Association between interleukin 6 promoter variants and chronic hepatitis B progression

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Abbreviations: HBc, hepatitis B core antigen; HBs, hepatitis B surface antigen; HCV, hepatitis C virus; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LD, linkage disequilibrium; SBE, single base extension; SNP, single nucleotide polymorphism

Abstract

Interleukin 6 (IL6) plays an essential role in the regulation of immune response to chronic disease. In this study, the three known single nucleotide polymorphisms (SNPs) in the IL6 promoter region were genotyped in a large chronic hepatitis B cohort to evaluate the effects of IL6 promoter variants. The single base extension method was used for this genotyping. Haplotypes were constructed by the three SNPs in IL6. Allele frequencies were compared for; i) patients with chronic hepatitis (CH) and chronic carriers vs. chronic hepatitis patients with clinical evidence of liver cirrhosis (LC) (i.e., portal hypertension), ii) cirrhotic patients with hepatocellular carcinoma (HCC) vs. without HCC by logistic regression, and iii) with respect to the time intervals from the onset of infection to HCC. Results were analyzed by Cox relative hazard analysis on the assumption that all the patients were infected during early infancy. The frequencies of each SNP were 0.002 (IL6-597 G>A), 0.25 (IL6-572 C>G) and 0.002 (IL6-174 G>C), respectively, in the Korean population ($n = 1,046$). No significant associations were detected between IL6-572 C>G and chronic hepatitis B outcome in this study; i.e.,

LC occurrence on CH (OR = 0.16-1.27, $P = 0.13-0.71$) and HCC occurrence on LC (OR = 1.04-1.23, $P = 0.89-0.60$) of heterozygotes and homozygotes for G allele in referent comparison to homozygotes for common allele (C/C genotype), and time interval to HCC (RH = 0.67-1.00; $P = 0.14-0.99$). In conclusion, there appeared to be no significant associations between IL6 promoter variants and disease outcome in chronic hepatitis B.

Keywords: carcinoma, hepatocellular; haplotypes; hepatitis B virus; interleukin-6; polymorphism, single nucleotide; promoter regions (genetics)

Introduction

Korea is an endemic area of chronic hepatitis B virus (HBV) infection. The sequelae of chronic HBV infection vary from an inactive carrier state to the development of cirrhosis and hepatocellular carcinoma (HCC). Chronic carriers of HBV have a high risk of developing HCC compared with uninfected individuals (Blumberg, 1984; Huh *et al.*, 1998; Merican *et al.*, 2000). HCC is the most common primary liver cancer and is one of the ten most common carcinomas in the world. The incidence of HCC shows geographical variation and is most common in Asia and Africa, where HBV infection is highly endemic and where chronic infection is usually acquired in early infancy (Yu *et al.*, 2000a). Typically, the development of HCC occurs 30-50 years after the onset of HBV infection; consequently HCC is far more likely to be seen in individuals exposed to HBV perinatally rather than horizontally in adult life.

The outcomes of HBV infection do not appear to be determined by the viral strains in themselves (Cacciola *et al.*, 2002). However, the factors involved in the progression of chronic HBV infection to LC and to HCC among chronic HBV carriers require investigation. It has been established through twin studies that chronic HBV infection outcome has a strong genetic component (Shimbo *et al.*, 1997). Moreover, allelic variants in the human genome, e.g., N-acetyltransferase (Yu *et al.*, 2000b), vitamin D receptor (Bellamy *et al.*, 1999), and HLA (Almarri and Batchelor, 1994) have been implicated in disease progression after HBV infection (Thursz, 1997; Thursz, 2001).

Interleukin 6 (IL6) is a multifunctional cytokine es-

essential for the regulation of immune response (Kakumu *et al.*, 1993; Galun *et al.*, 2000). It exerts its actions by binding to its cell-surface receptor. Serum levels of IL6 were found to be significantly increased in patients with chronic hepatitis B (Malaguarnera *et al.*, 1997; Song *et al.*, 2000) and in patients with chronic hepatitis C patients (Wang *et al.*, 1999). However, the association between polymorphisms of *IL6* gene and chronic hepatitis B progression has not been studied, nonetheless the importance of IL6 in conjunction with progression of chronic hepatitis disease remains.

