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ORIGINAL ARTICLE

Genetic variation in *IL28B* is associated with the development of hepatitis B-related hepatocellular carcinoma

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Abstract To evaluate the role of host *IL28B* (interleukin 28B; interferon lambda 3) single nucleotide polymorphisms (SNPs) in predicting hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) susceptibility, three SNPs in the *IL28B* gene (rs12979860C/T, rs8099917G/T and rs12980275G/A) were examined in 330 subjects (including 154 HBV-related HCC patients, 86 non-HCC patients with chronic hepatitis B (CHB), 43 HBV self-limited infections and 47 healthy controls). Notably, the frequency of CC homozygosity was 91.5% in healthy controls and 72.9% in CHB, the difference being statistically significant ($\chi^2 = 6.40$, $P = 0.01$). The statistically difference was seen between healthy controls (91.5%) and HCC (74.7%) ($\chi^2 = 6.05$, $P = 0.01$). However, this significant finding was not seen between HBV self-limited and healthy controls. Carriers of the minor T allele in rs12979860 had a higher risk of HCC compared with non-carriers ($\chi^2 = 4.44$, $P = 0.04$). Haplotype analyses revealed significant association between haplotype C–T–A and healthy controls, but not with the HCC group (96.6 vs. 82.0%, $\chi^2 = 6.08$, $P = 0.01$). Analyses of genotype combination and gene–gene interaction showed that there was a positive interaction between rs12979860 and rs12980275, with an OR rate of 11.79 (likelihood test, $P = 0.04$). Our results suggest that the *IL28B* rs12979860 C/T polymorphism might affect susceptibility to the chronic HBV infection and progression of HCC. Of note, the T allele and non-CC genotypes have strong predictive effect of increasing susceptibility of chronic HBV infection and HCC.

Keywords Hepatitis B virus (HBV) · Interleukin 28B · Hepatocellular carcinoma · Genetic polymorphism · Interferon-λ

Introduction

Hepatocellular carcinoma (HCC) ranks as the sixth most common cancer and the third most common cause of cancer death, accounting for more than 600,000 deaths worldwide each year [1]. The majority of HCC cases occur in China. The risk of developing HCC varies according to the particular etiology of a liver disease, with chronic hepatitis B virus (HBV) infection being the most frequent underlying cause [2, 3]. With antiviral, immunomodulatory and perhaps antitumor activities, standard or pegylated interferon-alpha (IFN-α) is the current therapeutic option of choice for patients with chronic hepatitis B (CHB). There is strong evidence from prospective cohort studies that IFN-α treatment, by suppressing HBV replication, decreases overall HCC incidence, with a more marked effect in sustained responders [4–7]. However, the putative beneficial effect of IFN treatment is difficult to prove due to the wide variability of individual outcomes in the natural course of these diseases, in which genetic factors are likely to play a role [8, 9].

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As a therapeutic agent, IFN-λ might have longer and more potent effects than IFN-α. IFN-λ interacts with a transmembrane receptor to induce potent antiviral responses that are mediated through the activation of the JAK-STAT and MAPK pathways [10–12]. In vitro and in vivo models have shown the importance of IFN-λ in the immune response to several viral pathogens, including hepatitis C virus (HCV) and HBV [13]. Several genome-wide association studies (GWAS) have identified a strong association between single nucleotide polymorphisms (SNPs) in and near *IL28B* (which encodes IFN-λ3) and response to therapy [14–16] and with spontaneous HCV clearance [17]. It is possible that similar effects occur in patients with chronic HBV infection, since IFN-λ inhibits HBV and HCV replication in an experimental model [18].

Based on this hypothesis, we evaluated whether specific *IL28B* risk genotypes associate with HBV-related HCC, because *IL28B* polymorphic alleles affect responses to IFN, and IFN therapy changes the prognosis of chronic hepatitis B (CHB).

