Influence of cytotoxic T lymphocyte-associated antigen 4 polymorphisms on the outcomes of hepatitis B virus infection

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Abstract. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates T-cell activation and Th1/Th2 cytokine production and is involved in the immune response against Hepatitis B virus (HBV) infection. To detect the association of the CTLA-4 gene polymorphisms with susceptibility to HBV infection a hospital-based case-control study was conducted. A total of 1,119 unrelated individuals were recruited. The CTLA-4 variants rs5742909, rs231775 and rs3087243 were genotyped via the TaqMan method in this cohort. A comparison with a chronic active hepatitis B group revealed that the SNP rs231775 exhibited significant susceptibility to HBV progression, with the highest odds ratio (OR) reaching 1.659 and P=0.009-0.049. Although an HBV clearance group was used as a control, results of the present study demonstrated an association of rs5742909 with viral persistence [OR=1.694, 95% confidence intervals (CI)=1.124-2.553 and P=0.012]. Subsequent analyses revealed risk haplotypes (C-A-A and T-A-G, for which the highest OR reached 1.865) compared with the protective haplotype C-G-G. Therefore, SNPs in the CTLA-4 gene may be associated with HBV progression and viral persistence which is consistent with its emerging role in the T regulatory cells in the pathogenesis of disease.

Introduction

Hepatitis B virus (HBV) infection is a worldwide health problem and more than two billion people have been infected with HBV (1). Although many individuals eventually achieve a state of non-replicative infection, the prolonged immunological response to infection leads to the development of cirrhosis, liver failure or hepatocellular carcinoma (HCC) in up to 40% of patients (2). In China, ~120 million people are HBV chronic carriers, and 50-80% of cirrhosis patients are infected with HBV (1). Persistent HBV infection has been considered a multifactorial and polygenic disequilibrium among viral, environmental and host genetic components (3). Single-nucleotide polymorphisms (SNPs) are the most abundant form of DNA variation in the human genome and contribute to human phenotypic differences (4). A number of studies have demonstrated the role of host genetic factors and their interactions with environmental factors leading to various outcomes following HBV infection (5-8). Understanding the key factors that influence the clinical outcomes of HBV infection is crucial for early diagnosis and optimal treatment (9).

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), mapped to chromosome 2q33 (10) and expressed in the activated and regulatory T cells, regulates T-cell activation and tolerance (11), Th1/Th2 differentiation and cytokine production (12) following B7 engagement. CTLA-4 has been established as a significant negative regulator of T-cell responses, leading to the preservation of T-cell homeostasis (13). Previously, it was reported that CTLA-4 may downregulate the immune response strength of tumor immunity (14), allergy (15), autoimmune disease (16), infection (17) and vaccination (18). In addition to inhibiting T-cell activation and proliferation, CTLA-4 may also induce Fas-independent apoptosis of activated T cells (19). Cytokines including TNF-α and IFN-β are critical, not only for viral clearance, but also for the immunopathogenesis of HBV infection (20,21). Therefore, the interaction of CTLA-4 with B7 molecules in regulating T-cell activation and Th1/Th2 cytokine production is involved in the immune response against HBV infection (22).

SNPs in CTLA-4 have been connected to the susceptibility to autoimmune disease and various types of cancer (23). Findings from recent studies have demonstrated that CTLA-4 gene polymorphisms may affect the susceptibility and chronicity of the disease in patients with HBV infection (24-29). However, the results remain controversial and whether CTLA-4 gene polymorphisms are associated with the status of HBV infection including chronic carriers and cirrhosis, and hepatitis B e antigen (HBeAg) positivity in the infected patients remains to be determined. In addition, the criteria for the control and case groups were not completely equal among these studies. Thus, whether there is an association between

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CTLA-4 genetic variations and HBV infection in the Chinese population using equal criteria remains to be clarified. In the present study, three SNPs were selected from the CTLA-4 gene: rs5742909 (-318 C/T) at the promoter region, rs231775 (+49 A/G), a non-synonymous at the exon 1 and rs3087243 (+6230 G/A) at the downstream in the 3' untranslated region (UTR). The three polymorphisms were genotyped in a hospital-based case-control study, including 1,119 unrelated Han Chinese subjects from the Hubei province (Central China).

