Phosphatase and Tensin Homologue Genetic Polymorphisms and their Interactions with Viral Mutations on the Risk of Hepatocellular Carcinoma

Yan Du[1], Yu-Wei Zhang[1], Rui Pu[1], Xue Han[2], Jian-Ping Hu[3], Hong-Wei Zhang[1], Hong-Yang Wang[4], Guang-Wen Cao[1]

[1]Department of Epidemiology, Second Military Medical University, Shanghai 200433, China
[2]Division of Chronic Diseases, Center for Disease Control and Prevention of Yangpu District, Shanghai 200090, China
[3]Department of General Surgery, The 85th Hospital of People Liberation Army, Shanghai 200052, China
[4]International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute/Hospital, Second Military Medical University, Shanghai 200043, China

Abstract

Background: Chronic hepatitis B virus (HBV) infection is the major cause of hepatocellular carcinoma (HCC). Some HBV mutants and dysregulation of phosphatase and tensin homolog (PTEN) may promote the development of HCC synergistically. We aimed to test the effects of PTEN genetic polymorphisms and their interactions with important HBV mutations on the development of HCC in HBV-infected subjects.

Methods: Quantitative polymerase chain reaction was applied to genotype PTEN polymorphisms (rs1234220, rs2299939, rs1234213) in 1012 healthy controls, 302 natural clearance subjects, and 2011 chronic HBV-infected subjects including 1021 HCC patients. HBV mutations were determined by sequencing. The associations of PTEN polymorphisms and their interactions with HBV mutations with HCC risk were assessed using multivariate logistic regression analysis.

Results: Rs1234220 C allele was significantly associated with HCC risk compared to healthy controls (adjusted odds ratio [AOR] = 1.35, 95% confidence interval [CI] = 1.07–1.69) and HCC-free HBV-infected subjects (AOR = 1.27, 95% CI = 1.01–1.57). rs1234220 C allele was significantly associated with increased frequencies of HCC-risk A1652G, C1673T, and C1730G mutations in genotype B HBV-infected subjects. Rs2299939 GT genotype was inversely associated with HCC risk in HBV-infected patients (AOR = 0.75, 95% CI = 0.62–0.92). The interaction of rs2299939 variant genotypes (GT+TT) with A3054T mutation significantly increased HCC risk (AOR = 2.41, 95% CI = 1.08–5.35); whereas its interaction with C3116T mutation significantly reduced HCC risk (AOR = 0.34, 95% CI = 0.18–0.66). These significant effects were only evident in males after stratification.

Conclusions: PTEN polymorphisms and their interactions with HBV mutations may contribute to hepatocarcinogenesis in males. The host-virus interactions are important in identifying HBV-infected subjects who are more likely to develop HCC.

Key words: Hepatitis B Virus; Hepatocellular Carcinoma; Polymorphism; Phosphatase and Tensin Homolog; Viral Mutation

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers and chronic infection with hepatitis B virus (HBV) is the most common cause of HCC worldwide. The mainland of China, an area endemic for HBV genotypes B and C, accounts for one-third of global HBV-infected subjects and half of global HCC cases.[1,2] According to the differences in cancer-inducing capacities of HBV genotypes B and C,[3] the endemicity of HBV genotypes in the mainland of China,[2] and the incidence of HCC in a cohort study carried out in Taiwan (China) where genotype B is endemic,[4] we estimated that 30% of male and 10% of female HBV-infected subjects in the mainland of China will develop HCC before 75 years old. Thus, at least 20 million HBV-caused HCC cases will be diagnosed in the mainland of China within next 50 years, even though, postnatal HBV infection has been greatly diminished by HBV vaccination since 1992 and other effective prophylaxis. HCC is a highly fatal malignancy, with a 5-year survival rate of 10% for patients without surgical treatment and 30% for those who received curative surgery.[5] The occurrence of HBV-related HCC in chronic HBV carriers can be greatly reduced via active prophylaxis using anti-HBV treatments.[6] It is, therefore,

Address for correspondence: Prof. Guang-Wen Cao,
Department of Epidemiology, Second Military Medical University,
800 Xiangyin Road, Shanghai 200433, China
E-Mail: gcao@smmu.edu.cn

a great challenge to identify the HBV-infected subjects who are more likely to develop HCC and need the specific prophylaxis in advance.

Epidemiological studies have shown that increasing age, male gender, HBV genotype C (vs. genotype B), high levels of circulating HBV DNA (>10[4] copies/ml),[3] hepatitis B e antigen (HBeAg) expression,[7] and certain HBV mutations are significantly associated with an increased risk of HCC in HBV-infected subjects. We and others have reported that HBV mutations C1653T, T1753V, A1762T/G1764A, T1674C/G, and C1766T/T1768A in the enhancer II/basal core promoter (EnhII/BCP) region; G1899A, C2002T, A2159G, A2189C, and G2203A/T in the precore/core region; as well as T53C, preS2 start codon mutation, preS1 deletion, C2964A, A2962G, C3116T, and C7A in the preS region of HBV genome are significantly associated with an increased risk of HCC.[8-13] HBV reverse transcriptase lacks proofreading activity, resulting in viral nucleotide substitution mutations during viral replication. Moreover, inflammatory factors promote HBV mutations, possibly through inducing expression of cytidine deaminases.[14] Chronic inflammatory microenvironment can facilitate the selection of HCC-related HBV mutations by host innate and adaptive antiviral immunity. The selected HCC-related HBV mutations in combination promote the hepatocarcinogenesis.[14,15] However, the effect of HBV mutations on HCC development might be influenced by their interactions with host genetic susceptibility.

