RESEARCH ARTICLE

Combined Effects of Six Cytokine Gene Polymorphisms and SNP-SNP Interactions on Hepatocellular Carcinoma Risk in Southern Guangxi, China

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Abstract

Cytokine gene single nucleotide polymorphisms (SNPs) are involved in the genesis and progression of hepatocellular carcinoma (HCC). We hypothesized that combined effects of cytokine gene SNPs and SNP-SNP interactions are associated with HCC risk. Six SNPs in cytokine genes (IL-2, IFN-γ, IL-1β, IL-6, and IL-10) were genotyped in a study of 720 Chinese HCC cases and 784 cancer-free controls. Although none of these SNPs individually had a significant effect on the risk of HCC, we found that the combined effects of these six SNPs may contribute to HCC risk (OR=1.821, 95% CI=1.078-3.075). This risk was pronounced among smokers, drinkers, and hepatitis B virus carriers. A SNP-SNP interaction between IL-2-330 and IFN-γ-1615 was associated with an increased HCC risk (OR=1.078, 95% CI=1.022-1.136). In conclusion, combined effects of SNPs and SNP-SNP interactions in cytokine genes may contribute to HCC risk.

Keywords: Cytokine genes - polymorphisms - interaction - risk - hepatocellular carcinoma - China

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide, representing more than 85% of all primary liver cancers (Parikh et al., 2007). About half of all liver cancer deaths were reported to have occurred in China (Jemal et al., 2011). Southern Guangxi has one of the highest occurrences of liver cancer in China, which ranked first in all cancer-related deaths (Zhang et al., 2010). Chronic infection with hepatitis B virus (HBV) that is perinatally acquired from carrier mothers is a primary cause in China (Yeh et al., 1989). After initial HBV infection, 90% to 95% of adults can rely on their own immune systems to clear the virus, causing self-limiting hepatitis. Only 5% to 10% of infected individuals develop chronic hepatitis, and 10% to 25% of these patients eventually progress to HCC (Lok et al., 2001; Iino et al., 2002). This phenomenon suggests that the body's scavenging ability for HBV is based on individual differences. That is, although HBV infection is the major risk factor for HCC, only a fraction of HBV-infected individuals develops HCC. This development behavior suggests that genetic factors may also have important functions in HCC etiology.

Cytokines are a family of proteins that mediate numerous inflammation responses; these proteins contribute to the outcomes of immune-mediated diseases, including HCC (Budhu et al., 2006). Substantial evidence suggests that HCC is inherently associated with the inflammation and up-regulation of cytokines (Bidwell et al., 1999; Li et al., 2010). In the past two decades, epidemiological and genetic evidence has shown that high mutagen sensitivity is a risk factor for cancer development (Wu et al., 2013). Gene regulatory regions, such as promoter regions, contribute to gene functions by binding to specific transcription factors and by regulating gene transcription initiation (Guo et al., 2005). Interleukin 1 beta (IL-1β) is not only an important host genetic factor but also a key pro-inflammatory cytokine that regulates the expression of several inflammatory molecules (Hwang et al., 2002). Two potentially functional SNPs (-511T/C and -1464C/G) in the promoter region of IL-1β can increase IL-1β production (Zeng et al., 2003). The promoter region of IL-1β is reportedly associated with the risk of developing cancers of the lung (Zienolddiny et al., 2004), breast (Liu et al., 2006), liver (Hirankarn et al., 2006), and stomach (El-Omar et al., 2000). IL-2 exerts pleiotropic effects on the immune system by acting as a pro- and anti-inflammatory regulator. The IL-2-330 site is located near an important transcription factor, nuclear factor of

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activated T-cells (NFAT). Thus, this site may influence the activity of transcription factors and subsequently affect the transcription and translation levels of IL-2 (Crabtree et al., 1994; Pyo et al., 2003). Several studies have found that IL-2-330A/C is associated with the risk of genetic susceptibility to nasopharyngeal carcinoma (Wei et al., 2010) and gastric cancer (Sugimoto et al., 2010). Interferon-gamma (IFN-γ), a major cytokine secreted by T cells, is crucial for anti-viral immune response. The constitutive expression of IFN-γ in the liver causes chronic active hepatitis through the recruitment of lymphomononuclear cells (Toyonaga et al., 1994). The expression of IFN-γ is slightly higher in HCC tissue than in adjacent normal tissue (Chia et al., 2002). Su et al. (2012) found that IFN-γ-1615 GA/AA genotypes are significantly related to an elevated risk of breast cancer. IL-10 is a potent immunosuppressive anti-inflammatory cytokine that is highly expressed in HCC tissue and adjacent normal tissue (Chia et al., 2002). IL-10 is the most studied anti-inflammatory cytokine involved in cancer research. The promoter region is highly polymorphic and is associated with gastric cancer and HCC (Shin et al., 2003; Pan et al., 2013). IL-6 is a multifunctional cytokine that serves pro- and anti-inflammatory functions. The level of IL-6 is higher in HCC patients than in healthy individuals (Giannitrapani et al., 2011).

