Polymorphisms near *Interleukin 28B* Gene Are Not Associated with Hepatitis B Virus Clearance, Hepatitis B e Antigen Clearance and Hepatocellular Carcinoma Occurrence

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Key Words

IL28B • Single nucleotide polymorphism • Haplotype • Hepatitis B virus • Hepatocellular carcinoma

Abstract

Background: Polymorphisms near the *IL28B* gene have been proposed to be strongly associated with treatment response and the rate of spontaneous clearance of hepatitis C virus infection, and treatment response of hepatitis B virus (HBV) infection. In this study, we aimed to determine whether these polymorphisms could affect natural courses of HBV infection. **Methods:** Genetic variations were identified through direct DNA sequencing using TaqMan assay in 1,439 patients with past or present HBV infection. Subjects included 404 spontaneously recovered patients, 313 chronic hepatitis B (CHB) patients, 305 liver cirrhosis (LC) patients and 417 hepatocellular carcinoma (HCC) patients. Three polymorphisms near the *IL28B* gene, rs8099917T>G, rs12979860C>T and rs12980275A>G, were identified. Associations between these polymorphisms and HBV clearance, hepatitis B e anti-

gen (HBeAg) clearance as well as HCC occurrence among patients were analyzed using logistic regression analyses adjusted for age and gender. **Results:** There were no significant associations between these polymorphisms and the HBV clearance both in CHB and LC groups. Similarly, these polymorphisms showed no significant associations with HBeAg clearance and the occurrence of HCC either. **Discussion:** No significant association was identified between polymorphisms near the *IL28B* gene and the natural courses of chronic HBV infection, including the HBV clearance and HCC occurrence.

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Introduction

Infection with hepatitis B virus (HBV) is one of the great global public health problems. The clinical outcome of HBV infection varies, and ranges from spontaneous recovery to chronic carriers (CC) or even death from hepatocellular carcinoma (HCC). Although a vaccine

against HBV is available, more than 350 million people worldwide are still in a CC state of HBV [1]. Chronic HBV infection is a major risk factor for several liver diseases, such as chronic hepatitis B (CHB), liver cirrhosis (LC) and HCC [2], which was the twelfth most common cancer and the eighth leading cause of cancer-related mortality in the USA in 2011 [3].

The molecular mechanisms that induce the different outcomes of HBV infection still remain undetermined. However, some family and twin studies show that host genetic factors are the probable cause influencing the outcome of HBV infection [4, 5]. Indeed, our previous studies have demonstrated that genetic variations in histone deacetylase 10, Fas/Fas ligand, transforming growth factor- $\alpha/\beta 1$, interleukin (IL)10, monocyte chemotactic protein-1, cyclin D2, DNA methyltransferase 1, insulin-like growth factor 2 and secreted phosphoprotein-1 are related to HBV clearance [6–8], HCC progression [9–12], or both [13, 14]. In spite of these results, associations of HBV-related liver diseases with other host genetic diversities still need further investigation.

The *IL28B* gene, which forms a cytokine gene cluster on chromosome 19q13, encodes for interferon-lambda (IFN- λ). IFN- λ activates a cascade through the JAK/STAT pathway, which induces IFN-stimulated genes [15, 16]. This cytokine has been certified as an important modulator of the immune response to hepatitis C virus (HCV). Genetic polymorphisms near the *IL28B* gene are associated with the response to pegylated interferon (PEG-IFN)- α and ribavirin therapy for chronic hepatitis C [17]. Furthermore, the same polymorphisms are associated with spontaneous clearance of HCV [18]. The exact biologic mechanism or pathway of this phenomenon remains unclear, but the influence of single nucleotide polymorphisms (SNPs) near the *IL28B* gene might not be limited in chronic HCV clearance. It is known that IFN- λ exhibits antitumoral activities in several experimental studies in cell lines and animal models [19–22]. In addition, higher spontaneous recovery rates of HCV infection in patients with favorable genotypes might lead to lower risk of HCC development. Several studies have already shown that the polymorphisms near the *IL28B* gene are associated with HCC occurrence in patients with chronic HCV infection [23].