Phosphatase and tensin homolog (PTEN), which is located at chromosome 10q23.3, spans 105 kb and includes 9 exons and 8 introns.[16] PTEN is a well-established tumor suppressor that negatively regulates AKT signaling pathway.[17] It involves in a variety of biological processes including regulation of cell growth, proliferation, migration, apoptosis, and cell cycle control. PTEN protein is a dual phosphatase that acts at both serine-threonine and tyrosine sites. In addition, PTEN also exerts anti-inflammatory activity.[18] Somatic mutations of PTEN have been reported in many types of cancers. Although PTEN mutation is a rare event in HCC, the signaling affected by PTEN, namely PI3K/AKT/mammalian target of rapamycin, is aberrantly regulated in half of HCC cases.[19] PTEN may affect the development of HCC via modulating HBV X protein-mediated signaling.[20] Single nucleotide polymorphisms (SNPs) of PTEN have been linked to multiple histotypes of cancers including nasopharyngeal carcinoma.[21] However, the effects of PTEN SNPs on HCC risk and their interactions with HBV mutations on HCC risk have not been reported.

We hypothesized that PTEN genetic polymorphisms may play a role in chronic HBV infection, HBV-related inflammation, and hepatocarcinogenesis, alone or possibly through interacting with HBV mutations. In this large epidemiological study, we investigated the effects of several PTEN polymorphisms and their interactions with important HBV mutations on the risk of HCC in chronic HBV-infected subjects. This study should be helpful in identifying HBV-infected subjects who are more likely to develop HCC and need active prophylaxis.

METHODS

Study population

Healthy controls and HBV-infected subjects with or without HCC enrolled in this study have been previously described.[22] In brief, 1012 healthy controls, 316 asymptomatic hepatitis B surface antigen (HBsAg) carriers, 316 chronic hepatitis B (CHB) patients, 358 liver cirrhosis patients, and 1021 HCC patients were recruited from our community-based epidemiological study in Yangpu district of Shanghai and our collaborative hospitals in Shanghai, Shandong province, Jiangsu province, and Chongqing between September 2009 and September 2011. We also enrolled 302 natural clearance subjects from our epidemiological survey in Yangpu district during this period. The definition and diagnostic criteria have also been reported.[23] Subjects who were positive for antibodies against hepatitis C virus (HCV), hepatitis delta virus (HDV), and/or human immunodeficiency virus (HIV) were excluded. All participants were self-identified Han Chinese and provided written informed consent. The study was approved by the Human Research Ethics Committee of the Second Military Medical University, and the protocol was conformed to the ethical guidelines of the 1975 *Declaration of Helsinki*.

Serological hepatitis B virus parameter examination, hepatitis B virus genotyping, and viral mutation analysis

Sera were prepared and stored at −80°C until use. Briefly, 5 ml fasting blood samples were obtained with a vacuum blood collection tube without anticoagulant during recruitment before any treatment. The serum was separated by centrifugation at 4°C and stored in a sterile tube at −80°C within 6 h of sample collection. HBV serological markers, including HBeAg, antibody to HBe, HBsAg, antibody to HBs were examined by Architect-i2000 (Abbott Laboratories, North Chicago, IL, USA).[24] Liver function tests including alanine aminotransferase (ALT) were conducted by serum chemistry autoanalyzer (Model 7600, Hitachi, Tokyo, Japan) using commercial reagents (Wako, Japan); alpha-fetoprotein (AFP) was tested by Bayer ACS-180 (Bayer, Germany) using commercial reagents (Bayer).[25] Antibody to HDV was examined using commercial kits (Wantai Bio-Pharm, Beijing, China). Serum anti-HCV and anti-HIV were examined in the recruitment hospitals. Serum viral load was measured in the LightCycler (Roche, Mannheim, Germany), using the Quantitative HBV polymerase chain reaction (PCR) Fluorogence Diagnostic Kit (PG Biotechnology, Shenzhen, China). HBV was genotyped by a multiplex PCR and nested multiplex PCR.[3,26] The EnhII/BCP/PC region and preS region of HBV genome were amplified by nested-PCR as previously described.[2] The amplicons were directly sequenced in both forward and reverse directions using ABI PRISM BigDye sequencing kits and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).[2]

The HBV sequences were aligned and analyzed by MEGA 5.0 (http://www.megasoftware.net/) and Bioedit 7.0 software packages (Ibis Therapeutics Carlsbad, CA, USA).[8] The mutations of HBV genotype B (B2) and genotype C (C2) were defined, respectively. HCC-related HBV mutations in the EnhII/BCP/precore region and the preS region of the HBV genome were evaluated, as previously characterized.[22,23]

Polymorphism selection and genotyping

Phosphatase and tensin homolog SNPs in the promoter, intronic, intron/exon boundary, exonic and 3'UTR regions were initially screened using the International HapMap Project tag SNP information in the Han Chinese population (http://hapmap.ncbi.nlm.nih.gov/). The SNPs in the intron (rs1234213, rs1234220, rs2299939, rs532678, rs1234219, rs12572106, and rs2299941), at intron/exon boundaries (rs1903858), and in the 3'UTR (rs701848) were selected based on the HapMap screening and previous literatures regarding the associations of PTEN SNPs with cancers or inflammation-related diseases.[27-29] We excluded SNPs if they were monomorphic or had low frequencies in our samples (rs12572106, rs1234219, and rs701848) or their surrounding sequences were not suitable for designing the minor groove binder (MGB) probes (rs532678, rs2299941, and rs1903858). Three intronic SNPs rs1234220 (T>C, in the intron 3), rs2299939 (G>T, in the intron 5), and rs1234213 (C>T, in the intron 7) were finally selected in this study. Genomic DNA was extracted from 200 μl peripheral blood using a QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. SNPs were genotyped using fluorescent probe real-time quantitative PCR in a LightCycler™ 480 (Roche, Basel, Switzerland). Primers and MGB probes [Table 1] were designed and synthesized by GeneCore BioTechnologies (Shanghai, China). Each reaction mixture contained 0.2 μmol/L of primers and probes, 1–4 ng/μl purified templates in Premix Ex Tag reaction system (Takara, Dalian, China). The conditions for PCR were: Initial denaturalizing at 95°C for 10 s, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s, then 40°C for 1 s. For quality control purpose, one blank control (ddH₂O) was included in each 384-well plate. 10% of the samples were randomly selected for duplication, yielding 100% concordance.

