Clinical Study

Rather than Rs1800796 Polymorphism, Expression of Interleukin-6 is Associated with Disease Progression of Chronic HBV Infection in a Chinese Han Population

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Interleukin-6 plays an important role in chronic inflammation as well as tumor growth and progression. Here, a case-control study was undertaken to investigate the association of rs1800796 polymorphism of IL-6 gene and serum levels with disease progression of chronic HBV infection. Rs1800796 polymorphism was genotyped in 641 Chinese Han patients with chronic HBV infection, including 23 IT, 25 IC, 292 CHB, 153 LC, and 148 HCC patients and 265 healthy controls. Serum IL-6 levels were measured in 23 IT, 25 IC, 47 CHB, 41 LC, and 49 HCC patients and 45 healthy controls, and the classifications of HCC were accorded to BCLC staging system. We found no significant association between rs1800796 polymorphism and disease progression of chronic HBV infection; however, serum IL-6 levels showed significant statistical differences between patients with CHB, LC, and HCC. Moreover, statistical differences can be observed in patients with terminal stage HCC compared with those of early to intermediate or advanced stage HCC. Our findings suggest that rs1800796 polymorphism unlikely contribute significantly to affect the progression of chronic HBV infection, and serum IL-6 levels can act as a useful indicator for disease progression and severity of chronic HBV infection.

1. Introduction

Hepatitis B virus (HBV) infection is a global public health problem. It is estimated that over 2 billion people have had contact with the HBV, and that 350–400 million people are chronically infected [1–3]. The clinical course of HBV infection varies from spontaneous recovery after acute hepatitis to a chronic persistent infection that may progress to cirrhosis or hepatocellular carcinoma (HCC). The outcomes of chronic HBV infection do not appear to be determined by the viral strains in themselves [4]. It has been established through twin studies that chronic HBV infection outcome has a strong genetic component [5]. Moreover, allelic variants in the human genome have been implicated in disease progression after HBV infection [6–8]. Thus, it is conceivable that genetic differences play an additional role in the influence of disease progression.

Recently, compelling evidence has shown that inflammation has an important role in initiation, promotion, and progression of HBV infection [9, 10]. As a well-recognized inflammatory cytokine, interleukin-6 (IL-6) plays a central role in the regulation of immune response. Previous studies have found that serum level of IL6 was increased in patients with chronic hepatitis B (CHB) and HCC [11–13]. Moreover, *in vitro* and animal models studies have shown that IL-6 could be essential in the initiation and development of experimental liver cancer, probably because of its contribution in promoting hepatocellular damage and compensatory proliferation [14, 15]. Therefore, high IL-6 levels might reflect more active hepatic necroinflammation and be associated with the presentation and severity in chronic HBV infection.

Several single nucleotide polymorphisms (SNPs) within the noncoding promoter region of IL-6 gene, including $-174G > C$ (rs1800795), $-572C > G$ (rs1800796), and

−596G > A (rs1800797), have been reported to be related to a range of diseases including cancer [16–20]. However, a different genetic background of ethnic diversity with respect to the allele frequencies of IL-6 promoter SNPs can be observed. The allele frequencies of −174G > C and −596G > A are extremely rare in Asian populations, and unlikely to be contributing significantly to disease susceptibility [21, 22]. As high frequency population specific alleles are particularly useful for mapping genes that are responsible for disease susceptibility, the SNP of IL6 gene rs1800796 can be selected as a more useful marker for an association study in Chinese population.

In light of the important role of IL-6 in HBV infection, we hypothesized that genetic polymorphisms of IL-6 and serum levels of the cytokine were associated with disease progression of chronic HBV infection. To test this hypothesis, we performed a case-control study to investigate an eventual correlation between the allelic variations, the circulating levels of the cytokine, and the risk of disease progression.