Although the mechanism by which *IL28B* influences outcome of HCV infection remains elusive, it is likely that the relationship is not specific to HCV infection. IFN- λ has been shown previously to be active against several other viruses, including HBV [24], and HBV is also an IFN- α -responsive chronic viral illness. Indeed, the rela-

tionship between genetic variation of the *IL28B* gene and serologic response to PEG-IFN in patients with hepatitis B e antigen (HBeAg)-positive CHB has been recently determined [25]. However, the association of polymorphisms near the *IL28B* gene with natural courses in patients with chronic HBV infection, such as spontaneous recovery, HBeAg clearance or HCC occurrence, has not been previously characterized. Accordingly, this study was conducted to determine whether polymorphisms near the *IL28B* gene could affect HBV clearance and HCC occurrence in chronic HBV-infected patients.

Materials and Methods

Study Patients

A total of 1,439 individuals having either past or present evidence of HBV infection were recruited from patients enrolled in the outpatient clinic of the Liver Unit and the Center for Health Promotion at Seoul National University Hospital and Ajou University Medical Center. Diagnoses of chronic carriers and spontaneously recovered individuals were established by repeated seropositivity for hepatitis B surface antigen (HBsAg; Enzygnost1 HBsAg 5.0; Dade Behring, Marburg, Germany) over a 6-month period, and for both anti-HBs (antibody to hepatitis B surface antigen; Enzygnost Anti-HBs II; Dade Behring) and anti-HBc (antibody to hepatitis B core antigen; AB-Corek; DiaSorin, Saluggia, Italy) of the IgG type without HBsAg, respectively. The chronic carrier group was assessed further for disease progression to cirrhosis or HCC. All patients in the chronic carrier group had undergone regular medical follow-ups and had been evaluated with serum alpha-fetoprotein level, abdominal ultrasonography and/or a 4-phase spiral liver CT scans more than twice a year to detect early stages of HCC. Dynamic contrast-enhanced abdominal MRI, bone scan, chest CT, brain MRI, brain CT, hepatic angiography or PET scan was also carried out in some patients based on the clinical decision.

Diagnosis of HCC was based on image findings of nodules larger than 1 cm showing intense arterial uptake followed by washout of contrast in the venous-delayed phases in a 4-phase multidetector CT scan or dynamic contrast-enhanced MRI and/or biopsy [26]. Cirrhosis of the liver, on the other hand, was diagnosed by biopsy or based on the clinical evidence of portal hypertension such as visible collateral vessels on the abdominal wall, esophageal varices on esophagogastroscope, palpable splenomegaly and sonographically definite findings of cirrhotic liver or ascites. The age of onset of HCC was determined according to the date of initial diagnosis.

Exclusion criteria for the study patients were the following: (i) testing positive for anti-HBs but not for anti-HBc; (ii) testing positive for anti-HCV or anti-HIV (GENEDIA®; Greencross Life Science Corp., Yongin-shi, Korea, HCV® 3.2; Dong-A Pharmaceutical Co., Seoul, Korea); (iii) average alcohol consumption of ≥ 10 g/day or an average number of ≥ 1 cigarette pack/s smoked daily assessed through individual interviews, and (iv) occurrence of other types of liver diseases such as autoimmune hepatitis, toxic hepatitis, hemochromatosis, Wilson's disease, nonalcoholic ste-

atohepatitis, primary biliary cirrhosis or Budd-Chiari syndrome. None of the patients had a previous history of immunosuppressant or anti-viral treatment, including interferon and/or nucleos(t)ide analogues. Finally, informed written consent was obtained from the patients prior to conducting the study, and ethical approval was obtained from the Institutional Review Board for Human Research at Seoul National University Hospital and Ajou University Medical Center.

