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Genetic Variation in the *NBS1* Gene Is Associated with Hepatic Cancer Risk in a Chinese Population

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NBS1 plays important roles in maintaining genomic stability as a key DNA repair protein in the homologous recombination repair pathway and as a signal modifier in the intra-S phase checkpoint. We hypothesized that polymorphisms of *NBS1* are associated with hepatic cancer (HCC) risk. The *NBS1* rs1805794 C/G polymorphism has been frequently studied in some cancers with discordant results, but its association with HCC has not been investigated. Moreover, studies of the 3'UTR variant rs2735383 have not touched upon HCC. This study examined the contribution of these two polymorphisms to the risk of developing HCC in a Chinese population. *NBS1* genotypes were determined in 865 HCC patients and 900 controls and the associations with risk of HCC were estimated by logistic regression. Compared with the rs1805794 GG genotype, the GC genotype had a significantly increased risk of HCC (adjusted odds ratios [OR] = 1.41; 95% confidence interval [CI] = 1.11–1.80), the CC carriers had a further increased risk of HCC (OR = 2.27; 95% CI = 1.68–3.14), and there was a trend for an allele dose effect on risk of HCC ($p < 0.001$). Also, we found that the risk effect of rs1805794 CC + CG was more pronounced in HCC patients that drank (OR = 2.28, 95% CI = 1.55–3.29 for drinkers; OR = 1.31, 95% CI = 1.00–1.77 for nondrinkers). However, there was no significant difference in genotype frequencies of rs2735383 G/C site between cases and controls. These findings suggest that rs1805794 C/G polymorphism in *NBS1* may be a genetic modifier for developing HCC.

Introduction

Liver cancer, also known as hepatic cancer (HCC), is reported at less than 30 cases per 100,000 inhabitants in most of the world, with higher rates observed in parts of Africa and eastern Asia (Parkin, 2001; Shibuya *et al.*, 2002). Unlike many other common malignancies, HCC occurs largely within the realms of known risk factors. They include chronic hepatitis B and C infection, cirrhosis of the liver, diabetes mellitus, smoking, alcohol consumption, and exposure to toxins such as certain types of fungi, vinyl chloride, and anabolic steroids (El-Serag and Mason, 2000). These environmental factors can potentially cause DNA damage and then lead to a higher risk of HCC. However, only a small portion of exposed individuals develops HCC, which implies influence of host factors on individual susceptibility. Therefore, it will surely be sensible to identify the at-risk populations so that they may be targeted for prevention and early detection. These interindividual differences in susceptibility to HCC may be attributed to genetic polymorphisms in some critical genes, including those involved in DNA repair.

A DNA double-strand break (DSB) is a relatively dangerous form of DNA lesion and, without successful repair, will lead to genomic instability and probably cancer (Gollin, 2005). There exist two distinct and complementary pathways for DSB repair: homologous recombination and nonhomologous end joining (Matsuura *et al.*, 2004). NBS1 plays a role in both pathways as a component of the MRN (a protein complex consisting of MRE11, RAD50 and NBS1) complex, which accounts for the recognition and signaling of DNA DSBs in the initial step of both pathways (Kobayashi, 2004). NBS1 acts either by modulating the DNA damage signal sensing by recruiting PIKK protein family members ATM, ATR, and probably DNA-PKcs to the DNA damage sites and activating their functions or by recruiting MREl1 and RAD50 to the proximity of DSBs by an interaction with H2AX through the BRCT/FHA domain at its C-terminus (Zhang *et al.*, 2006).

The *NBS1* gene is located in chromosome 8q21, spans over 50 kb, contains 16 exons, and encodes the 754-amino acid protein (Varon *et al.*, 1998; Kobayashi *et al.*, 2004). There are 84 common polymorphisms (with a minor allele frequency of >5%) in *NBS1* according to the Environmental Genome Project. A missense mutation rs1805794 C/G has been frequently studied in different tumors with discordant results (Lu *et al.*, 2009); however, its association with HCC has not been studied. Another polymorphism (rs2735383 C > G) in

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the 3'UTR of *NBS1* may change certain microRNA binding sites (http://snpinfo.niehs.nih.gov/), which may affect translation of *NBS1* mRNA and possibly influence the function of NBS1 (Zheng *et al.*, 2011b).Our study aims at finding the correlation between the *NBS1* rs1805794 and rs2735383 polymorphisms and risk of HCC.