2. Subjects and Methods

2.1. Subjects. A total of 641 subjects with chronic HBV infection, including 23 patients in the immune tolerant (IT) stage, 25 patients in the HBeAg-negative inactive carrier (IC) stage, 292 patients with CHB, 153 patients with liver cirrhosis (LC), 148 patients with HCC, and 265 healthy controls, were periodically enrolled between January 2008 and December 2012 at the Zhongnan Hospital of Wuhan University. The subjects were exclusively unrelated Han Chinese and recruited without restriction on gender and age. Patients with HBV infection were confirmed to be HBsAg (hepatitis B virus surface antigen) positive, HBcAb (hepatitis B virus core antibody) positive, and HBeAg (hepatitis B virus e antigen) or HBeAb (hepatitis B virus e antibody) positive for at least 6 months. IT was defined as being HBsAg-positive, HBeAg-positive, HBV-DNA levels >1×10^7 copies/mL, and having normal ALT levels on three or more occasions during at least 1 year of followup. IC was defined as being HBsAg-positive on two occasions at least 6 months apart, HBeAg-negative, HBeAb-positive with persistently normal ALT levels, and HBV-DNA levels <1×10^4 copies/mL. CHB was diagnosed by elevation of alanine aminotransferase (ALT) (≥2 times the upper limit of normal) at least once during the follow-up period, as well as positivity for HBV-DNA. LC was diagnosed pathologically or based on the clinical evidence of portal hypertension such as visible collateral vessels on the abdominal wall, esophageal varices on esophagogastroscopy, palpable splenomegaly, and sonographically definite findings of cirrhotic liver or ascites. HBV-related HCC was diagnosed based on (i) positive findings on cytological or pathological examination; and/or (ii) positive results on computed tomography (CT), magnetic resonance imaging (MRI), or ultrasonography combined; and/or (iii) α-fetoprotein (AFP) level >400 ng/mL [23]. The classifications of HCC were accorded to Barcelona Clinic Liver Cancer (BCLC) staging system. Additionally, the patients who had (i) coinfection with human immunodeficiency virus (HIV), hepatitis C virus (HCV), or hepatitis D virus (HDV); (ii)

previous antiviral agents, that is, interferon or nucleoside analogues, immunomodulatory, or antitumor agent; (iii) alcoholic liver disease, autoimmune hepatitis, or drug-induced liver disease; (iv) any concomitant illness, that is, diabetes or hypertension; or (v) acute inflammation within 2 weeks were excluded from the current study. The selection criteria for healthy controls were absence of evidence of any personal or family history of cancer or other serious illness, including HBV infection. Written informed consent was obtained from all subjects and the study was performed with the approval of the ethical committee of Zhongnan Hospital of Wuhan University, Wuhan, China.

2.2. DNA Extraction and Genotyping. Genomic DNA was extracted from peripheral whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Samples were stored at −80°C until genetic polymorphism analyses were performed. Rs1800796 polymorphism genotyping was performed using the TaqMan $5'$ allelic discrimination assay technology in a 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Genotyping was performed without knowing the subjects' case or control status; more than 10% of samples were randomly selected for repeat analysis (which yielded 100% concordance). Finally, 10% of samples were analyzed using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) to confirm the accuracy of this method.

2.3. Measurement of Serum IL-6 Levels. Venous blood samples were obtained from 23 IT, 25 IC, 47 CHB, 41 LC, 49 HCC patients and 45 healthy controls, respectively. After centrifugation, the serum sample was stored at −80 degree and IL-6 concentrations checked within 2 weeks using commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA). Results were expressed in picograms per millilitre (pg/mL) by reference to a standard curve obtained with recombinant IL-6.

2.4. Statistical Analyses. Continuous variables were expressed as mean ± SD. Deviation from Hardy-Weinberg equilibrium was tested by using the χ^2 test for goodness of fit. The differences of genotypes frequencies between groups were determined using a standard χ^2 test. Odds ratio (OR) and 95% confidence interval (CI) were calculated to assess the relative risk conferred by a particular genotype. To evaluate the effect of the genotype containing the SNP variant, we also conducted analyses under dominant model. Levels of serum IL-6 between groups/subgroups were compared using ANOVA, and Bonferroni test was used for comparison between two groups. A P value <0.05 was defined as statistically significant. All statistical analysis was two-tailed and performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. General Characteristics of the Subjects. General characteristics of the subjects are summarized in Table 1. Among the

separated groups, patients with HCC were older than those with LC or CHB ($P < 0.01$; 60.2 ± 11.5 versus 51.6 ± 12.3 versus 43.9 ± 11.9, resp.), which was compatible with the distribution of natural course of HBV infection. Although the HBV-related HCC patients had a higher proportion of males than patients with LC or CHB, the difference was not statistically significant ($P > 0.05$; 74.3% versus 72.5% versus 72.7%, resp.).