In the present study, as one of efforts to discover additional polymorphism(s) in genes of which variant(s) have been implicated in chronic hepatitis B progression, we examined the genetic effects of the all of the three known polymorphisms in the *IL6* promoter (-597 G>A, -572 C>G, -174 G>C) by identifying the naturally occurring haplotypes in a large scale HBV cohort.

Material and Methods

Study subjects and serological tests

1,046 Korean subjects were enrolled at an out-patient liver unit clinic and at the Center for Health Promotion at Seoul National University Hospital between January 2001 and August 2001. Subjects were categorized into three groups: chronic hepatitis (CH), LC and HCC. All patients included in this study were hepatitis B surface antigen (HBsAg) positive over a 6-month period. Patients who were positive for anti-HBs without anti-HBc (serological status by hepatitis B vaccination), and patients who were positive for anti-HCV or anti-HIV were excluded. Informed consents were obtained from each patient, and the Institutional Review Board for Human Research at Seoul National University Hospital approved the study protocol. The characteristics of the subjects are shown in Table 1.

Serologic tests were performed on every subject

using commercially available products for HBsAg (Enzygnost HBsAg 5.0, Dade Behring, Marburg, Germany), anti-HBs (Enzygnost Anti-HBs II) and anti-HBc (AB-Corek, DiaSorin s.r.l., Saluggia, Italy). Patients who were positive for anti-HCV or anti-HIV by ELISA (GENEDIA, Greencross Life Science Corp., Yongin-shi, Korea; HCV3.2, Dong-A Pharmaceutical Co., Seoul, Korea, respectively) and patients whose average alcohol consumption assessed by interview was ≥ 10 g/day were excluded (Yu *et al.*, 1988). The consumption and types of alcohol drinking were asked and the alcohol content of each drink was calculated. Weekly alcohol intake was converted into alcohol g/day. Patients having any other types of liver disease, such as autoimmune hepatitis, toxic hepatitis, primary biliary cirrhosis or Budd-Chiari syndrome were excluded. We also excluded subjects who had a previous history of immunosuppression or anti-viral treatment. The diagnosis of HCC or LC was made as described previously (Lee *et al.*, 1993).

Genotyping of SNPs in the *IL6* promoter region

All of the three known single nucleotide polymorphisms (SNPs) in the *IL6* promoter region were genotyped. The locations of these SNPs in relation to the genomic structure of the *IL6* gene are shown in Figure 1. Sequences of amplifying and extension primers for SNP genotyping using the single base extension method for *IL6*-597 G>A, *IL6*-572 C>G and *IL6*-174 G>C were forward 5'-GCAAAGTCCTCACTGGGAGGA-3', reverse 5'-GGGCTGCGATGGAGTCAGA-3', extension 5'-ATTATAATCAATGATGATTGAAGTAACTGCACGAAATTTGA GG-3'; forward 5'-GCAAAGTCCTCACTGGGAGGA-3', reverse 5'-GGGCTGCGATGGAGTCAGA-3', extension 5'-ATCAATGATGATCCAGGCA GTCTACAAC AGCC-3'; forward 5'-AATGACGACCTAAGCTGCAC-3', reverse 5'-TTGATAAATCTTTGTTGAGGGTG-3', extension 5'-TCATGATTATAATCAATGATGATTTCCCCCTAGTTGTGTCTTGC-3', respectively. PCR was performed in a mixture containing 1.25 pmol of each primer, 50 ng of genomic DNA, 250 μ M dNTPs, and 0.15 U *Taq* DNA Polymerase (Applied Biosystems, Foster City, CA) in a buffer provided by the manufacturer. Amplification was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) under touchdown conditions (Don *et al.*, 1991).

To clean up the PCR reaction for the primer extension reaction, one unit of Shrimp Alkaline Phosphatase (SAP; Amersham Life Sciences, Cleveland, OH) and two units of *Exo* I (Amersham Life Sciences) were added to the PCR products. The mixture was incubated for 1 h at 37°C and 15 min at 72°C to inactivate the enzymes.

Primer extension reactions were performed using a SNaPshot ddNTP Primer Extension Kit (Applied Bio-

Table 1. The characteristics of subjects.