Materials and Methods

Study subjects

All of the subjects in this study were ethnically homogenous Han Chinese. Patients with newly diagnosed HCC ($n = 865$) were consecutively recruited from April 2006 to March 2011, at The HuaiAn No.1 Hospital Affiliated to NanJing Medical University (HuaiAn). All the eligible patients diagnosed at the hospital during the study period were recruited, with a response rate of 91%. Patients were from HuaiAn city and its surrounding regions, and there were no age, stage, and histology restrictions. The clinical features of the patients are summarized in Table 1. Population controls ($n = 900$) were cancer-free people living around HuaiAn region; they were selected from a nutritional survey conducted in the same period as the cases were collected (Xiao *et al.*, 2010). The selection criteria included no history of cancer and frequency-matching of cases with respect to sex and age. Mean age was 48 years for case patients, and 47 years for control subjects ($p = 0.531$). At recruitment, informed consent was obtained from each subject. This study was approved by the Medical Ethics Committee of NanJing Medical University.

Genotyping analysis

Genotypes of the DNA samples were analyzed using PCR-RFLP methods. Genotyping was performed without knowledge of subjects' case/control status. A 30% masked, random sample of cases and controls was tested twice by different persons and the results were concordant for all masked duplicate sets.

Table 1. Demographic Characteristics Among Hepatic Cancer Cases and Controls

	Cases (n = *865*)		*Controls* (n = *900*)	
Characteristics	*No.*	*%*	*No.*	*%*
Age (years)				
≤48	526	60.8	549	61.0
>48	339	39.2	351	39.0
Sex				
Male	683	79.0	694	77.1
Female	182	21.0	206	22.9
Smoking status				
Smoker	518	59.9	461	51.2
Nonsmoker	347	40.1	439	48.8
Drinking status				
Drinker	407	47.1	233	25.9
Nondrinker	458	52.9	667	74.1
Family history of cancer				
Positive	95	11.0	70	7.8
Negative	770	89.0	830	92.2
HBV infection status				
HBsAg (+)	719	83.1	117	13.0
HBsAg (−)	146	16.9	783	87.0
Stage				
I	502	58.0		
II	312	36.1		
III	37	4.3		
IV	14	1.6		

The primers designed to amplify the target DNA fragment containing the rs1805794 C/G polymorphism were 5' ACCT TTCAATTTGTGGAGGC 3' (forward) and 5' GCAGTGAC CAAAGACCGACT 3' (reverse), which produced a fragment of 289 bp. Primers designed for rs2735383 were 5' TGCAG TGTTCTACACCTTGCTT 3' (forward) and 5' AGGTGACA TCTGCACCACTG 3' (reverse), producing a fragment of 156 bp. PCR was performed in 25 μL reaction systems containing 5 mM $MgCl_2$, 0.1 mM deoxynucleotide triphosphates, 3.0 units of *Taq* polymerase (Fermentas), and the manufacturer's buffer. The PCR consisted of an initial melting step at 94°C for 5 min, 35 cycles of 94°C for 45 s, annealing (62.9°C for rs1805794, 61.0°C for rs2735383) for 45 s, and 72°C for 45 s, and a final extension step of 72°C for 7 min. There was a native *Hin*f1 (Takara) site in the amplified fragment containing the rs1805794 polymorphism. After digestion by *Hin*f1 at 37°C for 3 h, the major G allele produced a single 289 bp band, whereas the minor C allele produced two bands, 30 bp and 259 bp. The two bands were separated by 3% agarose gel electrophoresis. The amplified fragment containing the rs2735383 polymorphism was digested by *Cvi*QI (NEB) at 25°C for 3 h. The major C allele produced two bands, 57 bp and 99 bp, whereas the minor G allele produced a single 156 bp band. The genotype identified by PCR-RFLP was confirmed by DNA sequencing.

