

# A Functional Insertion/Deletion Polymorphism in the Proximal Promoter of *CD3G* Is Associated with Susceptibility for Hepatocellular Carcinoma in Chinese Population

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Hepatocellular carcinoma (HCC) represents the most common primary malignancy of the liver with a worldwide increasing incidence. Although the risk factors for HCC are well characterized, the molecular mechanisms responsible for malignant transformation of hepatocytes are not well understood. In this study, a case-control study including 291 HCC patients and 294 healthy controls was conducted to investigate the association between HCC susceptibility and with a 4-bp insertion/deletion polymorphism (rs66465034) in the proximal promoter of *CD3G*. Logistic regression analysis showed that the heterozygote and the homozygote 4-bp ins/ins confer a significantly increased risk of HCC after controlling for other covariates (adjusted odds ratio [OR]=1.51, 95% confidence interval [C.I.] 1.01–2.27,  $p=0.040$ ; OR=1.71, 95% C.I. 1.07–2.89,  $p=0.025$ , respectively). Carriage of the 4-bp insertion allele was associated with a greatly increased risk of developing the disease (OR=1.30, 95% C.I. 1.02–1.64,  $p=0.027$ ). Moreover, hepatitis B virus (HBV) stratification analysis showed that the differences between cases and controls were more obvious in HBV-positive than in the HBV-negative population, suggesting a possible role of this polymorphism in the immune regulation during HBV infection. Further, luciferase-based transient transfection assays revealed that rs66465034 can affect promoter activity of *CD3G*, indicating its possible functional significance. Our data suggested that common genetic polymorphisms in *CD3G* may influence HCC risk in Chinese population. Considering the relative small sample size, replication in other populations with larger sample size and further functional analysis are required for fully understanding the roles of *CD3G* polymorphisms in predisposition for HCC.

## Introduction

**H**EPATOCELLULAR CARCINOMA (HCC) represents the most common primary malignancy of the liver with a worldwide increasing incidence (Rahbari *et al.*, 2011). It ranks third in annual cancer mortality rates and has the highest annual fatality ratio of any tumor. Carcinogenesis of HCC is a multistep and complex process, and it is widely recognized that multiple risk factors, such as hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, and exposure to dietary aflatoxin, contribute to hepatocarcinogenesis. Chronic HBV infection is by far the most important risk factor for HCC in humans, accounting for 55% of cases worldwide and 80% or more of those in China and sub-Saharan Africa, the highest incidence regions of HCC (Kew, 2010). Although the risk factors for HCC are well characterized, the molecular mechanisms responsible for malignant transformation of

hepatocytes are not well understood. Epidemiological studies have provided evidences that genetic factor is one of important variants for mediating an individual's susceptibility to HCC (Mínguez *et al.*, 2009). Recently, significant progress has been made in our understanding of the genetic predisposition to HCC, thanks to the implementation of genome-wide association studies (GWASs) that are currently routinely being used to identify common polymorphisms that underlie disease susceptibility in the large population (Zhang *et al.*, 2010). However, the genetic basis of susceptibility to HCC is still poorly understood and early detection of HCC is seldom available because of the lack of reliable biological markers.

One recent GWAS has identified genetic variations in the "antigen presentation and processing" pathway as being highly significantly associated with HCC, which suggests that polymorphisms affecting the immune response and

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differences in T-cell receptor (TCR) processing are involved in HCC susceptibility (Clifford *et al.*, 2010). *CD3G* gene encodes CD3-gamma polypeptide, which together with CD3-epsilon, -delta, and -zeta, and the TCR alpha/beta and gamma/delta heterodimers, forms the TCR-CD3 complex. This complex plays an important role in coupling antigen recognition to several intracellular signal-transduction pathways. The CD3 gamma chain has long been considered to mediate targeting to lysosomes for degradation (Dietrich *et al.*, 1994; Dietrich and Geisler, 1998). Defects in this gene are associated with T cell immunodeficiency (Guy and Vignali, 2009). Further, it has been proposed as one of the important candidate genes for cancer development due to its pivotal roles in TCR signaling pathway (Landi *et al.*, 2008). The "GGCT" 4-bp insertion/deletion polymorphism (rs66465034) is located in the proximal promoter of *CD3G*, where is the potential source of polymorphism affecting gene expression. The aim of present study was to investigate whether rs66465034 is associated with the risk of HCC in a Chinese population and to assess the possible functional significance of the polymorphism.

