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ORIGINAL ARTICLE

Association of TNF-α genetic polymorphisms with hepatocellular carcinoma susceptibility: a case-control study in a Han Chinese population

Xiangmei Chen[1], Ling Zhang[2], Yibin Chang[1], Tao Shen[1], Ling Wang[1], Hui Zhuang[1], Fengmin Lu[1]

[1]Department of Microbiology & Infectious Disease Center, Peking University Health Science Center, Beijing - PR China
[2]Department of Hepatobiliary Pancreatic Surgery, Henan Cancer Hospital, Zhengzhou - PR China

ABSTRACT

The single nucleotide polymorphisms (SNPs) within the tumor necrosis factor-α (TNF-α) gene promoter region have been reported to be associated with susceptibility to various types of cancers. A case-control study (126 hepatocellular carcinoma [HCC] patients and 126 normal controls) was conducted to elucidate their possible association with the risk of hepatitis B virus (HBV)-related HCC in a Han Chinese population. TNF-α polymorphisms -1031T/C, -863C/A, -857C/T, -308G/A, and -238G/A were genotyped by polymerase chain reaction (PCR) and direct DNA sequencing. Disease associations were analyzed by the chi-square test or Fisher's exact test. When analyzed by overall groups, no significant differences in genotype and allele distributions were observed between the control and cases. However, stratified analysis according to sex showed that the frequency of the homozygous C allele of the -857 polymorphism was lower in female cases than in female controls (62.9% vs. 88.9%, p=0.026). In addition, further haplotype analysis revealed that the TCCGA (-1031/-863/-857/-308/-238) was more frequent in controls than cases (p=0.018; odds ratio = 0.266; 95% confidence interval, 0.083–0.857). These results indicated that the TNF-α-857C/T polymorphism may modify HBV-related HCC risk among women, and the haplotype TCCGA (-1031/-863/-857/-308/-238) may account for a decreased susceptibility to HCC development in the Han Chinese population. Additional studies in patients with different ethnic backgrounds are needed to validate these finding and to further explore the genetic pathogenesis of HBV-related HCC.

Key words: Hepatitis B virus, Hepatocellular carcinoma, Polymorphism, Tumor necrosis factor-alpha

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers and the third leading cause of cancer mortality worldwide (1). It has been ranked as the second leading cause of cancer death in China since the 1990s (2). In hepatitis B virus (HBV) infection endemic areas such as China and other parts of Southeast Asia, chronic HBV infection has been identified as a distinct causative factor of HCC (3-5). Chronic hepatitis B patients had a greater than 100-fold increased risk of HCC development compared with individuals who are uninfected (6). Despite recent advances in chemotherapy such as the use of the multitargeting drug Sorafeinib,the long-term survival rate for HCC patients is still very low. Surgical excision is still the most effective treatment so far, which requires diagnosis of HCC patients at an early stage of the disease. Identifying individuals at high risk and applying enhanced surveillance to the high-risk population should catch HCC patients at an early stage.

It has been noticed for a long time that not all individuals with comparable exposure risk factors develop HCC eventually, suggesting a possible role of genetic factors in the etiology of this most lethal malignancy. Indeed, a number of studies have reported the potential role of single nucleotide polymorphisms (SNPs) in liver cancer (7-10). Tumor necrosis factor-α (TNF-α), a multifunctional cytokine, is involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune, and malignant diseases. It has been suggested that circulating TNF-α levels increase during HBV infection, and the elevated levels of TNF-α correlate with the severity of hepatic inflammation, fibrosis, cirrhosis, and even liver malignancy (11-14). TNF-α can induce the production of oxygen free radicals and other reactive oxygen species. These compounds are important mediators of genetic mutations and gross chromosomal alterations that may contribute to the development of HCC (15). Thus, it is conceivable that TNF-α might influence the severity of

liver inflammation and thereby be an excellent candidate as a susceptibility gene for HBV-related HCC.

The TNF-α gene is located within the class III region of the major histocompatibility complex (MHC) between the human leukocyte antigen-B (HLA-B) and HLA-DR. Furthermore, several SNPs have been described within the promoter region of the TNF-α gene, which have been associated with altered TNF-α promoter activity (16-18). Although several association studies between TNF-α and HBV infection, in relation to the outcome, progression, or prognosis of the disease, have been published, few genetic studies concerning the correlation between TNF-α and HBV-related HCC have been reported. This present case-control study was performed to assess the association of HCC and 5 TNF-α gene polymorphisms (-1031T/C, -863C/A, -857C/T, -308G/A, and-238A/G) in ethnic Han Chinese from northern China.

