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, Chengzhong Li, Jun Zhao, Hongyang Wang and Guangwen Cao

Department of Epidemiology, Department of Infectious Diseases, the 1st Affiliated Hospital, Department of Hepatobiliary Surgery and 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 21 6078 7106
Email: gcao@smmu.edu.cn

Genetic predisposition of nuclear factor-kappa B (NF-kB)-signaling pathways linking inflammation to hepatit 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 IkappaBalpha (I\(\kappa\)B) 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 \(-\dagger94\)ATTG2 allele and GG allele in the 3'-untranslated region of NFKBIA were more prevalent in HCC patients than in the healthy controls. NFKBIA \(-881\)AG and NFKBIA \(-881\)AG allelic carriages were more prevalent in HCC patients than in the non-HCC subjects with HBV infection. The estimated haplotype frequency of NFKBIA promoter \(-881\)G-\(-826\)T-\(-519\)C 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 \(-881\)AG allelic carriages were only associated with HCC risk in the subjects with HBV genotype C. The association of NFKBIA \(-881\)AG 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 \(-\dagger94\)ATTG2, NFKBIA \(-886\)T, NFKBIA \(-881\)AG and HBV genotype C were independently associated with an increased risk of HCC. In conclusion, NFKB1 \(-\dagger94\)ATTG2 allele and haplotype \(-881\)G-\(-826\)T-\(-519\)C in NFKBIA promoter were associated with hepatocarcinogenesis. NFKBIA \(-881\)AG and NFKBIA \(-826\)T and \(-881\)AG 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 (HBsAg) 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-kB), a hallmark of inflammatory response that is frequently detected in tumors, may constitute a missing link between inflammation and cancer (8,9). NF-kB was originally identified as a nuclear factor specific to B cells bound to the B site of the k-light chain gene enhancer. NF-kB is a heterodimer in Rel family with five members: RelA, RelB, c-Rel, p50/105 (NF-kB1) and p52/p100, and the dimeric form of NF-kB p50/RelA is the most common form. NF-kB 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-kB and liver neoplastic progression and have demonstrated that NF-kB 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-kB is inactivated in the cytoplasm by binding to its inhibitors, IkB. When IkB proteins are phosphorylated and degraded, NF-kB is subsequently released and further translocated into the nucleus, where the gene transcription is initiated (14). The IkB family includes IkappaBalpha (I\(\kappa\)B\(\alpha\)), I\(\kappa\)B\(\beta\), I\(\kappa\)B\(\gamma\), I\(\kappa\)B\(\epsilon\), I\(\kappa\)B-R, Bcl-3, p100 and p105 that are all constitutively expressed except for I\(\kappa\)B\(\zeta\), which is inducibly expressed. I\(\kappa\)B\(\zeta\) is a classic form of the IkB 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-kB1 gene NFKB1 and I\(\kappa\)B\(\alpha\) 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; HBsAg, hepatitis B e antigen; HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; I\(\kappa\)B\(\alpha\), IkappaBalpha; LC, liver cirrhosis; NF-kB, nuclear factor-kappa B; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism; 3'-UTR, 3'-untranslated region; WT, wild type.
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 and third affiliated hospitals 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 ethnic 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 history of HCC or other cancer at diagnosis 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 4 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, gastric varices, liver 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 3000 g for 20 min and stored in a sterile tube at −80°C within 4 h of sample collection. Serological testing for hepatitis B surface antigen, HBcAg, alpha-fetoprotein 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 NFKBIA –94 indel ATTG (rs 28720239), NFKBIA 3′-UTR A/G (rs 5789859), NFKBIA –519 CT (rs 2233408), NFKBIA –826 CT (rs 2233406) and NFKBIA –881 A/G (rs 3138053) was conducted using PCR–restriction fragment length polymorphism method. Primers for NFKBIA –94 were 5′-TGGCCCAACAGTGTGTTATGTA-3′ and 5′-CTGGAGCCGGTAAAGGAG-3′, amplifying a fragment of 281 bps or 285 bps (23). Primers for NFKBIA 3′-UTR were 5′-GGCTGAAAGAACATGGACTTG-3′ and 5′-AGCACCTTACGAGGG-3′ which amplify a fragment of 424 bps (23). Primers for NFKBIA –519 were 5′-CTGCCTCATTACAAGCTCAGT-3′ and 5′-AGAGTTGAAGGATGGCC-3′, which amplify a fragment of 188 bps (24). Primers for NFKBIA –826 CT and –881 A/G were 5′-GTCCTTAAGGTCATACCTG-3′ and 5′-GATGATCGCATGACTA-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 (NFKBIA –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, Hamburg, Germany). The PCR product of NFKBIA –94 was digested with Van91 I (New England Biolabs, Ipswich, 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 of sample collection. The HBV-infected subjects were stratified into sex- or age-matched groups. In women, NFKBIA –94ATTG2, NFKBIA –826T and NFKBIA –881G allelic carriages were 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 was true for NFKBIA –826T (CT+TT) (26.2 versus 15.7%, AOR = 2.19, 95% CI: 1.29–3.71, P = 0.003). As compared with ASCs, NFKBIA 3′-UTR GG allelic carriage was significantly associated with an increased risk of HCC (OR = 4.02, 95% CI: 1.60–10.13, P = 0.006). The estimated haplotype frequencies were determined by the estimating haplotype-frequeny program (http://linkage.rocketefeller.edu/slof). 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 (2), 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 HBcAg seropositivities and ALT levels.

