Carcinogenesis vol.30 no.11 pp.1916–1922, 2009
doi:10.1093/carcin/bgp226
Advance Access publication October 1, 2009

IkappaBalpha gene promoter polymorphisms are associated with hepatocarcinogenesis in patients infected with hepatitis B virus genotype C

Yongchao He, Hongwei Zhang, Jianhua Yin, Jiaxin Xie, Xiaojie Tan, Shijian Liu, Qian Zhang[1], Chengzhong Li[1], Jun Zhao[2], Hongyang Wang[3] and Guangwen Cao*

Department of Epidemiology, [1]Department of Infectious Diseases, the 1st Affiliated Hospital, [2]Department of Hepatobiliary Surgery and [3]Laboratory for Signal Transduction, the 3rd Affiliated Hospital, Second Military Medical University, Shanghai 200433, People's Republic of China

*To whom correspondence should be addressed. Tel/Fax: +86 818 71060; Email: gcao@smmu.edu.cn

Genetic predisposition of nuclear factor-kappa B (NF-κB)-signaling pathways linking inflammation to hepatitis B virus (HBV)-induced hepatocellular carcinoma (HCC) remains unresolved. We conducted a case–control study to determine the associations of the polymorphisms within the promoter regions of *NFKB1* encoding NF-κB1 and *NFKBIA* encoding IkappaBalpha with the development of HCC. A total of 404 healthy controls, 482 non-HCC subjects with HBV infection and 202 patients with HCC were included. *NFKB1* −94ATTG2 allele and GG allele in the 3′-untranslated region of *NFKBIA* were more prevalent in HCC patients than in the healthy controls. *NFKBIA* −826CT and *NFKBIA* −881AG allelic carriages were more prevalent in HCC patients than in the non-HCC subjects with HBV infection. The estimated haplotype frequency of *NFKBIA* promoter −881G−826T−519C was significantly higher in the patients with HCC than in the HBV-infected subjects without HCC (odds ratio = 3.142, *P* = 0.002). As compared with the HBV-infected subjects without HCC, *NFKBIA* −826 T and *NFKBIA* −881AG allelic carriages were only associated with HCC risk in the subjects with HBV genotype C. The association of *NFKBIA* −881AG allelic carriage with HCC risk was not affected by liver cirrhosis (LC) status, alanine aminotransferase level and hepatitis B e antigen status. By multivariate regression analysis, *NFKB1* −94ATTG2, *NFKBIA* −826T, *NFKBIA* −881AG and HBV genotype C were independently associated with an increased risk of HCC. In conclusion, *NFKB1* −94ATTG2 allele and haplotype −881G−826T−519C in *NFKBIA* promoter were associated with hepatocarcinogenesis. *NFKBIA* −826T and −881AG were associated with the risk of HCC in the subjects infected with HBV genotype C.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Most HCC cases (>80%) occur in either Eastern Asia or sub-Saharan Africa (1). Major risk factors for the development of HCC are chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), liver cirrhosis (LC), exposure to aflatoxin B1 and diabetes. It has been estimated that 80% of HCC worldwide is etiologically associated with HBV (2). Expression of hepatitis B e antigen (HBeAg) and a high serum level of HBV (i.e. a viral load ≥10 000 copies/ml) are associated with an increased risk of HCC (3,4). HBV genotype C, genotype mixture and viral mutations in the PreS, basal core promoter and enhancer II regions of HBV are also associated with an increased risk of HCC (5–7). HCC is widely accepted to be the outcome of chronic inflammation. However, molecular mechanisms linking chronic inflammation to HBV-induced hepatocarcinogenesis remain largely unresolved.

Activation of nuclear factor-kappa B (NF-κB), a hallmark of inflammatory response that is frequently detected in tumors, may constitute a missing link between inflammation and cancer (8,9). NF-κB was originally identified as a nuclear factor specific to B cells bound to the B site of the κ-light chain gene enhancer. NF-κB is a heterodimer in Rel family with five members: RelA, RelB, c-Rel, p50/105 (NF-κB1) and p52/p100, and the dimeric form of NF-κB p50/RelA is the most common form. NF-κB has a central role in coordinating the expression of a wide variety of genes that control innate and adaptive immune responses and also has a critical role in cancer development and progression (10). Recent studies have provided a causal link between constitutive activation of NF-κB and liver neoplastic progression and have demonstrated that NF-κB is essential for promoting inflammation-associated liver cancer and plays important roles in hepatic inflammation, fibrosis and the development of HCC—a process called the 'inflammation-fibrosis-cancer axis' (8,11–13). In normal cells, NF-κB is inactivated in the cytoplasm by binding to its inhibitors, IκB. When IκB proteins are phosphorylated and degraded, NF-κB is subsequently released and further translocated into the nucleus, where the gene transcription is initiated (14). The IκB family includes IkappaBalpha (IκBα), IκBβ, IκBγ, IκBδ, IκBϵ, IκBζ, IκB-R, Bcl-3, p100 and p105 that are all constitutively expressed except for IκBα, which is inducibly expressed. IκBα is a classic form of the IκB family that can be found in cytoplasm and nucleus (15).

Allelic variants in human genome are probably to affect hepatitis B progression after infection and are associated with poor prognosis of chronic HBV infection. Several single-nucleotide polymorphisms (SNPs) or haplotypes have reportedly been associated with an increased or reduced risk of HCC occurrence in patients with HBV infection (16,17). Polymorphic variations in the promoter regions of NF-κB1 gene *NFKB1* and IκBα gene *NFKBIA* and in the 3′-untranslated region (3′-UTR) of *NFKBIA* were associated with the risks of Hodgkin lymphoma, multiple myeloma, breast cancer, prostate cancer, gastric cancer, colorectal cancer and melanoma (18–24). Kim *et al.* (25) sequenced six exons and introns including −1000 promoter region of *NFKBIA* from 16 healthy volunteers and identified 10 polymorphisms. Six of 10 polymorphisms including two SNPs (−673 A>T and −642 C>T) in the promoter region were selected for HCC association study. They could not find any significant association of *NFKBIA* variants with the development of HCC among chronic hepatitis B (CHB) patients. Except Kim's study, there are no report focusing on the association of the polymorphisms of *NFKB1* and *NFKBIA* and the development of HCC. In this study, we evaluated the associations of the polymorphisms of *NFKB1* and *NFKBIA* with the development of HCC as compared with either healthy controls without HBV infection or HBV-infected subjects without HCC. To our knowledge, it is the first study that clearly demonstrates significant associations of the *NFKB1* and *NFKBIA* polymorphisms with HBV-induced hepatocarcinogenesis.

