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Original Article

Genetic polymorphisms of C-reactive protein increase susceptibility to HBV-related hepatocellular carcinoma in a Guangxi male population

Xianjun Lao*, Shan Ren*, Yu Lu, Dongmei Yang, Xue Qin, Shan Li

*Department of Clinical Laboratory, First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi, China. *Equal contributors and co-first authors.*

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Abstract: C-reactive protein (CRP) is a biomarker of inflammation and the production has been shown to be influenced by genetic variation in *CRP* gene. HBV-related hepatocellular carcinoma (HCC) is a typical inflammation-related disease occurs mainly in men. The present study was designed to investigate the association between *CRP* polymorphisms and HBV-related HCC risk in a Chinese male population. The *CRP* rs2794521 and rs3093059 SNPs were genotyped by polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) in 158 HBV patients with HCC, 207 HBV patients without HCC, and 150 unrelated healthy controls. A significant increased HCC risk in HBV patients were observed for the rs3093059 SNP comparing with those without HCC (C allele vs. T allele: adjusted OR=1.56, 95% CI, 1.07-2.29, *P*=0.021; TC vs. TT: adjusted OR=1.77, 95% CI, 1.13-2.76, *P*=0.012; TC/CC vs. TT: adjusted OR=1.76, 95% CI, 1.14-2.71, *P*=0.011). However, we did not observe any significant association of rs3093059 polymorphism with HCC when compared with healthy controls. With respect to rs2794521 polymorphism, no significant associations of this polymorphism with HCC risk were found in this population. In haplotype analysis between HBV patients with HCC and HBV patients without HCC, the TC haplotype was found correlated with a significant increased HCC risk (OR=1.803, 95% CI, 1.237-2.335, *P*<0.001). We concluded that the CRP rs3093059 polymorphism may play a significant role in the development of HBV-related HCC in the Guangxi male population.

Keywords: Hepatocellular carcinoma, chronic hepatitis B, C-reactive protein, polymorphism, male

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world (the fifth in males and eighth in females) and the third leading cause of cancer-related death [1]. Despite the high prevalence rate, the incidence of HCC is not uniform but varies across the world. According to statistics, about 80% of global HCC cases occurred in sub-Saharan Africa and Eastern Asia, and China alone accounted for over 50% of the world's cases [2, 3]. Guangxi is a district with high prevalence rate of HCC in southwest of China. The proportion of HCC incidence between men and women in Guangxi is 3.8 to 1 [4, 5]. HCC is a typical inflammation-related malignancy [6]. It develops frequently within the context of chronic hepatitis, which is characterized by liver inflammation and hepatocyte apoptosis [2]. Inflammation has been suggested to promote carcinogenesis by inhibiting apoptosis, damaging DNA, and stimulating angiogenesis and cell proliferation.

C-reactive protein (CRP) is a sensitive but non-specific biomarker of systemic inflammation, and it is mainly produced by hepatocytes under transcriptional regulation of interleukin-6 in inflammatory reaction [7]. The CRP levels have been routinely measured as markers for acute and chronic inflammation in inflammatory diseases as well as cancers [8, 9]. Furthermore, some studies have previously accessed the relationship between circulating CRP levels and HCC in different populations [10, 11]. Several case-control studies have indicated that HCC patients had a significantly higher CRP level in preoperative blood compared with healthy con-