3.2. Rs1800796 Polymorphism of the Subjects. Genotype and allelic frequencies of rs1800796 polymorphism of the subjects are presented in Table 2. The genotype distributions were in Hardy-Weinberg equilibrium in each group. As shown in Table 2, the CC genotype of rs1800796 prevailed in all groups. As for the frequency of CG and GG genotype, a weak increasing trend can be observed in HCC group when compared with LC and CHB groups (CG: 34.5% versus 30.1%, 34.5% versus 29.8%; GG: 4.7% versus 3.9%, 4.7% versus 3.8%, resp.), while there were no statistically significant differences regarding the genotype frequencies of rs1800796 polymorphism between the three groups. As the low frequency of GG genotype, the CG and GG genotype were combined for analysis in the dominant model, although an increasing trend can also be observed in HCC and LC groups compared with CHB group regarding the frequency of genotype with G allele (39.2% versus 34.0% versus 33.6% resp.), the statistical level was not significant, indicating that there was no significant association between rs1800796 polymorphism and the progression of chronic HBV infection in all subjects.

3.3. Comparison of IL-6 Levels in Different Groups. As shown in Figure 1, there were significantly strong IL-6 expressions in the CHB, LC, and HCC groups compared with healthy control group (3.58 ± 1.39 pg/mL versus 1.15 ± 0.59 pg/mL, $P = 0.034$; 13.38 ± 2.72 pg/mL versus 1.15 ± 0.59 pg/mL, $P < 0.001$; 17.81 ± 3.86 pg/mL versus 1.15 ± 0.59 pg/mL, $P < 0.001$ resp.). However, the serum levels of IL-6 were comparable in IT, CHB, and IC groups (1.75 ± 0.67 pg/mL versus 3.58 ± 1.39 pg/mL versus 2.08 ± 0.81 pg/mL, resp.). We next evaluated the association of changes in serum IL-6 concentrations with the progression of chronic HBV infection. Serum IL-6 levels were significantly higher in HCC and LC groups compared to that in CHB group (17.81 ± 3.86 pg/mL versus 3.58 ± 1.39 pg/mL, $P < 0.001$; 13.38 ± 2.72 pg/mL versus 3.58 ± 1.39 pg/mL, $P < 0.001$ resp.). Meanwhile, IL-6 also showed significantly higher expression in patients with HCC than those of LC (17.81 ± 3.86 pg/mL versus 13.38 ± 2.72 pg/mL, $P = 0.039$) (Figure 1).

3.4. Comparison of IL-6 Levels in Subgroups of HCC. Forty nine patients with HCC were classified according to BCLC staging system. IL-6 showed higher expression and significantly statistical differences in patients with terminal stage HCC (stage D, $n = 23$) than those with early to intermediate stages (stage A and B, $n = 11$) or advanced stage HCC (stage C, $n = 15$) (23.85 ± 6.79 pg/mL versus 7.89 ± 1.63 pg/mL, $P < 0.001$; 23.85 ± 6.79 pg/mL versus 14.40 ± 3.71 pg/mL, $P = 0.037$ resp.). Meanwhile, patients with advanced stage HCC
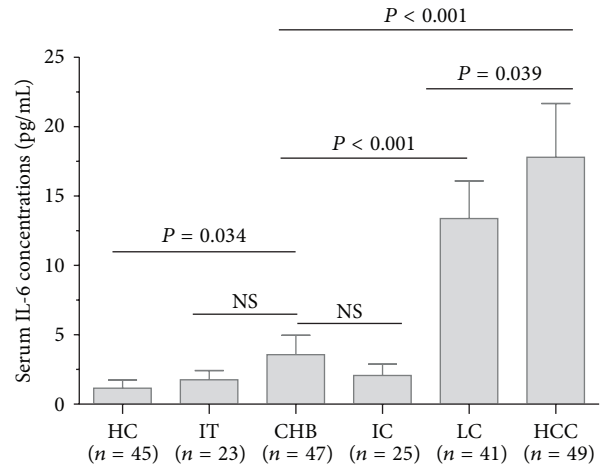