Materials and methods

Study subjects. In this hospital-based case-control study, a total of 1,119 unrelated Han Chinese individuals were recruited from Tongji Hospital and Union Hospital, Wuhan, China, between July 2007 and September 2009. All the subjects were divided into four groups: i) the HBV clearance group (clear); ii) the chronic active hepatitis B group (CHB); iii) the HBV-related liver cirrhosis group (LC) and iv) the HBV-related hepatocellular carcinoma group (HCC). The diagnostic criteria for study inclusion were described previously (6). Moreover, patients with positive laboratory tests for human immunodeficiency virus (HIV), alcoholic liver disease, suspected autoimmune diseases or schistosomiasis were excluded from the study.

All the study subjects were of unrelated ethnic background, Han Chinese who lived in Wuhan or the surrounding region. Informed consent was obtained from each participant for study enrollment. An information questionnaire was used and the demographic information included gender, age and place of origin. The study was approved by the local Research Ethics Committee at the Tongji Hospital, Huazhong University of Science and Technology in accordance with the principle of the Helsinki Declaration II.

DNA isolation and genotyping. Genomic DNA was isolated from the peripheral whole blood using a TIANamp blood DNA kit [Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. The concentration and purity of the DNA were determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilimington, DE, USA), then diluted to a final concentration of 8 ng/ml and distributed to a 96-well plate. Genotyping of genetic polymorphisms was performed via the TaqMan method according to the instructions of TaqMan®R SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA, USA). To detect the three SNPs (rs5742909, rs231775 and rs3087243), TaqMan®R MGB Probes and the primers for PCR amplification (Table I) were customized. The probes were labeled with FAM and VIC dyes to denote the two different alleles, respectively, and the allelic category was measured automatically using the Sequence Detection System 2.3 software (Applied Biosystems) according to the intensity of the VIC and FAM dyes.

Statistical analysis. A statistical analysis was conducted using Arlequin 3.5 (30), haploview 4.2 (Cambridge, MA, USA) and SPPS 17.0 (SPSS, Inc., Chicago, IL, USA) softwares. The Hardy-Weinberg equilibrium was tested separately for cases and controls by Arlequin 3.5. Linkage disequilibrium (LD) and haplotypes were assessed by the haploview 4.2 software. Genotypic analyses included allele, dominant, recessive and additive genetic models. The χ^2 test or Fisher's-exact test was applied to a row-by-column contingency table in the four genetic models. Age- and gender-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated on the basis of the unconditional binary logistic regression model. The strength of association between the genotypes or alleles and HBV infection was estimated by the SPSS17.0. ORs and 95% CIs were calculated using the major allele as a reference. All the tests were two sided with $P<0.05$ considered to indicate a statistically significant difference.

Results

Clinical and demographic characteristics. The clinical and demographic characteristics of the case-control study included gender, age, drinking habits, serum α-fetoprotein level, serum total bilirubin level, alanine transaminase, HBV-DNA load and serum markers of hepatitis B virus (Table II). Although an effort was made to obtain a good match on the age and gender, there were more male subjects in the three HBV infection groups (CHB+LC+HCC; average 78.7%) compared with those in the clear group (59.3%, $P<0.05$). The individuals in the CHB group were younger compared with those in the LC and HCC groups ($P<0.001$). However, populations in the LC and HCC groups demonstrated no significant difference with the clear group with regard to age ($P>0.05$). No significant difference was observed in the percentage of hepatitis B e antigen (HBeAg)-positive ($P>0.05$) between the patients in the LC group (11.74%) and those in the HCC group (10.24%). In addition, there was more alcohol consumption in patients ($P<0.05$) with the LC (29.11%) and HCC (34.19%) groups compared with those in the clear (13.17%) and CHB (18.63%) groups. The difference in the alcohol consumption status may be due to the limited number of drinkers within the Chinese female population.

Hardy-Weinberg equilibrium test. Hardy-Weinberg equilibrium was estimated by the Fisher's exact test via Arlequin 3.5 software. The allele and genotype distributions are shown in Tables III and IV. No significant difference was revealed between the observed and expected frequencies of each genotype in these groups ($P>0.05$). All the genotype distributions of the three SNPs (rs231775, rs5742909 and rs3087243) conformed to the Hardy-Weinberg equilibrium ($P>0.05$) in all the groups, making it suitable for subsequent statistical analysis.

