Promoter polymorphisms of DNA methyltransferase 3B and risk of hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide. Epigenetic changes in gene expression, including DNA methylation and histone modifications, may contribute to the development of HCC. Polymorphisms of the *DNA methyltransferase 3B (DNMT3B)* gene may affect the activity of this enzyme and increase the susceptibility to several types of cancer, including HCC. To confirm this hypothesis, we investigated the association between single-nucleotide polymorphisms-149C>T (rs2424913) and -579G>T (rs1569686) in the promoter region of DNMT3B and the risk of HCC. *DNMT* single-nucleotide polymorphisms (SNPs) were genotyped by polymerase chain reaction-restriction fragment length polymorphism in 108 HCC patients and 240 healthy controls matched for age, gender and ethnicity. The DNMT3B-149 TT genotype was not significantly associated with an increased risk of HCC. The frequency of DNMT3B-149C was 0.46% in HCC patients and 1.39% in healthy individuals, whereas the frequency of DNMT3B-579G was 8.33% in HCC patients and 10.42% in healthy individuals. No significant differences were observed in the genotype or allelic distribution between HCC patients and controls. In conclusion, DNMT3B-149C>T and -579G>T polymorphisms are not significantly associated with an increased risk of HCC. These results demonstrated that these particular SNPs may not be used as biomarkers to predict susceptibility to HCC.

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Abbreviations: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; OR, odds ratio; CI, confidence interval; HCC, hepatocellular carcinoma; SNP, single-nucleotide polymorphism

Key words: hepatocellular carcinoma, DNA methyltransferase 3B, single-nucleotide polymorphism, susceptibility

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer, the third leading cause of cancer-related mortality worldwide and the second leading cause of cancer-related mortality in China (1,2). The cause of HCC is a complex interplay between numerous factors (3). Accumulating evidence in molecular genetics indicate that single-nucleotide polymorphisms (SNPs) in immune response-, angiogenesis- and tumorigenesis-related genes are associated with susceptibility to HCC (4-7). Recent advances in genome-wide association studies also identified new susceptibility loci for HCC (8,9), which may help elucidate the underlying mechanism of genetic variations in the development of HCC.

Recent investigations on gene methylation utilizing genome- wide techniques revealed that a large number of genes exhibit aberrant DNA methylation profiles in cancer (10). These changes may be used to stratify cancer subtypes and predict cancer outcomes (11,12). DNA methyltransferase 3B (DNMT3B) plays a crucial role in embryonic development and aberrant DNA methylation in carcinogenesis. Polymorphisms of the *DNMT3B* gene may regulate gene expression, affect enzymatic activity and contribute to the susceptibility to cancer. It was previously demonstrated that certain SNPs in the *DNMT3B* gene may affect DNMT3B activity on DNA methylation, thereby modulating the susceptibility to cancer (13). The DNMT3B-149C>T polymorphism confers a 30% increase in promoter activity *in vitro* (14,15). DNMT3B promoter polymorphisms were previously reported to be associated with the risk of lung, colorectal and head and neck cancer (14,16). Based on the evidence mentioned above, we used a candidate gene strategy and screened variations in the *DNMT3B* gene to investigate the association between these variations and HCC susceptibility in a Chinese population.

Materials and methods

Subjects. The patients and control subjects in this study were recruited between September, 2006 and June, 2008 from the Jiangsu Tumor Hospital in the Jiangsu province. Cases and controls were matched by age and gender. The characteristics of the cases and controls are presented in detail in

Table I. Characteristics of the study population.

A, DNMT3B-149C>T

Variables	HCC cases (%) (n=108)	Controls (%) (n=216)	P-value
Age (years)			
<50	35 (32.41)	56 (25.93)	0.221
≥50	73 (67.59)	160 (74.07)	
Gender			
Male	85 (78.70)	152 (70.37)	0.111
Female	23 (21.3)	64 (29.63)	

B, DNMT3B-579G>T

Variables	HCC cases (%) (n=114)	Controls (%) (n=240)	P-value
Age (years)			
<60	62 (54.39)	122 (50.83)	0.532
≥60	52 (45.61)	118 (49.17)	
Gender			
Male	90 (78.95)	170 (70.83)	0.106
Female	24 (21.05)	70 (29.17)	

DNMT3B, DNA methyltransferase 3B; HCC, hepatocellular carcinoma.

Table I A and B. Samples were obtained following written consent and analyzed anonymously. This study was performed with the approval of the Medical Ethics Committee of the Medical School of Southeast University.

DNA extraction. Venous blood samples (5 ml) were collected from HCC patients and healthy control subjects in EDTA vacuum tubes. Genomic DNA was extracted from white blood cells within 1 week after sample collection by proteinase K digestion, as previously described (17).