MATERIALS AND METHODS

Study population

A total of 126 HCC patients with chronic HBV infection were enrolled in this study: 99 men and 27 women. The mean age of patients was 49.6 years. All HCC patients were diagnosed on the basis of histology or the combination of typical radiological findings of HCC, and underwent surgery in a cancer hospital in Zhengzhou, Henan province of China (Tab. I). The control population consisted of 126 age- and sex-matched healthy subjects who had visited outpatient clinics in Zhengzhou for their routine health checkups from December 2006 to June 2007. For the control subjects, all were both serum HBsAg and anti-HCV negative, and showed no clinical evidence of space-occupying lesion in the liver and no symptoms, signs, or biochemical evidence of liver disease, or other medical illness. All study subjects gave consent according to a protocol approved by the ethics committee of Peking University Health Science Center.

DNA extraction

Genomic DNA was isolated from EDTA-preserved whole blood by standard proteinase K digestion and phenol-chloroform methods. After ethanol precipitation, the DNA was dissolved in double-distilled water and frozen at −20°C until use.

Polymorphism genotyping

For determination of TNF-α polymorphisms of the gene promoter region at position -1031T/C, -863C/A, -857C/T, -308G/A, and -238A/G, a 895-bp fragment spanning positions from -1057 to -162 of the promoter region of the TNF-α gene was amplified using primers: TNF-α sense (5′-GGGAAGCAAAGGAGAAGC-3′) and antisense (5′-CTCGGTTTCTTCTCCATCG-3′). Polymerase chain reaction (PCR) was carried out in a 50-μL reaction mixture containing 100 ng of genomic DNA, 10 mM/mL of each primer 1 μL, 10 mM/mL total dNTP 1 μL, 10 × mixture buffer 5 μL, and 2 U of Taq plus polymerase under the reaction conditions of 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, annealing at 52°C for 1 minute and 72°C for 1 minute, and finally at 72°C for 10 minutes. The PCR products were purified by use of a Gel Purify Recovery Kit (Axygen Biotechnology, Hang Zhou, China) according to the manufacturer's recommendation, and the purified products were sequenced with the sequencing primer (5 -CCCAGGGAGTGAA A GAGC-3′) (-833˜-816) for the forward sequence and (5 -ATGCTGGTTTCAGTCTTGG-3′) (-679˜-697) for the reverse sequence, using an ABI 310 DNA Sequencer (Applied Biosystems). The acquired sequences were analyzed using the BioEdit program through the DNA Sequencing Electropherogram.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 10.0). Differences in the distributions of demographic characteristics between the cases

TABLE I - BASIC CHARACTERISTICS OF THE SUBJECTS STUDIED

Parameters	Cases (n=126)	Controls (n=126)	p Value*
Sex			NS
Male, no. (%)	99 (78.6%)	99 (78.6%)	
Female, no. (%)	27 (21.4%)	27 (21.4%)	
Age, mean years ±SD	49.6 ± 10.46	49.8 ± 9.93	NS
Smoking status			NS
Never	66	66	
Ever	60	60	
Alcohol status			NS
Nondrinker	78	77	
Drinker	48	49	
Viral infection			
HBsAg positive	126	-	
Anti-HCV Ab positive	-	-	
Stages of HCC			
I or II	42	-	
III or IV	84	-	

HCC = hepatocellular carcinoma; NS = not significant.
*p values were derived from the chi-square test except age, which was based on Student's *t*-test.

and controls were evaluated using Student's t-test (for continuous variables) and the chi-square test (for categorical variables). The frequencies of polymorphism distribution among cases and controls were compared using the chi-square test, and p values <0.05 were considered to indicate statistical significance. Adjusted odds ratios (OR) with 95% confidence intervals (95% CI) were calculated by logistic regression analysis to quantify the association between HCC and TNF-α polymorphisms. The analyses of linkage disequilibrium, haplotype construction, and deviation from Hardy-Weinberg equilibrium (HWE) were tested using a software platform, SHEsis (19).

