Interleukin 10 haplotype associated with increased risk of hepatocellular carcinoma

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Interleukin 10 (IL10) is a powerful Th-2 cell cytokine produced by lymphoid cells that exerts its functions by inhibiting macrophage/monocyte and T-cell lymphocyte replication and secretion of inflammatory cytokines (IL1, TNFA, TGFB, IL6, IL8 and IL12). Genetic association analysis of a well-characterized HBV cohort revealed that one of IL10 haplotypes, IL10-ht2, was strongly associated with hepatocellular carcinoma (HCC) occurrence in gene dose-dependent manner. The frequency of susceptible IL10-ht2 was much higher in HCC patients and significantly increased in order of susceptibility to HBV progression from chronic hepatitis to liver cirrhosis and HCC among hepatitis B patients. In addition, survival analysis clearly showed that the onset age of HCC was also accelerated among chronic hepatitis B patients who were carrying IL10-ht2. Increased IL10 production mediated by IL10-ht2 suggests that up-regulated IL10 accelerates progression of chronic HBV infection, especially to HCC development.

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major infectious diseases with more than 360 million chronic carriers worldwide. It is a major risk factor for chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC), which is one of the 10 most common carcinomas (1). The association between HBV infection and HCC is now well established. The risk of developing HCC among the HBsAg carriers had been proved to be more than 100 times greater than among the HBsAg non-carriers (2). Typically the development of HCC occurs 30–50 years after infection; consequently HCC is far more likely to be seen in individuals exposed to HBV in early life rather than in adult life. The outcomes of HBV infection do not appeared to be determined by viral strains. Instead, allelic variants in human genome are likely to affect the viral hepatitis progression after infection (3). A strong genetic component determining the outcomes of HBV infection has been established through twin studies (4).

As one of the efforts to discover polymorphism(s) in genes of which variant(s) have been implicated in HBV progression/ HCC occurrence, we scrutinized the single nucleotide polymorphisms (SNPs) in IL10 gene, located on chromosome 1q31–32 with total size ~10 kb as a potent candidate gene for HBV. We screened 1078 patients with chronic HBV infection, including patients without any evidence of portal hypertension (CH), patients with LC and patients with HCC.

RESULTS

The frequencies of rare alleles of four IL10 SNPs were 0.07, 0.30, 0.30 and 0.03, respectively, in the Korean population (n = 1078). Four haplotypes were identified without any ambiguous phasing due to LDs among SNPs (Fig. 1B). In the initial analysis, the rare allele (C allele) of IL10-592A > C showed weak and susceptible association with HCC occurrence (OR = 1.37–1.57; Table 1), whereas all other loci showed no significant associations. Similarly, by Cox analysis of age to HCC, IL10-1082A > G showed weak protective effect in co-dominant and dominant models (RH = 0.70–0.68, P = 0.05–0.04). On the other hand, IL10-592A > C showed weak susceptible effect in all three analyzing models (RH = 1.22–1.39, P = 0.03–0.11; Table 2).

In further haplotype analysis, one of the common haplotypes (frequency = 0.23, n = 1078), IL10-haplotype2 (A-C-C-T; abbreviated as IL10-ht2), which is also common in Caucasians

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(0.29, \(n = 1813\)) and African Americans (0.41, \(n = 858\)) (5), revealed a dose-dependent effect on HCC occurrence among patients with chronic HBV infection, i.e. higher risk for homozygotes (OR = 1.91, \(P = 0.0001\)) than heterozygote (OR = 1.42, \(P = 0.05\)) by referent analysis (Table 1). Among three alternative analyzing models (co-dominant, dominant and recessive models), IL10-ht2 also showed highly significant and susceptible effects with most significance in a co-dominant model (OR = 1.65, \(P = 0.0002\)) and highest risk in a recessive model (OR = 3.08, \(P = 0.0005\)). The role of IL10-ht2 in HCC development was analyzed by the Cox relative hazards model among 937 chronic HBV patients (Table 2) and demonstrated by the Kaplan–Meier survival curve (Fig. 2). Highly significant acceleration to HCC outcome was apparent among the IL10-ht2-bearing patients. Homozygous IL10-ht2/ht2 (red line) showed a significantly different rate to HCC occurrence compared with heterozygous –/ht2 (blue line; RH = 1.85, \(P = 0.01\)) and wild-type (black line; RH = 2.35, \(P = 0.0004\)). Heterozygous –/ht2 also showed accelerated rate to HCC compared with –/– genotype (RH = 1.31, \(P = 0.04\); Fig. 2).