Table 1. Primer sequences and reaction conditions

SNPs	Primer sequence	Annealing temperature	Restriction enzyme	Product size (bp)
rs2794521	F: 5'-GTGATGTTCCCCTTCCTGTG-3'	58.0°C	Bsh1236 I	TT: 268 bp
	R: 5'-CCTGACTCCTGCCTGAAGC-3'			TC: 268+202+66 bp
				CC: 202+66 bp
rs3093059	F: 5'-CCTGACTCCTGCCTGAAGC-3'	58.0°C	Tas I	TT: 160+64 bp
	R: 5'-CCCATCTATGAGTGAGAACACG-3'			TC: 224+160+64 bp
				CC: 224 bp

trols [12, 13]. It has been showed that high CRP levels may be due to the progression of disease and an indicator of poor outcomes [14, 15]. The *CRP* gene is located on chromosome 1 (1q21-1q23) and is characterized by genetic variability. It was reported that genetic polymorphisms in *CRP* gene have been associated with changes in CRP serum or plasma concentrations. Rs2794521, and rs3093059 are two common SNPs in the *CRP* gene and both of them have been demonstrated to up-regulate CRP protein expression or production and associated with susceptibility to inflammatory diseases including cancers [16, 17]. In light of the important role of CRP in HCC development and progression, we hypothesized that genetic polymorphisms of the *CRP* gene were correlated with the susceptibility to hepatitis B virus (HBV) related HCC. To test this hypothesis, we conducted a case-control study to investigate the association between *CRP* rs2794521 and rs3093059 polymorphisms and HBV-related HCC susceptibility in a Guangxi male population.

Materials and methods

Study population

The research comprised a hospital-based case-control study of 515 Guangxi male subjects, including 158 HBV patients with HCC, 207 HBV patients without HCC, and 150 unrelated healthy controls. All study subjects were consecutively recruited between May 2009 and December 2010 from the First Affiliated Hospital of Guangxi Medical University, Guangxi, China. All HBV patients with and without HCC selected for this study were confirmed by being HBsAg (hepatitis B surface antigen) positive, HbcAb (hepatitis B virus core antibody) positive and HBeAg (hepatitis B e antigen) or HBeAb (hepatitis B e antibody) positive for more than six months. The HBV patients without HCC and the healthy controls were tested for the absence of HCC by histology, magnetic resonance imaging (MRI), computed tomography (CT), ultrasonography, and laboratory tests. Subjects with positive laboratory parameters for immunodeficiency virus, hepatitis C virus (anti-HCV and/or HCV-RNA), alcoholic liver disease, or autoimmune diseases were excluded. For each participant, a one-time sample of about 3-5 ml venous blood was collected and the following laboratory parameters were detected: aspartate aminotransferase (AST), lanine aminotransferase (ALT), albumin (ALB), serum total bilirubin (T-Bil), g-glutamyltransferase (GGT), alpha fetoprotein (AFP), C-reactive protein (CRP), and HBV-related index (including HBsAg, HBsAb, HbcAb, HbeAg). All participants involved in the study have signed a written informed consent and the study has been approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University.

DNA extraction and genotyping

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes with proteinase K digestion and phenol-chloroform method. The CRP rs2794521, and rs3093059 polymorphisms were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The primer sequences, reaction conditions and products were listed in **Table 1**. PCR reactions were performed in a total volume of 25 μl containing 100 ng genomic DNA, 12.5 pg of each primer, 200 μM of each dNTP, 10×PCR buffer supplied by Invitrogen Corp (10 mM Tris-HCl, pH 8.8, 50 mM KCl), 2.0 mM $MgCl_2$, and 2.5 U of DNA Taq polymerase (Shanghai Biocolor, Shanghai, China). The PCR protocol included an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 60 s at 95°C, 50 s at 58°C and 60 s of elongation at