Associations of the CTLA-4 polymorphisms with HBV progression. To investigate which genotypic models were significantly associated with various outcomes, a comparison of the four models was conducted (multiplicative, additive, dominant and recessive models) in the Hubei Han Chinese population (data not shown). The best-fitting genotypic effect of the three SNPs (rs5742909, rs231775, rs3087243) was observed in the dominant model (Table III). Distributions of the CTLA-4 polymorphisms in the case and control groups are summarized in Tables III and IV. At the SNP site rs231775, the significant difference in allele distribution was observed only between the CHB and LC groups (P=1.53*10E4). Following adjustment for age and gender and analysis by unconditional binary logistic

Table I. TaqMan® probe and primer for three SNPs.

SNP (NCBI reference no.)	rs5742909[a]	rs231775	rs3087243
Forward primer	Commercialized	GCACAAGGCTCAGCTGAAC	CCATCCTCTTTCCTTTTGATTTCTTCAC
Reverse primer	Commercialized	CAGAAGACAGGGATGAAGAGAAGAA	TGTGTTAAACAGCATGCCAATTGATT
MGB probe 1	Commercialized	VIC-CCAGGTCCTGGTAGCCA-MGB	VIC-TCTGTGTTAACCCATGTTATA-MGB
MGB probe 2	Commercialized	FAM-CAGGTCCTGGCAGCCA-MGB	FAM-TGTGTTAACCCACGTTATA-MGB

[a]The Taqman probes and primers for the SNP locus rs5742909 were commercialized (Applied Biosystems). SNPs, single nucleotide polymorphisms.

Table II. Clinical characteristics of the study subjects.

Characteristics	Clear, n=205	CHB, n=467	LC, n=213	HCC, n=234
Gender, no. (%)				
Male	121 (59.3)	343 (73.4)	184 (86.4)	192 (82.1)
Female	83 (40.7)	124 (26.6)	29 (13.6)	42 (17.9)
Age in years, mean ± SD	48.85±12.52	35.42±12.89	46.21±10.83	47.84±13.23
Drinkers, no. (%)	27 (13.17)	87 (18.63)	62 (29.11)	80 (34.19)
HBsAg	All$^-$	All$^+$	All$^+$	All$^+$
Anti-HBs IgG	All$^+$	All	All$^-$	All$^-$
HBeAg-positive, no. (%)	All$^-$	94 (20.13)	25 (11.74)	24 (10.24)
Anti-HBc IgG	All$^+$	All$^+$	All$^+$	All$^+$
Family history, no. (%)	No	69 (14.78)	27 (12.68)	47 (20.09)
ALT, U/l	No	420.33±399.23	110.46±115.24	90.63±75.17
TBil, mmol/l	No	180.23±171.45	132.28±120.86	64.36±60.74
HBV-DNA, copy/ml	No	(3.05±3.67) E7	(2.34±4.02) E6	(5.59±2.34) E6

Total number for gender was not in accordance with the sum of each group as not all of the information was completelely collected. No, non-detected. Drinkers, alcohol consumption of 40 g/week, which included occasional and daily drinkers. Clear, HBV clearance group; CHB, chronic active hepatitis B group; LC, HBV-related liver cirrhosis group; HCC, HBV-related hepatocellular carcinoma group. HbsAg, hepatitis B antigens; ALT, alanine transaminase; TBil, total bilirubin level; HBV, hepatitis B virus.

Table III. Association of three SNPs (rs5742909, rs231775 and rs3087243) with HBV infection progression and clearance in the Han Chinese populations (dominant model).

					Dominant model	
SNPs	Sample	Allele distribution	P-value[a]	Genotypes	P-value	OR (95%CI)[b]
rs5742909	(-318C>T)	C/T		CC/CT/TT	CC vs. CT+TT	CC vs. CT+TT
	Clear	358/42	Ref.	160/38/2	Ref.	Ref.
	CHB+LC	1126/206	**0.013**	478/170/18	**0.012**	**1.694 (1.124-2.553)**
rs231775	(+49A>G)	A/G		AA/GA/GG	GG vs. AG+AA	GG vs. AG+AA
	CH	320/610	Ref.	53/214/198	Ref.	Ref.
	LC	189/229	**1.53*10E4**	46/97/66	**0.009**	**1.659 (1.137-2.421)**
	HCC	184/284	0.071	42/100/92	0.537	1.119 (0.782, 1.602)
	HCC+LC	373/513	**0.001**	88/198/158	**0.049**	**1.353 (1.001-1.829)**
rs3087243	(+6230G>A)	G/A		GG/GA/AA	GG vs. GA+AA	GG vs. GA+AA
	CH	750/184	Ref.	301/148/18	Ref.	Ref.
	LC	314/108	**0.015**	121/72/18	0.167	1.294 (0.898-1.866)
	HCC	349/113	**0.041**	134/81/16	0.235	1.247 (0.866-1.771)
	LC+HCC	663/221	**0.007**	255/153/34	0.191	1.224 (0.904-1.656)