Numerous cytokines participate in the construction of a highly complex and coordinated network in which they modulate their own synthesis and that of other cytokines (Balkwill et al., 1989; Elias et al., 1992). Recent studies have evaluated the effects of individual SNPs but failed to consider the potential combined effects and SNP-SNP interactions in two or more loci. Therefore, one-on-one association cannot sufficiently explain the complexity of disease causality (Onay et al., 2006; Lin et al., 2013). Therefore, we hypothesized that not only the individual effects but also the combined effects of SNPs and SNP-SNP interactions in cytokine genes may increase HCC risk. Cytokine gene functional SNPs (rs2069762, rs2069705, rs1143623, rs16944, rs1800769, and rs1800872) were selected to test our hypothesis in this study.

Materials and Methods

Study population

All HCC patients and cancer-free controls were recruited from First Affiliated Hospital of Guangxi Medical University, First Affiliated Hospital of Guangxi College of Traditional Chinese Medicine, and First Affiliated Hospital of Guilin Medical University from August 2007 to November 2010. The subjects received a detailed description of the study protocol and signed informed consent forms that were approved by the institutional review board for each medical center. The patients were all newly diagnosed with HCC but had not been treated with radiation, chemotherapy, or surgical therapy before enrollment in the study. The cancer-free controls were recruited in the same period from the Department of Orthopedics and Ophthalmology of the same hospitals. The controls were genetically unrelated visitors or companions of patients and were frequency matched to the cases by age (±5 years), gender, and nationality (Han, Zhuang, or others). The study protocol was approved by the institutional review board of the Guangxi Cancer Institute.

Data collection

After informed consent was obtained, a structured interview was conducted by trained interviewers. Data were collected from interviews and reviews of medical records on the demographic characteristics, recent and prior tobacco use, alcohol consumption, and HBV virus markers. Upon consent, 5mL of non-heparinized blood was collected from each participant for cytogenetic and molecular genetic analyses. All laboratory and questionnaire data were coded, entered by two investigators, and verified using EpiData 3.1 (www.epidata.dk/download.php). Neither the laboratory nor the data entry personnel had any knowledge of the subjects' case-control status.

Genotyping

Genomic DNA was extracted from leukocytes using the phenol-chloroform method and stored at -80°C. Four SNPs (Table 1) were selected through the SNP info platform (http://snpinfo.niehs.nih.gov/) with the following criteria: the genes were the main cytokine genes involved in the pro-or anti-inflammatory pathways of liver disease, the SNPs were in the transcription factor binding site of the 5' promoter region, and the pairwise linkage disequilibrium (LD) had a r^2 threshold of 0.8 and a minor allele frequency of 5% or higher in Han Chinese in Beijing (CHB). Considering these criteria, we selected six loci SNPs for genotyping (Table 1). The polymorphisms were genotyped using high-flux TaqMan MGB, a fluorescent quantitative real-time polymerase chain reaction (PCR) method. Each PCR reaction mixture (25 μL) contained 1.00 μL of DNA template, 10.25 μL of H_2O, 12.50 μL of 2×TaqMan universal PCR mix, and 1.25 μL of 20×SNP genotyping assay mix. The genomic DNA was amplified at 95°C for 10min, followed by 40cycles of 92°C for 15s and 60°C for one min. All genotyping reagents and analytical software were purchased from Applied Biosystems. Genotyping was performed according to the manufacturer's instructions. Approximately 5% of the samples were randomly repeated to validate the genotyping procedures, and the concordance rate was 100%.