Groups*	No. of subjects			Age ($\pm$ SD)	
	Total	Male	Female	Male	Female
CH	295	227	68	46.4 ($\pm$ 0.9)	49.2 ($\pm$ 8.9)
LC	514	371	143	49.1 ($\pm$ 8.6)	52.3 ($\pm$ 9.6)
HCC	237	193	44	56.2 ($\pm$ 8.56)	53.1 ($\pm$ 9.6)
Total	1046	791	255	50.0 ($\pm$ 10.0)	51.6 ($\pm$ 9.5)

*CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

systems). To clean up the primer extension reaction, one unit of SAP was added to the reaction mixture and the mixture was incubated at 37°C for 1 h, followed by 15 min at 72°C for enzyme inactivation. The DNA samples, containing extension products, and Genescan 120 Liz size standard solution was added to Hi-Di formamide (Applied Biosystems) according to the manufacturer's recommendations. The mixture was incubated at 95°C for 5 min, placed on ice for 5 min and then electrophoresed using an ABI Prism 3100 Genetic Analyzer. The results obtained were analyzed using ABI Prism GeneScan and Genotyper program (Applied Biosystems).

Statistics

The χ^2 test was used to compare the observed numbers of each genotype with those expected for a population complying with the Hardy-Weinberg equilibrium. Heterozygosity for each locus with allele frequencies p and $q = 1-p$ is given by $H = 1-p^2-q^2 = 2p(1-p)$. Widely used measures of linkage disequilibrium (LD) between all pairs of biallelic loci, Lewontin's D' ($|D'|$) and the measure d^2 (Hedrick, 1987) were calculated. Odds ratios with a 95% confidence interval and P values of logistic regression models, after controlling for the effects of age (continuous values) and

Figure 1. Map of human *IL6* on chromosome 7p21. Exons, UTR, and SNPs are shown with the putative transcriptional factor-binding site. Coding exons are marked by shaded blocks and 5' and 3' UTR by white blocks. The first base of the transcriptional start site is denoted nucleotide+1. The values of D' and d^2 between SNPs are also shown (See text).

Table 2. SNPs of the human *IL6* promoter and frequencies in the Korean population.

Locus	SNP site ^a	Genotype			Frequency ^b	Heterozygosity
<i>IL6</i> -597 G>A	-597	A	AG	G	0.002	0.005
		0	5	1019		
<i>IL6</i> -572 C>G	-572	G	G/C	C	0.25	0.375
		62	371	557		
<i>IL6</i> -174 G>C	-174	C	G/C	G	0.002	0.005
		0	5	1032		

^aCalculated from the transcriptional start site. ^bFrequencies of rare alleles. Genotype distributions in all loci were in Hardy-Weinberg equilibrium ($P > 0.05$). *IL6*-597 G>A and *IL6*-174 G>C are in absolute LD ($d^2 = 1$). *IL6*-572 C>G was in complete LD ($D' = 1$) with *IL6*-597 G>A and *IL6*-174 G>C. For the calculation of allele frequencies, HBsAg negative individuals in cohort are also included.

sex, were computed. Cox models were used to calculate the relative hazards and P values to evaluate the effects of genotypic variables on the time interval to HCC after HBV infection adjusted by gender.

Results

Allele and haplotype frequencies of IL6 promoter SNPs

The allele frequencies of the SNPs in Korean population were; 0.002 (*IL6-597 G>A*), 0.25 (*IL6-572 C>G*) and 0.002 (*IL6-174 G>C*) (Table 2). Genotype distributions were in Hardy-Weinberg equilibrium ($P > 0.05$). *IL6-597 G>A* and *IL6-174 G>C* were found to be totally linked together and to have showed very low allele frequencies (0.002). These SNPs are commonly found in Caucasians (0.4-0.44). On the other hand, the frequency of C allele of *IL6-572 C>G* was

much higher (0.75) than reported in a Caucasian population (0.06) (Terry *et al.*, 2000). Only 3 haplotypes were observed out of 8 possible haplotypes; these frequencies are shown in Table 3. The genotype combinations were phased without any ambiguity. Two major haplotypes were found to account for more than 99% of three haplotypes observed.

