Influence of *Angiotensin I-Converting Enzyme* Gene Polymorphism on Hepatocellular Carcinoma Risk in China

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Growing evidence suggests that the *angiotensin-converting enzyme* (ACE) and *endothelial nitric oxide synthase* (eNOS) genes are associated with risk in a wide range of cancers. The objective of this study was to examine whether two DNA polymorphisms at the ACE insertion/deletion (I/D) and the variable number of tandem repeats in NOS intron 4 (4a/4b) were linked to the risk of developing hepatocellular carcinoma (HCC) in a Chinese population. The polymorphisms at ACE I/D and eNOS 4a/4b were genotyped in 293 HCC patients and 384 healthy control subjects using polymerase chain reaction. The frequencies of the D allele ($p=0.003$, OR=0.72, 95% CI=0.58–0.90) in the ACE gene of HCC patients were significantly different from the healthy controls, and a significantly decreased HCC risk was associated with the DD genotype in both the recessive ($p<0.001$, OR=0.19, 95% CI=0.11–0.34) and codominant models ($p<0.001$, OR=0.26, 95% CI=0.14–0.48). This study provided evidence that the ACE I/D polymorphism is associated with HCC, indicating that the ACE I/D polymorphism contributes to HCC progression in the Chinese population.

Introduction

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignancies in the world and frequently occurs in Southeast Asia, especially in China. It ranks second and accounts for ~42.5% of all malignancies worldwide (Tang, 2001). The HCC incidence is variable worldwide; it is ~150 cases per 100,000 individuals in sub-Saharan Africa and Southeast Asia (Yoshiji *et al.*, 2002). The carcinogenesis of HCC is a complex, multistep process that is associated with various risk factors, including chronic hepatitis B virus (HBV) and hepatitis C virus infections, a family history of HCC, cirrhosis, exposure to carcinogens, excessive alcohol consumption, and cigarette smoking (El-Serag and Rudolph, 2007; Gomaa *et al.*, 2008). Although many individuals are exposed to these risk factors, HCC develops in only a small group of exposed people, suggesting that genetic factors might contribute to the carcinogenic mechanism. Complex interactions between many genetic and environmental factors might be the major cause of HCC (Sumbul *et al.*, 2012).

The *angiotensin-converting enzyme* (ACE) is a major component of the *renin-angiotensin system* (RAS) and plays a crucial role in the regulation of circulatory homeostasis. The human ACE gene is located on chromosome 17q23 (Zintzaras *et al.*, 2008) and contains a polymorphism based

on the presence (insertion [I]) or absence (deletion [D]) of a 287-base pair (bp) nonsense DNA domain within an intron (Rigat *et al.*, 1990; Rigat *et al.*, 1992), resulting in three genotypes (DD and II homozygotes and DI heterozygotes). Previous studies showed that genetic variants of ACE, especially the DD genotype, might increase the risk of the tumorigenesis in several cancers (Medeiros *et al.*, 2004; Yaren *et al.*, 2006; Carl-McGrath *et al.*, 2007). Recent studies determined that plasma ACE levels were highest in subjects with the ACE DD genotype, lowest in the II genotype, and intermediate in ID heterozygotes, suggesting that the ACE polymorphism is associated with plasma ACE levels (Rigat *et al.*, 1990; Zintzaras *et al.*, 2008). Moreover, a recent study has suggested that *angiotensin II* (AT-II)-induced angiogenesis contributes to liver fibrogenesis, carcinogenesis, and HCC growth (Yoshiji *et al.*, 2002). Considering the role of ACE, which converts *angiotensin I* into AT-II, we hypothesized that the I/D polymorphism may play an important role in HCC.