Statistical analysis

The differences in the distributions of categorical variables, including demographic characteristics, tobacco smoking, and alcohol drinking, and genotype/allele frequencies of selected SNPs between the patients and controls were evaluated using chi-square tests. Crude or adjusted (for age, gender, nationality, smoking, drinking, and HBsAg status) odd ratios (ORs) and 95% confidence intervals (CIs) were obtained from unconditional univariate and multivariable logistic regression analyses to evaluate the associations between SNPs and HCC risk in the case-control analysis. Data were further stratified by age, gender, nationality, smoking, drinking, and

Table 1. Candidate Genes and Target SNPs

Gene	Name	SNP ID	Chromosome position	Polymorphism	MAF[a]
IL2	Interleukin-2	rs2069762	4q26-q27	-330A > C	C:0.239
IFN-γ	Interferon-gamma	rs2069705	12q14	-1615G > A	A:0.238
IL1B	Interleukin-1 beta	rs1143623	2q13	-1464C > G	G:0.298
		rs16944	2q13	-511G > A	A:0.458
IL6	Interleukin-6	rs1800796	7q21	-572C > G	G:0.233
IL-10	Interleukin-10	rs1800872	1q31-32	-592T > G	G:0.250

*[a]Minor allele frequency in CHB according to HapMap database (HapMap Data Release 27 Phase I and II, February 2009, on NCBI B36 assembly, dbSNP126)

HBsAg status. Continuous variables were analyzed using Student's t-test and presented as mean±SD. HaploView 4.2 was used to test Hardy-Weinberg equilibrium in the control subjects and LD between SNPs in the same gene. The interactions between environmental factors and genomic variants, as well as those between SNPs were qualitatively analyzed with a logistic regression model. All statistical tests were two-sided with a 0.05 significance level, and all data were analyzed with SPSS 13.0 for Windows.

Results

Characteristics of the study population

This study included 720 HCC patients and 784 controls with available DNA samples. Table 2 presents the frequency distribution of the selected patient and control characteristics. No statistical differences in age and gender distributions were detected between the cases and controls because of the frequency matching by design. The mean age was 47.72 years (±11.16, range: 19 years to 81 years) for the controls and 48.65 years (±11.03, range: 18 years to 78 years, P=0.111) for the HCC patients. Approximately 83.3% and 16.7% of the controls and 86.0% and 14.0% of the patients were male and women, respectively (P=0.150). No significant difference in ethnicity distribution was detected between the cases and controls (P=0.911). However, the prevalence of smoking, drinking, and HBsAg positivity was significantly higher in the patients than in the controls (P<0.001 for each). Therefore, smoking, drinking, and HBsAg status were adjusted in the subsequent multivariate logistic regression analyses.

Genotype distribution of polymorphisms of cytokines between patients and controls

The allele and genotype distributions of the studied SNPs in the patients and controls and their associations with HCC risk are summarized in Table 3. All observed genotype distributions among the controls agreed with the Hardy-Weinberg equilibrium (P>0.05). No significant difference in the genotype frequencies of the studied SNPs was found between the patients and controls (P=0.874 for IL-2-330A/C, P=0.606 for IFN-γ-1615G/A, P=0.643 for IL-1β-511C/T, P=0.272 for IL-1β-1464C/G, P=0.786 for IL-6-572C/G, and P=0.101 for IL-10-592T/G). Although none of the variant genotypes was individually associated with a significantly altered risk, the IL-2-330 AC and CC genotypes were associated with a non-significantly reduced HCC risk (adjusted OR=0.93; 95% CI=0.72 to 1.19 for AC, and adjusted OR=0.99; 95% CI=0.68 to 1.45 for CC). The AA genotype of IFN-γ-1615 was associated with a non-significantly increased HCC risk (OR=1.36; 95% CI=0.92 to 2.01). The CT and TT genotypes of IL-1β -511 were associated with a non-significantly increased HCC risk (adjusted OR=1.31; 95% CI = 0.98 to 1.74 for CT, and adjusted OR=1.17; 95% CI=0.84 to 1.63 for TT). The GG genotype of IL-1β-1464G/C was associated with a non-significantly reduced HCC risk (adjusted OR = 0.84; 95% CI=0.60 to 1.17). The genotypes of IL-6-572C/G and IL-10-819T/G were associated with a non-significantly increased HCC risk (adjusted OR=1.05; 95% CI=0.82 to 1.34 for CG/GG, and adjusted OR=1.03; 95% CI=0.82 to 1.30 for TG/GG, respectively).

