women; average age, 44.2 years) recruited from Infectious Disease Department were arranged as experimental group and 301 acute HBV infection individuals (188 men and 143 women; average age, 44.9 years) were considered as controls. All the patients with chronic HBV infection fulfilled the diagnostic criteria of the Proposal of Prevention and Treatment of Viral Hepatitis, 2005, issued by the Chinese Society of Infectious Diseases and

Parasitology and the Society of Hepatology of the Chinese Medical Association.¹⁷ Clinical criteria for acute HBV infection were positive for HBsAb and HBcAb or positive for HBcAb, negative for HBsAg, and no history of HBV vaccination.

Tag SNP Selection

SNPs in IL-4 were analyzed and compared for minor allele frequencies (MAFs), haplotype frequencies, linkage disequilibrium patterns, and tag SNPs by using CHB HapMap data of the HaploView 4.2 program. Eligible SNPs were screened using quality control criteria of genotyping call rate of $> 80\%$, HardyWeinberg equilibrium (HWE; $P > 0.05$), and minor allele frequency (MAF) of > 0.1 . Tag SNPs were selected from eligible SNPs using the criteria of $r^2 \geq 0.8$ and pairwise log odds ratio of ≥ 3 and compared among the CHB samples. Common tag SNPs for CHB were directly reported by Haploview program or identified from SNPs that were highly related to the tag SNPs directly reported by Haploview program. Three tag SNPs of the IL-4 gene (rs2227284, rs2243283 and rs2243288) were selected (Figure 1).

Genotyping

The three SNPs were genotyped using the Multiplex Snapshot technique. The primers and probes

Figure 1. IL-4 gene locus and linkage disequilibrium (LD) structure.

Notes: The deepening of color implies higher LD among the SNPs. SNP rs2227284 or rs2243283 or rs2243288 is a tag SNP ($r^2 > 0.8$) for rs2243250, rs2070874, rs2227284, rs2227282, rs2243267, rs2243268, rs2243270, rs2243283, rs2243288 and rs2243290.

SNPs	Genotypes	Groups			
		Chronic HBV infection cases		Acute HBV infection controls	
		Number	<i>P</i> values for HWE	Number	<i>P</i> values for HWE
rs2227284	TT	372	0.396	229	0.969
	GT	122		67	
	GG	7		5	
rs2243283	CG	166	0.512	91	0.416
	CC	317		196	
	GG	18		14	
rs2243288	GG	326	0.687	200	0.685
	GA	158		92	
	AA	17		9	

SNPs	Minor allele frequencies (MAF)		<i>P</i> values
	Chronic HBV infection cases	Acute HBV infection controls	
rs2227284T/G	G (0.136)	G (0.128)	0.655
rs2243283C/G	G (0.202)	G (0.198)	0.849
rs2243288G/A	A (0.192)	A (0.183)	0.659

SNPs	Minor alleles	Additive (<i>P</i> values)	Dominant (<i>P</i> values)	Recessive (<i>P</i> values)
rs2227284T/G	G	0.771	0.563	0.766
rs2243283C/G	G	0.571	0.599	0.071
rs2243288G/A	A	0.902	0.691	0.555

SNPs	Haplotypes	Frequencies		OR	P values
		Chronic HBV infection cases	Acute HBV infection controls		
rs227284/ rs2243283/ rs2243288	GCA	0.135	0.128	1.067	0.670
	TCA	0.054	0.055	0.997	0.989
	TCG	0.609	0.620	0.964	0.733
	TGG	0.198	0.197	1.008	0.950

5'-CAGTCATGCAGAAGGCCCAAGTA -3', reverse primer: 5'-GGCCAGCAGGTTTGCCTATTT-3', and extended primer: 5'-TTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTCCCCSGCGCCACC CACCCC-3'. The PCR amplification conditions were: a 15 µl final volume containing 10 µl × 1.5 µL buffer, 0.3 µl dNTPs (10 mmol/L), 0.9 µl MgCl₂ (25 mmol/L), 0.1 µl HotstarR Taq DNA polymerase, 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°C for 15 min, denaturation at 94°C for 40 s,

annealing at 63°C for 1 min and recursive-descent 0.5°C, followed by extension at 72°C for 1.5 min for a total of 15 cycles. This was followed by 25 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, extension at 72°C for 1.5 min, with a final extension at 72°C for 8 min. Amplified samples were stored at 4°C. After amplification, 1.5 µl PCR product was examined on an agarose gel to test for the expected specific products.

PCR products (1.5 ml) were mixed with 5 U shrimp alkaline phosphatase (SAP) and 2 U *Exo I*; votexed; and maintained at 37°C for 1 h and 75°C for 15 min in order to inactivate SAP and *Exo I*. The purified templates could be preserved at 4°C for 24 h or at 20°C for long-term preservation. The Snapshot product was diluted 20 times. Then 8.6 ml of HiDiFormamide (high-purity formamide) and 0.9 ml of GeneScan-120 LIZ Size Standard were added to 0.5 ml of Snapshot purification products, followed by denaturation at 95°C for 5 min, quick cooling for 4 min, and loaded on ABI 3730 Genetic Analyzer (Applied Biosystem, Shanghai).

Statistical analyses

If a random sample population, with no selection or immigration, was assumed, the allele frequency from one generation to the next would have remained stable and reached equilibrium. This suggested that the population was at Hardy-Weinberg disequilibrium (HWD). HWE test was a statistic method to test this possibility in this study population. Fisher's exact test was used to perform the HWE test. SNPs in the chromosomal loci would produce different alleles, including the wild-type and mutant ones. MAF was the frequency at which less common alleles occur in a given population. The MAF was analyzed using the chi-test.