Abbreviations: ALT, alanine aminotransferase; AOR, adjusted odds ratios; ASC, asymptomatic hepatitis B surface antigen carriers; CHB, chronic hepatitis B; CI, confidence intervals; HBeAg, hepatitis B e antigen; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; IκBα, IkappaBalpha; LC, liver cirrhosis; NF-κB, nuclear factor-kappa B; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; 3′-UTR, 3′-untranslated region; WT, wild type.

© The Author 2009. Published by Oxford University Press.

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.5/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Patients and methods

Clinical data

The study involved 404 HBV-infected patients treated at the first and third affiliated hospitals of this university from October 2006 to October 2008, 404 healthy controls without HBV infection from the physical examination center of the First affiliated hospital from January 2007 to October 2008 and 280 asymptomatic hepatitis B surface antigen carriers (ASCs) recruited in an epidemiological survey from September to December 2006. All participants were ethic Chinese. Diagnostic criteria of ASCs, the patients with CHB and the patients with HCC, and exclusion criteria of the subjects were as described previously (6). Only newly diagnosed HCC patients were included; patients with a prior history of HCC or other cancers were excluded. LC was diagnosed by using a Philips iU22 scanner (Philips Medical Systems, Best, The Netherlands) equipped with a 2–4 MHz variable convex probe. The ultrasonography scoring system consisting of liver surface, parenchyma, vascular structure and splenic size was used to describe the existence and severity of cirrhosis. The scores ranged from four for a normal liver to 11 for advanced cirrhosis (26). A score of eight or more was used as the cutoff point for HBV-related ultrasonographic cirrhosis. In addition to ultrasonographic findings, esophageal of gastric varices, low platelet count and cirrhotic complications such as ascites or encephalopathy were used for the diagnosis of clinical cirrhosis. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the ethics committees of the institutions, and an informed consent was obtained from each participant.

Examination of HBV serological parameters

Before any treatment, 5 ml fasting blood was split into two blood collection tubes with or without anticoagulant. The serum was separated by centrifugation at 4°C and stored in a sterile tube at −80°C within 4 h of sample collection. Serological testing for hepatitis B surface antigen, HBeAg, alphafetoprotein and liver function examination including alanine aminotransferase (ALT) was performed as described previously (6). Upper limit of normal ALT was 45 U/l. HBV DNA concentration was measured in the LightCycler™480 (Roche, Basel, Switzerland) using quantitative HBV polymerase chain reaction (PCR) fluorescence diagnostic kits (Fuxun Diagnostics, Shanghai, China). The kit has a certified lower limit of detection of 500 copies/ml. HBV genotype and subgenotype were determined by a multiplex PCR assay (6,27).

SNP analysis

Genomic DNA was extracted from a 200 μl peripheral blood sample using a QIAamp blood kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Genotyping for the polymorphisms of *NFKB1* −94 *ins/del* ATTG (rs 28720239), *NFKBIA* 3′-UTR A/G (rs 57898959), *NFKBIA* −519 C/T (rs 2233408), *NFKBIA* −826 C/T (rs 2233406) and *NFKBIA* −881 A/G (rs 3138053) was conducted using PCR–restriction fragment length polymorphism method. Primers for *NFKB*1 −94 were 5′-TGGGCACAAGTCGTT-TATGA-3′ and 5′-CTGGAGCCGGTAGGGAAG-3′, amplifying a fragment of 281 bps or 285 bps (23). Primers for *NFKBIA* 3′-UTR were 5′-GGCTGAAAGAACATGGACTTG-3′ and 5′-GTACACCATTTACAGGAG-GG-3′ that amplify a fragment of 424 bps (23). Primers for *NFKBIA* −519 C/T were 5′-GCTTTCACAACTTCTACCTG-3′ and 5′-AGAGTGGAAAT-GATGGCTG-3′, which amplify a fragment of 188 bps (24). Primers for *NFKBIA* −826 C/T and −881 A/G were 5′-GGTCCTTAAGGTCCAATCG-3′ and 5′-GTTGTGGATACCTTGCACTA-3′ (underlined, mismatched nucleotide), which amplify a fragment of 200 bps (28). PCR was performed in 25 μl mixture containing 50–100 ng DNA, 1× PCR buffer, 1 U Taq polymerase (TaKaRa Biotechnology, Dalian, China), 0.5 μM each primer (synthesized by Sangon Biotechnology, Shanghai, China) and 0.25 mM deoxynucleoside triphosphate (TaKaRa Biotechnology). The amplification was performed with annealing temperature of 60°C (*NFKB*1 −94 or *NFKBIA* 3′-UTR) or 55°C (*NFKBIA* −519 or *NFKBIA* −826 and −881) for 30 s for 35 cycles by using an Autorisierter thermocycler (Eppendorf AG, Humburg, Germany). The PCR product of *NFKB1* −94 was digested with Van91 I (New England Biolabs, Ipswitch, MA) at 37°C for 18 h. The PCR product of *NFKBIA* 3′-UTR was digested with HaeIII (TaKaRa) at 37°C for 6 h. The PCR product of *NFKBIA* −519 was digested with MnlI (New England Biolabs) at 37°C for 6 h. The PCR product of *NFKBIA* −826 was digested with XspI (TaKaRa) at 37°C for 8 h, whereas that of *NFKBIA* −881 was digested with TspRI (New England Biolabs) at 65°C for 5 h. Both the PCR and restriction products were visualized in 2% agarose gel with 0.2 μg/ml ethidium bromide, together with the DNA ladders, as shown in supplementary Figure 1 (available at *Carcinogenesis* Online). Genotypes of *NFKB1* polymorphisms were distinguished as wild type (WT) (281 bps, ATTG1/ATTG1), heterozygote (ATTG1/ATTG2) and polymorphic homozygote (45 and 240 bps, ATTG2/ATTG2). Genotypes of *NFKBIA* 3′-UTR polymorphisms were determined as WT (424 bps, AA), heterozygote (AG) and polymorphic homozygote (108 and 316 bps, GG). Genotypes of *NFKBIA* −519 polymorphisms were determined as WT (188 bps, CC), heterozygote (CT) and polymorphic homozygote (121 and 67 bps, TT). Genotypes of *NFKBIA* −826 polymorphisms were determined as WT (200 bps, CC), heterozygote (CT) and polymorphic homozygote (180 and 20 bps, TT). Genotypes of *NFKBIA* −881 polymorphisms were determined as WT (200 bps, AA), heterozygote (200, 129 and 71 bps, AG).