Total number for the polymorphism was not in accordance with the sum of each genotype as not all the samples were successfully genotyped. [a]P-values were obtained by the χ^2 test. [b]The P-values, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated on the basis of the binary logistic regression analysis and adjusted for gender and age. Bold text denotes statistically significant differences. SNPs, single-nucleotide polymorphisms; HBV, hepatitis B virus.

Table IV. Alleles and genotypes distribution of the three SNPs in the cases and controls.

SNP ID	Clear	CHB	LC	HCC
rs5742909				
C	358 (89.5)	796 (85.8)	330 (81.7)	400 (89.3)
T	42 (10.5)	132 (14.2)	74 (18.3)	48 (10.7)
CC	160 (80.0)	342 (73.7)	136 (67.3)	176 (78.6)
CT	38 (19.0)	112 (24.1)	58 (28.7)	48 (21.4)
TT	2 (1.0)	10 (2.2)	8 (4.0)	0 (0.0)
Hardy-Weinberg equilibrium	0.88	0.82	0.57	0.07
rs231775				
A	142 (34.8)	320 (34.4)	189 (45.2)	184 (34.4)
G	266 (65.2)	610 (65.6)	229 (54.8)	284 (60.7)
AA	20 (9.8)	53 (11.4)	46 (22.0)	42 (17.9)
GA	102 (50.0)	214 (46)	97 (46.4)	100 (42.7)
GG	82 (40.2)	198 (42.6)	66 (31.6)	92 (39.3)
Hardy-Weinberg equilibrium	0.15	0.67	0.36	0.11
rs3087243				
G	311 (76.6)	750 (80.3)	314 (74.4)	349 (75.5)
A	95 (23.4)	184 (19.7)	108 (25.6)	113 (24.5)
GG	116 (57.1)	301 (64.5)	121 (64.5)	134 (58.0)
GA	79 (38.9)	148 (31.7)	72 (34.1)	81 (35.1)
AA	8 (3.9)	18 (3.9)	18 (8.5)	16 (6.9)
Hardy-Weinberg equilibrium	0.22	0.97	0.13	0.44

All groups conformed to the Hardy-Weinberg equilibrium: P>0.05. Clear, HBV clearance group; CHB, chronic active hepatitis B group; LC, HBV-related liver cirrhosis group; HCC, HBV-related hepatocellular carcinoma group. SNPs, single nucleotide polymorphisms.

regression, the difference remained significant (P=0.009; OR=1.659 and 95% CI=1.137-2.421). At SNP site rs3087243, compared with the CHB group, the allele distributions revealed significant differences in the LC and HCC groups (P=0.015 and 0.041, respectively). However, no differences were evident following the adjustment for age and gender. Although there was no difference between the CHB and HCC groups at the SNPs rs231775 and rs3087243 following adjustment for age and gender, a trend for the correlation was observed between the polymorphisms and HCC susceptibility. The present study found that subjects with an A allele of the two polymorphisms appeared to have a greater susceptibility to HBV-related LC and HCC compared with those with a G allele.

According to the clinical considerations, LC and HCC could be lumped together since they are involved in different steps in HBV progression. In the present study, these two HBV infection populations were combined into one group by using the CHB group as the reference. At the SNP site rs231775, a significant difference was found, not only in the allele frequencies (P<0.05), but also in the genotype distributions (P<0.05) when the combined group (LC+HCC) was compared with the CHB group (P=0.049, OR=1.353 and 95% CI=1.001-1.829). However, for the SNP site rs3087243, the significant difference only appeared in the χ^2 test for allele (P=0.007) and genotype distribution (P=0.017; data not shown). Although they were adjusted for age and gender and analysed by unconditional binary logistic regression, the difference in the CHB and the combined group (LC+HCC) was not significant in the site rs3087243, and a trend (OR=1.224 and CI=0.904-1.656)) was still observed. From these data, the subjects with A allele appeared to have a greater susceptibility to HBV progression compared with those with G allele in the two CTLA-4 polymorphisms, in particular in the rs231775. In addition, no association of the CTLA-4 polymorphism rs5742909 was found with HBV progression.