Statistical analysis

Each SNP was tested for deviation from the Hardy–Weinberg equilibrium (HWE) in healthy controls using the exact test. Student's t-test and χ^2 test were used to compare continuous variables and categorical variables, respectively. Unconditional logistic regression model was applied to test the effects of PTEN SNPs on HCC risk and other HBV-related characteristics, and to calculate odds ratios (ORs) and their 95% confidence intervals (CIs). Multivariate logistic regression analysis was applied to evaluate the effect of multiplicative interactions of SNPs with HBV mutations on HCC development, adjusting for age and gender. All statistical tests were two-sided, and a $P < 0.05$ was considered statistically significant. All analyses were performed by SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Characteristics of study participants

Table 2 summarizes the baseline characteristics of the study participants. Briefly, the group of HCC patients had a higher proportion of males compared to other groups. Healthy controls and HBV natural clearance subjects were relatively older than HBV-infected patients including HCC patients. HCC patients were older than other HBV-infected patients without HCC. HBV genotype C was more prevalent in HCC patients than in those without HCC. There was a larger portion of HBeAg negativity in HCC patients compared to HBV-infected patients without HCC.

Associations of phosphatase and tensin homolog single nucleotide polymorphisms with the risk of hepatocellular carcinoma

All three SNPs were conformed to HWE in healthy controls ($P > 0.05$ for all). We first evaluated the associations of PTEN SNPs with the risk of HCC [Table 3]. Compared with healthy controls, rs1234220 CT genotype and C (CT+CC) allele were significantly associated with increased risks of HCC (adjusted

Table 1: Primers and probes for genotyping the polymorphisms

SNP	Names	Sequence (5'-3')	Alleles
rs1234220	Forward	ATTACATTTCATAGACAAAGAAATTGAGAGTC	T/C
	Reverse	ATAGAAAAACCTTAATTTCCTCTCGTTG	
	Probe-P1	FAM-ACTGGGTAATTTGCCCAA-MGB	
	Probe-P2	HEX - CTGGGTAACTTGCCCAA-MGB	
rs2299939	Forward	TTGTCTCAAAAGGACTCTGAGTACCTC	G/T
	Reverse	GGGTGATCCATCTGTCTCGG	
	Probe-P1	FAM - AAACTTCGCATTCCATAA-MGB	
	Probe-P2	HEX - AAACTTCTCATTCCATAAC-MGB	
rs1234213	Forward	GCACATATATGCATCCTTGCTAGTAAT	T/C
	Reverse	GTACCAAAGAAAGTGTAAAATGTGACTGT	
	Probe-P1	FAM - TCATACCCATTGACATGA-MGB	
	Probe-P2	HEX - CATACCCACTGACATGA-MGB	

SNP: Single nucleotide polymorphism.

Table 2: Baseline characteristics of the study population

Characteristics	Healthy controls (n = 1012)	HBV natural clearance subjects (n = 302)	HBV-infected subjects without HCC			HBV-infected subjects with HCC (n = 1021)	P
			ASCs (n = 316)	CHB (n = 316)	LC (n = 358)		
Male, n (%)	763 (75.40)	169 (55.96)	186 (58.86)	230 (72.78)	264 (73.74)	864 (84.13)	<0.001*,†,‡, 0.001§
Age, years (mean ± SD)	59.56 ± 15.10	58.40 ± 11.72	45.08 ± 10.61	44.18 ± 14.51	50.68 ± 11.34	52.92 ± 11.17	<0.001*,†,§,‡
HBV genotype, n (%)							
B	ND	ND	97 (34.28)	52 (25.00)	56 (22.86)	107 (16.39)	<0.001†
C	ND	ND	186 (65.72)	156 (75.00)	189 (77.14)	546 (83.61)	
HBeAg, n (%)							
Positive	ND	ND	130 (41.14)	132 (45.36)	107 (35.55)	241 (25.08)	<0.001†
Negative	ND	ND	186 (58.86)	159 (54.64)	194 (64.45)	720 (74.92)	
HBV DNA (log$_{10}$ copies/ml)	ND	ND	3.88 ± 1.80	4.43 ± 1.67	4.13 ± 1.37	3.83 ± 1.18	<0.001†
ALT (log$_{10}$ U/L)	ND	ND	1.36 ± 0.21	1.97 ± 0.54	1.75 ± 0.44	1.66 ± 0.35	<0.001†

*HBV-infected subjects with HCC versus healthy controls; †HBV-infected subjects with HCC versus HBV-infected subjects without HCC; ‡All HBV-infected subjects including HCC versus HBV natural clearances; §HBV-infected subjects without HCC versus healthy controls. For multiple comparisons, P value was corrected by the Bonferroni correction (P = 0.010). ALT: Alanine aminotransferase; ASC: Asymptomatic hepatitis B surface antigen carrier; CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; LC: Liver cirrhosis; ND: No data; SD: Standard deviation.