Statistical analysis

The frequencies of all SNPs in health controls were tested for conformation to Hardy–Weinberg equilibrium online (http://ihg.gsf.de/ihg/snps.htm1). Statistical analyses were performed using the Statistical Program for Social Sciences (SPSS15.0 for Windows, SPSS, Chicago, IL). Categorical variables, like the frequencies of SNPs and HBV genotypes, were tested by χ^2 test. Continuous variables, like serum viral load and ALT level with skewed distribution, were adjusted to normal distribution by transformation into logarithmic function and then tested by Student's *t*-test. To determine the factors contributing independently to HCC, forward stepwise multivariate regression analysis ($P_{entry} = 0.05$, $P_{removal} = 0.10$) was performed to obtain the adjusted odds ratios (AOR) of polymorphisms for HCC risk and their 95% confidence intervals (CI). The estimated haplotype frequencies were determined by the estimating haplotype-frequencies program (http://linkage.rockefeller.edu/soft/). A P value of <0.05 was considered as statistically significant. All statistical tests were two sided.

Results

The characteristics of the participants are presented in Table I. Healthy controls without HBV infection, the patients with LC and those with CHB were age and sex matched to the patients with HCC. The patients with HCC were significantly older and there were higher proportion of men than ASCs. HBV genotype C (C2, 95.5%) was more frequent in the patients with HCC than in the HBV-infected participants without HCC ($P < 0.001$). Serum viral load of HBV was significantly higher in the HBV-infected participants without HCC than in the patients with HCC, and the same were true for the frequencies of HBeAg seropositivities and ALT levels.

Genotype data of all loci in the healthy controls were conformed to Hardy–Weinberg equilibrium ($P > 0.05$ for each). Genotype data of *NFKB1* −94, *NFKBIA* 3′-UTR and *NFKBIA* −826, except *NFKBIA* −519 and *NFKBIA* −881, in the non-HCC patients with HBV infection did not conformed to Hardy–Weinberg equilibrium (Table II). After being adjusted with age and sex, *NFKB1* −94 ATTG2/ATTG2 allelic carriage was more prevalent in the patients with HCC than in the patients with CHB (41.1 versus 28.2%, AOR = 2.17, 95% CI: 1.26–3.75, $P = 0.006$), and the same was true for *NFKBIA* −881AG allele (18.8 versus 6.9%, AOR = 3.288, 95%CI: 1.71–6.33, $P < 0.001$). As compared with ASCs, *NFKBIA* 3′-UTR GG allelic carriage was significantly associated with an increased risk of HCC (32.2 versus 13.6%, AOR = 2.44, 95%CI: 1.36–4.36, $P = 0.003$) after the adjustment with age and sex, and the same were true for *NFKBIA* −826T (CT+TT) (26.2 versus 15.7%, AOR = 2.19, 95%CI: 1.29–3.71, $P = 0.003$) and *NFKBIA* −881AG alleles (18.8 versus 6.8%, AOR = 3.20, 95%CI: 1.63–6.28, $P = 0.001$). Interestingly, we also found that *NFKB1* −94ATTG2 allelic carriage (ATTG1/ATTG2+ ATTG2/ATTG2) was more prevalent in ASCs than in the healthy controls (77.9 versus 69.3%, OR = 1.670, 95%CI: 1.10–2.52, $P = 0.016$). Table II shows the frequencies of all polymorphisms in the healthy controls and the HBV-infected patients with and without HCC. As compared with the age-, sex-matched healthy controls, *NFKB1* −94ATTG2 and *NFKBIA* 3′-UTR GG alleles were more prevalent in the HBV-infected patients with HCC. *NFKB1* −94ATTG2, *NFKBIA* −826CT and *NFKBIA* −881AG allelic carriages were more prevalent in the HBV-infected patients with HCC than in the HBV-infected subjects without HCC.

The HBV-infected subjects were stratified into sex- or age-matched groups. In women, *NFKB1* −94ATTG2, *NFKBIA* −826T and *NFKBIA* −881G allelic carriages were significantly associated with the risk of HCC, as compared with the HBV-infected subjects without HCC. In men, *NFKBIA* −881AG allelic carriage was significantly associated with an increased risk of HCC, as compared with the HBV-infected subjects without HCC. In those <53 years (average age of the HBV-infected patients with HCC), *NFKBIA* −826T and

NFKBIA −881AG allelic carriages were significantly associated with the risk of HCC, as compared with the HBV-infected subjects without HCC. In those at the age of ≥53 years, *NFKBIA* −881AG allelic carriage was significantly associated with an increased risk of HCC, as compared with the HBV-infected subjects without HCC. The results are presented in the supplementary Table I and II (available at *Carcinogenesis* Online), respectively.