Genotyping of IL28B Gene Polymorphisms

Using the Wizard genomic DNA purification kit (Promega, Madison, Wisc., USA), genomic DNA was extracted from patients' peripheral blood samples. In addition, SNP genotyping was performed using the TaqMan [27] assay in the ABI prism 7900HT sequence detection system (Applied Biosystems, Foster City, Calif., USA). Genotyping quality control was performed in 10% of the samples by duplicate checking (rate of concordance in duplicates = 100%). The *IL28B* gene polymorphisms, rs8099917, rs12979860 and rs12980275, were genotyped as described previously [28]. In more detail, Assays-On-Demand probe was used for rs8099917 (Applied Biosystems ref. C_11710096_10), and probes of rs12979860 and rs12980275 were custom designed. For rs12979860, primer sequences were: forward TGTACTGAACCA-GGGAGCTC and reverse GCGCGGAGTGCAATTCAAC, and probe sequences were: Vic-TGGTTCGCGCCTTC and Fam-TG-GTTCACGCCTTC. For rs12980275, primer sequences were: forward GTGCTGAGAGAAGTCAAATTCC and reverse CCGC-TACCCGGCAAATATT, and probe sequences were: Vic-AGA-CACGTCTGTTTCTA and Fam-ACACGTCCGTTTCTA.

Statistical Analyses

To determine the association of rs8099917, rs12979860 and rs12980275 with HBV clearance as well as HCC occurrence, the odds ratio (95% confidence interval) was calculated using logistic analysis adjusted for age (continuous value) and gender (male = 0, female = 1) as covariates to eliminate or reduce any confounds that might influence the findings. Data was managed and analyzed using the Statistical Analysis System (SAS) version 9.1 (SAS Inc., Cary, N.C., USA). Using the PHASE algorithm, version 2.0 [29], haplotypes were then inferred from the genotyped SNPs. Lewontin's D' ($|D'|$) and the linkage disequilibrium (LD) coefficient r^2 were then examined using the Haploview algorithm [30] to measure the LD between all pairs of biallelic loci.

Results

Polymorphisms near the IL28B Gene

By direct sequencing, three SNPs were identified near the region of the *IL28B* gene on chromosome 19q13.13. They were rs8099917T>G, rs12979860C>T and rs12980275A>G (fig. 1a). Minor allele frequencies of these SNPs were 0.053, 0.060 and 0.066 among Korean subjects (table 2). Genotype distributions in all loci were in Hardy-Weinberg equilibrium ($p > 0.05$). Five haplotypes were inferred from three polymorphisms, and the

Fig. 1. Physical map, haplotypes and linkage disequilibrium of *IL28B* gene polymorphisms. **a** Location of *IL28B* gene polymorphisms on chromosome 19q13.13. The black block represents the *IL28B* gene. The frequency in parentheses was based on the genotyping data ($n = 1,439$). **b** Haplotypes of rs8099917, rs12979860 and rs12980275. **c** Linkage disequilibrium coefficient ($|D'|$) and r^2 among the three SNPs.

most common haplotype was T-C-A (fig. 1b). The frequency of T-C-A was 0.933, while those of other haplotypes were below 0.050. The SNPs rs8099917 and rs12979860 were in strong linkage disequilibrium ($r^2 = 0.874$; fig. 1c). The linkage disequilibrium between rs8099917 or rs12979860 and rs12980275 was smaller ($r^2 = 0.715$ and $r^2 = 0.827$).

Clinical Profiles of the Study Patients

A total of 1,439 Korean subjects with past or present evidence of HBV infection were enrolled in the study and classified into 2 subgroups: (1) 404 spontaneously recovered subjects as controls, and (2) 1,035 chronic HBV-infected patients (table 1). Chronic HBV-infected patients were composed of 313 CH subjects, 305 LC subjects and