RESULTS

The analysis included 126 cases and 126 controls, all of whom were Han Chinese. Table I shows the demographic characteristics such as age, sex, smoking status, alcohol use, marker of hepatitis in the study subjects. The cases and controls were matched by age, sex, smoking status, and alcohol use. HCC was more predominantly found in male than in female patients (99 vs. 27).

The alleles at TNF-α-1031T/C, -863C/A, -857C/T, -308G/A, and -238G/A in all study subjects were in accordance with the Hardy-Weinberg equilibrium (data not shown), suggesting that there was no population stratification and no sampling bias. The genotypic distributions in all study subjects are presented in Table II. No significant difference was observed in the genotype and allele frequencies of -1031T/C, -863C/A, -857C/T, -308G/A, and -238G/A polymorphisms between controls and patients with HCC. Further, we looked for the association between the 5 SNPs of TNF-α and HCC by age, sex, smoking status, alcohol use, and clinical stages. As shown in Table III, a relative lower frequency of homozygous –857C alleles was found in female HCC patients than in female controls (62.9% vs. 88.9%; p=0.026; OR=0.213; 95% CI, 0.051-0.890). No other appreciable differences for -1031T/C, -863C/A, -308G/A, and -238G/A polymorphisms were observed.

Using SHEsis software analysis, pairwise linkage disequilibrium (LD) analysis between the different SNPs was performed (Fig. 1). A strong LD ($p<0.001$; $D'=1.0$, $r^2=0.73$) between the TNF-α-863 and -1031 polymorphisms was observed in our study population. TNF-α-863(C/A) and -1031(T/C) were shown as a single block. In contrast, no significant LD was identified between other polymorphisms. Haplotypes were constructed using the accelerated Expectation Maximization algorithm implemented in the SHEsis software. Five haplotypes were found to occur in more than 3% of the study population and were further analyzed. Estimated haplotype frequencies between healthy controls and HCC patients for all of the 5 haplotypes are listed in Table IV. The frequency of the haplotype TCCGA (-1031/-863/-857/-308/-238) in the HCC group was significantly lower than that in the healthy controls (p=0.018; OR=0.590; 95% CI, 0.083-0.857).

TABLE II - GENOTYPE AND ALLELE FREQUENCIES OF TNF-α POLYMORPHISMS IN CHINESE PATIENTS WITH HCC AND IN CONTROLS

SNP	TNF-α genotype frequency			p Value	TNF-α allele frequency		OR (95% CI)*	p Value
T-1031C	TT	CT	CC		T	C		
Control	89 (71.6)	32 (25.4)	5 (4.0)	0.921	210 (83.3)	42 (16.7)	1.118 (0.655-1.911)	0.682
HCC	84 (66.7)	35 (27.8)	7 (5.6)		203 (80.6)	49 (19.4)		
C-863A	CC	CA	AA		C	A		
Control	92 (73.0)	31 (24.6)	3 (2.4)	0.498	215 (85.3)	37 (14.7)	1.196 (0.741-1.930)	0.465
HCC	85 (67.5)	39 (31.0)	2 (1.6)		209 (82.9)	43 (17.1)		
C-857T	CC	TC	TT		C	T		
Control	98 (77.8)	24 (19.0)	4 (3.2)	0.553	220 (87.3)	32 (12.7)	0.965 (0.615-1.744)	0.894
HCC	95 (75.4)	29 (23.0)	2 (1.6)		219 (86.9)	33 (13.1)		
G-308A	GG	GA	AA		G	A		
Control	111 (88.1)	14 (11.1)	1 (0.8)	0.241	236 (93.7)	16 (6.3)	0.884 (0.203-1.151)	0.19
HCC	118 (93.7)	8 (6.3)	0 (0.0)		244 (96.8)	8 (3.2)		
G-238A	GG	GA	AA		G	A		
Control	115 (91.3)	11 (8.7)	0 (0.0)	0.233	241 (95.6)	11 (4.4)	0.989 (0.145-1.653)	0.240
HCC	120 (95.2)	6 (4.8)	0 (0.0)		246 (97.6)	6 (2.4)		

HCC = hepatocellular carcinoma; 95% CI = 95% confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism; TNF-α = tumor necrosis factor-α.
*Adjusted for age and sex by multivariate logistic regression.