We stratified the HBV-infected subjects into HBeAg-positive and HBeAg-negative groups and then evaluated the association of the polymorphisms with the development of HCC between the HCC patients with HBV infection and the non-HCC patients with HBV infection. *NFKBIA* −881AG allelic carriage was associated with an increased risk of HCC, either in HBeAg-positive group (22.9 versus 8.1%, OR = 3.36, 95% CI: 1.46–7.75, P = 0.003) or in HBeAg-negative group (17.5 versus 5.9%, OR = 3.42, 95% CI: 1.78–6.57, P = 0.000). We then stratified the HBV-infected subjects into normal ALT (<45 U/l) and abnormal (≥45U/l) groups and evaluated the association of the polymorphisms with the development of HCC

Table I. Characteristics of study subjects and their association with HCC

Characteristic	Healthy controls (N = 404)	Non-HCC subjects with HBV infection (N = 482)			HCC patients with HBV infection (N = 202)	P value
		ASC (n = 280)	CHB (n = 110)	LC (n = 92)		
Sex (male)	318 (78.7)	160 (57.1)	87 (79.1)	72 (78.3)	159 (78.7)	0.001[a]
Age (year)	52.0 ± 9.61	43.7 ± 7.94	50.1 ± 12.28	50.5 ± 8.46	52.9 ± 9.76	0.000[a]
Genotype						
B	ND	87 (31.1)	22 (21.8)	15 (17.9)	11 (5.8)	0.000[b]
C	ND	180 (64.3)	79 (78.2)	69 (82.1)	175 (91.6)	<0.01[c]
Others	ND	13 (4.6)	ND	ND	5 (2.6)	
HBeAg						
Positive	ND	129 (46.1)	40 (36.4)	40 (43.5)	48 (23.8)	0.018[d]
Negative	ND	151 (53.9)	70 (63.6)	52 (56.5)	154 (76.2)	0.000[e]
HBV DNA (Log_{10} copies/ml)	ND	ND	4.70 ± 1.67	4.17 ± 1.35	3.92 ± 1.17	0.000[f]
ALT (Log_{10} U/l)	ND	ND	2.09 ± 0.48	1.85 ± 0.49	1.69 ± 0.31	<0.01[c]

Data shown as N (%), except age, ALT and HBV DNA; ND, no data.
[a]Between ASC and HCC.
[b]Between ASC and HCC.
[c]Between HCC and CHB and between HCC and LC.
[d]Between CHB and HCC.
[e]Between HCC and ASC and between HCC and LC.
[f]Between CHB and HCC.

Table II. The frequencies of the polymorphisms at *NFKB1* −94 and *NFKBIA* 3′-UTR, −519, −826 or −881 in the participants

Polymorphisms (accession code)	Alleles	Healthy controls[a]	Non-HCC with HBV infection[a]	HCC with HBV infection[a]	AOR1 (95% CI)	OR2 (95% CI)	AOR2 (95% CI)
NFKB1 −94 (rs28720239, ATTG1 > ATTG2)	ATTG1/ATTG1	124 (30.7)	111 (24.1)	35 (17.3)	1.00		1.00
	ATTG1/ATTG2	183 (45.3)	192 (41.7)	84 (41.6)	1.60 (1.01–2.53)	1.39 (0.88–2.19)	1.30 (0.80–2.10)
	ATTG2/ATTG2	97 (24.0)	157 (34.1)	83 (41.1)	3.01 (1.87–4.85)	1.68 (1.05–2.67)	1.62 (1.00–2.64)
	ATTG2 (ATTG1/ATTG2+ ATTG2/ATTG2)	280 (69.3)	349 (75.8)	167 (82.7)	2.09 (1.37–3.19)	1.52 (0.99–2.32)	1.44 (0.93–2.25)
	HWE P value[b]	0.07	0.00				
NFKBIA 3′-UTR (rs57898959, A>G)	AA	138 (34.2)	186 (38.6)	70 (34.7)	1.00	1.00	1.00
	AG	185 (45.8)	193 (40.0)	67 (33.2)	0.71 (0.47–1.06)	0.92 (0.62–1.36)	1.01 (0.67–1.53)
	GG	81 (20.0)	103 (21.4)	55 (32.2)	1.61 (1.04–2.50)	1.68 (1.11–2.54)	1.49 (0.96–2.31)
	G (AG+GG)	266 (65.8)	296 (61.4)	122 (65.4)	0.98 (0.68–1.39)	1.19 (0.84–1.67)	1.19 (0.83–1.71)
	HWE P value[b]	0.19	0.00				
NFKBIA −519 (rs2233408, C>T)	CC	321 (79.5)	368 (77.8)	162 (80.2)	1.00	1.00	1.00
	CT	74 (18.3)	95 (20.1)	31 (15.3)	0.84 (0.53–1.33)	0.74 (0.48–1.16)	0.80 (0.50–1.28)
	TT	9 (2.2)	10 (2.1)	9 (4.5)	1.98 (0.77–5.09)	2.04 (0.82–5.13)	1.64 (0.62–4.34)
	T (CT+ TT)	83 (20.5)	105 (22.2)	40 (19.8)	0.96 (0.63–1.47)	0.87 (0.58–1.30)	0.89 (0.58–1.38)
	HWE P value[b]	0.06	0.19				
NFKBIA −826 (rs2233406, C>T)	CC	291 (72.0)	394 (81.7)	149 (73.8)	1.00	1.00	1.00
	CT	105 (26.0)	76 (15.8)	52 (25.7)	0.98 (0.66–1.44)	1.81 (1.21–2.70)	1.89 (1.24–2.89)
	TT	8 (2.0)	12 (2.5)	1 (0.5)	0.25 (0.03–2.00)	0.22 (0.03–1.71)	0.25 (0.03–2.01)
	T (CT+ TT)	113 (28.0)	88 (18.3)	53 (26.2)	0.92 (0.63–1.35)	1.59 (1.08–2.35)	1.67 (1.11–2.53)
	HWE P value[b]	0.68	0.00				
NFKBIA −881 (rs3138053, A>G)	AA	331 (81.9)	449 (93.2)	164 (81.2)	1.00	1.00	1.00
	AG	73 (18.1)	33 (6.8)	38 (18.8)	1.06 (0.69–1.64)	3.15 (1.91–5.20)	3.32 (1.95–5.65)
	GG	0	0	0	ND	ND	ND
	HWE P value[b]	0.06	1.00				