Materials and methods

Subjects

In accordance with the principle of informed consent, the research comprised a hospital-based case–control study of 330 subjects, including 154 HBV-related HCC patients, 86 non-HCC patients with CHB, 43 HBV self-limited infections and 47 healthy controls. The subjects were exclusively Han Chinese, attending Beijing You'an Hospital from August 2010 to September 2011. The diagnosis of HCC was confirmed by pathological examination or α-fetoprotein elevation (>400 ng/ml) combined with imaging examination (magnetic resonance imaging [MRI] and/or computed tomography [CT]). All patients with HBV infection selected for the study (including HCC and non-HCC patients) were further 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. Patients with positive laboratory tests for human immunodeficiency virus (HIV), hepatitis C virus (HCV; anti-HCV and/or HCV-RNA), alcoholic liver disease or suspected autoimmune diseases with antinuclear antibody titer greater than 1:160 were excluded from the study. Demographic and laboratory parameters of the subjects are presented in Table 1.

DNA extraction and genotyping

A single sample of approximately 3–5 ml of venous blood was collected from each participant into EDTA-containing tubes. Genomic DNA was extracted from a leukocyte pellet by traditional proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. The three SNPs in *IL28B* (rs12979860C/T, rs8099917G/T and rs12980275G/A) selected for the present study are recorded in the public dbSNP database. The SNP ID numbers and detailed sequence information are available at http://www.ncbi.nlm.nih.gov/SNP/. The three SNPs were genotyped using the TaqMan allelic discrimination assay on an ABI 7900FQ system (Applied Biosystems, Foster City, CA). 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) to confirm the accuracy of this method. The details of the genotyping experiments are summarized in Table 2.

Statistical analysis

Differences in laboratory parameters, such as albumin (ALB), total bilirubin (T-Bil), alanine aminotransferase (ALT), alphafetoprotein (AFP) and platelet (PLT), were compared between groups using one-way ANOVA to determine their associations with the presence of HBV-related HCC. Student's t test or the χ^2 test was used to evaluate differences in the distributions of demographic characteristics, selected variables and *IL28B* genotypes among HCC, CHB, HBV self-limited infections and healthy controls. Whether the studied SNPs were in Hardy–Weinberg equilibrium was analyzed by the χ^2 test. Multiple logistic regressions were performed to evaluate whether there were differences between each SNP after adjustment for age, sex and presence of HBV DNA. Linkage disequilibrium (LD) values (D'), r^2 values and haplotypes were estimated using the online software, SHEsis (Shi and He 2005). Multiple logistic regressions were also performed to evaluate the combined genotypes of the three SNPs in *IL28B* and gene–gene interactions. All statistical tests were 2-tailed. P values less than .05 were considered statistically significant. The analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 11.5.

Results

Demographic and clinical characteristics

Table 1 includes the demographic and laboratory parameters for cases within different disease groups and healthy controls, including gender, age, ALB, T-Bil, ALT, AFP and PLT. The HBV-related HCC patients were older than

Table 1 Demographic and laboratory parameters of the subjects enrolled in the study

Groups	HBV-related HCC ($n = 154$)	Non-HCC patients with CHB ($n = 86$)	HBV self-limited infections ($n = 43$)	Healthy controls ($n = 47$)
Demographic parameters				
Gender (M/F)	101/53	46/40	23/20	26/21
Age (Y) ($\chi \pm$ SD)	51.2 ± 10.96	42.3 ± 10.02	33.5 ± 9.68	33.8 ± 12.33
History of alcohol intake	58/96	21/65	11/32	10/37
Presence of cirrhosis	57/97	19/67	0/43	0/47
Laboratory parameters ($\chi \pm$ SD)				
T-Bil (μ mol/l)	33.06 ± 10.77	18.67 ± 6.57	14.77 ± 4.23	15.34 ± 6.56
ALB (g/l)	30.12 ± 6.55	42.11 ± 8.96	43.56 ± 2.71	42.18 ± 3.44
ALT (U/l)	118.67 ± 102.45	44.67 ± 31.05	20.26 ± 12.37	25.13 ± 11.11
AFP (ng/ml)	543.45 ± 520.67	2.14 ± 1.11	2.33 ± 2.20	3.12 ± 2.97
PLT (10^9/l)	114 ± 64.56	187.05 ± 66.57	196.78 ± 78.55	181.09 ± 54.32