Analysis for linkage disequilibrium

IL6-597 G>A and *IL6-174 G>C* were in absolute LD ($D' = 1$, $d^2 = 1$), and they had identical allele frequencies, with only two of the four possible haplotypes (A-C and G-G); i.e. the allele at *IL6-597 G>A* was found to occur only with the C allele at *IL6-174 G>C* and the G allele at *IL6-597 G>A* to occur only with the G allele at *IL6-174 G>C*, as indicated by a d^2 value of one. *IL6-597 G>A* and *IL6-174 G>C* had the same LD ($D' = 1$, complete LD) with *IL6-572 C>G*; only three of the four possible haplotypes were observed in the population.

Table 3. *IL6* promoter haplotypes and their frequencies in Korean population ($n = 1,046$) with three *IL6* promoter SNPs (*IL6-597 G>A*; *IL6-572 C>G*; *IL6-174 G>C*).

Haplotype ^a	SNPs in <i>IL6</i> promoter			Frequency
	<i>IL6-597 G>A</i>	<i>IL6-572 C>G</i>	<i>IL6-174 G>C</i>	
GCG	G	C	G	0.758
GGG	G	G	G	0.240
AGC	A	G	C	0.002

^aHaplotypes were constructed by pair-wise tables of three loci. There was no ambiguous haplotype phasing due to absolute LD ($d^2 = 1$) and complete LD ($|D'| = 1$) between SNPs. To calculate haplotype frequencies, HBsAg negative individuals were also included in the cohort.

Association analysis with chronic hepatitis B outcome

The loci, *IL6-597 G>A* and *IL6-174 G>C* were not analyzed statistically because of their allele frequencies in the population were too low. *IL6* haplotypes were not analyzed because they had almost the same distributions (>99.9%) as *IL6-572 C>G*. *IL6-572 C>G* associations with chronic HBV infection outcome were analyzed in a Korean chronic hepatitis B cohort. The results of the statistical analysis are summarized in Tables 4 and 5. Allele frequencies were compared for; i) patients with CH and chronic carriers vs. chronic hepatitis patients those with clinical evidence

Table 4. Analysis for two different phenotypes in the progression to hepatocellular carcinoma (HCC); i) occurrence of LC vs. no LC occurrence based on CH and ii) occurrence of HCC versus No HCC based on LC as functions of *IL6-572 C>G*.

Locus	Genotype	LC occurrence on CH				HCC occurrence on LC			
		LC N (%)	No-LC N (%)	OR ^a (95%CI)	P ^a	HCC N (%)	No-HCC N (%)	OR ^a (95%CI)	P ^a
<i>IL6-572 C>G</i>	C	391 (69.1%)	175 (30.9%)	1.0		117 (53%)	274 (57.7%)	1.0	
	GC	261 (74.8%)	88 (25.2%)	1.27 (0.94-1.73)	0.13	92 (41.6%)	169 (35.6%)	1.04 (0.56-1.95)	0.89
	G	44 (72.1%)	17 (27.9%)	1.06 (0.78-1.43)	0.71	12 (5.4%)	32 (6.7%)	1.23 (0.57-2.69)	0.60

^aOdds ratio (95% CI) and P value calculated by difference of allele frequency between each group. The homozygotes for common alleles were used as referent to heterozygotes and homozygotes of rare allele. Statistical analysis. Logistic regression models were used to calculate the odds ratios (95% confidence interval) and corresponding P values for *IL6-572 G>C* controlling age (continuous) and sex as covariates. Age was highly associated with the occurrence of LC and HCC ($P < 0.0001$) as expected. Sex was associated with the occurrence of HCC based on LC ($P = 0.0004$). All patients included in this study were HBsAg positive.

Table 5. Survival analysis of time to HCC after HBV infection as functions of *IL6-572 G>C*.

Locus ^a	Models	N	Events	P	RH
<i>IL6-572 C>G</i>	Co-dominant	1,028	226	0.62	0.95
	Dominant			0.90	1.02
	Recessive			0.17	0.66

^a*IL6-597 G>A*, *IL6-174 G>C* and *IL6* haplotypes were not analyzed due to low frequencies in the Korean population.