## Materials and Methods

### Study populations

The case-control study was performed on genomic DNA extracted from peripheral blood of newly diagnosed incident HCC cases together with controls matched for sex and age after obtaining informed consent. All subjects recruited were unrelated ethnic Han Chinese. The case series comprised of 291 HCC patients diagnosed, hospitalized, and treated in the affiliated hospitals of Soochow University from 2005 to 2008. A total of 294 controls were cancer-free individuals selected from a community nutritional survey that was conducted in the same regions during the same period as recruitment of cancer patients. The diagnosis of the cases, the inclusion and exclusion criteria for the cases and controls, and the definition of smokers and drinkers were described in detail previously (Gao *et al.*, 2009; Chen *et al.*, 2010; He *et al.*, 2010). Briefly, the diagnosis of these patients was confirmed by a pathological examination combined with positive imaging (magnetic resonance imaging and/or computerized tomography). Patients were excluded who were suffering from (1) primary or secondary biliary cirrhosis or Budd-Chiari syndrome, (2) autoimmune hepatitis or toxic hepatitis, (3) recurrence of HCC, (4) other tumors except HCC, and (5) liver disease due to parasitosis, diabetes, fatty liver, metabolism disorders, and severe cardiovascular diseases. Tumor stages were determined according to a modified American Joint Committee on Cancer and international union against cancer standard. The subjects who smoked more than two cigarettes per day for more than 1 year were classified as smokers. Others were defined as non-smokers. Subjects were considered as alcohol drinkers, if they drank at least once per week. All participants were negative for antibodies to HCV, hepatitis D virus, or HIV. The design of the study was approved by the Ethical Committee of Soochow University.

### DNA extraction and genotyping

A Chelex method was used for extracting genomic DNA of blood samples (Walsh *et al.*, 1991). DNA fragments con-

taining the polymorphism were amplified with the forward primer 5'-TGGGTTCTTGCCTTCTCTCAA-3' and reverse primer 5'-CATGTCAGTCTCTGTCCCTCCG-3'. Polymerase chain reaction (PCR) was performed in a total volume of 20  $\mu$ L, including 2.0  $\mu$ L 10 $\times$ PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 0.5 mM each primer, 50 ng of genomic DNA, and 1.0 U of Taq DNA polymerase. The PCR conditions were 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, with a final elongation at 72°C for 5 min. The PCR products were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining (Allen *et al.*, 1989). The allelic discrimination of rs66465034 was determined by the numbers and the positions of the band on the gels. The 4-bp deletion allele yielded a 132-bp band and the insertion allele yielded a 136-bp band. To validate the genotyping method, we analyzed 20 randomly selected DNA samples by both direct sequencing and PCR method; the concurrence rate of these two methods was 100%, suggesting that the PCR method is reliable. Genotyping was performed without knowledge of the case or control status. A 10% random sample was tested in duplicate by different researchers, and the reproducibility was 100%.

### Construction of reporter plasmids and luciferase assays

The promoter region (from -467 to +32 bp relative to the translation start site) containing either insertion or deletion allele of rs66465034 was amplified with forward primer 5'-ACAAGATCTCCTGAATGAAGGCCTGGACTGAGGTGG-3' and reverse primer 5'-CTAAAGCTTGTGTCAGTCTCTGTCCTCCGGCAAAGCG-3' from two homozygous human genomic DNA samples. The PCR products were separated in agarose gel and extracted, purified, and cloned with TA cloning kit (Promega). Finally, the above-prepared fragments were subcloned into pGL3-basic vector using restriction enzymes *Bgl*III and *Hind*III. The resulting constructs were verified by sequencing.

HepG2 and sk-Hep-1 cells were maintained in Dulbecco's modified Eagle's medium with high glucose (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 50  $\mu$ g/mL streptomycin (Gibco) at 37°C in an incubator supplemented with 5% CO<sub>2</sub>. Cells were seeded at 1  $\times$  10<sup>5</sup> cells per well in 24-well plates (BD Biosciences). Sixteen hours after the plating, cells were transfected by lipofectamin 2000 according to manufacturer's suggestion. In each well, 500 ng of constructed pGL3-basic vector and 50 ng of pRL-TK vector (Promega) were co-transfected into cells. The un-constructed pGL3-basic vector was added as negative control. After transfection for 24 h, cells were harvested by the addition of 100  $\mu$ L passive lysis buffer. Firefly luciferase activities in cell lysate were measured with the Dual Luciferase assay system (Promega) in TD-20/20 luminometer (Turner Biosystems) and were normalized with the Renilla luciferase activities. Six replicates for each group and the experiments were repeated at least three times.