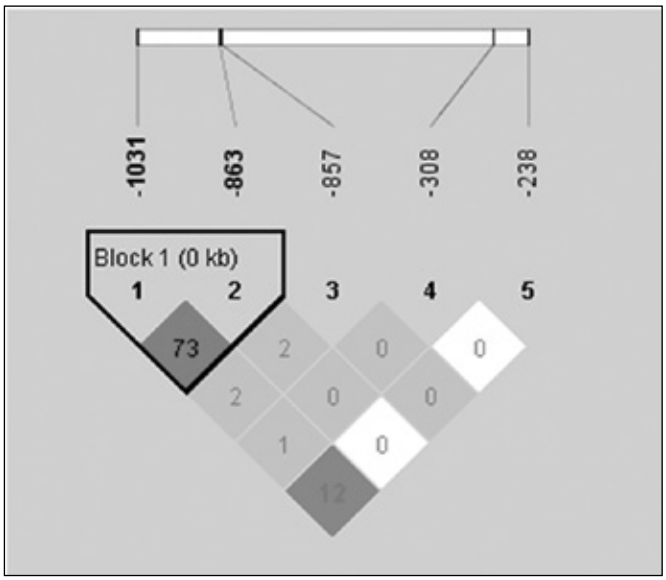

Fig. 1 - *Linkage disequilibrium (LD) tests of the tumor necrosis factor-α (TNF-α) gene. The haplotype block pattern was constructed using SHEsis software with data from cases and controls. The number in each cell represents the LD parameter D′ (x100). Each cell is color graduated relative to the strength of LD between 2 markers. Black cells indicate pairs of single nucleotide polymorphisms (SNPs) in significant and maximum LD (i.e., D′ = 1, log of the odds [LOD] ≥2). Gray cells also represent pairs of SNPs with D′ = 1, although in these cases, the observed LD levels are not statistically significant. White cells represent pairs of SNPs in strong recombination (D′ < 1, LOD < 2). A block has been created between the -1031 and -863 loci, and 95% of the informative comparisons there are "strong LD".*

DISCUSSION

HBV hepatocarcinogenesis is not only a multistage process but also involves multiple factors. Increasing knowledge about host genetic factors that predispose individuals to HCC will facilitate both identification and prevention for high-risk individuals. It is well known that a persistent inflammatory condition in liver tissue is the most important risk fact for HCC among chronic HBV carriers, which is closely related to an individual duration of HBV infection. Prolonged exposure to HBV elicits a cellular immune response through activation of tissue macrophage Kupffer cells, which in turn release an array of cytokines, including TNF-α, transforming growth factor-β, platelet-derived growth factor, and other factors that act on hepatic satellite cells (20, 21). Among antiviral cytokines, TNF-α plays an important role in the immune pathogenesis of HCC (22). The expression of the TNF-α gene is tightly controlled at the transcriptional and posttranscriptional levels. TNF-α is known to have 5 biallelic SNPs in the promoter region at -1031T/C, -863C/A, -857C/T, -308G/A, and -238G/A. Previous reports have shown that such SNPs may affect its transcription activity (17). Thus, efforts have been focused to determine the effect of TNF-α polymorphisms on the risk for HCC. However, the results have been controversial, and the association between TNF-α polymorphisms and HCC remains to be confirmed (23-26). Here, we studied the distribution of the 5 SNPs among chronic HBV infection–related HCC patients. To our knowledge, there has been no study of the association between the TNF-α polymorphisms in the promoter region and the risk of HCC in Han Chinese populations with chronic HBV infection.

Among the 5 SNPs in the promoter region, the most extensively investigated polymorphism of TNF-α is -308G/A. Higher transcriptional activity has been demonstrated for the -308A allele in comparison with the -308G variant, with the former associated with a variety of infectious diseases and some malignant tumors (27-30). As to HCC susceptibility, Jeng et al (23) found a significant association of the -308A allele with an increased risk of development of HCC in a Taiwanese population. In the present study, we failed to detect any difference in the genotypic and allelic frequencies of this polymorphism between HCC patients with chronic HBV infection and controls in Han Chinese populations. These results are consistent with those of other previous reports, which also failed to demonstrate the association between TNF-α-308G/A allele polymorphism and HCC, in Japanese, Hong Kong Chinese, and Thai populations (25, 26, 31, 32). The reasons for the different results between ours and those obtained by Jeng et al are unknown; different sample size and ethnicity of study cohorts could be an explanation.