OR [AOR] = 1.37, 95% CI = 1.08–1.73 and AOR = 1.35, 95% CI = 1.07–1.69, respectively), after adjusting for age and gender. When compared to HBV-infected patients without HCC, rs1234220 CT genotype and C (CT+CC) allele were significantly associated with increased risks of HCC (AOR = 1.28, 95% CI = 1.02–1.61 and AOR = 1.27, 95% CI = 1.01–1.57, respectively), whereas rs2299939 GT genotype and T (GT+TT) allele were significantly associated with reduced risks of HCC (AOR = 0.75, 95% CI = 0.62–0.92, and AOR = 0.79, 95% CI = 0.65–0.96, respectively). Compared with all subjects without HCC, rs1234220 CT genotype and C (CT+CC) allele were significantly associated with increased risks of HCC (AOR = 1.30, 95% CI = 1.08–1.56 and AOR = 1.29, 95% CI = 1.07–1.54, respectively), while rs2299939 GT genotype was significantly associated with a reduced risk of HCC (AOR = 0.84, 95% CI = 0.71–0.99). However, after stratifying the participants by gender, the significant results were only evident in males, but not in females [Table 3].

As HBV genotypes and HBeAg status are potential confounders in elucidating the associations of PTEN SNPs with the risk of HCC, we performed further stratified analyses. After stratifying by HBV genotypes, we found that rs1234220 CT genotype and C (CT+CC) allele were significantly associated with increased risks of HCC (AOR = 2.02, 95% CI = 1.14–3.59 and AOR = 2.02, 95% CI = 1.14–3.59, respectively) in genotype B HBV subjects, compared with genotype B HBV-infected patients without HCC; however, these effects did not exist in genotype C HBV-infected subjects [Table 4]. As compared with HBV-infected patients without HCC, rs2299939 GT genotype and T (GT+TT) allele were significantly associated with reduced risks of HCC (AOR = 0.68, 95% CI = 0.53–0.87 and AOR = 0.72, 95% CI = 0.57–0.92, respectively) in HBeAg-negative HBV-infected subjects. However, these effects were not evident in HBeAg-positive ones.

Associations of phosphatase and tensin homolog single nucleotide polymorphisms with the risks of other hepatitis B virus-related characteristics

We then investigated the associations of PTEN SNPs with HBV-related characteristics in HCC-free HBV-infected subjects. As shown in Table 5, none of the three PTEN SNPs were significantly associated with HBV persistence compared to the HBsAg seroclearance subjects or healthy controls. rs2299939 T (GT+TT) allele was significantly associated with high ALT level (AOR = 1.26, 95% CI = 1.01–1.57); rs2299939 GT genotype and T (GT+TT) allele were significantly associated with high viral load (≥10^4 copies/ml) (AOR = 1.33, 95% CI = 1.06–1.67 and AOR = 1.29, 95% CI = 1.03–1.60, respectively) after adjusting for age and gender. Interestingly, rs2299939 GT genotype and T (GT+TT) allele were significantly associated with high ALT level (AOR = 1.35, 95% CI = 1.04–1.75 and AOR = 1.36, 95% CI = 1.06–1.74, respectively) and high viral load (≥10^4 copies/ml) (AOR = 1.38, 95% CI = 1.07–1.78 and AOR = 1.29, 95% CI = 1.01–1.66, respectively) in males. No other significant associations were observed.

Interactions of phosphatase and tensin homolog single nucleotide polymorphisms with hepatitis B virus mutations and their associations with hepatocellular carcinoma risk

The associations of the PTEN SNPs with the HCC-related HBV mutations that were described in our previous studies[8,9,11,22,23,30] were assessed using the data of the HBV-infected patients including those with HCC. It was found that the variant genotype (TC) of rs1234220 was significantly associated with increased frequencies of HBV mutations A1652G (AOR = 4.16, 95% CI = 1.64–10.55), C1673T (AOR = 2.40, 95% CI = 1.02–5.66), and C1730G (AOR = 2.34, 95% CI = 1.02–5.39) in genotype B HBV-infected subjects.