Table 2 Profile of SNP genotyping

Locus *IL28B*	Primer (5′–3′) (sense/antisense)	Probe
rs12979860	GACTCGGCTCCAGGTCGG	FAM-CTGGTTCGCGCCTT-MGB
	CGGTCGTGCCTGTCGTGTA	HEX-TGGTTCACGCCTTC-MGB
rs8099917	TCACTGTTCCTCCTTTTGTTTTCC	FAM-TCTGTGAGCAATTTCACCCAAATTGG-BHQ
	TGAGAGATAATGGTAAGACATAA	HEX-CTGTGAGCAATGTCACCCAAATTGG-BHQ
rs12980275	TTCCTATTAACCCCTCCCGCT	FAM-ACACGTCTGTTTCTAG-MGB
	ACAAATGCTGTATGATTCCCCCT	HEX-ACACGTCCGTTTCTA-MGB

patients in the other three disease groups and had a higher proportion of males. The HBV-related HCC group and the other three groups without HCC (healthy controls, non-HCC patients with CHB, HBV self-limited infections) had statistically different laboratory results for ALB, T-Bil, ALT, AFP and PLT ($P < 0.001$).

Genotype distribution

Genotype distribution analysis showed that the studied SNPs were in Hardy–Weinberg equilibrium. No departure from the Hardy–Weinberg distribution was observed for each genotype (P value never significant) in control subjects and in patients with HCC. No significant SNP-specific deviation ($P > 0.05$) was observed.

IL28B polymorphism is associated with the development of hepatitis B-related hepatocellular carcinoma

The allele frequencies and genotype distributions of SNPs were compared among HCC, CHB, HBV self-limited infections and healthy controls groups. There were no significant differences in allele frequencies and genotype distributions of the *IL28B* rs8099917 and rs12980275 SNPs among these groups (see Table 3). Based on previous reports suggesting modest differences between CT and TT genotypes at rs12979860 in relation to SVR, genotypes were collapsed into a recessive model and comparisons were performed as CC versus CT or TT (non-CC) genotypes. There were significant differences among HCC, CHB and healthy controls groups ($\chi^2 = 6.82$, $P = 0.03$). Notably, the frequency of CC homozygosity was 91.5% in healthy controls and 72.9% in CHB, the difference being statistically significant ($\chi^2 = 6.40$, $P = 0.01$). And then, the frequency of CC was 91.5% in healthy controls and 74.7% in HCC, the difference being statistically significant ($\chi^2 = 6.05$, $P = 0.01$). However, this significant finding was not seen between HBV self-limited and healthy controls. In the analyses by genotypes, TT homozygous and CT heterozygous patients both had a higher risk of developing chronic hepatitis B and hepatitis B-related hepatocellular carcinoma compared with patients carrying the common genotype (CC). Minor T allele carriers had a higher risk of CHB and HCC than healthy controls ($\chi^2 = 4.44$, $P = 0.04$), and the T allele was therefore defined as the risk allele.

Associations between IL28B haplotypes and development of hepatitis B-related hepatocellular carcinoma

To understand whether the three associated *IL28B* SNPs were in strong linkage disequilibrium (LD) with each other

Table 3 Genotype distribution and allele frequencies of SNPs in *IL28* genes with the development of hepatitis B-related hepatocellular carcinoma

SNPs ID (gene)	Genotype (*n*, %)			Allele (*n*, %)	
rs12979860C/T	TT	TC	CC	T	C
HBV-related HCC	10 (6.5)	29 (18.8)	115 (74.7)	25 (15.9)	129 (84.1)
Hepatitis B	6 (7.1)	17 (20.0)	62 (72.9)	15 (17.1)	70 (82.9)
HBV self-limited	3 (7.0)	7 (16.3)	33 (76.7)	6 (15.1)	37 (84.9)
Control	0 (0)	4 (8.5)	43 (91.5)	2 (4.3)	45 (95.7)
Total	19 (6.1)	57 (18.8)	253 (75.1)	48 (15.5)	281 (84.5)
rs8099917G/T	TT	TG	GG	T	G
HBV-related HCC	127 (80.2)	21 (16.3)	6 (3.5)	137 (89.28)	17 (10.71)
Hepatitis	70 (82.4)	10 (11.8)	5 (5.9)	75 (88.2)	10 (11.8)
HBV self-limited	33 (76.7)	6 (14.0)	4 (9.3)	36 (83.7)	7 (16.3)
Control	44 (93.6)	2 (4.3)	1 (2.1)	45 (95.7)	2 (4.3)
Total	274 (82.8)	39 (12.3)	16 (5.0)	293 (88.9)	36 (11.1)
rs12980275G/A	AA	GA	GG	A	G
HBV-related HCC	131 (85.1)	21 (13.6)	2 (1.3)	141 (91.88)	13 (8.12)
Hepatitis	74 (87.1)	9 (10.6)	2 (2.4)	79 (92.4)	6 (7.6)
HBV self-limited	37 (86.0)	5 (11.6)	1 (2.3)	40 (91.9)	3 (8.1)
Control	43 (91.5)	3 (6.4)	1 (2.1)	45 (94.7)	2 (5.3)
Total	285 (87.4)	38 (11.1)	6 (1.5)	305 (92.9)	24 (7.1)