Nitric oxide (NO) is a gaseous-free radical that has an impact on multiple physiologic processes, including vasodilatation, neuronal transmission, smooth muscle relaxation, immunity, and carcinogenesis (Bredt, 1999; Xu *et al.*, 2002). Recently, studies have implicated NO in the pathogenesis of HCC and have suggested that it plays a protective role in

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HCC progression in an animal model of HCC (Goh and Beatrice, 2009, "Nitric Oxide in Liver Cancer," Kent Ridge: National University of Singapore, PhD thesis). NO is produced from L-arginine by *endothelial constitutive NO synthase (eNOS)*. *eNOS* is encoded by a gene located on chromosome 7q35–36 and is expressed in the endothelium (Nadaud *et al.*, 1994). Three isoforms of this enzyme have been identified: *inducible NO synthase*, *brain NO synthase*, and *endothelial cell NO synthase (ecNOS)*. Previous studies found that *ecNOS* is widely expressed in various tumor tissues and regulates bone marrow endothelial progenitor cell mobilization, endothelial cell damage, and homing. Furthermore, it also participates in the formation of blood vessels through NO generation, which can affect the growth and metastasis of tumors (Nadaud *et al.*, 1994). Two alleles have been identified in intron 4 of the *eNOS* gene: the larger allele (4b) consists of five tandem 27-bp repeats, and the smaller one (4a) has four repeats. Thus, individuals carry one of the genotypes 4b/4b, 4a/4b, or 4a/4a (Wang *et al.*, 1999; Yilmaz *et al.*, 2009). The 4a allele in *eNOS* polymorphisms has been reported to be associated with a number of disorders, including ischemic heart disease, diabetic nephropathy, and coronary artery disease (Neugebauer *et al.*, 2000; Casas *et al.*, 2004; Rao *et al.*, 2005). Previous studies have also demonstrated that *eNOS* 4a/4b polymorphisms could increase susceptibility in patients with prostate cancer and colon cancer (Medeiros *et al.*, 2002; Yeh *et al.*, 2009).

Because *ACE* and *eNOS* participate in the pathology of carcinomas, including HCC (Haswell-Elkins *et al.*, 1994; Mirvish, 1995; Murphey *et al.*, 2000; Yoshiji *et al.*, 2002; Suzuki *et al.*, 2003; Deshayes and Nahmias, 2005; Goh and Beatrice, 2009), we performed this study to determine whether these polymorphisms (*ACE* I/D and *eNOS* 4a/4b) were potential factors in the susceptibility to HCC.

Materials and Methods

Subjects and samples

HCC patients were pathologically confirmed and consecutively recruited between June 2008 and October 2010 in the West China Hospital of Sichuan University. The HCC diag-

nosis was based on α -fetoprotein elevation (>400 ng/mL) combined with an imaging examination (magnetic resonance imaging or computed tomography), and the patients were proven to be free of any other cancers. HCC was histologically confirmed in sonography-guided fine-needle biopsy specimens from all patients. Patients who had any other types of liver disease, such as autoimmune hepatitis, toxic hepatitis, or primary biliary cirrhosis, were excluded. Blood samples were collected before the initial treatment. Clinical characteristics, including gender, age, family history of HCC, serum Alpha-fetoprotein (AFP) levels, and HBV serological markers (hepatitis B surface antigen [HBsAg]), were collected. The control group was recruited from patients attending routine health surveys. The selection criteria for the control subjects included no medical history of any cancer or other serious diseases, especially liver diseases. All subjects were genetically unrelated ethnic Han Chinese living in the Sichuan Province of southwestern China. All individuals gave their consent before participating in the study, which was approved by the Ethics Committee of the West China Hospital of Sichuan University.

A total of 293 HCC patients and 384 control cases were included in this study. There were no significant differences between the two groups in terms of gender or age distribution, which suggested that matching the subjects based on these variables was adequate. The male-to-female ratio of HCC patients was 6:1, with a median age of 52 years. Among the patients, 219 were HBsAg-positive, and 27 had family histories of HCC. The characteristics of all subjects are summarized in Table 1.

Genotyping

Blood samples were collected in sterile tubes with the EDTA- Na_2 anticoagulant and stored at -20°C . Genomic DNA was extracted from the stored blood using a commercial extraction kit (Bioteke Corporation, Beijing, China) according to the manufacturer's instructions. The *ACE* and *eNOS* polymorphisms were identified based on polymerase chain reaction (PCR) amplification of the respective fragments. The primer sequences and amplification conditions were previously reported (Medeiros *et al.*, 2004; Yeh *et al.*, 2009).