Table 3: Association of PTEN polymorphisms with the risk of HCC

SNP	Genotype/ allele	Healthy controls	HBV natural clearance subjects	HBV-infected subjects without HCC	HBV-HCC patients	AOR (95% CI)			
						HBV-HCC patients versus healthy controls	HBV-HCC patients versus HBV clearance subjects	HBV-HCC patients versus HBV-infected subjects without HCC	HBV-HCC patients versus all of the controls
rs1234220	TT	816	235	770	766	1.00	1.00	1.00	1.00
Total	CT	181	61	184	233	1.37 (1.08–1.73)	1.17 (0.82–1.67)	1.28 (1.02–1.61)	1.30 (1.08–1.56)
	CC	12	5	13	15	1.05 (0.48–2.33)	0.73 (0.25–2.16)	1.10 (0.50–2.41)	1.12 (0.60–2.12)
	C (CT + CC)	193	66	197	248	1.35 (1.07–1.69)	1.13 (0.80–1.60)	1.27 (1.01–1.57)	1.29 (1.07–1.54)
	HWE P	0.584							
rs1234220	TT	199	106	240	124	1.00	1.00	1.00	1.00
Females	CT	46	24	61	36	1.46 (0.85–2.50)	1.38 (0.76–2.53)	1.20 (0.74–1.95)	1.30 (0.85–1.98)
	CC	2	2	5	2	0.93 (0.13–6.87)	0.75 (0.10–5.50)	0.91 (0.17–5.01)	0.92 (0.20–4.34)
	C (CT + CC)	48	26	66	38	1.43 (0.84–2.41)	1.33 (0.74–2.38)	1.18 (0.74–1.90)	1.27 (0.84–1.92)
rs1234220	TT	617	129	530	642	1.00	1.00	1.00	1.00
Males	CT	135	37	123	197	1.35 (1.04–1.75)	1.08 (0.70–1.66)	1.31 (1.01–1.70)	1.30 (1.06–1.60)
	CC	10	3	8	13	1.11 (0.46–2.65)	0.74 (0.20–2.71)	1.17 (0.47–2.88)	1.20 (0.59–2.42)
	C (CT + CC)	145	40	131	210	1.33 (1.03–1.71)	1.05 (0.69–1.60)	1.30 (1.01–1.67)	1.29 (1.06–1.58)
rs2299939	GG	624	188	587	660	1.00	1.00	1.00	1.00
Total	GT	337	96	358	306	0.91 (0.74–1.12)	0.98 (0.72–1.34)	0.75 (0.62–0.92)	0.84 (0.71–0.99)
	TT	35	17	34	45	1.13 (0.69–1.83)	0.82 (0.41–1.63)	1.19 (0.74–1.92)	1.10 (0.75–1.62)
	T (GT + TT)	372	113	392	351	0.93 (0.77–1.13)	0.96 (0.71–1.29)	0.79 (0.65–0.96)	0.86 (0.74–1.01)
	HWE P	0.200							
rs2299939	GG	142	85	187	102	1.00	1.00	1.00	1.00
Females	GT	92	41	111	55	0.86 (0.55–1.35)	1.14 (0.68–1.89)	0.87 (0.57–1.32)	0.92 (0.64–1.33)
	TT	11	7	9	5	0.62 (0.20–1.95)	0.75 (0.22–2.58)	1.18 (0.37–3.72)	0.75 (0.28–2.03)
	T (GT + TT)	103	48	120	60	0.84 (0.54–1.29)	1.09 (0.67–1.79)	0.89 (0.59–1.34)	0.90 (0.63–1.29)
rs2299939	GG	482	103	400	558	1.00	1.00	1.00	1.00
Males	GT	245	55	247	251	0.93 (0.74–1.16)	0.87 (0.59–1.30)	0.72 (0.57–0.90)	0.81 (0.68–0.98)
	TT	24	10	25	40	1.28 (0.74–2.23)	0.78 (0.34–1.80)	1.20 (0.71–2.02)	1.18 (0.78–1.80)
	T (GT + TT)	269	65	272	291	0.96 (0.77–1.20)	0.86 (0.59–1.25)	0.76 (0.62–0.95)	0.85 (0.71–1.01)
rs1234213	CC	248	71	237	253	1.00	1.00	1.00	1.00
Total	CT	518	158	494	486	0.94 (0.74–1.18)	0.86 (0.60–1.23)	0.93 (0.74–1.17)	0.92 (0.76–1.10)
	TT	238	72	253	276	1.12 (0.86–1.46)	1.12 (0.74–1.71)	1.03 (0.80–1.34)	1.08 (0.87–1.34)
	T (CT + TT)	756	230	747	762	0.99 (0.80–1.23)	0.94 (0.67–1.32)	0.97 (0.78–1.20)	0.97 (0.81–1.16)
	HWE P	0.311							
rs1234213	CC	61	33	81	34	1.00	1.00	1.00	1.00
Females	CT	123	65	146	77	1.22 (0.71–2.08)	1.15 (0.63–2.10)	1.32 (0.80–2.18)	1.29 (0.82–2.02)
	TT	63	35	82	51	1.42 (0.78–2.57)	1.35 (0.70–2.62)	1.52 (0.88–2.63)	1.49 (0.91–2.42)
	T (CT + TT)	186	100	228	128	1.29 (0.78–2.13)	1.24 (0.71–2.17)	1.39 (0.87–2.23)	1.37 (0.90–2.08)
rs1234213	CC	187	38	156	219	1.00	1.00	1.00	1.00
Males	CT	395	93	348	409	0.89 (0.69–1.14)	0.75 (0.48–1.18)	0.85 (0.66–1.10)	0.85 (0.69–1.05)
	TT	175	37	171	225	1.06 (0.79–1.42)	1.04 (0.61–1.78)	0.92 (0.69–1.24)	1.00 (0.79–1.27)
	T (CT + TT)	570	130	519	634	0.94 (0.74–1.20)	0.83 (0.54–1.28)	0.87 (0.68–1.11)	0.90 (0.74–1.09)

AOR: Adjusted odds ratio (adjusted for age and gender); *CI*: Confidence interval; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HWE: Hardy-Weinberg equilibrium; PTEN: Phosphatase and tensin homolog; SNP: Single nucleotide polymorphism.

We tested the interactions of PTEN SNPs with several important HCC-related HBV mutations on HCC risk. There were significant interactions between PTEN polymorphisms and A3054T or C3116T, important HBV mutations in the preS region [Table 6]. Although the variant genotype of rs2299939 was significantly associated with a reduced risk of HCC, the interaction of rs2299939 variant genotypes with A3054T was significantly associated with an increased risk of HCC. HBV C3116T mutation was a significant risk factor of HCC; however, its interaction with rs2299939 variant genotypes was significantly associated with a reduced risk of HCC. We also found that the interaction of rs1234213 variant genotypes with HBV C3116T mutation significantly increased the risk of HCC in the HBV-infected subjects. After stratifying by gender, it was found that the interaction between the polymorphism of rs2299939 GT+TT and C3116T significantly reduced the risk of HCC in male HBV-infected subjects (*AOR* = 0.27, 95% *CI* = 0.13–0.57).