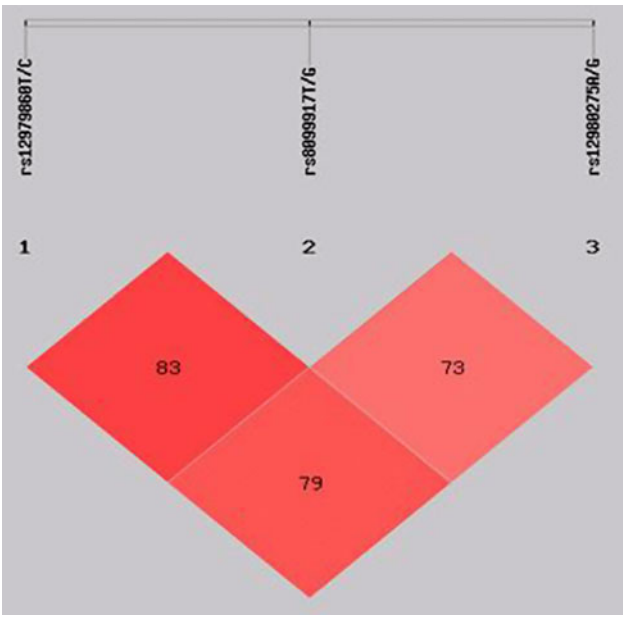

Fig. 1 Haplotype block structure of the *IL28B* gene region in the study cohort. The LD block structure was analyzed using SHEsis software, online. SNPs in the *IL28B* region clustered into *two blocks* of linkage disequilibrium

or whether each contributed independently to the association of treatment response, we performed LD analysis. As shown in Fig. 1, the three SNPs were slightly associated with each other (D ranges from 0.73 to 0.85; r^2 ranges from 0.33 to 0.47). There was no evidence of apparent LD. Thus, it was likely that some of the three SNPs may independently contribute to the association.

Because all of the three SNP associations were within the *IL28B* gene region, we focused on haplotype analysis, including all genotyped SNPs in the region of interest. Three *IL28B* haplotypes were identified in our study cohort with frequencies exceeding 5%. The global tests of *IL28B* haplotype analysis demonstrated significant associations between haplotypes and risk to HCC (see Table 4). The most common haplotype C–T–A (major alleles of all genetic variants) was associated with healthy controls, rather than with the HCC group ($\chi^2 = 6.08$, $P = 0.01$). In contrast, haplotype T–G–G was associated with development of HCC, compared with healthy controls, although the difference was not remarkable ($\chi^2 = 2.56$, $P = 0.11$).

Genotype combination and interaction between IL28B alleles

There were no significant differences in allele frequencies and genotype distributions of the three SNPs, except for rs12979860, between the HCC and healthy control groups. Genotype combination analysis was performed in a case–control study to investigate whether the *IL28B* gene contributed to HCC, whether there were gene–gene interactions contributing to the response and, if so, whether a dose-dependent effect existed.

We regarded patients with the HBV-related HCC as the case group, the healthy controls as the control group and the patients carrying no protective genotypes as the reference group (see Table 5). Genotypes of rs12979860 CC and rs8099917 TT were compared with other genotypes as protective reference genotypes to the development of HCC.