AOR, odds ratio adjusted by age and sex; AOR1: healthy controls versus HCC with HBV infection; OR2 and AOR2: non-HCC with HBV infection versus HCC with HBV infection; HWE, Hardy–Weinberg equilibrium; ND, no data.
[a]Data were presented as n (%).
[b]Significance probability determined by HWE test.

between the HCC patients with HBV infection and the non-HCC patients with HBV infection. *NFKBIA* −881AG allelic carriage was significantly associated with an increased risk of HCC either in normal ALT group or in abnormal ALT group. *NFKB1* −94ATTG2, *NFKBIA* 3′-UTR and *NFKBIA* −826T allelic carriages were significantly associated with the risk of HCC in normal ALT group, as shown in the supplementary Table III (available at *Carcinogenesis* Online).

We stratified the HBV-infected subjects into HBV genotype B- and HBV genotype C-infected groups. After the adjustment with age and sex, *NFKBIA* −826T and *NFKBIA* −881AG allelic carriages were significantly associated with the risk of HCC in the groups infected with HBV genotype C, as compared with the HBV genotype C-infected subjects without HCC (Table III).

We also found that the estimated haplotype frequency of *NFKBIA* promoter −881G−826T−519C was significantly higher in the patients with HCC than in the HBV-infected subjects without HCC (Table IV).

There were no significant differences in the frequencies of the polymorphisms in all loci between the non-HCC patients with and without LC, and the same was true for the difference between HCC patients with and without LC. The frequency of *NFKBIA* −881AG allele was significantly higher in the patients with HCC than in those without HCC, regardless of LC status (Figure 1). As compared with the non-HCC patients with LC, the HCC patients had significantly higher frequencies of *NFKBIA* −881AG (18.8 versus 6.5%, OR = 3.32, 95% CI: 1.35–8.17, $P = 0.006$) and *NFKB1* −94ATTG2 alleles (82.7 versus 71.7%, OR = 1.88, 95% CI: 1.05–3.36, $P = 0.032$).

Independent factors contributing to HCC were evaluated in the forward stepwise multivariate regression analysis (Table V). *NFKB1* −94ATTG2 allelic carriage was independently associated with an increased risk of HCC, as compared with the healthy controls. Age, male, *NFKBIA* −816T allelic carriage and HBV genotype C were independently associated with an increased risk of HCC, whereas *NFKBIA* −519T allelic carriage was inversely associated with an increased risk of HCC, if ASCs were used as controls. *NFKB1* −94ATTG2 allele, *NFKBIA* −881AG allele and HBV genotype C were independently associated with an increased risk of HCC if the patients with CHB and those with LC served as controls. *NFKB1* −94ATTG2 allelic carriage and *NFKBIA* −881AG allelic carriage and genotype C were independently associated with increased risks of HCC if combining ASCs, the patients with CHB and those with LC as controls (supplementary Table IV is available at *Carcinogenesis* Online).

Discussion

This study firstly demonstrated that the ATTG2 (ATTG1/ATTG2 + ATTG2/ATTG2) allelic carriage at −94 site of *NFKB1* promoter significantly increased the risk of HCC either compared with the healthy controls (Table II) or compared with the patients with CHB after being adjusted with age and sex. This study also revealed that *NFKB1* −94ATTG2 allelic carriage was more prevalent in ASCs than in the healthy controls (OR = 1.67; 95% CI: 1.10–2.52) and more frequent in the HBV-infected patients without HCC than in the healthy controls (Table II), indicating that *NFKB1* −94ATTG2 allelic carriage might also contribute to the risk of becoming chronic HBV infection. AOR of the ATTG2 allele for HCC was higher in HCC versus the healthy controls than in HCC versus CHB+LC (Table V). *NFKB1* −94ATTG2 allelic carriage was independently associated with an increased risk of HCC in the multivariate analysis using ASCs, the patients with CHB and those with LC as controls (supplementary Table IV is available at *Carcinogenesis* Online). Based on these observations, we hypothesize that *NFKB1* −94ATTG2 allelic carriage might play a role from HBV chronic infection to HBV-induced hepatocarcinogenesis. Insertion/deletion of ATTG at −94 site of *NFKB1* promoter is a functional *NFKB1* polymorphism. The ATTG insertion at the *NFKB1* promoter significantly increased the promoter activity, as demonstrated by the promoter-luciferase reporter assay in transient transfection and differential nuclear protein binding assays (29), indicating that the individuals with the ATTG2 allele usually had higher levels of *NFKB1* transcript and further increase in production of NF-κB protein. Thus, *NFKB1* −94ATTG2 allelic carriage-associated up-regulation of NF-κB1 might play a role from chronic inflammation to hepatocarcinogenesis caused by HBV chronic infection.

