Association of the –183 Polymorphism in the IFN- γ Gene Promoter With Hepatitis B Virus Infection in the Chinese Population

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Interferon- γ (IFN- γ) is a pleiotropic cytokine that plays an important role in regulating cellular immune responses. Regulation of IFN- γ expression is considered to be strictly controlled at the transcriptional level. Two single-nucleotide polymorphisms (SNPs) within the human IFN- γ promoter (at positions –183 and –155) are considered to influence the promoter activity by altering the acting transcription factor-1 (AP-1) binding. We sought to assess the association between the SNPs of the IFN- γ promoter and the host susceptibility to hepatitis B virus (HBV) infection, as well as its interaction with age and gender. No polymorphism at position –155 was detected in any of the participants, but a

significant difference was found in the polymorphism at position –183 between the cases and controls (G/T and T/T vs. GG; $P < 0.01$, odds ratio (OR) = 4.50 (95% confidence interval (CI) = 2.23–9.09). A susceptibility analysis revealed a gradually increased trend of the OR value from the young to the old group (OR = 3.03, 4.17, and 5.56). Similarly, the association of the –183 polymorphism was markedly different in females (OR = 5.71). Our data suggest that the polymorphism at position –183 of the IFN- γ gene promoter may be associated with susceptibility to HBV infection, and age and gender factors are coordinative risk factors. *J. Clin. Lab. Anal.* 19: 276–281, 2005. © 2005 Wiley-Liss, Inc.

Key words: hepatitis B virus; IFN- γ ; promoter; polymorphism; susceptibility

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most common infectious diseases in the world. Approximately 400 million people worldwide are affected, and it is estimated that 470,000 deaths occur each year as a consequence of progressive liver disease, including fulminant hepatic failure, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) (1). Epidemiologic data reveal that the incidence of HBV carriers has a different endemicity. The prevalence of chronic HBV infection varies widely in the world from a high of 8% to a low of 2%. According to the map of the geographic distribution of HBV infection, this infection is concentrated in Africa, Asia, and the Western Pacific (2). Because of the central location of Asia, it is estimated

that HBV is present in more than 120 million chronic carriers, carrying more than 10% of the Chinese population (3). The type B hepatitis is the most common contagious disease in China, and is expected to lead to a

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series of hepatic malignant transformations and high morbidity and mortality rates. HBV is a noncytotoxic hepatotropic DNA virus and may cause either acute or chronic infection in humans (4). Susceptibility to viral infectious diseases is a dynamic interactive process that depends on the balance between invasion of the pathogens and immunity of the host (5). A series of studies have indicated that pathogens and host genetic factors both play important roles in resistance to and control of infection (6–8). Both the direct way of inhibiting the viral replication and the indirect way of inducing the predominant host response have been performed with the use of various cytokines to eliminate the HBV infection (9). One of the most representative cytokines is IFN- γ , which plays a pivotal role in defending against the invasion of intracellular pathogens (especially virus) and the induction of an immune-mediated inflammatory response (10,11). It has been reported that disruption of the IFN- γ gene leads to susceptibility to intracellular pathogens (12). Similarly, there is also some evidence that SNPs of the IFN- γ gene are involved in human susceptibility to viral diseases, such as HIV and HCV (13,14).

Because the regulation of IFN- γ gene transcription is complex, and several transcription factors interact with the proximal promoter (15), in this study we selected two polymorphisms in the promoter area of the IFN- γ gene that had been reported previously (16). The first polymorphism located 183 bp upstream from the transcription start site of the IFN- γ gene may alter the activating transcription factor-1 (AP-1) binding domain and regulate the transcription of the IFN- γ gene (17). The other polymorphism site located 155 bp upstream of the IFN- γ gene promoter area is close to the nuclear factors-activated T-cell site (NFAT site, –168 taaacgcaaaa-160) and may affect the stability of this region (18). Given the diverse distribution of SNP among different races, in this study we investigated the distribution of these two polymorphisms located in the IFN- γ gene promoter area between HBV-infected patients and normal individuals in the Chinese population to elucidate whether they would increase the susceptibility to HBV infection.