### Statistical analysis

Hardy-Weinberg equilibrium was assessed using a goodness-of-fit  $\chi^2$  test for biallelic markers. The adjusted odds ratios (ORs) with their 95% confidence intervals (C.I.s)

TABLE 1. DEMOGRAPHIC CHARACTERISTICS AMONG HEPATOCELLULAR CARCINOMA CASES AND CONTROLS

Characteristics	Cases		Controls		p-Value
	N=291	Frequencies (%)	N=294	Frequencies (%)	
Age (mean ± SD)		50.9 ± 8.7		51.1 ± 9.2	0.89 <sup>a</sup>
Gender					
Male	190	65.3	197	67.0	0.66 <sup>b</sup>
Female	101	34.7	97	33.0	
Smoking status					
Smokers	92	31.6	91	31.0	0.86 <sup>b</sup>
Nonsmokers	199	68.4	203	69.0	
Drinking status					
Drinker	123	42.3	120	40.8	0.72 <sup>b</sup>
Nondrinker	168	57.7	174	59.2	
Tumor stages					
Ia + Ib	202	69.4			
IIa + IIb	56	19.2			
IIIa + IIIb	33	11.3			
HBsAg, N (%)					
Positive	216	74.2	34	11.6	<0.0001 <sup>b</sup>
Negative	75	25.8	260	88.4	

Cases indicate patients with HCC and controls are non-cancerous patients.

<sup>a</sup>Two-sided two-sample *t*-test between cases and controls.

<sup>b</sup> $\chi^2$  test for differences between cases and controls.

SD, standard deviation; HCC, hepatocellular carcinoma.

of the association between polymorphism and HCC risk were estimated by multiple logistic regression models after controlling for sex, age, smoking status, drinking status, tumor stage, and HBV infection. In all cases, homozygosity for the most common allele (i.e., del/del) was used as the reference category. As HBV infection was one of the major risk factors for HCC, a stratified analysis by HBV infection status was performed using binary logistic regression model. Student's *t* test was used to examine the differences in luciferase reporter gene expression. The statistical analyses were implemented in Statistic Analysis System software (version 8.0; SAS Institute).  $p < 0.05$  was used as the criterion of statistical significance. The statistical power was calculated by PS software (<http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize>) and Epi Info software (<http://open.epi.com>).

## Results

The statistical analysis of demographic characteristics of the 291 HCC patients and 294 controls was summarized in Table 1. There were no statistically significant differences between cases and controls in terms of the frequency distribution of sex, age, smoking, and drinking status. As expected, HBV infection was a significant risk factor for HCC. About 74.2% of the cases were HBsAg positive, which was significantly higher than that of the controls (11.6%,  $p < 0.0001$ ). PAGE analysis of the polymorphism was shown in Figure 1. Genotype distributions had no deviation from Hardy-Weinberg equilibrium in both case and control groups ( $p = 0.088$  and  $p = 0.591$ , respectively). We use a C.I. at 95% to obtain a power of 0.65 by Epi Info software and an OR of 1.6 with  $\alpha$  set at 0.05 to obtain a power of 0.66 by PS software.

Our results showed that rs66465034 was significantly associated with HCC susceptibility, at both the allele and genotype levels (Table 2). In the co-dominant model, compared

with the reference (del/del), we found that the heterozygote and homozygote ins/ins of rs66465034 were associated with a significantly increased risk of HCC after controlling for other covariates, such as age, sex, drinking status, smoking status, and HBV infection (adjusted OR = 1.51, 95% C.I. 1.01–2.27,  $p = 0.040$ ; OR = 1.71, 95% C.I. 1.07–2.89,  $p = 0.025$ , respectively). In the dominant model (ins/ins + ins/del vs. del/del), significant associations were also observed between cases and controls (adjusted OR = 1.56, 95% C.I. 1.07–2.27,  $p = 0.018$ ). Meanwhile, frequencies of 4-bp deletion or insertion allele were significantly different between HCC and control groups. In the additive model, each additional copy of the 4-bp insertion allele was associated with a 30% increased risk of developing the disease (OR = 1.30, 95% C.I. 1.02–1.64,  $p = 0.027$ ). Further, based on HBV stratification analysis, the overall trend is that the differences between cases and controls were more obvious in HBV-positive than in the HBV-negative population (Table 3).