Statistical analysis

Two-sided χ^2 tests were used to assess differences in the distributions of age, sex, and family history of HCC between cases and controls as well as the genotypes. The Hardy–Weinberg equilibrium (HWE) was tested by a goodness-of-fit χ^2 test to compare the expected genotype frequencies ($p^2 + 2pq + q^2 = 1$)with observed genotype frequencies in cancer-free controls. The association between case–control status and each single-nucleotide polymorphism (SNP), measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI), was estimated using an unconditional logistic regression model, with and without adjustment for age, sex, and family history of cancer. Logistic regression modeling was also used for the trend test. The data were further stratified by age, sex, smoking and drinking status, family history, hepatitis B virus (HBV) infection, and clinical stage of HCC to evaluate the stratum variable-related ORs among the *NBS1* genotypes. The 2LD program and the PROC ALLELE statistical procedure in SAS/Genetics (SAS Institute, Inc., Cary, NC) software were used to detect the linkage disequilibrium (LD) of the two SNPs. The statistical power was calculated using the PS Software (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). The tests were all two-sided and analyzed using the SAS software (version 9.1; SAS Institute, Inc.). A p-value of < 0.05 was considered statistically significant.

Results

The genotype results are shown in Table 2. The allele frequencies for rs1805794 C and rs2735383 C were, respectively, 0.426 and 0.412 in controls and 0.529 and 0.418 in

Table 2. Genotype and Allele Frequencies of *NBS1* Among Cases and Controls and Their Association with Risk of Hepatic Cancer

Genotype	*Cases* (n = *865*) *No. (%)*	*Controls* (n = *900*) *No. (%)*	*Crude OR*	*OR*[a] *(95% CI)*
rs1805794 (G/C)				
GG	194 (22.4)	288 (32.0)	1.00 (reference)	1.00 (reference)
GC	427 (49.4)	457 (50.8)	1.39 (1.10–1.75)	1.41 (1.11–1.80)
CC	244 (28.2)	155 (17.2)	2.34 (1.77–3.09)	2.27 (1.68–3.14)
C allele frequency	52.9	42.6		
rs2735383 (G/C)				
GG	295 (34.1)	304 (33.8)	1.00 (reference)	1.00 (reference)
GC	417 (48.2)	461 (50.1)	0.93 (0.75–1.15)	0.90 (0.68–1.21)
CC	153 (17.7)	145 (16.1)	1.09 (0.82–1.45)	1.07 (0.72–1.49)
C allele frequency	41.8	41.2		

[a]OR was adjusted by age, sex, smoking status, alcohol use, family history of cancer, and HBV infection status in a logistic regression model. CI, confidence interval; OR, odds ratio.

cases. The observed genotype frequencies of rs1805794 and rs2735383 polymorphisms in healthy controls did not deviate from those expected from the HWE ($\chi^2 = 1.266$, $p = 0.261$ for rs1805749; and $\chi^2 = 1.871$, $p = 0.171$ for rs2735383). The frequencies for the −8360 GG, GC, and CC genotypes in the cases differed significantly from those in controls ($p < 0.001$). Multivariate analysis found that the rs1805794 CC genotype carriers had a 2.3-fold elevated risk for developing HCC (crude OR = 2.34, 95% CI: 1.77–3.09; adjusted OR = 2.27, 95% CI: 1.68–3.14) compared with the noncarriers. However, the difference in genotype frequencies at the rs2735383 C/G site between cases and controls was not significant ($p = 0.747$).

The LD analyses in controls showed that the linkage between two loci were relatively weak ($D' = 0.114$ and $r^2 = 0.071$), suggesting each may have an independent effect on the risk of HCC. The risk of HCC related to *NBS1* genotypes were further examined with stratification by age, sex, smoking status, drinking status, family history, HBV infection, and clinical stage. As shown in Table 3, we observed a significant difference in the genotype frequency between drinking patients and nondrinking patients ($p = 0.03$). Compared with the GG genotype, the C allele carriers (CC + CG) had 2.28-fold increased risk for developing HCC in drinking patients. As for the nondrinking patients, the increased risk

Table 3. Stratification Analysis of the *NBS1* rs1805794 C > G Genotypes by Selected Variables in Hepatic Cancer Patients and Controls