Associations of the CTLA-4 polymorphisms with viral persistence. According to the clinical considerations of this study and our previous study (6), CHB and LC could be lumped together, since both of them were chronic HBV carriers. The populations of the CHB and LC were combined in a similar manner to the HBV persistence group by using the clear group as the reference. Following a series of statistical analyses, the associations of the CTLA-4 polymorphisms with viral persistence were noted only at the SNP site rs5742909 of these three SNPs (Table III). In the HBV persistence group (including CHB and LC), the proportions of the C and T alleles were 89.5 and 10.5%, respectively, which were significantly different from those observed in the clear group (P=0.013). Following adjustment for age and gender and analysis by unconditional binary logistic regression, the statistical level remained significant (P=0.012, OR=1.694 and 95% CI=1.124-2.553) compared with the clear group under the best-fitting model (dominant model). In addition, under the additive model, the frequencies of the C/T and T/T genotypes in HBV persistence patients were higher compared with those in the clear subjects

Table V. Associations of the three SNPs (rs5742909, rs231775 and rs3087243) with HBV infection progression and clearance in the Han Chinese populations (recessive and additive models).

		Recessive model		Additive model		
SNP ID	Group	P-value	OR (95%CI)	Genotypes	P-value	OR (95%CI)
rs5742909		CC+CT vs TT	CC+CT vs TT	CC/CT/TT		CC/CT/TT
	Clear	Ref.	Ref.	Ref.		Ref.
	CHB+LC	0.25	2.412, (0.537, 10.824)	(CC vs. CT)	0.022	1.636, (1.074, 2.553)
				(CC vs. TT)	0.196	2.695, (0.599, 12.131)
rs231775		GG+GA vs. AA	GG+GA vs. AA	GG/GA/AA		GG/GA/AA
	CHB	Ref.	Ref.	Ref.		Ref.
	LC	0.017	1.78 (1.111-2.852)	(GG vs.GA)	P1=0.054	1.487 (0.993-2.226);
				(GG vs.AA)	P1=0.003	2.212 (1.310-3.735)
	HCC+LC	0.017	1.78 (1.111-2.852)	(GG vs. GA)	P2=0.172	1.252 (0.907-1.728)
				(GG vs. AA)	P2=0.022	1.673 (1.079-2.595)
rs3087243		GG+GA vs. AA	GG+GA vs. AA	GG/GA/AA		GG/GA/AA
	CHB	Ref.	Ref.	Ref.		Ref.
	LC	0.18	1.667 (0.789-3.519)	(GG vs. GA)	P1=0.432	1.169 (0.791-1.728)
				(GG vs. AA)	P1=0.146	1.757 (0.822-3.754)
	LC+HCC	0.303	1.410 (0.734-2.709)	(GG vs. GA)	P2=0.427	1.138 (0.827-1.568)
				(GG vs. AA)	P2=0.251	1.474 (0.760-2.858)

P-value is for Pearson's χ^2 test. P1, LC vs. CHB; P2, LC+HCC vs. CHB. P1 and P2 are in the genotype ratios of GG/GA and GG/AA. The P-values, odds ratios (ORs), and 95% confidence intervals (CIs) were calculated on the basis of the binary logistic regression analysis, adjusted for gender and age. SNPs, single-nucleotide polymorphisms.

Table VI. Results of the association test for the three SNPs haplotypes in the Han Chinese populations.

Haplotype/SNP	-318C>T	+49A>G	+6230G>A	Clear (2n=400)	CH (2n=928)	LC (2n=404)	CH+LC (2n=1332)	HCC+LC (2n=852)
1	C	G	G	261 (65.2)	609 (65.6)	219 (54.3)	828 (62.6)	491 (59.9)
2	C	A	A	93 (23.3)	186 (20.0)	103 (25.4)	289 (21.9)	211 (25.7)
3	T	A	G	46 (11.5)	133 (14.3)	72 (17.9)	205 (15.5)	118 (14.4)
P1-value[a]				Ref.	0.283	0.102	0.849	
OR (95%CI)				1	0.854	1.320	0.974	
					(0.640-1.139)	(0.946-1.841)	(0.742-1.279)	
P2-value[a]				Ref.	0.246	**0.003**	**0.054**	
OR (95%CI)				1	1.246	**1.865**	1.406	
					(0.861-1.778)	**(1.236-2.814)**	(0.992-1.993)	
P1-value[b]					Ref.	**0.003**		0.004
OR (95%CI)					1	**1.546**		1.403
						(1.161-2.058)		(1.114-1.767)
P2-value[b]					Ref.	**0.014**		0.512
OR (95%CI)					1	**1.503**		1.096
						(1.085-2.082)		(0.833-1.443)