Table 1. Clinical profiles of the study patients

Clinical profile	Spontaneously recovered	Chronic carrier		
		hepatocellular carcinoma	liver cirrhosis	chronic hepatitis
Patients, n	404	417	305	313
Mean age (range), years	53.1 (28–81)	57.5 (25–82)	50.8 (22–90)	44.4 (18–79)
Gender (male/female)	272/132	279/48	231/74	238/75
HBeAg-positive rate, %	0	25.7	29.7	37.1
HBeAb-positive rate, %	37.7	65.8	50.2	44.9
HBsAg-positive rate, %	0	100	100	100
HBsAb-positive rate, %	100	0	0	0

Table 2. Genotypes and minor allele frequencies of SNPs in the *IL28B* gene among Korean subjects (n = 1,439)

rs	Chr	Coordinate ¹	Location relative to <i>IL28B</i>	Alleles	Genotype (samples, n)	MAF	Heterozygosity	HWE ²
rs8099917	19	39743165	Promoter	T>G	TT (1,253) TG (137) GG (6)	0.053	0.101	0.963
rs12979860	19	39738787	Promoter	C>T	CC (1,242) CT (155) TT (6)	0.060	0.112	0.770
rs12980275	19	39731783	3' downstream	A>G	AA (1,234) AG (167) GG (9)	0.066	0.123	0.669

¹ Human genome build 37.² p values of deviation from Hardy-Weinberg equilibrium among a spontaneously recovered group.

417 HCC subjects. Subjects with more progressive disease tended to be older and had a higher male to female ratio, as well as lower positive rate of HBeAg.

Association Analysis of Polymorphisms near the IL28B Gene with Spontaneous Clearance of HBV

We analyzed the association of polymorphisms near the *IL28B* gene with the rate of spontaneous clearance of HBV among Korean subjects with CH (table 3). Polymorphism rs8099917T>G had no significant association with the clearance of HBV (OR = 1.02, p = 0.90 in a codominant model; OR = 1.01, p = 0.97 in a dominant model; OR = 1.58, p = 0.68 in a recessive model). Similarly, neither did rs12979860C>T (OR = 1.03, p = 0.89 in a codominant model; OR = 1.01, p = 0.94 in a dominant model; OR = 1.57, p = 0.68 in a recessive model), nor rs12980275A>G (OR = 1.09, p = 0.60 in a co-dominant model; OR = 1.06, p = 0.75 in a dominant model; OR = 2.86, p = 0.32 in a recessive model).

Association Analysis of Polymorphisms near the IL28B Gene with Risk of HCC Occurrence

There were no statistically significant associations between polymorphisms near the *IL28B* gene and the occurrence of LC and HCC. Polymorphisms rs8099917T>G (OR = 0.82, p = 0.37 in a codominant model; OR = 0.86, p = 0.51 in a dominant model), rs12979860C>T (OR = 0.80, p = 0.31 in a codominant model; OR = 0.84, p = 0.43 in a dominant model) and rs12980275A>G (OR = 0.72, p = 0.11 in a codominant model; OR = 0.73, p = 0.16 in a dominant model; OR = 0.16, p = 0.10 in a recessive model) did not show statistically significant association with the occurrence of HCC in CH and LC groups. Similarly, rs8099917T>G (OR = 0.77, p = 0.31 in a codominant model; OR = 0.84, p = 0.52 in a dominant model), rs12979860C>T (OR = 0.75, p = 0.22 in a codominant model; OR = 0.80, p = 0.38 in a dominant model), and rs12980275A>G (OR = 0.66, p = 0.07 in a codominant

Table 3. Association analysis of the *IL28B* gene polymorphisms with risk of liver disease in Korean subjects (n = 1,439)