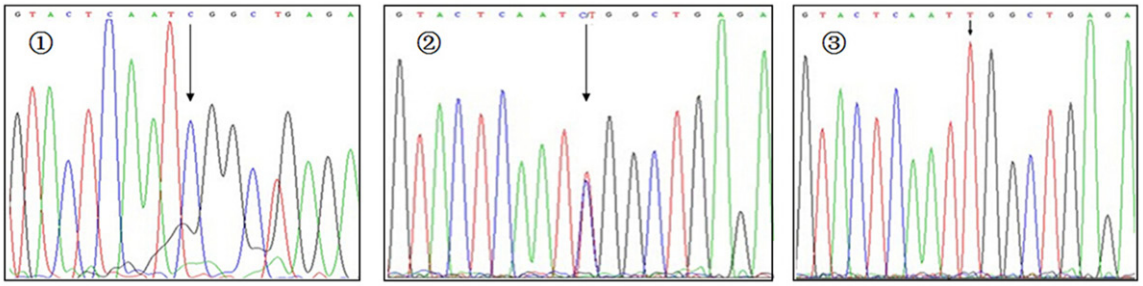

Figure 1. Sequencing map for genotypes of CRP rs3093059 polymorphism. The arrows in ①-③ show CC, TC, and TT genotypes, respectively.

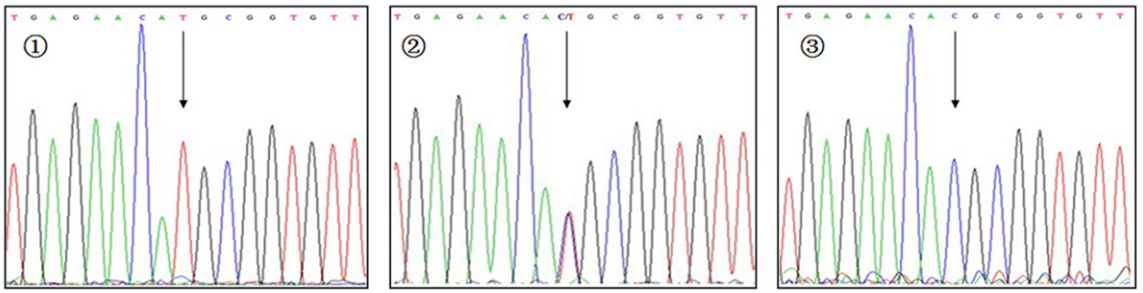

Figure 2. Sequencing map for genotypes of *CRP* rs2794521 polymorphism. The arrows in ①-③ show TT, TC, and CC genotypes, respectively.

72°C, followed by a final elongation step of 72°C for 10 min for both of the two SNPs. After amplification, the PCR products were digested at 37°C overnight with the corresponding restriction enzymes (Bsh1236 I for rs2794521, and Tas I for rs3093059). The cleaved DNA fragments were resolved on 2.5% agarose gels and stained with ethidium bromide for visualization under ultraviolet light. Two authors read the gel pictures independently and performed the repeated assays if they did not reach a consensus on the tested genotypes. To validate the results of genotyping assays, the sequences of 10% of the PCR samples were confirmed by direct sequencing in an ABI PRISM 3730 (Sangon Biotech, Shanghai), and the results were 100% concordant (**Figures 1** and **2**).

Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD). Differences in demographic characteristics and laboratory parameters were compared between groups with one-way ANOVA for continuous variables and χ^2 test for categorical variables. The association between genotypes and alleles of the CRP rs2794521 and rs3093059 polymorphisms and the presence of HBV-related HCC were determined using a standard χ^2 test. Binary logistic regression analysis and adjustment for age, smoking and drinking were used to calculate Odds ratio (OR) and 95% confidence intervals (CIs). To assess the effect of the genotypes carrying the variant allele we ran analysis under the dominant model. The Hardy-Weinberg equilibrium (HWE) was evaluated with a goodness of fit χ^2-test with one degree of freedom to compare the observed genotype frequencies among the subjects with the expected genotype frequencies. The haplotypes and their frequencies were estimated based on a Bayesian algorithm using the Phase program [18]. All tests were two-sided and *P* values less than 0.05 were considered statistically significant. The statistical analyses were performed with SPSS statistical software package version 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Population characteristics

Table 2 summarizes the demographic and laboratory parameters of all participants enrolled in