P1-value: CGG vs. CAA; P2-value: CGG vs. TAG. P1 and P2 represent P-value calculated on the binary logistic regression analysis and adjusted for sex and age. Haplotypes with frequencies <0.05 were not considered. [a]Three SNPs haplotypes C-G-G, C-A-A, T-A-G in the clear group compared with those in the HBV infection groups. [b]Three SNPs haplotypes C-G-G, C-A-A, T-A-A in the CHB group compared with those in the other HBV infection groups. SNPs, single-nucleotide polymorphisms.

(25.53 vs. 19% and 2.7 vs. 1%, respectively). Their ORs reached 1.636 (95% CI=1.074-2.492 and P=0.022) and 2.695 (95% CI=0.599-12.131 and P=0.196) compared with the C/C genotype (Table V). These results indicated that the T/T and C/T genotypes and T allele of the polymorphism rs5742909 may increase the risk of HBV persistence.

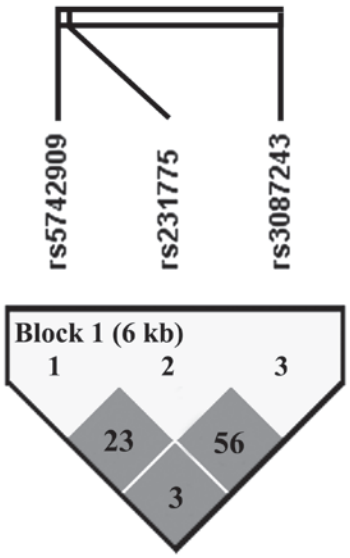

Figure 1. Linkage disequilibrium analysis of the SNPs rs5742909, rs231775 and rs3087243 in HBV clearance population (n=200) generated by HaploView 4.2 software. SNPs, single-nucleotide polymorphisms; HBV, hepatitis B virus.

Results of the haplotype analysis. In order to understand the contributions of these loci to the HBV susceptibility, three-locus haplotypes were constructed for the SNPs rs5742909, rs231775 and rs3087243 (Table VI). Pairwise LD analyses (Fig. 1) were performed using all the individuals from the clear group. Results of the analyses revealed that the SNPs rs5742909, rs231775 and rs3087243 were in LD with each other (D'=1, r^2=0.233 between rs5742909 and rs231775; D'=1, r^2=0.564 between rs231775 and rs3087243; D'=1, r^2=0.035 between rs5742909 and rs3087243). In order to derive HBV infection-specific haplotypes, haplotypes with frequencies <0.05 were not considered and three haplotypes were observed. When a protective haplotype C-G-G was selected as a baseline, haplotypes C-A-A and T-A-G exhibited an increased susceptibility to progressed hepatitis and the haplotype T-A-G revealed an increased risk for viral persistence (P-value and OR are shown in Table VI). Three-loci haplotyping was performed only for the subjects with complete genotyping.

Discussion

The outcomes of the HBV infection vary according to the vigor of the immune response, a process that is regulated by a number of molecules, including the cell surface receptor CTLA-4 (25). The CTLA-4 gene is expressed by T-lymphocytes and functions as an inhibitory receptor, acting as a negative regulator of T-cell responses (31). Mohammad Alizadeh *et al* (26) and Schott *et al* (27) demonstrated a significant association between the genotypes or alleles of the mutation rs5742909 and the susceptibility to chronic hepatitis B. In their studies, Gu *et al* and Hu *et al* demonstrated that the SNP site rs231775 was associated with HCC (23,29). However, Gu *et al* only identified the association in a male Chinese population and Hu *et al* included HCC subjects with a virus infection as well as with HBV. Thio *et al* (25) reported that presence of allele +6230A in the 3'UTR of the CTLA4 gene (at SNP site rs3087243) was suspected to have viral persistence and that allele +49G (at SNP site rs231775) was detected more often in individuals who recovered from HBV infection. Although the association between various outcomes of HBV infection and the CTLA-4 gene has become increasingly evident (24,28), their diagnosis criteria were not completely equal and the ethnic background difference should be considered. Different results are therefore likely to arise with different diagnosis criteria and ethic backgrounds although the control and case groups are similar.