The tendency of IL10-ht2-bearing genotypes to progress to LC and HCC rapidly was also apparent in defined disease categorical analysis (Fig. 3), which allow the inclusion of groups susceptible to LC. Although it was not possible to collect the exact onset age of LC (because a large proportion of patients had enrolled with LC), we could introduce a defined category of...
### Table 1. Analysis of progression to HCC from CH as functions of SNPs and haplotypes in IL10 gene

<table>
<thead>
<tr>
<th>Loci</th>
<th>Genotype</th>
<th>HCC</th>
<th>No HCC</th>
<th>Analyzing models</th>
<th>Co-dominant OR (95% CI)</th>
<th>P</th>
<th>Dominant OR (95% CI)</th>
<th>P</th>
<th>Recessive OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL10-1082</strong></td>
<td>A</td>
<td>201 (87.4%)</td>
<td>675 (85.2%)</td>
<td>1</td>
<td>0.73 (0.46–1.16)</td>
<td>0.18</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>28 (12.2%)</td>
<td>112 (14.1%)</td>
<td>0.9 (0.3–2.72)</td>
<td>0.85</td>
<td>0.9 (0.3–2.72)</td>
<td>0.85</td>
<td>0.9 (0.3–2.72)</td>
<td>0.85</td>
<td>0.9 (0.3–2.72)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>1 (0.4%)</td>
<td>5 (0.6%)</td>
<td>0.73 (0.46–1.16)</td>
<td>0.18</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
<td>0.86 (0.09–7.95)</td>
</tr>
<tr>
<td><strong>IL10-592 [ht1(A-T-A-T)]</strong></td>
<td>A</td>
<td>89 (41.2%)</td>
<td>384 (51.3%)</td>
<td>1</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>101 (46.8%)</td>
<td>299 (40%)</td>
<td>1.41 (1–1.98)</td>
<td>0.05</td>
<td>1.37 (1.08–1.75)</td>
<td>0.009</td>
<td>1.48 (1.07–2.05)</td>
<td>0.02</td>
<td>1.57 (0.94–2.6)</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>1 (0.4%)</td>
<td>5 (0.6%)</td>
<td>1.37 (1.08–1.75)</td>
<td>0.009</td>
<td>1.48 (1.07–2.05)</td>
<td>0.02</td>
<td>1.57 (0.94–2.6)</td>
<td>0.08</td>
<td>1.57 (0.94–2.6)</td>
</tr>
<tr>
<td><strong>IL10 + 117 [ht3(G-C-C-C)]</strong></td>
<td>T</td>
<td>199 (93.4%)</td>
<td>700 (93.1%)</td>
<td>1</td>
<td>0.95 (0.5–1.81)</td>
<td>0.87</td>
<td>0.88 (0.47–1.65)</td>
<td>0.7</td>
<td>0.91 (0.48–1.74)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>14 (6.6%)</td>
<td>51 (6.8%)</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
<td>0.86 (0.09–7.95)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0 (0%)</td>
<td>1 (0.1%)</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
<td>0.86 (0.09–7.95)</td>
</tr>
<tr>
<td><strong>ht2 (A-C-C-T)</strong></td>
<td><strong>/</strong>_</td>
<td>106 (50.2%)</td>
<td>448 (61.5%)</td>
<td>1</td>
<td>0.95 (0.5–1.81)</td>
<td>0.87</td>
<td>0.88 (0.47–1.65)</td>
<td>0.7</td>
<td>0.91 (0.48–1.74)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>__/ht2</td>
<td>85 (40.3%)</td>
<td>251 (34.4%)</td>
<td>1</td>
<td>0.95 (0.5–1.81)</td>
<td>0.87</td>
<td>0.88 (0.47–1.65)</td>
<td>0.7</td>
<td>0.91 (0.48–1.74)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>ht2/ht2</td>
<td>20 (9.5%)</td>
<td>30 (4.1%)</td>
<td>1</td>
<td>0.95 (0.5–1.81)</td>
<td>0.87</td>
<td>0.88 (0.47–1.65)</td>
<td>0.7</td>
<td>0.91 (0.48–1.74)</td>
<td>0.78</td>
</tr>
<tr>
<td><strong>ht4 (G-C-C-T)</strong></td>
<td><strong>/</strong>_</td>
<td>198 (93.8%)</td>
<td>672 (92.2%)</td>
<td>1</td>
<td>0.95 (0.5–1.81)</td>
<td>0.87</td>
<td>0.88 (0.47–1.65)</td>
<td>0.7</td>
<td>0.91 (0.48–1.74)</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>__/ht4</td>
<td>13 (6.2%)</td>
<td>56 (7.7%)</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
<td>0.86 (0.09–7.95)</td>
</tr>
<tr>
<td></td>
<td>ht4/ht4</td>
<td>0 (0%)</td>
<td>1 (0.1%)</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.75 (0.48–1.16)</td>
<td>0.19</td>
<td>0.86 (0.09–7.95)</td>
<td>0.89</td>
<td>0.86 (0.09–7.95)</td>
</tr>
</tbody>
</table>