Table 2. General characteristics of the subjects included in the study

Groups	Healthy controls (n=150)	HBV patients without HCC (n=207)	HBV patients with HCC (n=158)
Demographic parameters			
Age (years) (mean ± SD)	44.43±12.91	47.46±13.04	48.81±12.46
Drinking, n (%)			
Yes	138 (92.0)	199 (96.1)	151 (95.6)
No	12 (8.0)	8 (3.9)	7 (4.4)
Smoking, n (%)			
Yes	132 (88.0)	196 (94.7)	153 (96.8)
No	18 (12.0)	11 (5.3)	5 (3.2)
Laboratory parameters (mean ± SD)			
T-Bil (μmol/L)[a]	10.24±3.77	10.49±4.31	29.02±10.14
AST (IU/L)[b]	11.37±1.11	38.27±9.62	50.19±13.21
ALT (IU/L)[b]	13.15±0.98	49.17±13.05	60.33±15.42
ALB (g/L)[b]	47.16±14.89	42.58±17.51	31.28±16.33
GGT (IU/L)[b]	27.86±5.71	57.22±16.38	79.12±23.44
AFP (ng/ml)[b]	6.31±0.33	17.96±4.55	467.22±219.77

T-Bil serum total bilirubin level, AST serum aspartate aminotransferase level, ALT serum alanine aminotransferase level, ALB serum albumin level, GGT serum gamma-glutamyltransferase level, AFP serum alpha fetoprotein level; [a]Significant difference exists between the HCC group and non-HCC groups (P<0.001); [b]Significant difference exists between each group (P<0.001).

Table 3. Genotypes and alleles distribution of CRP rs2794521, and rs3093059 polymorphisms in HBV patients with HCC and healthy controls

SNPs	Healthy controls (n=150)	HBV patients with HCC (n=158)	OR (95% CI)[a]	*P*[a]
rs2794521	HWE=0.872	HWE=0.564		
Genotypes				
TT	84 (56.0)	86 (54.4)	1.00 (Ref)	
TC	56 (37.3)	63 (39.9)	1.11 (0.71-1.77)	0.695
CC	10 (6.7)	9 (5.7)	0.89 (0.35-2.28)	0.801
TC+CC	66 (44.0)	72 (45.6)	1.08 (0.69-1.68)	0.792
Allele				
T	224 (74.7)	235 (74.4)	1.00 (Ref)	
C	76 (25.3)	81 (25.6)	1.03 (0.72-1.46)	0.930
rs3093059	HWE=0.751	HWE=0.118		
Genotypes				
TT	97 (64.7)	94 (59.5)	1.00 (Ref)	
TC	48 (32.0)	60 (38.0)	1.30 (0.81-2.07)	0.293
CC	5 (3.3)	4 (2.5)	0.83 (0.23-3.18)	0.781
TC+CC	53 (35.3)	64 (40.5)	1.23 (0.78-1.96)	0.350
Allele				
T	242 (80.1)	248 (78.5)	1.00 (Ref)	
C	58 (19.9)	68 (21.5)	1.15 (0.77-1.70)	0.502

The most common genotype TT in healthy controls was used as reference. OR, odds ratio; CI, confidence interval; [a]Adjusted for age, drinking and smoking.

this study. The parameters included age, smoking, drinking, T-Bil, AST, ALT, ALB, GGT, and AFP. Briefly, the healthy controls were significantly younger than the two diseased groups ($P<0.05$). In addition, when compared with the two diseased groups, the healthy control group had a higher proportion of smokers and drinkers ($P<0.05$). Statistically significant different laboratory results for T-Bil, AST, ALT, ALB, GGT, and AFP ($P<0.001$) was observed between group of HBV patients with HCC and groups of HBV patients without HCC and healthy controls.