FIGURE 1: Serum IL-6 concentrations in HC, IT, CHB, IC, LC, and HCC groups according to different clinical-pathologic stages. NS: no significance; IT: immune tolerant; IC: inactive carrier; HC: healthy control; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma.
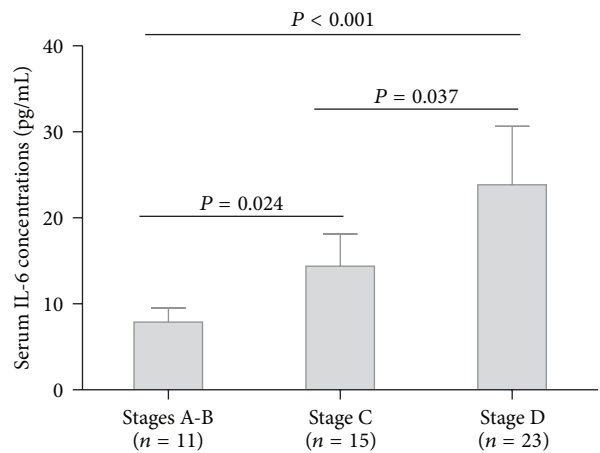

FIGURE 2: Serum IL-6 concentrations in different-stage HCC patients according to the classification of BCLC staging system. BCLC-Barcelona Clinic Liver Cancer; HCC: hepatocellular carcinoma.

had higher IL-6 levels than those with early to intermediate stages (14.40 ± 3.71 pg/mL versus 7.89 ± 1.63 pg/mL, $P = 0.024$) (Figure 2).

4. Discussion

Cytokine mediated immunity linking innate and adaptive immunities in host may play a crucial role in determining the outcome of HBV infection [24, 25]. IL-6, a well-recognized inflammatory cytokine, might be associated with the presentation and severity in HBV-infected or HCC studies [26, 27]. In the present study, we found no association of $-572C > G$ polymorphism in the IL-6 gene with the disease progression of chronic HBV infection; however, the results demonstrated positive correlation of serum IL-6 levels with the development of LC and HCC from chronic HBV infection

TABLE 1: General characteristics of subjects.

	HC ($n = 265$)	IT ($n = 23$)	CHB ($n = 292$)	IC ($n = 25$)	LC ($n = 153$)	HCC ($n = 148$)
Age (y) (mean ± SD)	41.6 ± 10.7	24.7 ± 10.2	43.9 ± 11.9	32.6 ± 11.3	51.6 ± 12.3	60.2 ± 11.5
Male, n (%)	193 (72.8)	16 (69.6)	212 (72.7)	17 (68.0)	111 (72.5)	110 (74.3)

HC: healthy control; IT: immune tolerant; IC: inactive carrier; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma.

and also the liver severity of HCC, indicating that rather than genetic variant of IL-6 gene, levels of cytokine expression may play an extremely important role to determine the progression of chronic HBV infection.

Previous association studies have revealed that host genetic variants are related to the susceptibility to HBV clearance or persistence [28–30]. Other clinical outcomes, such as disease severity or progression, may also be influenced by host genetics, and a few association studies have found evidences to support this theory [8, 31]. A large body of evidence confirms that IL-6 is a key regulator of inflammatory mechanisms that play an important role in the pathophysiology and development of liver carcinogenesis [32], indicating the crucial role of this cytokine in chronic inflammation. As a suitable candidate gene, IL-6 might influence the development of chronic HBV infection; therefore, SNPs of IL-6 gene can be selected as useful marker for association study. Recently, genetic polymorphisms of IL-6 have been extensively studied, and evidence demonstrates that several polymorphisms are associated with inflammatory diseases and cancer [16–18, 20]. HCC and LC usually arise after several years of chronic inflammation due to HBV infection. In the case-control study described here, regarding the frequency of rs1800796 CG and GG genotypes, an increasing trend can be observed in groups abided by the subsequent progression of naturally chronic HBV infection; however, we failed to demonstrate a significant association between rs1800796 polymorphism and the progression of chronic HBV infection, which is consistent with the results of previous study performed in the Korean population [22].

It has already been described that elevated IL-6 levels were detected in chronic liver disease [33–35] and a link between IL-6 and progression of HCV infection [36]. In the present study, we established that the serum IL-6 levels are significantly higher in patients with HCC than in LC and CHB patients, but also in LC patients when compared to CHB group. When the patients with HCC were divided according to classification of BCLC staging system, IL-6 values significantly increased as the disease worsened, imply positive correlation with severity of liver disease.