Concurrent findings of an association of TNFα-857C/T have more recently been made in a study of HCC cohorts in Korea. Subsequent analyses in that study found that serum TNF-α levels in HCC patients with CT or TT genotype of TNF-α-857 were significantly increased compared with those with the CC homozygous genotype, which may suggest that the TNF-α-857C/T polymorphism directly correlates to HCC via the increase in circulating TNF-α levels (33). The results in the current study showed no significant difference in the frequency of TNF-α-857C/T between overall control subjects and patients with HCC. However, we found that the frequency of the homozygous C allele of the -857 polymorphism was lower in female HCC patients than in female controls. This result might show that TNF-α-857C/T contributes to the development of HCC in women by stimulating the production of TNF-α. However, the current study cohort was too small to make a final conclusion, and a study with a large number of female HCC patients should be conducted in the future.

Haplotype is a set of statistically associated SNPs on a single chromosome. The results of the present study revealed that when analyzed independently, none of the

TABLE III - ASSOCIATION OF TNF-α GENOTYPE FREQUENCIES WITH HCC RISK FACTORS IN CHINESE CONTROL INDIVIDUALS AND PATIENTS WITH HCC

		T-1031C		p value	C-863A		p value	C-857T		p value	G-308A		p value	G-238A		p value
		TT	TC+ CC		CC	CA+AA		CC	CT+TT		GG	GA+AA		GG	GA+AA	
Age																
≥50 years	Control	50 (75.8)	16 (24.2)	0.82	51 (77.3)	15 (22.7)	0.241	47 (71.2)	19 (28.8)	0.55	62 (93.9)	4 (6.1)	0.51	63 (95.4))	3 (4.6)	0.93
	HCC	48 (72.7)	18 (27.3)		45 (68.2)	21 (31.8)		50 (75.8)	16 (24.2)		60 (90.9)	6 (9.1)		62 (93.9)	4 (6.1)	
<50 years	Control	39 (65.0)	21 (35.0)	0.89	41 (68.3)	19 (31.7)	0.845	51 (85.0)	9 (15.0)	0.17	52 (86.6)	8 (13.4)	0.12	52 (86.7)	8 (13.3)	0.34
	HCC	36 (60.0)	24 (40.0)		40 (66.7)	20 (33.3)		45 (75.0)	15 (25.5)		55 (91.6)	5 (9.4)		58 (96.6)	2 (3.4)	
Sex																
Male	Control	68 (68.7)	31 (31.3)	0.48	68 (68.7)	31 (31.3)	0.32	74 (74.7)	25 (25.3)	0.50	91 (91.9)	8 (8.1)	0.44	90 (90.9)	9 (9.1)	0.49
	HCC	60 (60.6)	39 (39.4)		62 (62.6)	37 (37.4)		78 (78.8)	21 (21.2)		94 (94.9)	5 (5.10)		94 (94.9)	5 (5.1)	
Female	Control	21 (77.8)	6 (22.2)	0.36	24 (88.9)	3 (11.1)	0.600	24 (88.9)	3 (11.1)	**0.026**	20 (74.1)	7 (25.9)	0.1600	25 (92.6)	2 (7.4)	0.55
	HCC	24 (88.8)	3 (11.2)		23 (85.2)	4 (14.8)		17 (62.9)	10 (37.1)		24 (88.8)	3 (11.2)		26 (96.3)	1 (3.7)	
Smoking																
Never	Control	43 (71.7)	17 (28.3)	0.55	45 (75.0)	15 (25.0)	0.41	51 (85.0)	9 (15.0)	0.25	52 (86.7)	8 (13.3)	0.38	54 (90.0)	6 (10.0)	0.51
	HCC	40 (66.7)	20 (33.3)		41 (68.3)	19 (31.7)		46 (76.7)	14 (23.3)		55 (91.7)	5 (8.3)		56 (93.3)	4 (6.7)	
Ever	Control	46 (69.7)	20 (30.3)	0.71	47 (71.2)	19 (28.8)	0.57	47 (71.2)	19 (28.8)	0.70	59 (89.4)	7 (10.6)	0.19	61 (92.4)	5 (7.6)	0.24
	HCC	44 (70.9)	22 (29.1)		44 (70.9)	22 (29.1)		49 (74.2)	17 (25.8)		63 (95.5)	3 (4.5)		64 (97.0)	2 (3.0)	
Alcohol																
Nondrinker	Control	59 (75.6)	19 (26.4)	0.16	55 (70.5)	23 (29.5)	0.86	60 (76.9)	18 (23.1)	0.85	68 (87.2)	10 (11.8)	0.29	71 (91.0)	7 (9.0)	0.19
	HCC	51 (65.4)	27 (34.6)		54 (69.2)	24 (30.8)		59 (75.6)	19 (24.4)		72 (92.3)	6 (7.7)		75 (96.2)	3 (3.8)	
Drinker	Control	30 (62.5)	18 (37..5)	0.52	37 (77.1)	11 (22.9)	0.17	38 (79.2)	10 (20.8)	0.63	43 (89.6)	5 (10.4)	0.23	44 (91.7)	4 (8.3)	0.69
	HCC	33 (68.8)	15 (31.2)		31 (64.5)	17 (35.5)		36 (75.0)	12 (25.0)		46 (95.8)	2 (4.2)		45 (93.8)	3 (6.2)	
Stages																
I or II		26 (61.9)	16 (38.1)	0.42	29 (69.0)	13 (31.0)	0.78	30 (71.4)	12 (28.6)	0.47	38 (90.5)	4 (9.5)	0.31	40 (95.2)	2 (4.8)	1.00
III or IV		58 (69.0)	26 (31.0)		56 (66.7)	28 (33.3)		65 (77.4)	19 (22.6)		80 (95.2)	4 (4.8)		80 (95.2)	4 (4.8)	