Comparison between HBV patients with HCC and healthy controls

Table 3 showed the genotype and allele frequencies of the CRP rs2794521 and rs3093059 polymorphisms between HBV patients with HCC and healthy controls. The genotype distributions of the two polymorphisms were consistent with HWE in both groups. Logistic regression analysis adjusted for age, smoking

Table 4. Genotypes and alleles distribution of CRP rs2794521, and rs3093059 polymorphisms between HBV patients with HCC and HBV patients without HCC

SNPs	HBV patients without HCC (n=207)	HBV patients with HCC (n=158)	OR (95% CI)[a]	*P*[a]
rs2794521	HWE=0.764	HWE=0.564		
Genotypes				
TT	111 (53.6)	86 (54.4)	1.00 (Ref)	
TC	80 (38.6)	63 (39.9)	1.01 (0.67-1.57)	0.938
CC	16 (7.7)	9 (5.7)	0.74 (0.32-1.74)	0.464
TC+CC	96 (46.4)	72 (45.6)	0.98 (0.65-1.48)	0.875
Allele				
T	302 (72.9)	235 (74.4)	1.00 (Ref)	
C	112 (27.1)	81 (25.6)	0.94 (0.68-1.31)	0.665
rs3093059	HWE=0.726	HWE=0.118		
Genotypes				
TT	149 (72.0)	94 (59.5)	1.00 (Ref)	
TC	54 (26.1)	60 (38.0)	1.77 (1.13-2.76)	0.012
CC	4 (1.9)	4 (2.5)	1.59 (0.39-6.38)	0.518
TC+CC	58 (28.0)	64 (40.5)	1.76 (1.14-2.71)	0.011
Allele				
T	352 (85.0)	248 (78.5)	1.00 (Ref)	
C	62 (15.0)	68 (21.5)	1.56 (1.07-2.29)	0.021

The most common genotype TT in HBV patients without HCC was used as reference. OR, odds ratio; CI, confidence interval; [a]Adjusted for age, drinking and smoking.

Table 5. Association of *CRP* polymorphisms with serum CRP levels in study subjects

SNPs	CRP serum level (mg/L)		
	Healthy controls (n=150)	HBV patients without HCC (n=207)	HBV patients with HCC (n=158)
rs2794521			
Genotypes			
TT	3.98±2.56	8.44±7.98	13.14±11.02
TC	4.09±3.51	9.97±7.32	14.87±12.83
CC	4.79±4.62	11.34±9.46	16.27±13.22
TC+CC	4.38±4.17	10.32±9.78	15.49±13.84
rs3093059			
Genotypes			
TT	3.77±3.58	9.48±8.57	12.01±10.87
TC	4.49±4.86	10.31±8.42	17.42±13.02*
CC	5.03±4.64	12.63±10.72	15.49±17.64
TC+CC	4.74±4.79	11.07±10.89	16.88±16.90*

*$P<0.05$ compared with rs3093059 TT genotypes.

and drinking revealed that there were no significant differences in the CRP rs2794521 and rs3093059 polymorphisms between the HBV patients with HCC and healthy controls.

Comparison between HBV patients with HCC and HBV patients without HCC

Table 4 showed the genotype and allele frequencies of the *CRP* rs2794521 and rs3093059 polymorphisms between HBV patients with HCC and HBV patients without HCC. The genotype distributions of the two polymorphisms were consistent with HWE in both groups. With respect to rs3093059, the frequencies of TT, TC, and CC genotypes were 59.5%, 38.0% and 2.5% in HBV patients with HCC, and were 72.0%, 26.1%, and 1.9% in HBV patients without HCC, respectively. The frequencies of the T and C alleles were 78.5% and 21.5% in HBV patients with HCC, and were 85.0% and 15.0% in HBV patients without HCC, respectively. We found that there were significant differences in the genotype and allele frequencies of the *CRP* rs3093059 polymorphism between the two groups. The rs3093059 C allele was associated with a significantly increased risk of HCC as compared with the T allele (adjusted OR=1.56, 95% CI, 1.07-1.29, *P*=0.021) and the rs3093059 TC genotype was associated with a significantly increased HCC risk when compared with the TT genotype (adjusted OR=1.77, 95% CI, 1.13-2.76, *P*=0.012) in binary logistic regression analyses adjusted for age, smoking and drinking. Furthermore, the rs3093059 TC/CC genotypes were found to be correlated with a significant increased HCC risk compared with the TT genotype in a dominant model in logistic regression analysis (adjusted OR=1.76, 95% CI, 1.14-2.71, *P*=0.011). However, with respective to *CRP* rs2794521 polymorphism, we did not