We combined the SNPs by the number of the putative risk genotypes (i.e., IL-2-330AA, IFN-γ-1615AG/AA, IL-1β-511CT/TT, IL-1β-1464CG/GG, IL-6-572GG/CG, and IL-10-592TG/GG) to assess their possible combined

Table 2. Demographic Characteristics of HCC Patients and Cancer-free Controls

Variables		Controls n (%)	Cases n (%)	t/χ^2	P^a
All Subjects		784 (100)	720 (100)		
Age		47.72±11.16	48.65±11.03	1.62	0.111
	< 40	147 (18.8)	156 (21.7)	2.50	0.287
	40 to 55	427 (54.4)	367 (51.0)		
	> 55	210 (26.8)	197 (27.3)		
Gender			2.07	0.150	
	Male	653 (83.3)	619 (86.0)		
	Female	131 (16.7)	101 (14.0)		
Nationality				0.19	0.911
	Han	557 (71.0)	512 (71.1)		
	Zhuang	218 (27.8)	198 (27.5)		
	Other	9 (1.2)	10 (1.4)		
Smoking			113.69	<0.001	
	No	669 (85.3)	440 (61.1)		
	Yes	115 (14.7)	280 (38.9)		
Alcohol drinking				125.66	<0.001
	No	671 (85.6)	432 (60.0)		
	Yes	113 (14.4)	288 (40.0)		
HBsAg status				265.14	<0.001
	(–)	494 (63.0)	154 (31.4)		
	(+)	290 (37.0)	566 (78.6)		

*a P value for two-sided Chi-square test.

Table 4. Association of Combined Risk Genotypes and HCC Risk

Number of combined risk genotype[a]	Control	Case	P	OR (95% CI)[b]
0-1	53 (6.76)	36 (5.00)	0.025	1
2-3	441 (56.25)	409 (56.81)		1.576 (0.945-2.628)
4-6	290 (36.99)	275 (38.19)		1.821 (1.078-3.075)

*a. Number of risk genotypes used in calculation and assumed risk genotypes used for calculation (i.e., IL-2-330AA, IFN-γ-1615AG/AA, IL-1β-511CT/TT, IL-1β-1464 CG/GG, IL-6-572 GG/CG, and IL-10 -592TG/GG). b. Adjusted for age, gender, nationality, smoking, alcohol drinking, and HBsAg status

Table 3. Genotype and Allele Frequency Polymorphisms Among Controls And Patients, and their Associations with HCC Risk

Variant genotypes		Controls n = 784 (%)	Cases n = 720 (%)	χ^{2a}	P^a	OR (95% CI)[b]
IL-2 -330	AA	311 (39.7)	292 (40.6)	0.27	0.874	1
	AC	373 (47.6)	333 (46.2)			0.93 (0.72-1.19)
	CC	100 (12.7)	95 (13.2)			0.99 (0.68-1.45)
	AC/CC	473 (60.3)	428 (59.4)			0.94 (0.74-1.20)
	A	573 (36.5)	523 (33.4)	0.02	0.899	
IFN-γ-1615	GG	384 (49.0)	357 (49.6)	1.01	0.606	1
	AG	318 (40.6)	278 (38.6)			0.95 (0.74-1.22)
	AA	82 (10.4)	85 (11.8)			1.36 (0.92-2.01)
	AG/AA	400 (51.0)	363 (50.4)			1.02 (0.81-1.29)
	G	1086 (65.5)	992 (63.3)	0.04	0.826	
IL1B -511	CC	206 (26.3)	174 (24.2)	0.88	0.643	1
	CT	384 (49.0)	363 (50.4)			1.31 (0.98-1.74)
	TT	194 (24.7)	183 (25.4)			1.17 (0.84-1.63)
	CT/TT	578 (73.7)	546 (75.8)			1.26 (0.96-1.65)
	C	772 (49.3)	729 (46.5)	0.58	0.446	
IL1B -1464	CC	238 (30.4)	214 (29.7)	2.6	0.272	1
	CG	367 (46.8)	363 (50.4)			1.28 (0.97-1.67)
	GG	179 (22.8)	143 (19.9)			0.84 (0.60-1.17)
	CG/GG	546 (69.6)	506 (70.3)			1.24 (0.95-1.64)
	C	843 (53.8)	791 (54.9)	0.41	0.521	
IL-6 -572	CC	523 (66.7)	485 (67.4)	0.48	0.786	1
	CG	232 (29.6)	213 (29.5)			0.88 (0.46-1.69)
	GG	29 (3.7)	22 (3.1)			1.07 (0.83-1.38)
	CG/GG	261 (33.3)	235 (32.6)			1.05 (0.82-1.34)
	C	1278 (81.5)	1183 (75.4)	0.21	0.605	
IL-10 -592	TT	392 (50.0)	356 (49.2)	4.58	0.101	1
	TG	313 (39.9)	312 (43.3)			0.67 (0.43-1.06)
	GG	79 (10.1)	52 (7.5)			1.01 (0.88-1.46)
	TG/GG	705 (89.9)	668 (92.5)			1.03 (0.82-1.30)
	T	1091 (69.6)	1020 (65.1)	0.56	0.453	