Table 4: Association of PTEN polymorphisms with the risk of HCC stratified by HBV genotypes

SNP	HBV genotype B			HBV genotype C		
	Non-HCC, *n*	HCC, *n*	*AOR* (95% *CI*)	Non-HCC, *n*	HCC, *n*	*AOR* (95% *CI*)
rs1234220						
TT	164	74	1.00	412	416	1.00
CT	37	32	2.02 (1.14–3.59)	95	124	1.24 (0.91–1.71)
CC	0	0	–	8	4	0.55 (0.15–1.99)
C (CT + CC)	37	32	2.02 (1.14–3.59)	103	128	1.20 (0.88–1.63)
rs2299939						
GG	124	67	1.00	303	337	1.00
GT	72	36	0.88 (0.53–1.48)	202	175	0.78 (0.60–1.02)
TT	8	3	0.67 (0.17–2.73)	18	27	1.51 (0.79–2.90)
T (GT + TT)	80	39	0.86 (0.52–1.43)	220	202	0.84 (0.65–1.08)
rs1234213						
CC	49	31	1.00	132	132	1.00
CT	109	47	0.67 (0.37–1.21)	266	272	1.01 (0.74–1.38)
TT	47	28	0.91 (0.46–1.78)	129	132	1.10 (0.76–1.59)
T (CT + TT)	156	75	0.75 (0.43–1.29)	395	409	1.04 (0.78–1.39)

AOR: Adjusted odds ratio (adjusted for age and gender); *CI*: Confidence interval; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; Non-HCC: ASC + CHB + LC; PTEN: Phosphatase and tensin homolog; SNP: Single nucleotide polymorphism; ASC: Asymptomatic hepatitis B surface antigen carrier; CHB: Chronic hepatitis B; LC: Liver cirrhosis.

Similarly, the interaction between rs1234213 CT+TT and C3116T also significantly increased HCC risk in male HBV-infected subjects (*AOR* = 3.61, 95% *CI* = 1.54–8.42) after stratifying by gender.

Haplotype analysis

The three PTEN SNPs were in linkage disequilibrium with each other in our study population. The estimated haplotype frequencies of HCC patients (*n* = 1016) and healthy controls (*n* = 1004) indicated that only one haplotype of rs1234220(C)-rs2299939(G)-rs1234213(T) was significantly more frequent in HCC patients compared to healthy controls after adjusting for age and gender (*AOR* = 1.32, 95% *CI* = 1.06–1.63, *P* = 0.012). The frequencies of other estimated haplotypes did not differ significantly between the two groups.

DISCUSSION

In the present study, we found that the variant genotypes or C allele of rs1234220 were significantly associated with increased risks of HCC compared to healthy controls and HCC-free HBV-infected subjects, whereas the variant genotypes of rs2299939 were significantly associated with a reduced risk of HCC compared to HCC-free HBV-infected subjects [Table 3]. The haplotype rs1234220(C)-rs2299939(G)-rs1234213(T) was more frequent in HCC patients compared to healthy controls. These PTEN SNPs have not been found as susceptible genetic loci of cancers especially HCC in genome-wide association studies carried out in Chinese.[31] We also found that three important interactions of genetic factors (SNPs) with environmental factors (HBV mutations) significantly affected the risks of HCC [Table 6]. Interestingly, the associations of the variant genotypes of rs1234220 and rs2299939 with the risk of HCC were solely evident in males but not in females. HBV-HCC occurs more

frequently in males than in females, with a male-to-female ratio of 3:1.[4] These findings should be of clinical and/or public health importance in determining the HBV-infected males who are more likely to develop HCC and need specific prophylaxis.

The mechanisms by which the two PTEN SNPs affected the risk of HCC remain to be determined. As PTEN is a well-known tumor suppressor gene, the variant genotypes or C allele of rs1234220 may predispose HBV-infected subjects to a reduced function of PTEN in their liver. The variant genotypes of rs2299939 were reversely associated with HCC risk in HBeAg-negative HBV-infected subjects; however, they were significantly associated with high viral load ($>10^4$ copies/ml) and ALT abnormality ($\geq$40 U/ml) [Table 5]. Our data were in contradiction with the observations in previous prospective studies that high viral load and hepatic inflammation predicted the occurrence of HCC in HBV-infected subjects.[3,4,7,10,12] Our study is of cross-sectional case-control design that can only indicate the statistical association between genetic polymorphisms and diseases. The results of our study indicate that rs2299939 variant genotypes may predispose the HBV-infected subjects to occur acute-on-CHB. It has been demonstrated that reduced PTEN expression level is correlated with tumor progression, high AFP levels, and poor prognosis of HCC patients.[32,33] However, somatic mutation frequencies of PTEN were rarely detected in HCC,[19,34] suggesting other mechanisms such as promoter methylation, decreased transcription/translation by noncoding RNA (ncRNA), and increased protein degradation/phosphorylation may be responsible for PTEN down-regulation. In this study, we found that 2 of the 3 intronic SNPs of PTEN affected the risk of HCC, and the effects were only evident in males. Although introns do not code for proteins, they may regulate gene expression, or generate ncRNAs. There should be inherent – as yet

Table 5: Associations of PTEN polymorphisms with HBV-related characteristics in HCC-free HBV-infected subjects