MATERIALS AND METHODS

Specimens

A polymorphism detection analysis was performed on subjects from north China. A total of 157 patients affected with HBV were recruited between 2003 and 2004 from Peking University First Hospital and Shandong Qianfo Hospital (75 females and 82 males; mean age = ($\pm$ SD) 38.26($\pm$ 6.88) years). For all patients the diagnosis was made on the basis of an enzyme-linked

immunosorbent assay (ELISA). A total of 210 volunteers (112 males and 98 females, mean age ($\pm$ SD) = 38.28($\pm$ 6.58) years) were found to be free from HBV by examination and were included simultaneously in the assay matched with the case group. Informed consent was obtained from each patient and control individual before 3-mL peripheral blood samples were collected. Genomic DNA from peripheral blood leukocytes was isolated by sodium dodecyl sulphate lysis and proteinase K digestion followed by standard phenol-chloroform extraction according to a standard protocol as described previously (19). DNA samples were quantified and subjected to specific PCR amplification as described below.

PCR Amplification

The primers for PCR amplification of promoter-specific fragments that covered these two polymorphic sites were designed from the wild-type IFN- γ sequence (GenBank accession number J00219). Details regarding the primer sequences, annealing temperatures, and fragment sizes for amplifying the IFN- γ promoter are provided in Table 1. The reaction was carried out in a 15- μ L reaction mixture containing genomic DNA 1 μ L, 10 $\times$ HotStar Taq PCR buffer (Qiagen, Inc., China) 1.5 μ L, Q solution for PCR (Qiagen, Inc., China) 3.0 μ L, 1.5 mM MgCl₂, 0.033 mM concentration of each dNTP, 5 pmol primer, and 1.0 U of Hotstar Taq polymerase (Qiagen, Inc., China). The reaction was started at 95°C for 2 min. Thirty rounds of thermal cycling were conducted under the following conditions: denaturation at 95°C for 40 sec; annealing at 56.5°C and 57.5°C for 45 sec, respectively; and extension at 72°C for 50 sec. The final extension was carried out at 72°C for 10 min on the Gene-Amp PCR System 9700 (PE Applied Biosystems). PCR products were purified with a Multi-Screen-PCR purifying plate (Millipore, Co., USA).

Genotype Analysis

For the detection of allelic polymorphisms in the IFN- γ promoter region at positions –155 and –183, the program NEB cutter V2.0 (<http://tools.neb.com/NEB-cutter2/index.php>) was used. After accurate scanning, the result revealed *AluI* and *EcoI47I* restriction sites. Two single-base polymorphisms at INF- γ promoter positions –155 and –183 were screened by using the restriction fragment length polymorphism (RFLP) analysis. A volume of 15 μ L of PCR product was digested with *AluI* and *EcoI47I* (New England Biolabs, Beverly, MA) under the conditions recommended by the manufacturer. After digestion, the PCR products were fractionated on 2% agarose Tris-borate-EDTA gel (Agarose-1000; Gibco BRL, Rockville, MD) and

TABLE 1. PCR Primer and Restricted Enzyme for analysis of the IFN- γ promoter region

Polymorphisms position	Base substitution	PCR Primers (sense/antisense)	Annealing temperature	Length	Restricted enzyme	Fragments length (bp)
–155	A $\rightarrow$ G	5'-GCAAATGATCAATGTGCTTTG-3' 5'-TTACATGGGTCCTGG-3'	56.5°C	559bp	<i>AluI</i>	193+366
–183	G $\rightarrow$ T	5'-AATGATCAATGTGCTTTGTG-3' 5'-TTAAGATGAGATGGTGCAG-3'	57.5°C	271bp	<i>EcoI</i> 471	163+108

stained with ethidium bromide (product size after digestion shown in Table 1). DNA direct sequencing was determined to confirm the RFLP analysis in 10 patients and controls for the two polymorphic sites. For DNA sequencing, the terminator ready sequencing reaction was performed with an ABI 3700 DNA sequencer (PE Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

All analyses were performed with SAS system 8.2 (SAS Institute, Cary, NC). For cases and controls, the distribution of gender and Hardy-Weinberg equilibrium equation of the IFN- γ genotype by using chi-square test, and the diversity of age by using Mann-Whitney *U*-test. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated to assess the relative risk of IFN- γ polymorphism by using unconditional logistic regression analysis adjusted by gender and age. The *P*-value was two-sided, and *P* < 0.05 was chosen as the level of significance.