In this study, *NFKBIA* promoter polymorphisms were selected based on their frequency determined in a preliminary screening of 200 normal subjects. The polymorphism at position −881 was further selected based on potential functional effects due to their location within transcription factor binding sites (http://www.cbrc.jp/research/db/TFSEARCH.html). From this website and literature (30), we created schematic diagrams to show several known transcription factor binding sites in *NFkB1* and *NFkBIA* promoters (supplementary Figure 2 is available at *Carcinogenesis* Online). The −881 locus is within a consensus binding site (TAAA/TNTAGGTCA) for the

Table III. Association of the polymorphisms with the development of HCC in different HBV genotype groups

Polymorphism	HBV genotype B			HBV genotype C		
	Non-HCC[a]	HCC[a]	AOR (95% CI)	Non-HCC[a]	HCC[a]	AOR (95% CI)
NFKB1 −94						
ATTG1/ATTG1	29 (24.8)	3 (27.3)	1.00	78 (24.8)	32 (18.3)	1.00
ATTG2 (ATTG1/ATTG2+ATTG2/ATTG2)	88 (75.2)	8 (72.7)	0.747 (0.18–3.11)	236 (75.2)	143 (81.7)	1.46 (0.90–2.37)
NFKBIA 3′-UTR						
AA	51 (41.1)	3 (27.3)	1.00	123 (37.5)	60 (34.3)	1.00
G (AG+GG)	73 (59.8)	8 (72.7)	2.05 (0.50–8.37)	205 (62.5)	115 (65.7)	1.13 (0.76–1.70)
NFKBIA −519						
CC	101 (82.8)	11 (100.0)	1.00	250 (77.9)	142 (81.1)	1.00
T (CT+TT)	21 (17.2)	0	ND	71 (22.1)	33 (18.9)	0.87 (0.53–1.42)
NFKBIA −826						
CC	104 (83.9)	9 (81.8)	1.00	267 (81.4)	129 (73.7)	1.00
T (CT+TT)	20 (16.1)	2 (18.2)	1.13 (0.22–5.74)	61 (18.6)	46 (26.3)	1.71 (1.07–2.73)[b]
NFKBIA −881						
AA	115 (92.7)	10 (90.9)	1.00	308 (93.9)	142 (81.1)	1.00
AG	9 (7.3)	1 (9.1)	1.15 (0.13–10.41)	20 (6.1)	33 (18.9)	4.02 (2.14–7.59)[c]

AOR, odd ratio adjusted by age and sex; ND, no data.
[a]Data were presented as *n* (%).
[b]$P = 0.024$.
[c]$P = 0.000$.

retinoic acid-related orphan receptor α (30). This study revealed that *NFKBIA* −881G allelic carriage was an independent risk factor of HCC, especially in the subjects infected with HBV genotype C. Although there is not direct evidence showing that the −881G at the promoter of *NFKBIA* decreases the promoter activity, indirect evidences indicate this possibility. For instance, NF-κB expression was twice as high in the patients with sarcoidosis compared with the control subjects, whereas the −881G allelic carriage and −826T allelic carriage were more prevalent in sarcoidosis than in the controls (31), indicating that the −881G and −826T allelic carriages might be associated with an increased activity of NF-κB. Since IkBα is an absolute requirement for normal termination of the NF-κB response (15), *NFKBIA* −881G and −826T allelic carriages are therefore speculated to retard efficient IκBα expression. *NFKBIA* −881G might alter the binding ability of retinoic acid-related orphan receptor α, a group of transcriptional factors with potential roles in osteoporosis, autoimmune diseases and cancer (30), and contribute to hepatocarcinogenesis. This study found that *NFKBIA* −881 G and *NFKBIA* −826 T allelic carriages were only associated with HCC in patients infected with HBV genotype C (Table III). We speculate that the large HBV surface protein and the X protein of genotype C might involve in this process. The PreS2 domain of the large HBV surface protein is able to activate a variety of promoter elements including NF-κB (32), whereas our recent meta-analysis have shown that viral mutations in PreS region are significantly higher in HBV genotype C than in genotype B during HBV-induced hepatocarcinogenesis (7). HBV X protein is able to activate NF-κB by directly interacting with IκBα at amino acids 249–253 of IκBα in the C-terminal part of the sixth ankyrin repeat (33), while nucleotide change of codon 38 in the X gene of HBV genotype C is independently associated with the development of HCC (34). The large HBV surface protein and the X protein of genotype C might specifically activate important transcriptional factors in NF-κB-signaling pathways. Moreover, genotype C products, e.g. X protein, might be able to bind *NFKBIA* −881G and

Table IV. The estimated haplotype frequencies of *NFKBIA* promoter polymorphisms in the HBV-infected patients with HCC and the HBV-infected subjects without HCC

NFKBIA promoter haplotype	HCC (*N* = 202)	Non-HCC (*N* = 482)	OR (95% CI)	*P* value
−881A−826C−519C	0.765	0.792	0.863 (0.582–1.280)	0.464
−881A−826C−519T	0.100	0.106	0.929 (0.538–1.602)	0.790
−881A−826T−519C	0.036	0.060	0.561 (0.242–1.302)	0.173
−881A−826T−519T	0.007	0.009	—[a]	—[a]
−881G−826C−519C	0.003	0.004	—[a]	—[a]
−881G−826C−519T	0.000	0.002	—[a]	—[a]
−881G−826T−519C	0.076	0.025	3.142 (1.443–6.838)	0.002
−881G−826T−519T	0.016	0.003	—[a]	—[a]

[a]OR and *P* value were not calculated due to small size of case number.

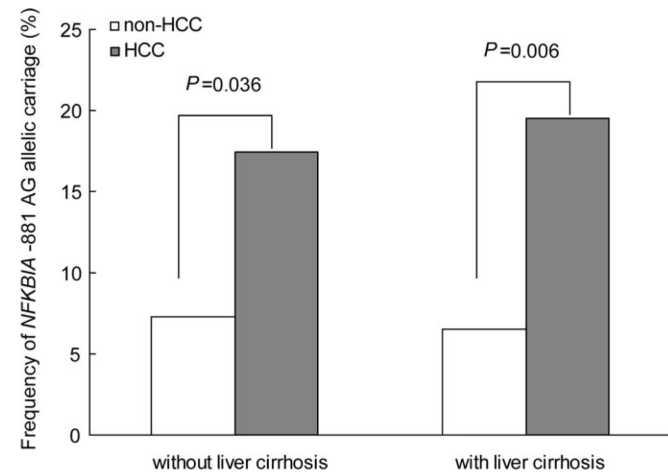

Fig. 1. *NFKBIA* −881AG allelic carriage was significantly associated with HCC as compared with non-HCC patients with HBV infection, regardless of LC state.