Table 6. Haplotype distribution of CRP rs2794521, and rs3093059 polymorphisms in HBV patients with HCC and HBV patients without HCC

Haplotype	HBV patients without HCC (2n=414) (%)	HBV patients with HCC (2n=316) (%)	OR (95% CI)	*P*
TT	252.02 (0.609)	167.05 (0.529)	0.732 (0.721-1.108)	0.127
TC	51.91 (0.125)	67.92 (0.215)	1.803 (1.237-2.335)	0.001
CT	100.07 (0.242)	81.02 (0.256)	1.049 (0.811-1.401)	0.779
CC	10.00 (0.024)	0.01 (0.000)	—	—

observe any significant differences between the HBV patients with HCC and the HBV patients without HCC in logistic regression analysis.

Association of CRP gene polymorphisms and serum CRP levels

The association of *CRP* gene polymorphisms and serum CRP levels of the study subjects were presented in **Table 5**. We found that there was no significant association between the *CRP* rs2794521 and rs3093059 polymorphisms and serum CRP levels in healthy controls and HBV patients without HCC. In addition, no significant association of the *CRP* rs2794521 polymorphism with serum CRP levels was observed in HBV patients with HCC. However, we found that the genotypes of the *CRP* rs3093059 polymorphism were significantly correlated with serum CRP levels in HBV patients with HCC. The serum CRP levels were significantly elevated in subjects with TC genotypes (17.42±13.02 mg/L, n=60) and TC+CC genotypes (16.88±16.90 mg/L, n=64) compared with TT genotypes. However, there was no significant difference in the serum CRP levels between the *CRP* rs3093059 TC and CC genotypes.

Haplotype analysis

Table 6 showed the haplotype analysis results between the HBV patients with HCC and HBV patients without HCC. Strong linkage disequilibrium (LD) was observed between the T allele at locus rs2794521 and the T allele at locus rs3093059 (|D'|=0.949). By haplotype analyses, an increased risk of HBV-related HCC was found in the TC haplotype (OR=1.803, 95% CI, 1.237-2.335, *P*=0.001).

Discussion

Accumulating evidences from preclinical and clinical studies have suggested that inflammation and inflammatory cells implicated in the process of tumor development and progression [19, 20]. Chronic inflammation increased the opportunities for DNA damage and mutation via induction of a high rate of cell turnover and a highly oxidative microenvironment, leading to carcinogenesis [21]. The inflammatory process is regulated by cytokines, many of which can exert a variety of biological responses in local tissues. HCC is a typical inflammation-related cancer occurs mainly in men [22]. In this study, we investigated whether *CRP* gene rs2794521, and rs3093059 polymorphisms are associated with HBV-related HCC susceptibility in a Guangxi male population. We observed that there were significant differences between the HBV patients with HCC and HBV patients without HCC in the genotype and allele frequencies of the *CRP* gene rs3093059 polymorphism. The rs3093059 C allele was associated with a significantly increased HCC risk compared with the T allele (adjusted OR=1.56, 95% CI, 1.07-1.29, *P*= 0.021). The rs3093059 TC genotype was correlated with a significantly increased HCC risk compared with the TT genotype (adjusted OR=1.77, 95% CI, 1.13-2.76, *P*=0.012). In addition, the rs3093059 TC/CC genotypes were found to correlate with a significantly increased HCC risk as compared with the TT genotype in the dominant model (adjusted OR=1.76, 95% CI, 1.14-2.71, *P*=0.011). However, we did not observe any significant effect of *CRP* rs2794521 polymorphism on HCC risk in this population. The findings suggest that *CRP* gene rs3093059 polymorphism could be used as genetic susceptibility marker of the HBV-related HCC in males.