Since it was a case-control study, susceptibility alleles of HBV were found using different genetic models, including dominant, recessive, and additive models. In population genetics, linkage disequilibrium (LD) is the non-random association of alleles at 2 or more loci, that is, the occurrence of some combinations of alleles in a population more or less often than would be expected from a random formation of haplotypes from alleles based on their frequencies. It was shown in Figure 1 that three blocks of IL-4 gene were identified using HaploView software, and the alleles in each block were highly linked; hence, one SNP per block were selected on behalf of the other SNPs called

tagSNPs for the next step.

Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between tag SNP genotypes and alleles with the susceptibility of the chronic HBV infection were estimated using conditional logistic regression. This study used the genetic model for modeling the number of minor alleles as a continuous variable. For the additive model, individuals who were homozygous and had zero copy of the minor allele were coded 0, and heterozygous individuals with one copy were coded 1, and individuals homozygous for the rare allele with two copies were coded 2. The coding of the dominant model was as follows: individuals who were homozygous had zero copy of the minor allele and were coded 1, and individuals who had 2 or 1 minor alleles were coded 2. In the recessive model, individuals who were homozygous had two copies of the minor allele were coded 2, and individuals with zero or 1 allele were coded 1. Haplotypes structured among the three tag SNPs were also analyzed. All analyses were performed using SPSS13 and PLINK software. Bonferoni correction was used to adjust the *P* value. An online software was used to test the power of this study (<http://www.divms.uiowa.edu/rlenth/Power/>). This study hypothesized that the difference of case and control groups was more than 5%. Hence, the power of this study would be not less than 0.7.

RESULTS

For the gender disequilibrium between chronic HBV infection cases and acute infection controls ($\chi^2 = 0.180$ and $P = 0.671$), the groups were not formed by sex. As shown in Table 1, this study had successfully genotyped a total of three tag SNPs. The three tag SNPs conformed to the HWE in each group.

Chronic HBV infection cases were compared with acute infection controls to identify the relation between the three tag SNPs and chronic HBV infection susceptibility. As shown in Table 2, none of the tag SNPs allele frequencies showed significant differences between the chronic HBV infection cases and the acute infection controls. In addition, the different genetic models in each subgroup showed no differences (Table 3). Finally, haplotype analysis showed no statistically significant association between the haplotypes and the susceptibility of chronic HBV infection (Table 4).

DISCUSSION

The IL-4 gene is located in the Th2 cytokine cluster on human chromosome 5q31.¹⁸ Genetic alterations may influence the expression and function of IL-4, which can lead to a weakened cell-mediated immune response, thereby increasing one's susceptibility to infections and liver diseases, including hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.¹⁹ Wang et al suggest that polymorphisms in IL-4 may be associated with hepatitis B vaccine-induced long-term immunity.²⁰ He found the T allele and the TT genotype in SNP rs2243250 and rs2070874 in IL-4, and the A allele in SNP rs2227284 in IL-4 were associated with poor humoral response to vaccine, while the C allele in SNP rs2243250, rs2070874 and rs2227284 were associated with good humoral response. Furthermore, the haplotype TT defined by SNP rs2243250 and rs2070874 in IL-4 was associated with poor antibody response. In a meta-analysis, eight studies were identified.²¹ When all the eligible studies were pooled into the meta-analysis, the results showed that T allele of rs2243250, T allele of rs2070874 and C allele of rs2227284 in the IL-4 gene were associated with a high response to hepatitis B vaccine, especially among Asian populations. Nevertheless, another report found no statistically significant association between IL-4 rs2243250 polymorphism and HBV infection risk, suggesting that IL-4 may not be critical in the development of chronic hepatitis.²² This controversy could be explained with several reasons, such as the differences in study designs, sample size, ethnicity, source of subjects and genotype methods.²¹

To identify the relationship between the SNPs of IL-4 gene and genetic susceptibility to chronic hepatitis B virus infection in Chinese patients, three SNPs in IL-4 (rs2227284, rs2243283 and rs2243288) were selected using genotype data from the panel (Han Chinese in Beijing) of the phase II HapMap Project. The three tag SNPs captured 100% of common SNPs (minor allele frequency > 0.1) in the HapMap Chinese database at $r^2 > 0.8$. The associations of the three SNP alleles with chronic HBV-infected patients compared to self-limiting HBV infection controls were analyzed. The results indicated that there were no significant differences between cases and controls in the genotype or haplotype frequencies of any of the three SNPs, suggesting that tag SNPs of IL-4 do not predict the outcome of CHB infection. There are several advantages that would further strengthen

this study compared to previous similar studies, and would add to 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 analysis and search more SNPs than ever. Second, we selected self-limiting HBV-infected subjects but not unexposed subjects as controls, so our results may be more reliable than other reports that recruited blood donors as controls. Third, we included only antiviral-naïve subjects, in contrast, the previous similar studies which investigated natural courses of chronic HBV infection did not set any limitation regarding antiviral treatment.

In conclusion, although IL-4 is a functional gene with a relevant role in HBV pathogenesis, our study demonstrated that the tag SNPs of IL-4 do not correlate with genetic susceptibility to chronic HBV infection in Chinese patients. Further genetic studies are needed to examine the polymorphisms in other IL-4 SNPs and the possible association with disease progress in chronic HBV infection.

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