Values are numbers (percentage), unless specified otherwise.
HCC = hepatocellular carcinoma; TNF-α = tumor necrosis factor-α.

TABLE IV - HAPLOTYPE ANALYSIS IN CHINESE PATIENTS WITH HCC AND IN CONTROLS

Haplotype					Frequency*		Chi-square test	OR	p Value
-1031	-863	-857	-308	-238	HCC	Control		(95% CI)	
C	A	C	G	G	0.138	0.121	0.326	1.194 (0.650-2.193)	0.568
C	C	C	G	A	0.015	0.038	1.665	0.412(0.103-1.651)	0.197
T	C	C	A	G	0.020	0.072	5.596	0.59 (0.083-0.857)	**0.018**
T	C	C	G	G	0.665	0.648	0.446	1.162 (0.748-1.803)	0.504
T	A	C	G	G	0.126	0.108	0.402	1.228 (0.650-2.319)	0.526

HCC = hepatocellular carcinoma; 95% CI = 95% confidence interval; OR = odds ratio.
*All those with frequency <0.03 were ignored in the analysis.

5 SNPs in the promoter region of the TNF-α gene could be associated with a susceptibility to HCC development. However, when those SNPs were analyzed together as haplotypes, a significant association of haplotype TCCGA (-1031/-863/-857/-308/-238) was found with protection against the development of HCC. Therefore, our study corroborates the idea that haplotypes are more powerful for detection of disease associations than individual polymorphisms, and previous reports and our data here may contribute to future investigations of the etiology and pathogenesis of HBV-related HCC (34). To better understand the underlying mechanism, it is worth assessing if such a haplotype is relevant to lower TNF-α expression, due to synergetic effects of each component SNP.

In summary, this study investigated the relationship between TNF-α gene polymorphisms and the risk of HBV-related HCC. For the first time to our knowledge, the frequency of the homozygous C allele of the -857 polymorphism was found to be lower in female HCC patients than in female controls. Moreover, the TCCGA (-1031/-863/-857/-308/-238) haplotype in the TNF-α gene was found to be associated with protection against HBV-related HCC in a Han Chinese population. However, a large-scale longitudinal study to further explore the polymorphisms of the TNF-α gene in different ethnic populations is needed, and controlling for all other risk factors relevant to hepatocarcinogenesis should be considered to validate the current observations.

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Address for correspondence:
Fengmin Lu, Hui Zhuang
Department of Microbiology & Infections Disease Center
Peking University Health Science Center
Xueyuan Road 38, Haidian District
Beijing 100191, PR China
e-mail: lu.fengmin@hsc.pku.edu.cn; zhuangbmu@126.com

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