Logistic regression models were used for calculating odds ratios (95% confidential interval) and corresponding P-values for each SNP sites and haplotypes controlling age, sex and LC as covariables using SAS. The common alleles were used as referent genotype to heterozygote and homozygote of rare allele. P-values of co-dominant, dominant and recessive models are also given. Age (continuous value), sex (male = 0, female = 1) and LC (LC = 1, no LC = 0) were highly associated with occurrence of HCC (P < 0.0001) as expected and adjusted by including in logistic analysis as co-variables. All patients included in study were HBsAg-positive (chronic hepatitis). The mean ages (±SD) were 46.9(±10.4), 49.9(±9.1) and 55.8(±8.8) for CH, LC and HCC groups, respectively. IL10-819 was not analyzed because it is in absolute linkage disequilibrium (r² = 1) with IL10-592. Haplotype analyses were performed only with IL10-h1 and ht4 because h1 and h3 are equivalent with IL10-592 and IL10 + 117, respectively. Recessive models of IL10-1082, IL10 + 117 and ht4 were not analyzed due to low frequencies.
LC (category II; susceptible group to LC occurrence from CH regardless of the onset age of LC). In that category, patients who had progressed to LC before the cutoff ages were included. The frequencies of susceptible IL10-ht2-bearing genotypes were increased in order of susceptibility to LC and HCC (Fig. 2A; $P = 0.005–0.00008$). In addition, separated genotype frequencies of homozygotes and heterozygotes were also compared (Fig. 3B and C). The frequencies of homozygote for IL10-ht2 showed increasing pattern with high significance ($P = 0.05–0.003$), whereas those of heterozygotes showed just increasing trend ($P = 0.04–0.44$). The overall analyses suggest that IL10-ht2 has dose-dependent effects on the onset age of HCC. The attributable fraction of IL10-ht2 on HCC, which is a parameter that combines the strength of the epidemiological influence (relative risk) and the frequency of genotype, was estimated. The attributable fraction calculated by relative risk (1.61, 95% CI; 1.16–2.23) combined with the IL10-ht2-containing genotype frequency (0.41) (Table 1, dominant model) indicates that 21% (95% CI; 6–34%) of HCC progression among chronic HBV patients can be attributed to the detrimental effect of IL10-ht2 allele.

**DISCUSSION**

In HBV endemic areas including Korea, infections occur either during the prenatal period or early in childhood (especially before the beginning of HBV vaccinations in Korea on 1983) (6), and that account for the high prevalence of chronic HBV infection in these areas. Perinatal acquisition differs clinically from acquisition later in life. Most newborns develop an asymptomatic carrier state, despite high levels of HBV replication due to their immature immune response, whereas most adults clear HBV (7). Persistent HBV infection is considered to be an essential factor in the development of HCC. Therefore, the vertical transmissions in early life from carrier mothers become the most important causal factor of LC and HCC.