TABLE 1. CHARACTERISTICS OF THE HEPATOCELLULAR CARCINOMA PATIENTS AND CONTROLS

Characteristics	Cases		Controls		OR (95% CI)	χ^2	p
	n = 293	%	n = 384	%			
Gender(n)							
Male	250	85.3	308	80.2	1		
Female	43	14.7	76	19.8	0.70 (0.46–1.05)	3.0	0.08
Age (years)							
Mean $\pm$ SD	51.8 $\pm$ 12.7		48.2 $\pm$ 15.8				
Family history of hcc							
Yes	27						
No	266						
HBV serological markers							
HBs Ag(+)	219						
HBs Ag(–)	74						

n corresponds to the number of individuals.

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen.

TABLE 2. INFORMATION OF SELECTED SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN HCC PATIENTS AND CONTROLS

Gene	Allele	Cases (%)	Controls (%)	OR (95% CI)	X ²	p
ACE	I	332 (57.4)	379 (49.3)	1.00 (reference)	8.66	0.003
	D	246 (42.6)	389 (50.7)	0.72 (0.58–0.90)		
eNOS	4b	521 (88.9)	670 (87.2)	1.00 (reference)	0.87	0.35
	4a	65 (11.1)	98 (12.8)	0.85 (0.61–1.19)		

n corresponds to the number of individuals.

Boldfaced values indicate a significant difference at the 5% level.

ACE, angiotensin-converting enzyme; eNOS, endothelial nitric oxide synthase.

The PCR products were distinguished with 6% polyacrylamide gel electrophoresis and visualized with silver staining to determine the genotypes. The PCR products of the two loci with different genotypes were confirmed with the direct sequencing method. To confirm the accuracy of the method, we randomly selected ~10% of the samples and repeated the experiments, and the results were 100% concordant.

Statistical analysis

The genotype and allele frequencies of the two loci were compared between HCC patients and control subjects with the chi-square test, and odds ratios (ORs) and 95% confidence intervals (CIs) were used to evaluate the relative risk conferred by a particular allele or genotype. Genotypic association tests in a case-control pattern assuming codominant, dominant, or recessive genetic models were performed. All data were analyzed with SPSS software for Windows, version 17.0 (SPSS, Inc., Chicago, IL). The haplotype frequency and estimates of pairwise linkage disequilibrium were determined by SHEsis software. A *p*-value <0.05 was considered statistically significant.

Results

A total of 293 HCC patients and 384 control cases were included in this study. Genotyping failed for a few samples

at a given locus, even after repeating the procedure three times. Therefore, the sample numbers were different for each polymorphism. The genotype frequencies of the two loci in the control subjects were both in agreement with the Hardy–Weinberg equilibrium.

As shown in Table 2, the frequencies of the D allele in the ACE gene were significantly decreased (*p*=0.003, OR=0.72, 95% CI=0.58–0.90) in patients compared to healthy controls. However, the allele frequency of eNOS indicated no difference between HCC patients and controls.

The genotype of these two genes was also evaluated in HCC patients and healthy controls. The significantly decreased HCC risk was associated with the DD genotype in a recessive model compared to I allele carriers (II/ID genotypes) (*p*<0.001, OR=0.19, 95% CI=0.11–0.34). Moreover, compared to the II genotype in a codominant model, significantly decreased HCC risk was associated with the DD genotype (*p*<0.001, OR=0.26, 95% CI=0.14–0.48). For genotypic association analysis, no statistically significant difference was observed between HCC patients and controls in the eNOS 4a/4b polymorphism (Table 3).

The result suggested that there was no linkage disequilibrium between the two polymorphisms (*r*²<0.8). A haplotype analysis was performed to evaluate the frequencies of haplotypes based on the ACE and eNOS polymorphisms. The haplotypes of the two polymorphisms did not show a significant association with HCC (Table 4).