Table V. Multivariate regression analysis for factors independently associated with the risk of HCC

Variable	HCC versus healthy controls		HCC versus ASC		HCC versus (CHB+LC)	
	AOR (95% CI)	*P*	AOR (95% CI)	*P*	AOR (95% CI)	*P*
Age		NS	1.12 (1.09–1.15)	0.000		NS
Sex						
Female			1.00			
Male		NS	3.86 (2.23–6.69)	0.000		NS
NFKB1 −94						
ATTG1/ATTG1	1.00				1.00	
ATTG2 (ATTG1/ATTG2+ ATTG2/ATTG2)	2.11 (1.39–3.22)	0.000		NS	1.84 (1.05–3.24)	0.034
NFKBIA −519						
CC			1.00			
T (CT+TT)		NS	0.52 (0.28–0.96)	0.038		NS
NFKBIA −826						
CC			1.00			
T (CT+TT)		NS	2.74 (1.48–5.08)	0.001		NS
NFKBIA −881						
AA					1.00	
AG		NS		NS	3.53 (1.54–8.08)	0.003
HBV genotype						
B	ND		1.00		1.00	
C	ND	ND	10.43 (4.75–22.88)	0.000	4.01 (1.80–8.92)	0.001
HBeAg						
Negative	ND		1.00		1.00	
Positive	ND	ND	0.39 (0.23–0.65)	0.000	0.50 (0.30–0.85)	0.010
ALT (Log_{10} U/l)	ND	ND	ND		0.18 (0.10–0.33)	0.000

ND, no data; NS, no statistical significance.

NFKBIA −826T alleles specifically and affect IκBα expression. These speculations remain to be investigated in future. In this study, multivariate analysis showed that *NFKBIA* −519T allelic carriage was inversely associated with HCC risk, whereas *NFKBIA* −881G and *NFKBIA* −826T allelic carriages were independently associated with an increased risk of HCC, as compared with the HBV-infected non-HCC subjects with or without clinical symptoms (Table V). These results were consistent with the observation that the estimated haplotype frequency of *NFKBIA* promoter −881G−826T−519C was significantly higher in the patients with HCC than in the subjects without HCC (Table IV). The polymorphisms in *NFKBIA* promoter may also affect the binding of other unknown transcription factors. Alteration of one or more of these transcription factor binding sites could reduce the levels of IκBα expression, increase NK-κB activity and contribute to HBV-induced hepatocarcinogenesis.

This study also showed that no significant differences in the frequencies of polymorphisms in all loci were found between the patients with and without LC and *NFKBIA* −881G allelic carriage was associated with an increased risk of HCC regardless of LC status, indicating that the polymorphisms were not associated with cirrhosis. Since NF-κB plays important roles in the 'inflammation-fibrosis-cancer axis' (13), the role of IκBα on HBV-induced hepatocarcinogenesis may be not limited to NK-κB regulation.

HBV genotype C, elevated ALT, high viral load of HBV and HBeAg positivity have been demonstrated to increase the risk of HCC (4,5,35). In this study, however, elevated ALT, viral load and HBeAg were inversely associated with the development of HCC, even in age-, gender-matched groups. This difference is possibly due to different study design and different study population recruited. In prospective studies, ALT, HBV viral load and HBeAg have been proved to be risk factors for HCC because these data are usually collected at the beginning of cohort establishment. Because viral load and HBeAg change dramatically during HBV-induced hepatocarcinogenesis (6), it is difficult to draw the same conclusion in prevalence case–control study (36). This study recruited the hospitalized patients with CHB and those with LC whose clinical symptoms and/or syndromes were usually severe. HBV-infected patients with severe symptoms usually accompany with high ALT level and high viral load. Nevertheless, the associations of the polymorphisms on the development of HCC should be reliable in this case–control study because the polymorphisms are germ line transmitted and do not change during HBV-induced hepatocarcinogenesis.

In conclusion, *NFKB1* −94ATTG2, *NFKBIA* −826T and *NFKBIA* −881G allelic carriages and HBV genotype C were independently associated with an increased risk of HCC, whereas *NFKBIA* −519T allelic carriage was inversely associated with an increased risk of HCC. The haplotype −881G−826T−519C in *NFKBIA* promoter were associated with HBV-induced hepatocarcinogenesis. *NFKBIA* −826T and *NFKBIA* −881G allelic carriages were associated with the risk of HCC in the subjects infected with HBV genotype C.

Supplementary material

Supplementary Tables I–IV and Figures I and II can be found at http://carcin.oxfordjournals.org/

Funding

Ministry of Health, the People's Republic of China (2008ZX10002-15 to G.C.); Shanghai Science and Technology Committee (08XD14001 to G.C.); Shanghai Board of Health (08GWD02, 08GWZX0201, 08GWZX0101 to G.C.).

Acknowledgements

The authors thank Ms Liping Lin and Mr Xingxing Xu (Department of Epidemiology, Second Military Medical University, Shanghai, China) for their technical assistance and Dr. Yaoliang Pan (Physical Examination Center, the First Affiliated Hospital, Second Military Medical University, Shanghai, China) for his help in recruiting healthy controls.

Conflict of Interest Statement: None declared.