	Patients (n = *865*)		*Controls* (n = *900*)		*Adjusted OR (95% CI)*[a]	
	GG No. (%)	*GC + CC No. (%)*	*GG No. (%)*	*GC + CC No. (%)*	*GC + CC vs. CC*	p-*Value*[b]
Age (years)						
≤ 48	120 (13.9)	406 (46.9)	167 (18.6)	382 (42.4)	1.45 (1.10–1.99)	
> 48	74 (8.6)	265 (30.6)	121 (13.4)	230 (25.6)	1.92 (1.37–2.71)	0.28
Sex						
Male	146 (16.9)	537 (62.1)	221 (24.6)	473 (52.6)	1.78 (1.32–2.31)	
Female	48 (5.5)	134 (15.5)	67 (7.4)	139 (15.4)	1.36 (0.80–2.20)	0.34
Smoking status						
Smoker	104 (12.0)	414 (47.9)	141 (15.7)	320 (35.6)	1.82 (1.35–2.39)	
Nonsmoker	90 (10.4)	257 (29.7)	147 (16.3)	292 (32.4)	1.40 (1.01–1.97)	0.36
Drinking status						
Drinker	77 (8.9)	330 (38.2)	79 (8.8)	154 (17.1)	2.28 (1.55–3.29)	
Nondrinker	117 (13.5)	341 (39.4)	209 (23.2)	458 (50.9)	1.31 (1.00–1.77)	**0.03**
Family history of cancer						
Positive	23 (2.7)	72 (4.3)	23 (2.6)	47 (5.2)	1.48 (0.75–3.32)	
Negative	171 (19.8)	599 (69.2)	265 (29.4)	565 (62.8)	1.69 (1.35–2.09)	0.85
HBV infection status						
HBsAg (+)	157 (18.1)	562 (65.0)	35 (3.9)	82 (9.1)	1.64 (0.98–2.51)	
HBsAg (−)	37 (4.3)	109 (12.6)	253 (28.1)	530 (58.9)	1.46 (0.90–2.17)	0.78
Stage						
I	107 (12.4)	395 (45.7)	288 (32.0)	612 (68.0)	1.84 (1.54–2.37)	
II + III + IV	87 (10.0)	276 (31.9)	288 (32.0)	612 (68.0)	1.47 (1.15–2.06)	0.94

[a]ORs were adjusted for age, sex, smoking status, alcohol use, family history of cancer, and HBV infection status in a logistic regression model.
[b]*p*-Value of the test for homogeneity between stratum-related ORs for *NBS1* gene (rs1805794 GC + CC vs. GG genotypes).
Bold-faced *p*-value is considered to be significant.

is only 1.31-fold. However, there were no differences in risk among age, sex, smoking status, family history, HBV infection, and clinical stage of HCC.

Discussion

Associations between HCC and *NBS1* polymorphisms have not been detected in any population using case–control studies. In this molecular epidemiological study, we sought to identify genetic factors that confer individual susceptibility to HCC. Our results obtained by analyzing 865 HCC patients and 900 controls showed that the functional polymorphism rs1805794 C/G in *NBS1* was associated with increased risk for developing HCC, in an allele–dose response manner. However, there was no significant difference in the susceptibility to HCC between different genotypes of the loci rs2735383. Consumption of alcohol is a major cause of cirrhosis (Li *et al.*, 1999), which may finally lead to liver cancer. The high risk effect of rs1805794 CC+CG was more pronounced in drinking HCC patients, demonstrating a role of this polymorphism as a relevant genetic factor in the major cause of death in HCC patients.

In African and Asian countries where the incidence of HCC is reported to be as high as 20–150 cases per 100,000 people, underlying cirrhosis occurs in more than half of those diagnosed with HCC, making cirrhosis the most common risk factor for HCC (Parkin *et al.*, 1984; Di Bisceglie *et al.*, 1988; Macdonald, 2001). Similarly, approximately 80% of patients demonstrate underlying cirrhosis, with chronic ethanol consumption being considered a major risk factor in North America and Western Europe, where the incidence of HCC is 1.5 to 3 cases per 100,0000 (Di Bisceglie *et al.*, 1988; Macdonald, 2001). Some epidemiologic studies have found that ethanol-dependent HCC patients with viral hepatitis, or before exposure to aflatoxin, have a poorer prognosis than that of patients who abstain from ethanol, demonstrating a vital role of alcohol consumption in HCC development (Voigt, 2005). After alcohol ingestion, ethanol metabolism in the liver by alcohol dehydrogenase leads to the generation of acetaldehyde and free radicals, which can bind rapidly to numerous cellular targets, including components of cell signaling pathways and DNA, resulting in DNA and cell damage (Crabb *et al.*, 1987). Chronic alcohol abuse will cause DNA and cell damage in the liver, possibly leading to cirrhosis and even liver cancer (McKillop and Schrum, 2005). As NBS1 can act in the repair of DNA damage caused by ethanol metabolism, we can deduce a tight association between NBS1, alcohol ingestion, and HCC, which is consistent with our stratified result that high risk effect of rs1805794 CC+CG was more pronounced in ever-drinking HCC patients.