Table 4 Comparison of haplotype frequencies among different groups

Haplotype	Case (freq)	Control (freq)	χ^2	Pearson's p	OR (95% CI)
C T A	252.56 (0.820)	87.98 (0.936)	6.08	0.01	0.25 (0.07–0.82)
T G G	18.87 (0.061)	1.98 (0.021)	2.56	0.11	3.16 (0.72–13.95)
T T A	16.30 (0.053)	1.02 (0.011)	3.27	0.07	5.28 (0.71–39.45)

Table 5 Contribution of genotype combination and gene–gene interaction of *IL28B* to the development to HCC in a case–control study

Genotype	Genotype	Case	Control	OR (95% CI)	*P* value	Likelihood test
rs12979860	rs8099917					
CC	TT	133	42	1		
TC + TT	TT	2	1	6.05 (1.25–7.85)	0.01	
CC	TG + GG	15	2	3.53 (1.05–4.44)	0.06	
TC + TT	TG + GG	4	2	6.10 (1.46–8.19)	0.04	
				ORint = 11.91		0.06
rs12979860	rs12980275					
CC	AA	112	42	1		
TC + TT	AA	18	1	5.44 (0.76–1.95)	0.02	
CC	GA + GG	5	1	1.28 (1.05–3.44)	0.26	
TC + TT	GA + GG	19	3	6.28 (1.46–7.19)	0.04	
				ORint = 11.79		0.04

For the rs12979860 TC/TT and rs8099917 TT (protective genotype) genotype combination, and the rs12979860 CC (protective genotype) and rs8099917 TG/GG genotype combination, the OR for development of HCC was 6.064 (95% CI: 1.25–7.85, $P = 0.01$) and 3.53 (95% CI: 1.05–4.44, $P = 0.06$), respectively. Interestingly, the OR was 6.10 with the rs12979860 TC/TT and rs8099917 TG/GG genotype combinations. The difference of OR between healthy controls and the HCC group was remarkable (95% CI: 1.46–8.19, $P = 0.04$). The results indicated that there was a dose-dependent effect between genotypes and clinical IFN treatment response. In addition, we found that the two SNPs presented a positive interaction, and the rate of OR was 11.91 (likelihood test, $P = 0.06$).

Likewise, rs12979860 and rs12980275 also presented a positive interaction (likelihood test, OR = 11.79, $P = 0.04$); if the two genotypes coexisted, their benefit to treatment response was increased (OR = 6.28, $P = 0.04$) and the OR difference between rs12979860 and rs12980275 genotypes was statistically remarkable.

Discussion

HBV infection accounts for most primary HCC, and treating HBV infection substantially reduces the risk of HCC development, as the viral load is found to be the most important factor leading to cirrhosis and cancer development in the liver [5, 6]. Although chronic HBV infection is recognized as the most important causal factor for HCC in humans, some HCC cases are without chronic HBV infection, suggesting the presence of important cofactors in HBV-related HCC. Different approaches have been used to identify genetic susceptibility factors for the natural course of, and treatment response in, hepatitis C and B. Recently, allelic variants in the *IL28B* gene have gained major interest as several GWAS identified a panel of SNPs to be strongly associated with treatment-induced and spontaneous clearance of hepatitis C. The associated region (19q13) encodes 3 cytokine genes (*IL28A*, *IL28B* and *IL29*) that belong to the IFN-λ (also named type III IFN) family. IFN-λs interact with a transmembrane receptor to induce potent antiviral responses [10–12]. This antiviral activity is mediated through the activation of the JAK-STAT (IFN-αs, IFN-γs and IFN-λs) and MAPK (IFN-αs and IFN-λs) pathways. In vitro and in vivo models have shown the importance of IFN-λs in the immune response and in the up-regulation of transcription of IFN-stimulated genes (ISGs) that are required to control viral infection, including herpes simplex virus [19, 20], HIV [21], and hepatitis B and C viruses [13, 18]. IFN-λ seems to inhibit HBV and HCV replication in an experimental model [18]. Therefore, in studies aimed at assessing the role of *IL28B* rs12979860 C/T polymorphism in patients with chronic HCV infection, similar effects could also be expected in patients with chronic HBV infection. However, such studies are sparse and are limited by small numbers and cohort samples restricted to Asian populations.