Data from previous epidemiological studies in diverse ethnic populations was inconsistent with the results of the present study. Zee et al. [23] reported that the frequency of rs3093059 C allele was 0.07 in an American white population. Motoyama et al. [24] showed that the rs2794521 C allele was 0.11 in a Japan population. However, in the current study, we found that the frequencies of the *CRP* rs3093059 C, and rs2794521 C alleles among the healthy

controls were 0.20, and 0.25, respectively, which were significantly higher than the frequencies observed in the above two studies. Moreover, we also observed that the rs2794521, and rs3093059 polymorphisms of the *CRP* gene were in strong LD (|D'|=0.949). The frequency of the main haplotype TT in healthy controls in this study was 0.55, which was significantly higher than that of study performed in Taiwan (0.49) [25], suggesting that the distribution of the *CRP* gene frequencies might vary among the different ethnic groups.

CRP is one of the acute-phase proteins that are mainly produced by hepatocytes in response to acute and chronic inflammation. Previous studies have shown that high circulating CRP correlates with T cell impairment and increased levels of serum angiogenic factors and shows resistance to chemotherapy in tumor patients [26]. In addition, serum high level of CRP was inversely associated with low tumor-infiltrating $CD4^+$ T-lymphocytes within the tumor microenvironment. In clinical studies, elevated circulating levels of CRP have been reported to associate with increased risk of cancers and poor cancer prognosis [26, 27]. It was reported that the transcription and expression of CRP was positively regulated by IL-6 in response to advanced cancer or chronic inflammation. Over expression of IL-6 can result in increased concentration of CRP [7]. However, IL-6 production was regulated by estrogen. The estrogen-mediated inhibition of IL-6 production can result in decreased CRP production [28, 29]. Therefore, the estrogen may affect the CRP expressions indirectly and responsible for decreased hepatic response to infections or systemic inflammation and reduced HCC risk in females. In this study, the significant association between CRP rs3093059 polymorphism and HCC susceptibility was detected in male subjects, which indicated that the effect of this polymorphism on developing HCC might be counteracted by the protective estrogenic hormone-related pathway in females or engendered by androgenic hormone-related pathway in males.

Haplotypes are a set of closely related genetic markers existed on one chromosome which tend to be inherited together and appear frequently in a block pattern due to the existence of LD [30]. Haplotype analysis was more informative than study of individual SNPs because it involves the parallel assay of several SNPs within the same gene. In the present study, we observed that the TC haplotype was significantly correlated with increased HBV-induced HCC risk in the Guangxi male population (OR=1.803, 95% CI, 1.237-2.335, *P*=0.001). The possible reason is that a high presence of the TC haplotype in the *CRP* gene may modulate the CRP production, resulting in upregulation of inflammation response induced by viral infection in HCC development. The results of our study suggested that the TC haplotype of *CRP* gene may play a facilitative effect in the development of HBV induced HCC.

Some limitations of this study should be noted. First of all, the study population was limited to Guangxi district, so it does not permit extrapolation of the results to other populations in other areas. Second, the potential selection bias should be noted since this is a hospital based case-control study. Third, only two SNPs in the *CRP* gene were studied in the current study. It would be important to identify more SNPs in the *CRP* gene and study their roles in HBV-related HCC. Therefore, the results of the present study should be interpreted with caution in light of the limitations.

In conclusion, our data demonstrated that the *CRP* rs3093059 polymorphism might play a facilitative role in the development of HBV-related HCC in the Guangxi male population. Further replication in other populations with larger samples for association study and biological functional study should be warranted in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Xue Qin and Shan Li, Department of Clinical Laboratory, First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning 530021, Guangxi, China. Tel: +86-771-5356052; Fax: +86-771-865353342; E-mail: qinxue919@126.com (XQ); lis8858@126.com (SL)

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