Mutation of the *NBS1* gene is responsible for a rare disease called Nijmegen breakage syndrome, in which a defective response to DNA damage is associated with chromosomal instability and a strong predisposition to develop malignancy (Weemaes *et al.*, 1981; Antoccia *et al.*, 2006). Experimental evidence that *NBS1* heterozygosity predisposes cells to malignancy comes from a study in which the mouse homolog of the human *NBS1* gene was disrupted in mice (Dumon-Jones *et al.*, 2003). $NBS1^{+/-}$ mice showed a significantly elevated risk of some spontaneous solid tumors including liver, prostate and mammary glands, and gonad malignancy. These data provide a tight relationship between *NBS1* heterozygosity and increased cancer risk.

The human NBS1 protein contains three functional regions: the N-terminus (amino acids 1–183), a central region (amino acids 278–343), and the C-terminus (amino acids 665–693) (D'Amours and Jackson, 2002). The N-terminal region contains an FHA domain (amino acids 24–109) and two breast cancer carboxy-terminus (BRCT) domain (BRCT1: amino acids 114–183; BRCT2: amino acids 221–291). The rs1805794 C/G polymorphism is located in the BRCT domain, through which NBS1 can interact with BRCA1 to form the BRCA1-associated genome surveillence complex (BASC) (Kobayashi *et al.*, 2002). The rs1805794 C/G polymorphism causes a nonsynonymous mutation of 185 amino acid from Glu to Gln, which may possibly change the function of the NBS1 protein and then probably the protein–protein interaction of NBS1 and BRCA1.

This *NBS1* polymorphism has been frequently studied in different cancer types and ethnicities; however, the results are not accordant. The rs1805794 C/G variant genotypes (G/C, C/C) are associated with an increased risk of several cancers, such as acute lymphoblastic leukemia (Jiang *et al.*, 2011), nasopharyngeal carcinoma (Zheng *et al.*, 2011a), and lung cancer (Ryk *et al.*, 2006). However, a recent study found no association between this variation and breast cancer risk in a European population (Kuschel *et al.*, 2002). Interestingly, the rs1805794 GG genotype was demonstrated to be associated with risk of lung cancer instead of the GC or CC genotype in a Chinese study (Lan *et al.*, 2005). Our present study also supported that *NBS1* rs1805794 C/G variant genotypes are related to increased risk of HCC.

There are some limitations in our present study. The sample size may not be large enough to detect gene–environment interactions. Moreover, selection bias and/or systematic error may have occurred, because the cases are from our hospital and the controls are from the community. However, the fact that the genotype frequencies among controls could fit the Hardy–Weinberg disequilibrium law suggested the randomness of subject selection; we have achieved a more than 90% study power (two-sided test, $\alpha = 0.05$) to detect an OR of 1.63 for the rs1805794 CC+CG genotypes (which occurred at a frequency of 68.0% in the controls) compared with the rs1805794 GG genotype, suggesting that this finding is noteworthy.

In conclusion, our study indicated that compared with carriers of *NBS1* rs1805794 GG genotype, the carriers of rs1805794 GC+CC genotypes had increased risk of HCC in a Chinese population. Moreover, the phenomenon is more obvious in ever-drinking patients. To our best knowledge, our study first demonstrated a significant association between the *NBS1* rs1805794 C/T polymorphism and risk of HCC. Larger, preferably population-based, case–control studies, as well as well-designed mechanistic studies, are warranted.

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Disclosure Statement

No competing financial interests exist.

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