The aim of this study was to analyze the association of the *IL28B* SNPs (rs12979860, rs8099917, rs12980275)

with the development of HBV-related HCC. We observed a trend for a relationship between rs12979860 and chronic hepatitis B and HBV-related HCC susceptibility. Furthermore, the frequency of the TC + TT genotype was significantly increased in CHB and HBV-related HCC patients compared with healthy individuals, while both C allele and CC genotype frequencies of healthy controls were protective, which indicates that the rs12979860 T/C polymorphism is associated with the carcinogenic process of chronic hepatitis B and HBV-related HCC. However, a significant difference was only identified in subjects between chronic HBV-infected individuals (including CHB and HCC) and healthy controls, possibly because of the small sample size of non-HCC patients with chronic HBV infection. The protective effect of the C allele in our study is consistent with the results of a GWAS [15], which identified a SNP (C>T; rs12979860) 3 kb upstream of *IL28B* that was associated with a favorable treatment response in patients with HCV genotype 1 infection. Of note, our study showed that the CC genotype was a stronger predictor of response than the viral genotype. Because the C allele at rs12979860 was already known to enhance the inhibitory effect on JAK-STAT and on ISGs activation of IFN-λ, the T/C variant was hypothesized to decrease the HBV clearance capability of the immune response and to up-regulate transcription of antiviral proteins.

A second potentially interesting finding of our paper concerns the demonstration of interaction between gene–gene and dose-dependent effects in the promoter and regulatory regions of the *IL28B* gene that are associated with the development of HBV-related HCC. We found that for *IL28B* rs12979860 TC/TT and rs12980275 GA/GG genotype combinations, the rate of development to HCC increased strikingly. Such a trend could also be seen in *IL28B* rs12979860 TC/TT and rs8099917 TG/GG genotype combination analyses. Based on these, a differential distribution of *IL28B* haplotype, namely C–T–A, resulting from the rs12979860, rs8099917 and rs12980275 SNPs in *IL28B*, was observed in HCC versus healthy controls, indicating the prediction of a favorable outcome. These results indicated that there was a dose-dependent effect between genotypes and development to clinical HCC. This may be due to the cumulative effect of each allele on different genes in the *IL28B* gene family, which when combined showed significant differences.

Recent experimental evidence is emerging suggesting that type III IFN possesses antitumor activity in a BNL hepatoma model [22]. *IL28* and *IL29* potently induced STAT signaling and antiproliferative effects in neuroendocrine BON1 tumor cells [23]. Interestingly, NK cells express IFNLR1, and their cytotoxicity against colon tumor cells is increased by IFN-λ treatment [24]. In other respects, IFN-λ can act on T cells as demonstrated by Chi et al. [25] who showed that both IFN-α and IFN-λ are critical mediators of the suppression of $CD4^+$ T cells associated with HBV infection. Like type I IFNs, IFN-λ exhibits an in vivo antitumor effect that is likely to be mediated by an action on the host rather than through its antiproliferative activity on tumor cells. The results presented here may provide an integrative hypothesis. The *IL28B* rs12979860 non-CC genotypes, associated with lower level ISG expression, have previously been associated with higher rates of chronic hepatitis B infection and development to HCC. Nevertheless, our study, which attempted to link these genetic components to HCC susceptibility, was limited due to its cohort-specific nature. GWAS have been utilized to perform large-scale interrogation of genetic variants in cancer. Zhang and colleagues [25, 26] have conducted the first liver GWAS for HCC in chronic HBV carriers of Chinese ancestry. They identified rs17401966, located on chromosome 1p36.22, as a new susceptibility SNP for HBV-related HCC. However, this SNP did not reach significance in unadjusted conditions in any of the five case–control studies or family trios used in the follow-up studies. It is not surprising, as noted in the literature and by ourselves that rs17401966 only had a statistical level of 10^{-6} in the initial GWAS scan, which is below what is considered to be of genome-wide significance ($P < 10^{-7}$), a cut-off level that takes multiple testing into account. This 'cherry-picking' approach of selecting this SNP raises the question of whether this study should be considered a GWAS approach since the selection of this SNP was not based on a global unbiased rule [27].

Taken together, we report that rs12979860 in the *IL28B* gene might be a candidate risk factor for chronic HBV infection and even developing HBV-related HCC, presumably by down-regulating ISG expression and then influencing the clearance of HBV. However, the present study genotyped only one polymorphism in the *IL28B* gene. Further investigation with a larger sample size and haplotype analysis with other SNPs may be required to confirm these results. Screening of these polymorphisms and functional studies would be useful to clinical practice for identifying groups at high risk of HCC and might help to modify the design of HCC surveillance programs for patients with chronic HBV infection. In addition to epidemiological analysis, functional studies are also warranted to further explore the role of *IL28B* in HCC carcinogenesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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