TABLE 3. ACE I/D AND eNOS GENE INTRON 4 VNTR GENOTYPES OF THE HCC PATIENTS AND CONTROLS

Genetic model	Genotype	Cases	Controls	OR (95% CI)	p
ACE					
Codominant	II	<i>n</i> = 289 (%) 59 (20.4)	<i>n</i> = 384 (%) 84 (21.9)	1.00 (reference)	0.060
	ID	214 (74.0)	211 (54.9)	1.44 (0.98–2.12)	
	DD	16 (5.6)	89 (23.2)	0.26 (0.14–0.48)	
Dominant	II	59 (20.4)	84 (21.9)	1.00 (reference)	0.647
	ID + DD	230 (79.6)	300 (78.1)	1.09 (0.75–1.59)	
Recessive	II + ID	273 (94.4)	295 (76.8)	1.00 (reference)	< 0.001
	DD	16 (5.6)	89 (23.2)	0.19 (0.11–0.34)	
eNOS					
Codominant	4b/4b	<i>n</i> = 293 (%) 231 (78.8)	<i>n</i> = 384 (%) 288 (75.0)	1.00 (reference)	0.192
	4b/4a	59 (20.1)	94 (24.5)	0.78 (0.54–1.03)	
	4a/4a	3 (1.1)	2 (0.5)	1.87 (0.31–11.29)	
Dominant	4b/4b	231 (78.8)	288 (75.0)	1.00 (reference)	0.242
	4b/4a + 4b/4b	62 (21.2)	96 (25.0)	0.81 (0.56–1.16)	
Recessive	4a/4a	3 (1.1)	2 (0.5)	1.00 (reference)	0.449
	4b/4b + 4b/4a	290 (98.9)	382 (99.5)	0.51 (0.08–3.05)	

n corresponds to the number of individuals.

Boldfaced values indicate a significant difference at the 5% level.

TABLE 4. ASSOCIATION OF HAPLOTYPES IN *ACE* AND *eNOS* WITH HCC

Haplotype	Case (freq)	Control (freq)	OR (95%CI)	p
D/4b	0.36	0.47	1.00 (reference)	
D/4a	0.04	0.09	0.58 (0.17–2.04)	0.391
I/4b	0.52	0.38	1.79 (0.98–3.27)	0.058
I/4a	0.08	0.06	1.74 (0.55–5.47)	0.338

Freq proportion of indicated haplotype (%).

p value was calculated by a χ^2 -test.

Discussion

There are many studies on the relationship between the polymorphisms *ACE* I/D and *eNOS* 4a/4b and the risk for human cancers. To our knowledge, this is the first study to investigate the *ACE* I/D and *eNOS* 4a/4b polymorphisms in relation to HCC in a Chinese population. In this study, we determined that the D allele of *ACE* was significantly associated with HCC. Additionally, the DD genotype of *ACE* was associated with significantly decreased HCC risk in both the recessive and codominant models. However, *eNOS* did not show any difference between HCC patients and controls. The haplotype analysis of the two polymorphisms suggested no significant association with HCC. The study power was determined with the Quanto 1.1.1 program. Using a dominant genetic model at the 0.05 level of significance with a two-sided test for these two polymorphisms, our study had 91.51% and 91.47% power (for the *ACE* I/D and *eNOS* 4a/4b polymorphisms, respectively), to detect an effect with a relative risk of 1.8 in HCC patients and healthy controls.

Several investigators have reported that the presence of the D allele or homozygosity for the D allele of the *ACE* I/D polymorphism is associated with an increased risk for coronary heart disease (Gardemann *et al.*, 1998), myocardial infarction (Cambien *et al.*, 1992), arterial hypertension (O'Donnell *et al.*, 1998), and left ventricular hypertrophy (Celentano *et al.*, 1999). The RAS is frequently activated in patients with chronic liver diseases. *AT-II*, which is produced by *ACE*, has physiological proangiogenic effects. *AT-II* mediates the angiogenic signal by *AT4-R* and induces the vascular endothelial growth factor (VEGF) in endothelial cells. The VEGF primarily utilizes the VEGF receptor 2 (VEGFR-2) and protein kinase-C as an intracellular signaling cascade.