Test group	rs	Minor allele frequency		Codominant		Dominant		Recessive	
		case	control	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
HBV infection	rs8099917	0.054	0.051	1.02 (0.71–1.48)	0.90	1.01 (0.68–1.49)	0.97	1.58 (0.18–13.64)	0.68
HBV (n = 1,035) vs.	rs12979860	0.060	0.057	1.03 (0.72–1.46)	0.89	1.01 (0.70–1.47)	0.94	1.57 (0.18–13.49)	0.68
SR (n = 404)	rs12980275	0.068	0.061	1.09 (0.78–1.53)	0.60	1.06 (0.74–1.51)	0.75	2.86 (0.35–23.11)	0.32
HCC occurrence	rs8099917	0.045	0.060	0.82 (0.52–1.27)	0.37	0.86 (0.54–1.37)	0.51	–	–
HCC (n = 417) vs.	rs12979860	0.052	0.066	0.80 (0.53–1.23)	0.31	0.84 (0.54–1.30)	0.43	–	–
CH and LC (n = 618)	rs12980275	0.053	0.077	0.72 (0.48–1.07)	0.11	0.73 (0.48–1.13)	0.16	0.25 (0.03–2.14)	0.20
HCC occurrence on LC	rs8099917	0.045	0.057	0.77 (0.47–1.27)	0.31	0.84 (0.49–1.44)	0.52	–	–
HCC (n = 417) vs.	rs12979860	0.052	0.066	0.75 (0.47–1.19)	0.22	0.80 (0.48–1.32)	0.38	–	–
LC (n = 305)	rs12980275	0.053	0.076	0.66 (0.43–1.03)	0.07	0.69 (0.42–1.12)	0.13	0.16 (0.02–1.40)	0.10
LC occurrence on CH	rs8099917	0.057	0.064	0.98 (0.61–1.58)	0.94	0.86 (0.51–1.47)	0.59	4.93 (0.54–44.66)	0.16
LC (n = 305) vs.	rs12979860	0.066	0.066	1.11 (0.70–1.75)	0.66	1.01 (0.61–1.68)	0.96	4.91 (0.54–44.52)	0.16
CH (n = 313)	rs12980275	0.076	0.077	1.08 (0.71–1.65)	0.72	1.00 (0.62–1.61)	1.00	2.85 (0.53–15.24)	0.22
HBsAg clearance	rs8099917	0.041	0.050	0.78 (0.50–1.22)	0.27	0.78 (0.49–1.24)	0.30	0.43 (0.03–6.97)	0.56
Chronic (n = 666) vs.	rs12979860	0.050	0.056	0.87 (0.57–1.32)	0.51	0.88 (0.57–1.35)	0.55	0.43 (0.03–6.85)	0.55
Cleared (n = 353)	rs12980275	0.056	0.061	0.91 (0.61–1.34)	0.62	0.89 (0.59–1.34)	0.57	1.27 (0.13–12.32)	0.84
HBeAg clearance	rs8099917	0.057	0.034	1.64 (0.90–2.97)	0.11	1.58 (0.85–2.94)	0.14	–	–
Chronic (n = 189) vs.	rs12979860	0.063	0.045	1.46 (0.84–2.55)	0.18	1.41 (0.79–2.50)	0.24	–	–
Cleared (n = 428)	rs12980275	0.069	0.051	1.33 (0.80–2.23)	0.27	1.40 (0.81–2.42)	0.23	0.95 (0.08–10.79)	0.97

Minor allele frequencies and p values for logistic analyses of three alternative models (codominant, dominant and recessive models) are shown.
SR = Spontaneously recovered.

model; OR = 0.69, p = 0.13 in a dominant model; OR = 0.16, p = 0.10 in a recessive model) had no statistically significant association with the occurrence of HCC in the LC group.

Effects of Polymorphisms near the IL28B Gene in Other Clinical Fields

We analyzed the effects of the SNPs near the *IL28B* gene to LC occurrence, HBsAg seroconversion and HBeAg clearance. However, the polymorphisms near the *IL28B* gene had no significant association with LC occurrence, HBsAg seroconversion or HBeAg seroconversion (table 3).

Discussion

The purpose of this study was to determine whether polymorphisms near the *IL28B* gene could affect natural courses of HBV infection. Three polymorphisms near the *IL28B* gene were identified and no significant association was identified between these 3 polymorphisms and the natural history of chronic HBV infection including the serologic response and HCC occurrence.