RESULT

Distribution of General Characteristics and Polymorphisms in Two Groups

A total of 157 cases with HBV infection, and 210 normal controls were enrolled in this case-control study. As a whole, no significant difference was observed in gender and age between the case and control groups evaluated by the χ^2 -test or Mann-Whitney *U*-test (*P* = 0.867, *P* = 0.567, respectively).

In our experiment the –183 position of SNP in the IFN- γ gene promoter region was carried out in 367 Chinese individuals, which the substitution of nucleotide from G to T was determined by DNA direct sequencing. The IFN- γ gene promoter–188 position polymorphism was analyzed by PCR amplification followed by restriction analysis using *EcoI*471 digestion. PCR products of 271 bp that were digested by restriction enzyme were categorized as “T” and showed up as 163 bp and 108 bp on agarose electrophoresis. The categorized “G” allele was undigestible and remained as 271 bp on

Fig. 1. Product of PCR-based restriction analysis of the IFN- γ gene promoter region –188 G/T polymorphism shown on agarose electrophoresis. Lane 1: marker, 100-bp ladder. Lane 2: GG homozygote, undigested, 271-bp. Lane 3: TT homozygote, 163 bp + 108 bp. Lane 4: G/T heterozygote.

agarose electrophoresis (Fig. 1). No polymorphism was detected at position –155 of the IFN- γ gene promoter in the same study population.

Association Analysis of the IFN- γ –183 Polymorphism With HBV Infection

The polymorphism of the IFN- γ gene promoter was analyzed after the subjects were assigned to one of two groups: 1) subjects carrying –183GG, and 2) subjects carrying –183GT or TT genotypes. As shown in Table 2, without consideration of age and gender factors, a preliminary case-control analysis of the population revealed significant difference in genotype frequency of –183 site polymorphism: the frequency of –183GT or

TABLE 2. Associated Risk Analysis of the HBV infection by the IFN- γ Gene promoter –183 site Polymorphism

Variable	Genotype	Case n(%)	Control n(%)	Case vs control	
				<i>P</i> *	OR* (95% CI)
All subjects	G/G	124(78.98)	198(94.29)	1.000	–
	G/T+T/T	33(21.02)	12(5.71)	<0.001	4.50(2.23–9.09)
Gender groups					
Male	G/G	65(79.27)	105(93.75)	1.000	–
	G/T+T/T	17(20.73)	7(6.25)	0.005	3.83(1.50–9.77)
Female	G/G	59(78.67)	93(94.90)	1.000	–
	G/T+T/T	16(21.33)	5(5.10)	0.002	5.71(1.95–16.95)
Age groups					
29	G/G	8(61.54)	11(68.75)	1.000	–
	G/T+T/T	5(38.46)	5(31.25)	0.909	3.03(0.75–1.12)
30–39	G/G	56(82.35)	98(95.15)	1.000	–
	G/T+T/T	12(17.65)	5(4.85)	0.012	4.17(1.37–12.50)
>40	G/G	55(72.37)	85(93.41)	1.000	–
	G/T+T/T	21(27.63)	6(6.59)	0.001	5.56(2.08–14.29)

*Logistic regression model with the genotype G/G as the reference group, adjusted by another non-included variable.

TT was significantly higher in cases than in controls with GG ($P < 0.01$, OR = 4.50, 95% CI = 2.23–9.09). Subsequently, to determine whether age was a cooperative factor with polymorphism, all participants were divided into three different age groups: <29, 30–39, and >39 years. Logistic regression analyses revealed that the –183 polymorphism had no association with HBV infection in the <29 years group ($P = 0.909$; Table 2). However, when the other two groups were assessed a remarkably statistically significant difference in the distribution of the –183 site polymorphism was observed between cases and controls ($P = 0.012$). In the HBV patients of the 30–39 years group, the frequency of –183GT or TT was higher than that of controls with GG (OR = 4.17, 95% CI = 1.37–12.50). A similar pattern emerged when the >39 years group was estimated (OR = 5.56; 95% CI = 2.08–14.29; $P = 0.001$). We also performed separate analyses for males and females to examine the association of gender and polymorphic genotype with HBV infection. The data shown in Table 2 suggest that the male group with –183GT or TT was statistically significant difference from the group with –183GG (OR = 3.83; 95% CI = 1.50–9.77; $P = 0.005$). An even more statistically significant difference was found in the female group (OR = 5.71; 95% CI = 1.95–16.95; $P = 0.002$).