Genotype data of all loci in the healthy controls were confirmed to Hardy–Weinberg equilibrium (P > 0.05 for each). Genotype data of NFKBIA –94 ATTG, NFKBIA 3′-UTR and NFKBIA –881, 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, NFKBIA –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 –881 AG allele (18.8 versus 6.9%, AOR = 3.28, 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 –881 AG allele (18.8 versus 6.8%, AOR = 3.20, 95% CI: 1.63–6.28, P = 0.001). Interestingly, we also found that NFKBIA –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, NFKBIA –94ATTG2 and NFKBIA 3′-UTR GG alleles were more prevalent in the HBV-infected patients with HCC. NFKBIA –94ATTG2, NFKBIA –826T and NFKBIA –881G 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, NFKBIA –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
Polymorphisms (accession code) Alleles Healthy controls (N = 404) Non-HCC subjects with HBV infection (N = 482) HCC patients with HBV infection (N = 202) P value

Sex (male) 318 (78.7) 160 (57.1) 87 (79.1) 72 (78.3) 159 (78.7) 0.001
Age (year) 52.0 ± 9.61 43.7 ± 7.94 50.1 ± 12.28 50.5 ± 8.46 52.9 ± 9.76 0.000
Genotype B ND 87 (31.1) 22 (21.8) 15 (17.9) 11 (5.8) 0.000
C ND 180 (64.3) 79 (78.2) 69 (82.1) 175 (91.6) <0.01
Others ND 13 (4.6) ND ND 5 (2.6) ND
HBV Positive ND 129 (46.1) 40 (36.4) 40 (43.5) 48 (23.8) 0.018
Negative ND 151 (53.9) 70 (63.6) 52 (56.5) 154 (76.2) 0.000
HBV DNA (Log10 copies/ml) ND ND 4.70 ± 1.67 4.17 ± 1.35 3.92 ± 1.17 0.000
ALT (Log10 U/l) ND ND 2.09 ± 0.48 1.85 ± 0.49 1.69 ± 0.31 0.000

Data shown as N (%), except age, ALT and HBV DNA; ND, no data.

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 Healthy controls with HBV infection Healthy controls with HBV infection 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 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 (1.05–2.70) 1.62 (1.00–2.64)
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 0.07 0.00 0.00 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)
HWE P value 0.19 0.00 0.00 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 (2.5) 1 (0.5) 0.25 (0.03–2.00) 0.22 (0.03–1.71) 1.64 (0.62–4.34)
HWE P value 0.06 0.19 0.00 0.89 (0.58–1.38)

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)
HWE P value 0.68 0.00 0.00 1.67 (1.11–2.53)

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 ND ND ND ND ND ND
HWE P value 0.06 1.00 0.00 0.00

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.

Data were presented as n (%).

Significance probability determined by HWE test.
between the HCC patients with HBV infection and the non-HCC patients with HBV infection. \textit{NFKBIA} –881AG allelic carriage was significantly associated with an increased risk of HCC either in normal ALT group or in abnormal ALT group. \textit{NFKB1} –94ATTG2, \textit{NFKBIA} 3′-UTR and \textit{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 \textit{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, \textit{NFKBIA} –826T and \textit{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 \textit{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 the HBV patients with and without LC. The frequency of \textit{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 \textit{NFKBIA} –881AG (18.8 versus 6.5%, OR = 3.32, 95% CI: 1.35–8.17, \( P = 0.006 \)) and \textit{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). \textit{NFKB1} –94ATTG2 allelic carriage was independently associated with an increased risk of HCC, as compared with the healthy controls. Age, male, \textit{NFKBIA} –816T allelic carriage and HBV genotype C were independently associated with an increased risk of HCC, whereas \textit{NFKBIA} –519T allelic carriage was inversely associated with an increased risk of HCC, if ASCs were used as controls. \textit{NFKB1} –94ATTG2 allele, \textit{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. \textit{NFKB1} –94ATTG2 allelic carriage and \textit{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 \textit{Carcinogenesis} Online).