To further investigate whether rs66465034 would likely affect the level of transcriptional activity of *CD3G*, two luciferase reporter gene constructs were generated by PCR,

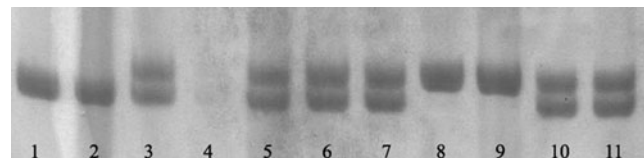


FIG. 1. Polyacrylamide gel electrophoresis analysis of 4-bp insertion/deletion *CD3G* polymorphism. The deletion allele corresponds to the 132-bp fragment, and the insertion allele corresponds to the 136-bp fragment. Lanes 1, 2: homozygous (deletion/deletion); lanes 3, 5–7, 10, 11: heterozygous (insertion/deletion); lanes 8, 9: homozygous (insertion/insertion); lane 4, negative control.

TABLE 2. GENOTYPIC AND ALLELIC FREQUENCIES OF rs66465034 IN CASES AND CONTROLS, AND RISK OF HEPATOCELLULAR CARCINOMA

Genotype/allele	Cases (n)	%	Controls (n)	%	OR <sup>a</sup> (95% CI)	p-Value
del/del	66	22.7	92	31.0	1.00 (Reference)	—
ins/del	160	55.0	149	50.7	1.51 (1.01–2.27)	0.040
ins/ins	65	22.3	53	18.0	1.71 (1.07–2.89)	0.025
<i>p</i> <sub>trend</sub>						0.022
del	292	50.2	333	56.6	1.00 (Reference)	—
ins	290	49.8	255	43.4	1.30 (1.02–1.64)	0.027

<sup>a</sup>Adjusted for sex, age, smoking status, drinking status, HBV infection, and tumor stage. OR, odds ratio; CI, confidence interval; HBV, hepatitis B virus.

and they were used to transiently transfect HCC cell lines. As shown in Figure 2, we found that CD3G promoter containing insertion allele drove a 1.48–1.52-fold increased reporter expression compared with the deletion allele containing counterpart in HepG2 and sk-Hep-1 cells.

**Discussion**

To the best of our knowledge, this is the first molecular epidemiological study to investigate the associations of CD3G polymorphism with risk of HCC in Chinese population. The genotyping of 291 HCC patients and 294 healthy control individuals showed significant associations with the 4-bp insertion/deletion variant within proximal promoter of CD3G with HCC susceptibility. Moreover, using *in vitro* assays, we found that the insertion variant allele significantly increased the transcription activity of the CD3G gene compared with the deletion allele.

Promoter sequences are potential sources of polymorphism affecting gene expression. In the current study, the luciferase-based transient transfection system is used to examine the effect of this polymorphism on the promoter activity. Significant difference is observed between two constructs concerning firefly luciferase activities. Therefore, it is possible that increased spacing by a 4-bp insertion may create flexibility in the DNA chain, facilitating the bindings of transcription factors, thus increasing the CD3G promoter activity.

The immune system plays a pivotal role in the development of HCC as chronic inflammation is one of the most important causes of HCC (Moradpour and Blum, 2005). Chronic hepatitis is characterized by an inefficient T cell response unable to completely clear HBV or HCV from the liver, which consequently sustains continuous cycles of low-level cell destruction (Guidotti and Chisari, 2006.). A three-