DNMT3B SNP genotyping. The *DNMT3B*-149C>T and -579G>T SNPs were analyzed by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs). The PCR was performed in a 25-μl volume containing 100 ng genomic DNA, 2.5 μl 10X PCR buffer, 2.0 mM $MgCl_2$, 0.1 mM dNTPs (mixture of dATP, dTTP, dCTP and dGTP), 10 pmol of each primer (*DNMT3B*-149C>T primer: forward 5'-TGCTGTGACAGGCAGAGCAG-3' and reverse 5'-GGTAGCCGGGAACTCCACGG-3'; *DNMT3B*-579G>T primer: forward 5'-GAGGTCTCATTATGCCTAGG-3' and reverse 5'-GGGAGCTCACCTTCTAGAAA-3'), 1 unit of *Taq* DNA polymerase (Biocolor BioScience and Technology Co., Shanghai, China). The PCR cycle conditions consisted of an initial denaturation step at 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, at 62˚C for 30 sec (-149C>T) or at 57˚C for 30 sec (-579 G>T) and at 72˚C for 30 sec, with a final elongation step at 72˚C for 5 min. The 380-bp (*DNMT3B*-149C>T) fragment was digested with *Bln*I for 5 min at 37˚C, the digested products were separated on 2.0% agarose gel and the PCR-RFLP bands were visualized under UV light with ethidium bromide staining. The T allele has a *Bln*I restriction site that resulted in two bands (207- and 173-bp) in the homozygous genotype, the heterozygote exhibited three bands (380-, 207- and 173-bp), whereas the variant C allele produced only one band representing the entire 380-bp fragment. The -579G>T polymorphism was determined by PCR-RFLPs. *Pvu*II was used to detect the G-T transition. The DNMT3B T/T genotype was expected to exhibit two DNA bands at the positions of 132 and 93 bp, whereas the G/G genotype was expected to exhibit a single band (at 225 bp) and the heterozygote was expected to exhibit 3 bands (at 225, 132 and 93 bp). PCR was conducted and the results were evaluated without knowledge of the case-control status. The samples were successfully genotyped. For genotyping quality control, 5% of the samples were randomly selected and directly sequenced to obtain 100% identical results.

Statistical analysis. Data were analyzed with SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Patients and controls were compared using the Student's t-test for continuous variables and the Chi-square (χ^2) test for categorical variables. Allele and genotype frequencies between control subjects and HCC patients were obtained using the Chi-square test and the standard goodness-of-fit test was used to assess the Hardy-Weinberg equilibrium. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The demographics of the cases and controls enrolled in this study are provided in Tables I A and B. No significant differences were observed in age and gender distribution between

Table II. DNMT3B-149C>T genotype and allele frequency and distribution.

A, DNMT3B-149C>T genotype and allele frequency among cases and controls

	HCC (n=108)		Control subjects (n=216)			
Genotype/allele	No.	(%)	No.	(%)	Crude OR (95% CI)	P-value[a]
Genotype						
TT	107	99.07	210	97.22	1	
CT	1	0.93	6	2.78	0.327 (0.039-2.752)	0.499[b]
CC	0[c]	0.00	0	0.00	-	
Allele						
C	1	0.46	6	1.39	1	
T	215	99.54	426	98.61	3.028 (0.362-25.313)	0.502[d]

B, Distribution of-149C>T DNMT3B genotypes and association with age and gender in HCC cases

	Genotype			Allele	
Groups	CC (%)	CT (%)	TT (%)	T (%)	P-value[e]
Age (years)	0 (0.00)	1 (0.93)	107 (99.07)	99.07	
<50	0 (0.00)	0 (0.00)	35 (32.40)	100.00	1.000[f]
≥50	0 (0.00)	1 (10.93)	72 (66.67)	98.63	
Gender					
Male	0 (0.00)	0 (0.00)	85 (78.70)	100.00	0.213[g]
Female	0 (0.00)	1 (0.93)	22 (20.37)	95.65	

[a]Chi-square (χ^2) test; [b]HCC cases vs. controls; [c]CC genotype not detected in HCC patients and control subjects; [d]T allele vs. C allele; [e]Chi-square (χ^2) test; [f]frequency of the T allele in individuals aged <50 vs. ≥50 years; [g]frequency of the T allele in male vs. female individuals. DNMT3B, DNA methyltransferase 3B; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

cases and controls, suggesting that the matching based on these two variables was adequate. There was no evidence of deviation from the Hardy-Weinberg equilibrium among the cases or controls. The genotypic and allelic frequencies of DNMT3B-149C>T are provided in Table II A. No significant differences were found in the genotypic and allelic frequencies between the two groups. Subsequently, we stratified the HCC cases by age and gender and no significantly different frequencies of -149C>T were observed (Table II B).

As regards DNMT3B-579G>T, the distributions of the polymorphism genotypes among the HCC cases (TT, 83.33%; GT, 16.67%; and GG, 0%) exhibited no significant differences from those among the controls (TT, 80.83%; GT, 17.50%; and GG, 1.67%; P>0.05). No significant differences in allelic frequencies were observed between the two groups (Table III A). The stratified analysis revealed no significant difference in the distribution of the -579G>T genotype according to age or gender in the HCC patients (Table III B).