### Discussion

This study firstly demonstrated that the ATTG2 (ATTG1/ATTG2 + ATTG2/ATTG2) allelic carriage at –94 site of \textit{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 \textit{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 \textit{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). \textit{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 \textit{Carcinogenesis} Online). Based on these observations, we hypothesize that \textit{NFKB1} –94ATTG2 allelic carriage might play a role from HBV chronic infection to HBV-induced hepatocarcinogenesis. Insertion/deletion of ATTG at –94 site of \textit{NFKB1} promoter is a functional \textit{NFKB1} polymorphism. The ATTG insertion at the \textit{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 \textit{NFKB1} transcript and further increase in production of NF-κB protein. Thus, \textit{NFKB1} –94ATTG2 allelic carriage-associated up-regulation of NF-κB might play a role from chronic inflammation to hepatocarcinogenesis caused by HBV chronic infection.

In this study, \textit{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 \textit{NFKB1} and \textit{NFKBIA} promoters (supplementary Figure 2 is available at \textit{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

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>HBV genotype B</th>
<th>HBV genotype C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-HCC(^a)</td>
<td>HCC(^a)</td>
</tr>
<tr>
<td>\textit{NFKBIA} –94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATTG1/ATTG1</td>
<td>29 (24.8)</td>
<td>3 (27.3)</td>
</tr>
<tr>
<td>ATTG2 (ATTG1/ATTG2+ATTG2/ATTG2)</td>
<td>88 (75.2)</td>
<td>8 (72.7)</td>
</tr>
<tr>
<td>\textit{NFKBIA} 3′-UTR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>51 (41.1)</td>
<td>3 (27.3)</td>
</tr>
<tr>
<td>G (AG+GG)</td>
<td>73 (59.8)</td>
<td>8 (72.7)</td>
</tr>
<tr>
<td>\textit{NFKBIA} –519</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>101 (82.8)</td>
<td>11 (100.0)</td>
</tr>
<tr>
<td>T (CT+TT)</td>
<td>21 (17.2)</td>
<td>0</td>
</tr>
<tr>
<td>\textit{NFKBIA} –826</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>104 (83.9)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td>T (CT+TT)</td>
<td>20 (16.1)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>\textit{NFKBIA} –881</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>115 (92.7)</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>AG</td>
<td>9 (7.3)</td>
<td>1 (9.1)</td>
</tr>
</tbody>
</table>

\(^a\)Data were presented as n (%).

\(^b\)AOR, odd ratio adjusted by age and sex; ND, no data.

\(^p\)\( P = 0.024 \).

\(^q\)\( P = 0.000 \).
retinoic acid-related orphan receptor \(\alpha\) (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-\(\kappa\)B 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-\(\kappa\)B. Since IkB\(\alpha\) is an absolute requirement for normal termination of the NF-\(\kappa\)B response (15), \(NFKBIA\) –881G and –826T allelic carriages are therefore speculated to retard efficient IkB\(\alpha\) expression. \(NFKBIA\) –881G might alter the binding ability of retinoic acid-related orphan receptor \(\alpha\), a group of transcriptional factors with potential roles in osteoporosis, autoimmune diseases and cancer (30), and contribute to hepatocarcinogenesis. This study found that \(NFKBIA\) –881G and \(NFKBIA\) –826T 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-\(\kappa\)B (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-\(\kappa\)B by directly interacting with IkB\(\alpha\) at amino acids 249–253 of IkB\(\alpha\) 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-\(\kappa\)B-signaling pathways. Moreover, genotype C products, e.g. X protein, might be able to bind \(NFKBIA\) –881G and

<table>
<thead>
<tr>
<th>Table IV. The estimated haplotype frequencies of (NFKBIA) promoter polymorphisms in the HBV-infected subjects without HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NFKBIA) promoter haplotype</td>
</tr>
<tr>
<td>–881A–826C–519C</td>
</tr>
<tr>
<td>–881A–826C–519T</td>
</tr>
<tr>
<td>–881A–826T–519C</td>
</tr>
<tr>
<td>–881A–826T–519T</td>
</tr>
<tr>
<td>–881G–826C–519C</td>
</tr>
<tr>
<td>–881G–826C–519T</td>
</tr>
<tr>
<td>–881G–826T–519C</td>
</tr>
<tr>
<td>–881G–826T–519T</td>
</tr>
</tbody>
</table>

*a*OR and \(P\) value were not calculated due to small size of case number.

<table>
<thead>
<tr>
<th>Table V. Multivariate regression analysis for factors independently associated with the risk of HCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>(NFKB1) –94</td>
</tr>
<tr>
<td>ATTTG2 (ATTTG1/ATTTG2+ ATTTG2/ATTTG2)</td>
</tr>
<tr>
<td>(NFKBIA) –519</td>
</tr>
<tr>
<td>T (CT+TT)</td>
</tr>
<tr>
<td>(NFKBIA) –826</td>
</tr>
<tr>
<td>T (CT+TT)</td>
</tr>
<tr>
<td>(NFKBIA) –881</td>
</tr>
<tr>
<td>HBV genotype</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>HBBeAg</td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>ALT (Log10 U/l)</td>
</tr>
</tbody>
</table>

ND, no data; NS, no statistical significance.
NFKBIA – 826T alleles specifically and affect IκBz 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 IκBz 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 IκBz 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

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