*a χ^2 and *P* value for two-sided Chi-square test. **b Adjusted for age, gender, nationality, smoking, alcohol drinking, and HBsAg status.

Table 5. Association and Stratification Analysis of Combined Genotypes of IL-2, IFN-γ, IL-1β, IL-6, and IL-10 Polymorphisms and HCC Risk

Variables	n, control/case		Number of combined risk genotypes of SNP on studied cytokine genes n, control/case			Adjusted OR (95% CI)[a]	
			0~1	2~3	4~6	0~1 verse 2~3	0~1 verse 4~6
Age (years)	< 40	147/156	29/22	52/68	66/66	1.092 (0.750-2.749)	2.767 (0.650-3.777)
	40-55	427/367	79/58	171/139	177/170	1.595 (0.784-3.244)	1.802 (0.869-3.736)
	> 55	210/197	33/32	84/82	93/83	1.223 (0.428-3.492)	1.565 (0.538-4.554)
Gender	Female	131/101	19/16	53/54	59/40	1.943 (0.503-7.512)	1.330 (0.331-5.345)
	Male	653/619	122/96	254/244	277/279	1.477 (0.846-2.577)	1.906 (1.077-3.371)
Nationality	Han	557/512	97/86	217/204	243/222	1.413 (0.804-2.481)	1.642 (0.921-2.928)
	Zhuang	227/208	44/26	90/85	93/97	2.165 (0.605-7.775)	2.502 (0.681-9.200)
Smoking	No	115/280	17/52	44/109	54//119	1.118 (0.448-2.789)	0.875 (0.342-2.240)
	Yes	669/440	124/60	263/180	282/200	1.892 (0.992-3.609)	2.497 (1.293-4.822)
Alcohol drinking	No	113/288	14/50	43/121	56/117	0.972 (0.371-2.552)	0.883 (0.327-2.384)
	Yes	671/432	127/62	264/168	280/202	2.019 (1.067-3.820)	2.483 (1.297-4.757)
HBsAg	(-)	290/566	57/94	112/235	121/237	0.847 (0.354-1.026)	1.052 (0.434-2.549)
	(+)	494/154	84/18	195/54	215/82	1.958 (1.067-3.594)	2.122 (1.134-3.971)

*a. Adjusted for age, gender, nationality, smoking, alcohol drinking, and HBsAg status

effects on HCC risk. The combined risk genotypes were statistically associated with an increased HCC risk (OR=1.821; 95% CI=1.078 to 3.075) (Table 4).

Association and stratification analysis forcombined genotypes of cytokine gene polymorphisms and HCC risk

After further stratification by age, gender, nationality, smoking, alcohol drinking, and HBsAg status, an increased risk associated with the combined genotype with four to six risk genotypes was pronounced in persistent smokers (OR=2.497; 95% CI=1.293 to 4.822), persistent drinkers (OR=2.483; 95% CI=1.297 to 4.757), and HBsAg-positive individuals (OR=2.122; 95% CI=1.134 to 3.971) (Table 5).