Statistical analysis. Cox models were used for calculating relative hazards and *P* values for *IL6-572 C>G* after controlling for age (adjusted age groups; <40, 40-60 and >60) and sex. Most patients in HBV endemic areas, such as Korea, acquire HBV virus vertically, and it progress to HCC after a period of persistent chronic disease (See text). Here, we used the variable 'time to HCC' on the assumption of early infection.

of LC (*i.e.*, portal hypertension), ii) cirrhotic patients with HCC vs. without HCC by logistic regression, and iii) the time interval from the onset of infection to HCC were analyzed by Cox relative hazard analysis, on the assumption that all the patients were infected during early infancy. No significant associations were detected between *IL6-572 C>G* and hepatitis B outcome; *i.e.*, LC occurrence on CH (OR = 1.27, *P* = 0.13; OR = 1.06, *P* = 0.71) and HCC occurrence on LC (OR = 1.04, *P* = 0.89; OR=1.23, *P* = 0.60) of heterozygotes and homozygotes for G allele in referent comparison to homozygotes for common genotype (C/C genotype), and time interval to HCC (RH = 0.67-1.00; *P* = 0.14-0.99).

Discussion

The different outcomes of HBV infection might be explained by virus-host interactions (Cruz *et al.*, 1987). However, little is known about the host genetic factors that are involved in the progress of HBV infection. *IL6* is one of the candidate genes, which might influence the outcome of chronic liver disease. Elevated *IL6* levels were detected in chronic liver disease (Sekiya *et al.*, 1994; Oyanagi *et al.*, 1999). During HBV infection progress, *IL-6* production was reported to be up-regulated by HBV-X in hepatic inflammation (Lee *et al.*, 1998). *IL6* is also associated with chronic immune-mediated diseases, such as systemic onset juvenile chronic arthritis (Fishman *et al.*, 1998). Moreover, it is known that differences in the *IL6* promoter haplotype may have an important role in determining levels of *IL6* gene transcription. The transcriptional control of this gene is complex, and subtle variations in the promoter are known to influence the regulation of this system (Terry *et al.*, 2000).

In the present study, we found a different genetic background in Koreans and Caucasians with respect

to the allele frequencies of the *IL6* promoter SNPs. The allele frequencies of *IL6-174* and *IL6-597* reported in Caucasian (0.40-0.45) (Terry *et al.*, 2000; Brull *et al.*, 2001; Burzotta *et al.*, 2001; Cavet *et al.*, 2001; Cox *et al.*, 2001; Fedetz *et al.*, 2001; Helmy *et al.*, 2001; Zhai *et al.*, 2001) are much higher than those found in Koreans in our study (0.002) or in the Chinese population (0.002) (Zhai *et al.*, 2001). On the other hand, the frequency of the C allele of *IL6-572* in Caucasians (0.04-0.06) is much lower than those of the Korean (0.75) and Japanese populations (0.816) (Ota *et al.*, 2001). High frequency population-specific alleles are particularly useful for mapping genes the responsible for disease susceptibility and other traits in a population (Parra *et al.*, 1998; Stephens *et al.*, 2001). The SNP of *IL6-572* might be a more useful marker for an association study in Asians than in Caucasians.

LD is becoming an important tool in genetic studies because it is applicable to a variety of topics, including disease-gene mapping (Rannala and Reeve, 2001; Riley *et al.*, 2000). The SNPs in the *IL6* promoter region are in strong LD, and only 3 haplotypes were observed out of eight possible haplotypes. Generally, haplotypes are more informative than single SNPs, but *IL6* haplotypes in Korean population were no more informative for association study of complex trait diseases than single SNPs due to too low frequencies of *IL6-597 G>A* and *IL6-174 G>C*.

In this study, we found that the three haplotypes constructed by the 3 SNPs in the *IL6* promoter region, and their frequencies in the Korean population, and variants in the *IL6* gene are not associated with subsequent HBV outcomes, though elevated *IL6* levels have been detected in several studies and have been found to functionally significant in other diseases. In conclusion, this study shows that although *IL6* may have important functions in the progress of chronic HBV infection, that its' genetic variants probably do not influence the development of LC and HCC from chronic HBV infection.

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