In the analysis of the present study, three SNPs sites (rs5742909, rs231775 and rs3087243) in the gene CTLA-4 were confirmed to be significantly associated with HBV infection. Additionally, CTLA-4 haplotypes are significant determinants of the HBV infection in the Hubei Han Chinese population. Haplotypes containing the +49G allele were protective against HBV progression and viral persistence. Since these haplotypes may alter the ability of CTLA-4 to downregulate the immune response, these data indicated that the vigor of counter-regulatory mechanisms contributes to HBV infection. Although the present study may not have indicated that the genotypes distribution in all these three sites (rs5742909, rs231775 and rs3087243) was significantly different between the control and case groups, the frequencies of susceptible alleles were similar compared with those in other Chinese populations. Thus, it may be confirmed that the polymorphisms of the CTLA-4 gene play a crucial role in HBV progression and viral persistence in the Hubei Han Chinese population.

Following HBV infection, the inflammatory immune response of the host induces hepatocellular damage and is followed by the pathogenesis of liver cirrhosis and cancer (32). Liver cancer arises most frequently in the setting of chronic liver inflammation (33). In the present study, the allele +49A was identified as a risk factor for HBV progression while the mutation allele +49G may be protective against HBV progression. Inherited changes or SNPs in CTLA-4 expression that presumably alter T-cell self-reactivity have been found to be associated with autoimmune disorders (10,34,35) including autoimmune liver disease (36-39). The A→G mutation which exists at position +49 in exon 1 (rs231775) leads to a non-synonymous amino acid change from threonine to alanine, thus changing the polarity of the amino acid and potentially altering the function of the protein. The +49A allele has been associated with a decreased risk for diseases resulting from a downregulated vigorous immune response (36,40-42). Similarly, in an earlier study, the allele +49G was also associated with improved clearance of an HCV infection resulting from α-interferon-based therapy (43). Lymphocytes from donors carrying +49G appear to express less CTLA-4 on their surfaces, proliferate more under conditions of suboptimal activation and exhibit less CTLA-4-mediated inhibition of the T-cell responses (44).

The SNP rs5742909 is an A→G mutation in the promoter region at position -318 of the CTLA-4 gene. It has been demonstrated that the -318T allele of CTLA-4 may result in increased levels of CTLA-4 mRNA compared with the -318C/-318C homozygote (45) and an increased expression of CTLA-4 following activation (46). Although in the present study, only the association between the mutation and viral persistence was observed, the haplotype containing -318C or -318T is associated with viral persistence and HBV progres-

sion, as mentioned in previous studies (24,27). In addition, individuals with the allele +6230A in the 3' UTR (at the site rs3087243) were also found to be more likely to have HBV progression. Ueda *et al* (10) reported that the SNP site rs3087243 determines the levels of the soluble isoform of CTLA-4 (sCTLA-4), which has been demonstrated *in vitro* to inhibit T-cell proliferation. This may partially account for the association of this haplotype containing the +6230A allele with HBV progression.

In the presence of two functional polymorphisms on the same LD block, when the predisposing allele of one is in LD with the protective allele of the other, the genetic effects of individual SNPs are likely to be blunted by an increase in the frequency of haplotypes that carry one predisposing and one protective allele (47). Chistiakov *et al* (48) suggested that whether the haplotype are likely to be susceptible, protective or neutral depends on a ratio between predisposing and protective alleles constituting a haplotype and an interaction between their functional significance and strength of their functional effects. Therefore, it is possible that the effects of the three SNPs in chronic HBV patients involved in the present study may be due to the linkage to each other, in particular to the linkage to +49 A/G.

In summary, in the present case-control study, the A allele of the rs231775 and rs3087243 SNPs sites in CTLA-4 was confirmed to be significantly associated with HBV progression in the Han Chinese population, and allele T in rs5742909 revealed a strong risk effect on viral persistence. Although HBV disease is not determined solely by genetic factors, the experimental results offer the foundation for further study of genetic variations in CTLA-4 for the prevention and therapy of chronic HBV infection. However, following adjustment for age and gender, the association among the three SNPs and the HBV-related HCC, excluding the (LC+HCC) group, was not observed. This may be due to the difference in criteria and ethnic background. Future investigations with a larger sample size, multi-center study and functional studies in this gene are required to confirm the results of the present study.

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