Discussion

HCC is one of the most common malignant tumors, has a poor survival rate and is particularly prevalent in China, as well as in the rest of Asia. The identification of biomarkers for the early diagnosis and accurate prognosis of HCC is crucial for improving patient survival. Numerous tissue and serum markers associated with invasiveness, metastasis, recurrence and potential prognostic significance have been identified in HCC thus far (18,19).

In mammals, DNMT3B plays an important role in carcinogenesis and DNMT SNPs are important indicators of the genetic susceptibility to cancer development. Therefore, genetic polymorphism assays have been used to investigate the aetiology of malignant diseases (20). The significance of DNMT3B promoter genetic polymorphisms for tumorigenesis has been extensively investigated, although no consensus was reached due to the differences in tumors, ethnic groups, geographical areas or sample size between individual studies. A meta-analysis demonstrated that the -149C>T polymorphism of DNMT3B was not associated with colorectal cancer risk (21). It has also been demonstrated that the -579G>T SNP of the DNMT3B promoter decreased the susceptibility to lung and colon cancer (22,23), suggesting that the DNMT3B promoter -579G>T polymorphism may be used as a genetic risk factor to evaluate the population susceptible to tumor development. Khorshied and El-Ghamrawy (24) and Zhao *et al* (25) demonstrated that the DNMT3B -579G>T polymorphism represents a novel genetic risk factor for idiopathic thrombocytopenic

Table III. DNMT3B-579G>T genotype and allele frequency and distribution.

A, DNMT3B-579G>T genotype and allele frequency among cases and controls

	HCC (n=114)		Control subjects (n=240)			
Genotype/allele	No.	(%)	No.	(%)	Crude OR (95% CI)	P-value[a]
Genotype						
GT (ref.)	19	16.67	42	17.50	1	
TT	95	83.33	194	80.83	1.082 (0.597-1.962)	0.794[b]
GG	0[c]	-	4	1.67	1.452 (1.227-1.719)	0.448
Allele						
G	19	8.33	50	10.42	1.000	
T	209	91.67	430	89.58	1.279 (0.735-2.225)	0.382[d]

B, Distribution of DNMT3B-579G>T genotypes and allele frequencies among HCC cases and association with age and gender

	Genotype			Allele	
Groups	GG (%)	GT (%)	TT (%)	T (%)	P-value[e]
Total	0 (0.00)	19 (16.67)	95 (83.33)	91.67	
Age(years)					
<50	0 (0.00)	12 (10.53)	50 (43.86)	90.32	0.423[f]
≥50	0 (0.00)	7 (6.14)	45 (39.47)	93.27	
Gender					
Male	0 (0.00)	14 (12.28)	76 (66.67)	92.22	0.919[g]
Female	0 (0.00)	5 (4.39)	19 (16.67)	89.58	

[a]Chi-square (χ^2) test; [b]HCC cases vs. controls; [c]GG genotype not detected in HCC patients; [d]G allele vs. T allele; [e]Chi-square (χ^2) test; [f]frequency of the T allele among individuals aged <50 vs. ≥50 years; [g]frequency of the T allele in male vs. female individuals. DNMT3B, DNA methyltransferase 3B; HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

purpura. However, it was also reported that there is no association between the -579G>T polymorphism and head and neck squamous cell carcinoma or esophageal carcinoma (26,27). A meta-analysis for -149C>T and -579G>T suggested that there was no evidence that individuals carrying the variant genotypes (CC+CT) have an increased risk of cancer compared to those carrying the wild homozygote TT genotype, although -579G>T was associated with a significantly decreased risk of cancer (28).

However, little is known about the association between the polymorphisms of the DNMT3B promoter and HCC. Therefore, we conducted a case-control study to investigate the association of the DNMT3B promoter -149C>T and -579G>T polymorphisms with genetic susceptibility to HCC and evaluate whether these SNPs may act as biomarkers to predict the susceptibility to HCC in a Chinese population. Wu and Lin (29) reported that DNMT3B-149C>T polymorphism was not associated with an increased risk of HCC in a Chinese population and Ezzikouri *et al* (30) demonstrated that the DNMT3B-149C>T genotype was not significantly associated with an increased risk of HCC in a Moroccan population. Our results are consistent with those of Wu and Lin (29) and Ezzikouri *et al* (30), with insignificant differences in genotype and allele frequency distribution between HCC patients and control subjects suggesting that these two particular SNPs are not associated with the risk of HCC in the study population.

To the best of our knowledge, this is the first study to investigate the association of DNMT3B-149C>T and -579G>T polymorphisms with genetic susceptibility to HCC. Our results suggest that the -149C>T and -579G>T polymorphisms do not affect the risk for HCC, at least in the Chinese population. This finding may provide valuable insight into hepatocellular carcinogenesis. However, a larger population has to be investigated and evaluated to elucidate the contribution of DNMT3B SNPs to the susceptibility to HCC in Chinese individuals. Furthermore, to elucidate the true effects of DNMT3B polymorphisms on determining the pathogenesis of HCC, investigations of other variants of DNMT3B (including promoter and coding regions) and their effects on the biological functions of DNMTs are also required.

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