IL10, produced mainly by macrophages, is a potent immunosuppressive cytokine by down-regulating the expression of Th1 cytokines and co-stimulatory molecules (8). The two SNPs, IL10-1082T $> C$ and -592A $> C$, contributing to IL10-ht2, were particularly interesting, because several previous reports have demonstrated quantitative differences in IL10 transcription and/or expression mediated by alternative alleles or haplotypes. Differences in nuclear-binding activity and IL10 production mediated by the IL10-592A $> C$ polymorphism (5,9), by IL10-1082 A $> G$ polymorphism (10,11) and by IL10 promoter haplotype (12–14) (IL10-ht2 was associated with increased production of IL10 production), have been reported. Several positive associations have also been detected: AIDS progression (5); HCV infection and resistance to antiviral therapy (13,15,16); advanced alcoholic liver disease (17); Epstein–Barrvirus infection (18); gastric cancer (19); cervical cancer (20); multiple myeloma (21); and cutaneous malignant melanoma (22). Recently, similar and concordant effects of IL10 haplotypes on progression to LC among chronic HBV patients have been reported (23). The frequency of IL10-ht2 was higher in patients with LC than in asymptomatic carriers (147 LC patients versus 77 asymptomatic carriers). Also, a higher frequency in patients with advanced fibrosis stage (21 subjects with F0–F2 versus 32 subjects with F3–F4 fibrosis stage) was observed.

The roles of IL10 in those diseases and chronic hepatitis B progression to LC and HCC occurrence in this study suggest that IL10 polymorphisms play a critical role in immunity, inflammation progress and cancer development. The greater susceptible effects of IL10 haplotype on chronic hepatitis B progression also suggest that the effect of IL10 polymorphism might be the result of combined genotype (haplotype) rather than single polymorphisms.

In summary, IL10-ht2 showed a strong association with occurrence of HCC (Table 1), onset age of HCC (Table 2 and demonstrated in Fig. 2) and disease progression (Fig. 3) in a gene dose-dependent manner among chronic hepatitis B patients. Therefore, it could be suggested that the inhibition of innate immunity by increased IL10 production in IL10-ht2-bearing individuals might accelerate the progression of chronic hepatitis B to LC and subsequently to HCC.

**MATERIALS AND METHODS**

**Study population and outcomes**

The study group included 1078 Korean subjects who were enrolled from the outpatient clinic of the liver unit and from the Center for Health Promotion of Seoul National University Hospital between January 2001 and August 2001. HBsAg positive patients were followed up for disease progression at least every 6 months. Subjects were categorized into three groups: (i) CH; (ii) LC; and (iii) 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

**Table 2. Cox relative hazards analysis for age of HCC occurrence as functions of SNPs and haplotypes in IL10 gene**

<table>
<thead>
<tr>
<th>Locus</th>
<th>n/events</th>
<th>Co-dominant</th>
<th>Dominant</th>
<th>Recessive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\chi^2$</td>
<td>RH $P$</td>
<td>$\chi^2$</td>
</tr>
<tr>
<td>IL10-1082</td>
<td>949/255</td>
<td>3.97</td>
<td>0.70</td>
<td>0.05</td>
</tr>
<tr>
<td>IL10-592 h(t1[A-T-A-T])</td>
<td>947/256</td>
<td>4.52</td>
<td>1.02</td>
<td>0.03</td>
</tr>
<tr>
<td>IL10 + 117 h(t3[G-C-C-C])</td>
<td>947/254</td>
<td>0.86</td>
<td>0.78</td>
<td>0.35</td>
</tr>
<tr>
<td>ht2[A-C-C-T]</td>
<td>937/253</td>
<td>12.57</td>
<td>1.43</td>
<td>0.0004</td>
</tr>
<tr>
<td>ht4[G-C-C-T]</td>
<td>937/253</td>
<td>1.92</td>
<td>0.69</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Cox models were used for calculating relative hazards and $P$-values for SNPs and haplotypes controlling sex and LC by SAS. The results of IL10-ht2 are visualized by Kaplan–Meier survival curve (Fig. 2).
anti-HBc (serological status by 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 of Human Research of Seoul National University Hospital approved the study protocol. The serologic tests of every subjects were performed by commercially available products for HBsAg (ELISA Processor III, Behring, Germany), anti-HBs (ELISA Processor III, Behring, Germany) and anti-HBc (ELISA Processor III, Behring, Germany). All patients were also tested for HIV and HCV antibodies using ELISA (ELISA Processor III, Behring, Germany). The diagnosis of HCC was based on: (i) positive findings on cytological