Evaluation of SNP-SNP interactions

Considering the possible interaction effects among the six SNPs and environmental factors on HCC risk, we further assessed the interactions of these SNPs

Table 6. Interaction Effects of IL-2, IFN-γ, IL-1β, IL-6, and IL-10 Polymorphisms on HCC Risk

SNP×SNP interaction	SE	Wald	*P*	OR (95% CI)[a]
*IL-2-330A > C * IFN-γ-1615G/A*	0.138	4.213	0.005	1.078 (1.022-1.136)
*IL-2-330A > C * IL-1β-1464C/G*	0.280	0.045	0.833	1.061 (0.613-1.836)
*IL-2-330A > C * IL-1β-511C/T*	0.265	1.375	0.241	1.364 (0.812-2.292)
*IL-2-330A > C * IL-6-572 C > G*	0.043	3.223	0.073	1.080 (0.993-1.175)
*IL-2-330A > C * IL-10-592 T > G*	0.040	0.192	0.662	0.982 (0.908-1.063)
*IFN-γ-1615G/A * IL-1β-1464C/G*	0.126	0.048	0.826	0.944 (0.567-1.573)
*IFN-γ-1615G/A * IL-1β-511C/T*	0.031	0.001	0.996	1.000 (0.942-1.062)
*IFN-γ-1615G/A * IL-6-572 C > G*	0.042	0.236	0.627	1.021 (0.940-1.109)
*IFN-γ-1615G/A * IL-10-592 T > G*	0.040	0.680	0.410	1.033 (0.896-1.117)
*IL-1β-1464C/G * IL-1β-511C/T*	0.092	0.524	0.469	1.069 (0.892-1.281)
*IL-1β-1464C/G * IL-6-572 C > G*	0.049	0.088	0.767	0.986 (0.896-1.085)
*IL-1β-1464C/G * IL-10-592 T > G*	0.043	0.264	0.688	0.978 (0.899-1.054)
*IL-1β-511C/T * IL-6-572 C > G*	0.046	0.041	0.841	1.009 (0.922-1.105)
*IL-1β-511C/T * IL-10-592 T > G*	0.052	0.504	0.479	1.029 (0.852-1.081)
*IL-6-572C > G * IL-10-592 T > G*	0.063	0.824	0.364	0.944 (0.834-1.069)

*a.Adjusted for age, gender, nationality, smoking, alcohol drinking, and HBsAg status.

and environmental factors, such as smoking, drinking, and HBsAg positivity in HCC. However, no statistical evidence was found for interactions among these variables in the multivariate logistic regression models (data not shown). In addition, SNP-SNP interaction between IL-2-330 and IFN-γ-1615 was associated with an increased HCC risk (OR=1.078, 95% CI=1.022 to 1.136). SNP-SNP interactions were not observed in any other SNPs (Table 6).

Discussion

In the 720 HCC patients and 784 cancer-free controls, none of the six SNPs in cytokine genes (IL-2-330A/C, IFN-γ-1615G/A, IL-1β-511C/T, IL-1β-1464C/G, IL-6-572C/G, and IL-10-592T/G) was individually associated with HCC risk. Rather, the combined effects of these SNPs increased HCC risk among smokers, drinkers, and HBsAg-positive individuals. These findings support our hypothesis that the effect of an individual SNP is generally not significant and that the genetic combined effects of SNPs and SNP-SNP interactions increase HCC risk.

Numerous studies have investigated the relations between cytokine polymorphisms and HCC risk; however, their results are controversial (Shin et al., 2003; Hirankarn et al., 2006; Slattery et al., 2007). Two studies have consistently noted that IL-1β-511 polymorphisms are significant risk factors for HCC progression in the Thai population and in Japanese patients (Tanaka et al., 2003; Hirankarn et al., 2006). However, a recent meta-analysis has reported that IL-1β-511C/T is not significantly associated with HCC risk in an Asian population (Yang et al., 2011). And a recent study has suggested the lack of association of selected seven cytokine gene polymorphisms with the risk of HCC recurrence in Han Chinese population (Wu et al., 2013). Previous studies evaluated the effects of individual SNP or single one gene on the HCC risk and failed to consider the potential combined effects of these SNPs on different genes (Tanaka et al., 2003; Hirankarn et al., 2006; Pan et al., 2013). In the present study, no individual SNP was significantly associated with HCC risk. It is likely that the effect of single SNP on HCC risk may be modest and undetectable. Alternatively, the effect of a single SNP may be influenced by that of other gene SNPs. In this study, we found that individuals with four to six risk genotypes (i.e., IL-2-330AA, IFN-γ-1615AG/AA, IL-1β-511CT/TT, IL-1β-1464CG/GG, IL-6-572GG/CG, and IL-10-592TG/GG) may have an increased HCC risk. Consistent with the result of Nieters et al. (2005), the present study found that the synergistic rather than the individual effects of cytokine gene SNPs were associated with an increased HCC risk.