DISCUSSION

It is well known that exposure to HBV can cause a broad spectrum of effects ranging from no infection to different clinical disease phenotypes (20). However, the reasons for individual or ethnic differences in acquiring

infection, disease severity, and clinical outcomes have not been completely clarified. It has long been realized that many human diseases arise from the complex interplay between environmental exposures and host genetic susceptibilities. In addition to environmental factors, such as viral load, genotype, and genetic divergence due to viral gene mutations, variations affected by HBV are closely related to the function of the host immune response.

Host immunological factors, such as cytokine, play a crucial role in the defense against viral infection, both indirectly (through determination of the predominant pattern of the host response) and directly (through inhibition of viral replication). Several proinflammatory cytokines, such as Th1 cytokines (including IL-2 and IFN- γ) and tumor necrosis factor- α (TNF- α), have been reported to participate in viral clearance and host susceptibility to HBV infection. During the early phase of the host immunological defense after viral invasion, IFN- γ produced by NK cells plays a pivotal role in causing acute inflammation. Then it acts as a regulator of antigen presentation and proliferation of the lymphocyte subpopulation in the subsequent antigen-specific phase of the immune response (21). Since individual variation in cytokine release is predominantly caused by polymorphisms near or within the genes, there is evidence that SNPs located within the promoter region of the IFN- γ gene may affect the transcriptional activation of this gene (22).

The present study suggests that the IFN- γ promoter polymorphisms may be an important factor in the etiology of HBV infection. In this study we investigated two promoter polymorphisms of the human IFN- γ gene

in the patients with HBV and a control population in China. The results demonstrate that the frequency of the IFN- γ gene position -183 polymorphism was significantly higher in patients with HBV than in controls. The risk of HBV infection in participants with a G/T or T/T allele was 4.50-fold higher ($P < 0.001$) compared to those with the G/G wild allele, which indicates that the -183 site of the IFN- γ gene promoter may be related to the susceptibility of HBV infection. However, there was no polymorphism at position -155 of the IFN- γ gene in this Chinese population, in contrast to other polymorphic research conducted in different ethnic populations (16). There is accumulating evidence that the IFN- γ -183 G/T polymorphism has functional importance. Kaminuma et al. (23) reported that transcription factor Stat4 enhanced IFN- γ promoter activity, but was downregulated by GATA-3 at the gene transcription level. These effects of GATA-3 or Stat4 on the IFN- γ promoter were proven to be lost upon removal of the region encompassing regions -257 to -172 (23). In addition, Barbulescu et al. (18) found that the -196 to -183 regions of the IFN- γ gene could be combined with AP-1. Nucleotide substitution of this area may alter AP-1 binding domain and promoter activity, which would influence the transcription and production of the IFN- γ gene and alter the host immune response to pathogens (18).

Further analysis indicated that age was an increased susceptibility factor for HBV, as reflected by individual genotypes that exhibited a gradual upward trend of the OR value from the young to the old group. Our data indicate that older people with -183 G/T or T/T polymorphism were more susceptible to HBV. In the <29, 30-39, and >39 years groups the OR was 3.03, 4.17, and 5.56, respectively ($P = 0.909$, 0.012 , and 0.001 , respectively). Further correlative analyses were performed in patients with HBV to assess the role of gender. The association of -183 G/T or T/T polymorphism with HBV was markedly different in females (OR = 5.71). So it would be concluded that females with this genotype allele are more likely to be infected with HBV.

In conclusion, we determined the existence of a polymorphism in position -183 of the IFN- γ gene promoter in a Chinese population. The results of an association analysis suggest that -183 polymorphism of the IFN- γ gene promoter is an important factor that independently influences host susceptibility to HBV. Similarly, age and gender factors are also interact with polymorphism in terms of HBV infection. From these findings, we can speculate that the risk of -183 polymorphism located in the IFN- γ gene promoter is implicated in the pathogenesis of HBV infection in Chinese people. However, future studies should be performed to confirm these results in different ethnic populations.

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