SNP	Genotypes/alleles	AOR (95% CI)				
		LC versus ASC + CHB	ALT ≥40 versus <40 U/L*	HBV DNA ≥10^4 versus <10^4 copies/ml*	HCC-free HBV-infected subjects versus HBV natural clearances*	HCC-free HBV-infected subjects versus healthy controls*
rs1234220	TT	1.00	1.00	1.00	1.00	1.00
Total	CT	0.93 (0.66–1.32)	0.88 (0.68–1.15)	0.83 (0.63–1.08)	0.82 (0.56–1.18)	0.99 (0.76–1.29)
	CC	1.94 (0.61–6.16)	0.97 (0.40–2.32)	0.31 (0.09–1.08)	0.85 (0.27–2.67)	0.90 (0.38–2.18)
	C (CT + CC)	0.98 (0.70–1.38)	0.89 (0.69–1.15)	0.79 (0.61–1.02)	0.82 (0.57–1.18)	0.99 (0.77–1.27)
rs1234220	TT	1.00	1.00	1.00	1.00	1.00
Females	CT	0.89 (0.45–1.75)	1.22 (0.69–2.16)	0.68 (0.37–1.24)	1.09 (0.60–1.96)	1.18 (0.70–1.99)
	CC	1.89 (0.25–14.17)	0.26 (0.03–2.59)	0.74 (0.07–8.29)	1.09 (0.18–6.64)	1.10 (0.19–6.48)
	C (CT + CC)	0.94 (0.49–1.81)	1.12 (0.64–1.95)	0.68 (0.37–1.23)	1.09 (0.62–1.92)	1.18 (0.71–1.95)
rs1234220	TT	1.00	1.00	1.00	1.00	1.00
Males	CT	0.95 (0.63–1.43)	0.81 (0.61–1.09)	0.88 (0.65–1.19)	0.72 (0.17–3.12)	0.93 (0.69–1.27)
	CC	2.21 (0.51–9.64)	1.32 (0.48–3.63)	0.25 (0.06–1.10)	0.68 (0.42–1.08)	0.85 (0.31–2.36)
	C (CT + CC)	1.00 (0.67–1.49)	0.84 (0.63–1.12)	0.83 (0.62–1.11)	0.68 (0.43–1.07)	0.93 (0.69–1.25)
rs2299939	GG	1.00	1.00	1.00	1.00	1.00
Total	GT	1.11 (0.84–1.48)	1.24 (0.99–1.56)	1.33 (1.06–1.67)	1.17 (0.85–1.60)	1.16 (0.93–1.43)
	TT	0.79 (0.36–1.72)	1.41 (0.80–2.49)	0.99 (0.58–1.70)	0.70 (0.34–1.45)	0.89 (0.51–1.53)
	T (GT + TT)	1.08 (0.82–1.42)	1.26 (1.01–1.57)	1.29 (1.03–1.60)	1.11 (0.82–1.50)	1.13 (0.92–1.39)
rs2299939	GG	1.00	1.00	1.00	1.00	1.00
Females	GT	0.89 (0.52–1.54)	0.92 (0.56–1.51)	1.14 (0.69–1.88)	1.33 (0.82–2.17)	0.95 (0.63–1.44)
	TT	0.40 (0.05–3.58)	1.37 (0.33–5.73)	2.72 (0.74–10.9)	0.73 (0.22–2.44)	0.50 (0.18–1.44)
	T (GT + TT)	0.85 (0.50–1.46)	0.96 (0.59–1.55)	1.24 (0.77–2.01)	1.26 (0.79–2.01)	0.90 (0.60–1.34)
rs2299939	GG	1.00	1.00	1.00	1.00	1.00
Males	GT	1.20 (0.86–1.67)	1.35 (1.04–1.75)	1.38 (1.07–1.78)	1.06 (0.70–1.61)	1.24 (0.97–1.60)
	TT	0.93 (0.40–2.18)	1.43 (0.77–2.65)	0.81 (0.44–1.48)	0.68 (0.27–1.69)	1.08 (0.58–2.05)
	T (GT + TT)	1.17 (0.85–1.62)	1.36 (1.06–1.74)	1.29 (1.01–1.66)	1.01 (0.67–1.50)	1.23 (0.96–1.57)
rs1234213	CC	1.00	1.00	1.00	1.00	1.00
Total	CT	0.93 (0.66–1.29)	0.91 (0.70–1.19)	0.94 (0.72–1.21)	0.83 (0.58–1.20)	0.99 (0.77–1.27)
	TT	0.82 (0.56–1.19)	0.87 (0.64–1.18)	0.76 (0.56–1.03)	0.96 (0.63–1.46)	1.04 (0.78–1.39)
	T (CT + TT)	0.89 (0.65–1.21)	0.89 (0.69–1.15)	0.87 (0.68–1.11)	0.87 (0.62–1.24)	1.01 (0.80–1.27)
rs1234213	CC	1.00	1.00	1.00	1.00	1.00
Females	CT	0.71 (0.38–1.30)	1.28 (0.71–2.33)	0.79 (0.44–1.41)	0.82 (0.47–1.42)	0.88 (0.55–1.42)
	TT	0.75 (0.37–1.52)	0.98 (0.51–1.87)	0.79 (0.42–1.50)	0.80 (0.43–1.49)	0.91 (0.53–1.57)
	T (CT + TT)	0.72 (0.41–1.28)	1.15 (0.66–2.00)	0.79 (0.47–1.36)	0.81 (0.49–1.36)	0.89 (0.57–1.39)
rs1234213	CC	1.00	1.00	1.00	1.00	1.00
Males	CT	1.04 (0.70–1.54)	0.83 (0.62–1.12)	0.97 (0.73–1.30)	0.86 (0.52–1.40)	1.04 (0.77–1.39)
	TT	0.85 (0.54–1.34)	0.84 (0.60–1.19)	0.76 (0.54–1.06)	1.12 (0.63–2.00)	1.10 (0.78–1.54)
	T (CT + TT)	0.97 (0.67–1.41)	0.83 (0.63–1.11)	0.89 (0.68–1.17)	0.93 (0.58–1.49)	1.06 (0.80–1.39)

*HCC-free HBV-infected subjects: ASCs, CHB patients, and LC patients. AOR: Adjusted odds ratio (adjusted for age and gender in the total subjects; adjusted for age after stratification by gender); PTEN: Phosphatase and tensin homolog; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism; ASC: Asymptomatic hepatitis B surface antigen carrier; CHB: Chronic hepatitis B; ALT: Alanine aminotransferase; CI: Confidence interval; ASC: Asymptomatic hepatitis B surface antigen carrier; CHB: Chronic hepatitis B; LC: Liver cirrhosis.

unknown – links between androgen/androgen receptor and PTEN signaling in HBV-induced hepatocarcinogenesis. The intronic SNPs may function in hepatocarcinogenesis via affecting gene expression as putative enhancers, altering alternative splicing, and/or generating ncRNAs upon HBV infection.