There have been only a few studies regarding associations of SNPs near the *IL28B* gene with HBV clinical outcomes and the results are somewhat contradictory. Recently, Sonneveld et al. [25] showed that genetic polymorphisms near the *IL28B* gene, including rs12980275 and rs12979860, are associated with higher rates of serologic response to PEG-IFN in patients with HBeAg-positive CHB. However, the results of studies on the association of SNPs near the *IL28B* gene with natural courses of chronic HBV infection are inconsistent, as contrasted with chronic HCV infection. Martin et al. [31] reported that the SNP upstream of the *IL28B* gene rs12979860 was not related to the immune response to HBV. On the contrary, Li et al. [32] described that the three polymorphisms dealt with in our study might affect the HBV clinical outcomes by suppression of viral load and hepatic inflammation among a Chinese Han population. Previous studies support the latter results by showing that IFN-λ suppresses HBV viral replication in vivo [16, 22].

The current study did not find a significant relationship between genetic variations near the *IL28B* gene and natural courses of chronic HBV 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, 1,439 individuals with chronic HBV infection, which is more than twice the subjects included in previous studies. This would further add to the statistical power of analysis and search more SNPs than ever. Second, this study was conducted in a place where almost all patients had their infections transmitted vertically [33]. The mode of transmission is relatively homogenous as contrasted with previous studies, where the impact of the mode of transmission as a potentially confounding factor could be minimized. Considering that the most significant factor affecting the chronicity of HBV infection is age at infection, the chronicity in Korean patients seems to be determined by host factors or genetic differences rather than viral factors such as variations in virulence of the viral strains [34–36]. 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.

The sustained virological response rates of HCV infection to PEG-IFN and ribavirin are higher in Asian patients compared with other ethnic groups. A previous study showed that Asian race is one of the independent predictors of sustained virological response in a multivariate logistic regression analysis [37]. The sustained virological response rates of Korean patients in particular are about 10% higher than those of Caucasian [38–40]. The causes of racial variation are still unclear. Some factors, like cytokine production and hepatic iron level, have been considered as the origin of inter-ethnic differences in prior investigations [41, 42]. Since the strong associations of *IL28B* gene polymorphisms with HCV clearance have been demonstrated, SNPs near the *IL28B* gene are thought to be the leading cause of racial variation of HCV clinical outcomes [43]. In this study, we have demonstrated the minor allele frequencies of those polymorphisms among the Korean population. The minor allele frequencies of Koreans are rarer than those of Caucasians, African-Americans, Hispanics and some Asians [44, 45]. Although SNPs near the *IL28B* gene have no association with HBV natural histories, their minor allele frequencies among Koreans might be important in the studies regarding chronic HCV infection and treatment response of HBV infection.

However, there are some limitations in this study. Disrelation between genetic variations of the *IL28B* gene and

HBV clinical outcomes does not mean that IFN- λ has no role in HBV pathophysiology. It has been already demonstrated that therapeutic responses to PEG-IFN are dependent on the polymorphisms near the *IL28B* gene in patients with HBeAg-positive CHB [25]. The polymorphisms near the *IL28B* gene may modify the immune functions of IFN- λ . In vitro IFN- λ functional assays with mutagenesis studies have not been done even in HCV fields [46]. Therefore, functional studies to evaluate the expression levels of IFN- λ and IFN-stimulated genes are needed in CHB patients with or without PEG-IFN treatment. Furthermore, we enrolled only patients with genotype C [47] and it is therefore unclear whether our findings can be extended to patients with other genotypes. Also, more than 90% of our cohort had favorable *IL28B* polymorphisms and the absence of a statistical relationship might be caused by this deviation. Therefore, the associations of *IL28B* gene polymorphisms with HBV outcome should be studied in other ethnic cohorts with different viral genotypes.

In conclusion, we have shown the frequencies of minor alleles and haplotypes near the *IL28B* gene in the Korean population. However, the genetic variations near the *IL28B* gene which have strong association with HCV natural history, therapeutic response rates and HBV therapeutic responses have demonstrated no effects on disease progression, HBeAg clearance and spontaneous recovery in chronic HBV infection.

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