As a proinflammatory cytokine, IL-6 is involved in the fibrotic response. Liver cirrhosis is associated with increased intrahepatic mRNA expression of IL-6 [37], and significant correlation between IL-6 production and the induction of hepatic stellate cell proliferation exists in the culture supernatants of LC patients, suggesting that IL-6 may participate in hepatic stellate cell proliferation in LC patients [38]. Previous studies also have suggested possible biological mechanisms for IL-6 in hepatocarcinogenesis. Experimental models of HCC in mice have suggested that chronic exposure to high IL-6 level is associated with increased liver injury and HCC

[14], and IL-6 gene expression is upregulated by the hepatitis B virus X-protein in HCC cells, leading to oncogenic transformation [39]. IL-6 also induces a high metastatic potential and decreases apoptosis of HCC cells [40, 41]. Moreover, HCC cells themselves might have been a source of IL-6, which stimulated tumor growth by an autocrine mechanism, as evidenced by high expression in human HCC samples [36]. Our findings further support the notion that IL-6 is involved in the pathogenesis of hepatitis B-related HCC.

Our study has some limitations. First, it is a case-control study and a selection bias could not be completely excluded for the group of patients with chronic HBV infection. Second, although the highly significant association between IL-6 and susceptibility to disease progression of chronic HBV infection derives from a prior hypothesis with substantially biological basis, our initial findings should be independently verified in large size of the Chinese population and other different ancestry. Finally, only one polymorphism has been determined at this time. It is likely that with a more refined technology, such as genome-wide association studies, additional polymorphisms will be identified.

From the current study we conclude that −572C > G polymorphism (rs1800796) of IL-6 gene is unlikely to contribute significantly to affect the progression of chronic HBV infection in Chinese population. Moreover, our findings lend support to the notion that serum IL-6 levels can act as a useful indicator for disease progression and severity of chronic HBV infection, which may assist clinicians in selecting high-risk patients for HCC surveillance program.

Abbreviations

AFP: α-fetoprotein
ALT: Alanine aminotransferase
BCLC: Barcelona Clinic Liver Cancer
CHB: Chronic hepatitis B
CI: Confidence interval
CT: Computed tomography
ELISA: Enzyme-linked immunosorbent assay
HBcAb: Hepatitis B virus core antibody
HBeAb: Hepatitis B virus e antibody
HBeAg: Hepatitis B virus e antigen
HBsAb: Hepatitis B virus surface antibody
HBsAg: Hepatitis B virus surface antigen
HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma
HCV: Hepatitis C virus
HDV: Hepatitis D virus
HIV: Human immunodeficiency virus
IT: Immune tolerant
IC: Inactive carrier

TABLE 2: Genotype distribution of rs1800796 in different groups.

rs1800796	HC (n = 265)	IT (n = 23)	CHB (n = 292)	IC (n = 25)	LC (n = 153)	HCC (n = 148)	LC versus CHB OR (95% CI)	P	HCC versus CHB OR (95% CI)	P	HCC versus LC OR (95% CI)	P
Genotype, n (%)												
CC	176 (66.5)	15 (65.2)	194 (66.4)	17 (68.0)	101 (66.0)	90 (60.8)	1.00		1.00		1.00	
CG	78 (29.4)	7 (30.4)	87 (29.8)	7 (28.0)	46 (30.1)	51 (34.5)	1.02 (0.66–1.56)	0.944	1.26 (0.83–1.94)	0.282	1.24 (0.76–2.03)	0.381
GG	11 (4.1)	1 (4.4)	11 (3.8)	1 (4.0)	6 (3.9)	7 (4.7)	1.05 (0.38–2.92)	0.929	1.37 (0.52–3.66)	0.526	1.31 (0.42–4.04)	0.638
Dominant model												
CC	176 (66.5)	15 (65.2)	194 (66.4)	17 (68.0)	101 (66.0)	90 (60.8)	1.00		1.00		1.00	
CG + GG	89 (33.5)	8 (34.8)	98 (33.6)	8 (32.0)	52 (34.0)	58 (39.2)	1.02 (0.67–1.54)	0.928	1.28 (0.85–1.92)	0.244	1.25 (0.78–2.00)	0.349
Hardy-Weinberg equilibrium (P)	0.529	0.874	0.749	0.797	0.791	0.947						

Note: P value obtained using a standard χ^2 test, and not adjusted for confounding factors. OR: odds ratio; CI: confidence interval; HC: healthy control; IT: immune tolerant; IC: inactive carrier; CHB: chronic hepatitis B; LC: liver cirrhosis; HCC: hepatocellular carcinoma.

IL-6: Interleukin-6
MRI: Magnetic resonance imaging
OR: Odds ratio
SNP: Single nucleotide polymorphism.

Conflict of Interests

All authors have no conflict of interests to declare.

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