Figure 3. Frequencies of susceptible IL10-h12 in three categories of progression of chronic hepatitis B. Each group is composed of as following in the order of susceptibility to progression of chronic hepatitis B to LC and HCC. (I) Patients who did not progress to LC and HCC with age greater than the cutoff age. (II) Patients who progressed to LC (without HCC) before the cutoff age. (III) Patients who progressed to HCC before the cutoff age. Patients who progressed to HCC without LC were excluded from categories III to eliminate the complexity of different progression of chronic hepatitis B other than CH→LC→HCC. Ages of 50, 55 and 60 were chosen for cutoff ages considering high occurrence ages of LC and HCC. (A) Frequencies of IL10-h12 and IL10-h12/h12 (dominant model). The numbers above bars are the total number of patients in each category. Mantel–Haenszel chi-square P-values are given. (B) Same as (A) for IL10-h12/h12. (C) Same as (A) for IL10-h12/h12 (recessive model). Frequencies of IL10-h12/h12 and IL10-h12/h12 of all HBsAg positive patients [n = 1078; 5.2% and 41.4% for IL10-h12/h12 and (IL10-h12/h12 or --/h12), respectively] are indicated by arrows. Frequencies of IL10-h12/h12 and h12/h12 of all HBsAg-positive patients were not different (P > 0.05; data not shown) from those of the high-risk HBV-uninfected group (n = 81) and our asthma cohort of the Korean population (n = 938).
or pathological examination; and/or (ii) positive images on angiogram, ultrasonography, and/or computed tomography, combined; and/or (iii) alpha-fetoprotein level >400 ng/ml.

Genotyping

Sequences of amplifying and extension primers for IL10 SNP genotyping for IL10-1082 A > G; IL10-819 T > C; IL10-592 A > C and IL10 –117 T > C are; forward-5‘-ccagaattcgtcccttactccttac-3‘, reverse-5‘-caggattcgtcccttacgttgtaa-3‘, extension-5‘-gtcaggatagtgtaa-3‘; forward-5‘-gggtgaaacatggcagcc-3‘, reverse-5‘-ggtagtgcacctgcagcc-3‘, extension-5‘-gtcactgacccagccc-ctt-3‘; forward-5‘-atactgtgccagccct-cct-3‘, reverse-5‘-atagctgacccagccc-ctt-3‘, extension-5‘-ctcattttaccttc-cagagaagtgtgtaa-3‘; forward-5‘-atactgtgccagccct-cct-3‘, reverse-5‘-atagctgacccagccc-ctt-3‘, extension-5‘-ctcattttaccttc-cagagaagtgtgtaa-3‘; forward-5‘-atactgtgccagccct-cct-3‘, reverse-5‘-atagctgacccagccc-ctt-3‘, extension-5‘-ctcattttaccttc-cagagaagtgtgtaa-3‘, respectively. Single-base extension methods were performed for genotyping of SNPs in IL10 as described previously (24).

Statistics

Logistic regression models were used for calculating odds ratios (95% confidential interval) and corresponding P-values controlling age (continuous value), sex (male = 0, female = 1) and LC (LC = 1, no LC = 0) as covariates. Cox models were used for calculating relative hazards and P-values controlling sex and LC. The results of Cox models were visualized by Kaplan–Meier survival curves. Mantel–Haenszel chi-square (MHC) tests were used for trend tests in categorical analysis.

The attributable fraction was computed by the formula as described previously (25).

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