HCC is a multi-factorial disease that results from complex interactions between environmental and genetic factors. Therefore, we speculated that hereditary factors and environmental factors (smoking, drinking, and HBsAg positivity) increase HCC risk. Compared with individuals who never smoked or drank, smokers and drinkers had a significantly higher HCC risk, which was associated with the combined effects of the putative high-risk genotypes in cytokine genes (Table 5). Similar conclusions have been previously attained. Chen et al. (2005) have reported that smoking modifies the combined effects of multiple loci in the cytokine and DNA repair genes. Cigarette smoking is reportedly associated with an increased HCC risk in HBsAg carriers, and this association is independent of geography and ethnicity (El-Zayadi et al., 2006; Koh et al., 2011). Cigarette consists of over 4000 toxic substances, which cause adverse effects on the immune system (Zhang et al., 2014). Excessive alcohol consumption is a well-established risk factor for liver disease and HCC (Morgan et al., 2004). These results, together with our findings, suggest that smoking and drinking promote HCC progression. Previous epidemiological studies have found that chronic infection with HBV is a major etiological risk factor for HCC in China (Yeh et al., 1986; Groopman et al., 1996). Expectedly, the combined effects of cytokine gene SNPs with HBV infection increased HCC risk. However, we did not find statistical evidence on the interactions among these variables possibly because of the limited sample size.

The present study showed that the interaction between IL-2-330A/C and IFN-γ-1615G/A was significantly associated with HCC risk. This result indicates that the individual effect of IL-2-330A/C or IFN-γ-1615 G/A is generally minimal and SNP-SNP interaction increases HCC risk. Ikeguchi et al. (2005) evaluated the gene expression levels of IL-2 in HCC patients and found that IL-2 expression may be an important prognostic biomarker for HCC. However, it was reported that the distribution of IL-2 -330A/C genotypes between HCC patients and healthy people had no statistical difference (Ognjanovic

et al., 2009). IFN-γ was detected in HCC, and the authors concluded that IFN-γ may not play a large role in liver inflammation (Tangkijvanich et al., 2000). Similar to that, no significant differences were found in IL-2-330 and IFN-γ-1615 genotype distribution in Guangxi people of China between HCC patients and controls in this study. Although IL-2-330 and IFN-γ-1615 are located on different genes, their positions are considerably near (rs2069762 and rs2069705). Therefore, their locations may affect their biological functions through interactions. To date, SNP-SNP interactions between different cytokine genes and HCC risk have not yet been reported. Further studies with large sample sizes are necessary to confirm our findings.

To the best of our knowledge, this study is the first to investigate the association between cytokine gene SNP-SNP interactions and HCC risk. We found that SNP-SNP interactions between SNPs that did not exert individual effects on HCC risk were associated with HCC occurrence. Previous studies investigated SNP-SNP interactions during the development of breast cancer (Sapkota et al., 2013) and colorectal cancer (Goodman et al., 2006). These reports support our findings regarding the effects of SNP-SNP interactions on HCC risk. That is, SNPs with negligible effects may interact with other SNPs to increase HCC risk. In this study, potential confounding factors, such as age, gender, and nationality, were matched between the patients and controls to decrease possible biases. Furthermore, our results were based on adjusted estimates. The analyses were adjusted for other covariates, including smoking, alcohol drinking, and HBsAg status. However, several potential limitations in this study should be considered. Firstly, six SNPs were included in the analyses, and the possibility of false-positive associations could not be ruled out because of multiple tests. Secondly, this study excluded several other cytokine SNPs, such as TNF-A-308 and IL-10-819 polymorphisms (Budhu et al., 2006), which might affect HCC risk. Thirdly, the number of current studies was relatively small. Therefore, further investigations involving subjects of different races must be conducted to clarify this relationship. Despite these limitations, our findings are biologically plausible. Therefore, more risk polymorphisms of HCC should be induced as covariants to conclude an accurate effect.

In summary, none of the six SNPs of IL-2-330A/C, IFN-γ-1615G/A, IL-1β-511C/T, IL-1β-1464C/G, IL-6-572C/G, and IL-10-592T/G was individually associated with HCC risk. However, their combined effects and interactions may have important functions in HCC development in the high-risk regions in China. The cytokine-coordinated network system is so comprehensive that analyzing only a single pathway may be limiting. Therefore, further independent studies of multiple pathways with large sample sizes and relatively functional studies are needed to validate the current findings.

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