Our stratification analysis indicated that rs1234220 CT genotype and C (CT+CC) allele significantly increased the risk of HCC only in HBV genotype B subjects, not in genotype C HBV-infected subjects [Table 4]. We also found that rs1234220 variant genotype was significantly associated with increased frequencies of HBV mutations A1652G,

C1673T, and C1730G in genotype B HBV-infected subjects. It is reasonable to speculate that rs1234220 variant genotypes increase HCC risk, possibly via facilitating the generation of A1652G, C1673T, and C1730G, the three HCC-risk HBV mutations in genotype B HBV-infected subjects.[23] In our previous studies, we found that the genetic polymorphisms of a group of the key immune and/or proinflammatory molecules facilitated the generation of HCC-risk mutations, possibly because these immune/proinflammatory molecules predispose the host immunity to select these HCC-risk mutations, especially in genotype C HBV-infected subjects.[22,23,30,35,36] To our surprise, the immune selection

Table 6: Interactions of PTEN polymorphisms with HBV mutations on HCC risk

SNP	Mutation	Non-HCC	HCC	OR (95% CI)	AOR (95% CI)
rs2299939	A3054T				
GG	A	159	202	1.00	1.00
GG	T	49	36	0.58 (0.36–0.93)	0.66 (0.39–1.10)
GT + TT	A	135	105	0.61 (0.44–0.85)	0.62 (0.44–0.87)
GT + TT	T	28	31	0.87 (0.50–1.51)	0.98 (0.73–1.32)
OR for interaction				2.46 (1.17–5.18)	2.41 (1.08–5.35)
rs2299939	C3116T				
GG	C	164	138	1.00	1.00
GG	T	51	113	2.63 (1.76–3.93)	2.17 (1.42–3.32)
GT + TT	C	102	95	1.11 (0.77–1.59)	1.08 (0.74–1.59)
GT + TT	T	61	45	0.88 (0.56–1.37)	0.89 (0.70–1.13)
OR for interaction				0.30 (0.16–0.56)	0.34 (0.18–0.66)
rs1234213	C3116T				
CC	C	56	71	1.00	1.00
CC	T	38	25	0.52 (0.28–0.96)	0.51 (0.27–0.97)
T (CT + TT)	C	210	163	0.61 (0.41–0.92)	0.66 (0.43–1.02)
T (CT + TT)	T	75	133	1.40 (0.89–2.19)	1.13 (0.90–1.43)
OR for interaction				4.40 (2.17–8.93)	3.68 (1.74–7.76)

AOR: Adjusted odds ratio (adjusted for age and gender); *CI*: Confidence interval; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCC: ASC + CHB + LC; PTEN: Phosphatase and tensin homolog; LC: Liver cirrhosis; SNP: Single nucleotide polymorphism; OR: Odds ratio.

milieu predisposed by the PTEN SNPs might be special in genotype B HBV-infected subjects who are more likely to develop HCC. As PTEN has an anti-inflammatory activity,[18] rs1234220 variant genotypes may be associated with reduced expression or activity of PTEN, thus facilitating the formation of active inflammation essential for the immune selection of HCC-risk mutation. Compared to HBV genotype C, genotype B is more apt to cause active inflammation and the occurrence at the young without cirrhosis background and recurrence of HCC.[24,25] Thus, the mechanisms by which the HCC-risk HBV mutations are selected might be different between HBV genotype B and genotype C.

Interestingly, the associations of the PTEN polymorphisms with the risk of HCC were significantly affected by the HBV mutations. The interactions of rs2299939 polymorphism with A3054T mutation or rs1234213 polymorphism with C3116T mutation significantly increased the risk of HCC in male HBV-infected subjects; whereas the interaction of rs2299939 polymorphism with C3116T mutation significantly decreased the risk of HCC [Table 6]. Thus, the effects of important HCC-related HBV mutations such as A3054T and C3116T on HCC susceptibility can be moderated by host genetic susceptibility such as PTEN polymorphisms. Functional studies of PTEN polymorphisms are necessary. Nevertheless, these interactions should be helpful in identifying HBV-infected subjects who are more likely to develop HCC.

To the best of our knowledge, this is the first study investigating the effects of PTEN genetic polymorphisms on the generation of HCC-related HBV mutations, and their interactions on the risk of HCC in HBV-infected subjects. Moreover, our epidemiological study with a relatively large sample size provided convincing data to support the role of PTEN polymorphisms in determining individual's genetic susceptibility to HCC. However, several limitations should be addressed. First, other important information such as environment risk factor, alcohol consumption, and family history of HCC were not collected, and we could not adjust for those covariates. Second, the current study is cross-sectional in nature, and our findings need to be validated in prospective cohort studies.

In conclusion, the variant genotypes of rs1234220 are significantly associated with increased risks of HCC, whereas the variant genotypes of rs2299939 are significantly associated with a reduced risk of HCC in male HBV-infected subjects. The effect of rs1234220 variant genotypes on HCC risk is only observed in genotype B HBV-infected subjects, possibly via facilitating the immune selection of A1652G, C1673T, and C1730G, the three HCC-risk mutations in genotype B HBV-infected subjects. The interactions of rs2299939 polymorphism with A3054T mutation or rs1234213 polymorphism with C3116T mutation significantly increased the risk of HCC in male HBV-infected subjects; whereas the interaction of rs2299939 polymorphism with C3116T mutation significantly decreased the risk of HCC. These findings should be of clinical and/or public health importance in determining the HBV-infected males who are more likely to develop HCC and, therefore, need specific prophylaxis. The effects of the interactions of genetic predisposition (PTEN SNPs) with environmental factors (HBV mutations) on HCC occurrence and progression should be validated in large prospective cohort studies.

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