Increasing evidence has shown that *ACE* participates in the pathology of carcinomas (Murphey *et al.*, 2000; Yoshiji *et al.*, 2002; Deshayes and Nahmias, 2005) and plays a role in tumor cell proliferation, migration, angiogenesis, and metastasis (Suzuki *et al.*, 2003). Several epidemiological studies have examined the relationship between the *ACE* I/D polymorphism and cancers, including breast, gastric, and prostate cancers (Medeiros *et al.*, 2004; Rocken *et al.*, 2005; Soda *et al.*, 2005; Sugimoto *et al.*, 2006; Yaren *et al.*, 2006; Carl-McGrath *et al.*, 2007). In this study, we determined that the D allele of *ACE* was significantly associated with HCC development in the Chinese population and that the DD genotype of *ACE* was associated with significantly decreased HCC risk in both recessive and codominant models. The data suggested that the D allele and DD genotype of *ACE* play a protective role in HCC. This result is consistent with previous studies (Rocken *et al.*, 2005; Soda *et al.*, 2005; Sugimoto *et al.*, 2006). We also compared genotype frequencies in our study to other populations worldwide (Table 5). There were significant differences in the frequencies of the *ACE* polymorphism between the Chinese population and Japanese, Italian, American, Canadian, African American, and Spanish populations (Alvarez *et al.*, 1999; Hu *et al.*, 1999; Farrer *et al.*, 2000; Narain *et al.*, 2000; Perry *et al.*, 2001; Fatini *et al.*, 2003), but there were no differences compared with the German and Russian populations (Gardemann *et al.*, 1998; Crawford *et al.*, 2000). The difference may be due to the disease conditions or the ethnic populations.

eNOS, a crucial factor for synthesizing NO, plays an important role in tumor growth and angiogenesis (Grande *et al.*, 2000). By analyzing the human *eNOS* gene structure, a five tandem repeat in a 27-bp consensus sequence in intron 4 can serve as a genetic marker (Miyahara *et al.*, 1994). NO and its

TABLE 5. COMPARISON OF THE FREQUENCY OF *ACE* GENE POLYMORPHISM

Country	N	Frequency of <i>ACE</i> gene polymorphism (%)			<i>p</i> value	References
		II	ID	DD		
Chinese	384	84 (0.22)	211 (0.55)	89 (0.23)	ref	
Japanese	257	98 (0.37)	114 (0.44)	45 (0.19)	<0.001	Hu <i>et al.</i> (1999)
German	1201	277 (0.23)	598 (0.50)	326 (0.27)	0.179	Gardemann <i>et al.</i> (1998)
Italian	378	106 (0.28)	171 (0.45)	101 (0.27)	0.024	Fatini <i>et al.</i> (2003)
American	175	33 (0.19)	68 (0.39)	74 (0.42)	<0.001	Crawford <i>et al.</i> (2000)
Canadian	169	31 (0.18)	64 (0.38)	74 (0.44)	<0.001	Farrer <i>et al.</i> (2000)
English	342	60 (0.18)	203 (0.59)	79 (0.23)	<0.001	Narain <i>et al.</i> (2000)
Russian	206	51 (0.25)	94 (0.46)	61 (0.30)	0.085	Crawford <i>et al.</i> (2000)
African American	85	17 (0.20)	30 (0.35)	38 (0.45)	<0.001	Alvarez <i>et al.</i> (1999)
Spanish	400	60 (0.15)	176 (0.44)	164 (0.41)	<0.001	Perry <i>et al.</i> (2001)

n corresponds to the number of individuals.

metabolic byproducts have been implicated in the multi-staged process of carcinogenesis (Haswell-Elkins *et al.*, 1994; Mirvish, 1995). On the one hand, NO promotes the tumor effect through regulating cell proliferation-related gene expression and promoting the formation of tumor blood vessels. On the other hand, NO also has an antitumor function through inducing tumor cell apoptosis (Wink *et al.*, 1996). In this study, we did not note any differences in the distribution of allele or genotype frequencies of this single nucleotide polymorphism (SNP) between HCC patients and control subjects, suggesting that the *eNOS* polymorphism is not associated with HCC risk. This result is consistent with studies on breast cancer (Hefler *et al.*, 2006; Lu *et al.*, 2006) and colorectal cancer (Conde *et al.*, 2006) in a European population.

Our study may have some limitations. The study subjects were all ethnic Han Chinese, and the results should not be extended to other populations. Another weakness of this study was the relatively small sample number, which may limit the statistical power of our analysis.

In summary, we found that the *ACE* I/D polymorphism was significantly associated with HCC risk in a Han population from southwest China, indicating that it may play a role in HCC development. Nevertheless, further studies should be conducted to confirm these findings in larger samples and diverse ethnic groups.

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Disclosure Statement

The authors have declared that no conflicts of interest exist.

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