step process of “hepatitis (liver inflammation)–liver fibrosis/cirrhosis–HCC” is believed to be involved in hepatocarcinogenesis although how fibrosis promotes HCC remains unexplained (Elsharkawy and Mann, 2007). It has been suggested that host genetics factors are associated with response to hepatitis B vaccine (Davila *et al.*, 2010). Of particular interest, the association between rs66465034 and HCC susceptibility was preferentially observed for HBV-positive patients in the present study. During HBV infection, T cells require an antigen receptor-driven signal to become activated, proliferate, and exert their effector function. These events are mediated by the mature TCR complex in which the CD3 complex plays a key role in transmitting signals after TCR engagement (Wange and Samelson, 1996). It has been demonstrated that T cell responses were reduced or abrogated in T cells derived from CD3-gamma null-mutant mice, probably because of decreased expression levels of the mature TCR complex lacking CD3-gamma (Haks *et al.*, 2001). Moreover, CD3 proteins are dispensable for NK cell development (Wang *et al.*, 1999), which has been suggested to be involved in the early, non-specific immune response to clear HBV virus (Li *et al.*, 2011). Therefore, it is possible that polymorphisms within CD3G may influence its expression levels which in turn disturb CD3 complex assembling.

On the other hand, host immunity can either protect or promote tumor growth by the predominance and activation of certain subsets of immune cells (Fatourou and Koskinas, 2009). It has been suggested that antigens, such as alpha-fetoprotein (AFP) and glypican 3, which are highly expressed in HCC, are potential targets for T-cell responses (Capurro *et al.*, 2003; Butterfield *et al.*, 2007). Several studies have come to the conclusion that cytotoxic T-cell infiltration of the tumors is indicative of a better survival, whereas the predominance of suppressor cells is associated with a worse

TABLE 3. LOGISTIC REGRESSION ANALYSES FOR THE ASSOCIATION BETWEEN rs66465034 AND RISK OF HEPATOCELLULAR CARCINOMA IN HEPATITIS B VIRUS POSITIVE AND NEGATIVE GROUPS

Genotype	HBV positive					HBV negative				
	Cases	%	Controls	%	OR <sup>a</sup> (95% C.I.)	Cases	%	Controls	%	OR <sup>a</sup> (95% CI)
del/del	40	18.5	14	41.2	1.00 (Reference)	17	22.7	81	31.2	1.00 (Reference)
ins/del	111	51.4	13	38.2	2.98 (1.21–7.46)	41	54.7	132	50.8	1.49 (0.78–2.95)
ins/ins	65	30.1	7	20.6	3.25 (1.11–9.84)	17	22.7	47	18.1	1.74 (0.79–3.97)
<i>p</i> <sub>trend</sub>					<i>p</i> = 0.014					<i>p</i> = 0.148

<sup>a</sup>Adjusted for sex, age, smoking status, drinking status, and tumor stage.



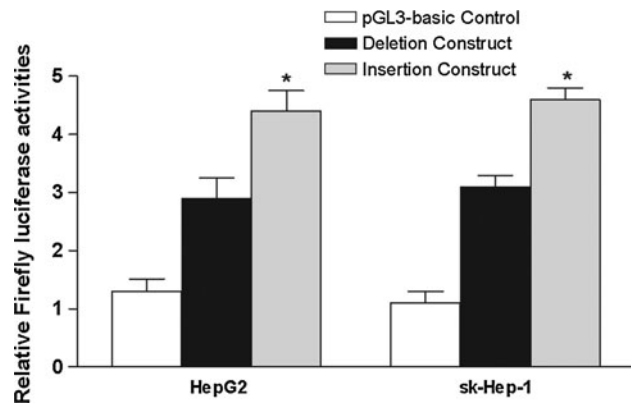


FIG. 2. Luciferase expression of two constructs in HepG2 and sk-Hep-1 cells co-transfected with pRL-TK to standardize transfection efficiency. \* $p < 0.01$  compared with deletion construct.

outcome and lower survival rates (Mizukoshi *et al.*, 2006; Gao *et al.*, 2007). In line of this, aberrant CD3-gamma expression potentially due to polymorphisms residing in the promoter region may contribute to host immunity against HCC.

Taken together, our data suggest that common genetic polymorphisms in *CD3G* may influence HCC risk in Chinese population. However, the presence of this HCC-associated polymorphism does not immediately imply that this polymorphism is causative since there are such possibilities that another genetic variation linked to rs66465034 is the true causal mutation driving the association. Finally, we have to acknowledge that the statistical power for the current study is not completely adequate because of relative small sample size, especially for stratification analysis, which would prevent us to effectively detect the association between HCC incidence and *CD3G* indel polymorphism. Replications in other populations with larger sample size and further functional analysis are required for fully understanding the roles of *CD3G* polymorphisms in predisposition for HCC.

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### Disclosure Statement

No competing financial interests exist.

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