References

1. El-Serag,H.B. *et al*. (2007) Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology*, **132**, 2557–2576.
2. Yu,M.C. *et al*. (2000) Epidemiology of hepatocellular carcinoma. *Can. J. Gastroenterol.*, **14**, 703–709.
3. Chen,C.J. *et al*. (2006) Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA*, **295**, 65–73.
4. Yang,H.I. *et al*. (2002) Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N. Engl. J. Med.*, **347**, 168–174.
5. Yu,M.W. *et al*. (2005) Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J. Natl Cancer Inst.*, **97**, 265–272.
6. Yin,J. *et al*. (2008) Role of hepatitis B virus genotype mixture, subgenotypes C2 and B2 on hepatocellular carcinoma: compared with chronic hepatitis B and asymptomatic carrier state in the same area. *Carcinogenesis*, **29**, 1685–1694.
7. Liu,S. *et al*. (2009) Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J. Natl Cancer Inst.*, **101**, 1066–1082.
8. Pikarsky,E. *et al*. (2004) NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature*, **431**, 461–466.
9. Kim,S.H. *et al*. (2009) Involvement of NF-kappaB and AP-1 in COX-2 upregulation by human papillomavirus 16 E5 oncoprotein. *Carcinogenesis*, **30**, 753–757.
10. Karin,M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature*, **441**, 431–436.
11. Tai,D.I. *et al*. (2000) Constitutive activation of nuclear factor kappaB in hepatocellular carcinoma. *Cancer*, **89**, 2274–2281.
12. Berasain,C. *et al*. (2009) Inflammation and liver cancer: new molecular links. *Ann. N. Y. Acad. Sci.*, **1155**, 206–221.
13. Elsharkawy,A.M. *et al*. (2007) Nuclear factor-kappaB and the hepatic inflammation-fibrosis-cancer axis. *Hepatology*, **46**, 590–597.
14. Gilmore,T.D. (2003) The Re1/NF-kappa B/I kappa B signal transduction pathway and cancer. *Cancer Treat. Res.*, **115**, 241–265.
15. Whiteside,S.T. *et al*. (1997) I kappa B proteins: structure, function and regulation. *Semin. Cancer Biol.*, **8**, 75–82.
16. Kim,Y.J. *et al*. (2005) Single nucleotide polymorphisms associated with hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *Intervirology*, **48**, 10–15.
17. Yoon,Y.J. *et al*. (2008) MDM2 and p53 polymorphisms are associated with the development of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *Carcinogenesis*, **29**, 1192–1196.
18. Chang,E.T. *et al*. (2009) Polymorphic variation in NFKB1 and other aspirin-related genes and risk of Hodgkin lymphoma. *Cancer Epidemiol. Biomarkers Prev.*, **18**, 976–986.
19. Spink,C.F. *et al*. (2007) Haplotypic structure across the I kappa B alpha gene (NFKBIA) and association with multiple myeloma. *Cancer Lett.*, **246**, 92–99.
20. Curran,J.E. *et al*. (2002) Polymorphic variants of NFKB1 and its inhibitory protein NFKBIA, and their involvement in sporadic breast cancer. *Cancer Lett.*, **188**, 103–107.
21. Zhang,P. *et al*. (2009) A functional insertion/deletion polymorphism in the promoter region of the NFKB1 gene increases susceptibility for prostate cancer. *Cancer Genet. Cytogenet.*, **191**, 73–77.
22. Lo,S.S. *et al*. (2009) Functional polymorphism of NFKB1 promoter may correlate to the susceptibility of gastric cancer in aged patients. *Surgery*, **145**, 280–285.
23. Bu,H. *et al*. (2007) Importance of polymorphisms in NF-kappaB1 and NF-kappaBIalpha genes for melanoma risk, clinicopathological features and tumor progression in Swedish melanoma patients. *J. Cancer Res. Clin. Oncol.*, **133**, 859–866.
24. Gao,J. *et al*. (2007) Association of NFKBIA polymorphism with colorectal cancer risk and prognosis in Swedish and Chinese populations. *Scand. J. Gastroenterol.*, **42**, 345–350.
25. Kim,L.H. *et al*. (2003) Identification of variants in NFKBIA and association analysis with hepatocellular carcinoma risk among chronic HBV patients. *Hum. Mutat.*, **21**, 652–653.

26. Hung,C.H. *et al*. (2003) Correlation between ultrasonographic and pathologic diagnoses of hepatitis B and C virus-related cirrhosis. *J. Gastroenterol.*, **38**, 153–157.
27. Chen,J. *et al*. (2007) Improved multiplex-PCR to identify hepatitis B virus genotypes A-F and subgenotypes B1, B2, C1 and C2. *J. Clin. Virol.*, **38**, 238–243.
28. Ou,T.T. *et al*. (2008) IkappaBalpha promoter polymorphisms in patients with primary Sjogren's syndrome. *J. Clin. Immunol.*, **28**, 440–444.
29. Karban,A.S. *et al*. (2004) Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. *Hum. Mol. Genet.*, **13**, 35–45.
30. Jetten,A.M. (2009) Retinoid-related orphan receptors (RORs): critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nucl. Recept. Signal.*, **7**, e003.
31. Abdallah,A. *et al*. (2003) Inhibitor kappa B-alpha (IkappaB-alpha) promoter polymorphisms in UK and Dutch sarcoidosis. *Genes Immun.*, **4**, 450–4.
32. Hildt,E. *et al*. (1996) The hepatitis B virus large surface protein (LHBs) is a transcriptional activator. *Virology*, **225**, 235–239.
33. Weil,R. *et al*. (1999) Direct association and nuclear import of the hepatitis B virus X protein with the NF-kappaB inhibitor IkappaBalpha. *Mol. Cell. Biol.*, **19**, 6345–6354.
34. Muroyama,R. *et al*. (2006) Nucleotide change of codon 38 in the X gene of hepatitis B virus genotype C is associated with an increased risk of hepatocellular carcinoma. *J. Hepatol.*, **45**, 805–812.
35. Wu,C.F. *et al*. (2008) Long-term tracking of hepatitis B viral load and the relationship with risk for hepatocellular carcinoma in men. *Carcinogenesis*, **29**, 106–112.
36. Yuen,M.F. *et al*. (2004) Role of hepatitis B virus genotypes Ba and C, core promoter and precore mutations on hepatocellular carcinoma: a case control study. *Carcinogenesis*, **25**, 1593–1598.

Received July 6, 2009; revised August 14, 2009; accepted September 8, 2009