TGF-β1 polymorphism 509 C>T is associated with an increased risk for hepatocellular carcinoma in HCV-infected patients

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ABSTRACT. Transforming growth factor-beta 1 (TGF-β1), a member of the transforming growth factor beta family, functions as a multi-functional cytokine and plays a key role in cellular growth, proliferation, and differentiation. The 509 C/T polymorphism in the *TGF-β1* gene has been implicated in the outcome of hepatitis C virus (HCV) infection; however, little is known regarding the relationship between *TGF-β1* gene mutations and the development of hepatocellular carcinoma (HCC) in HCV-infected patients. The aim of the study was to evaluate the effect of the *TGF-β1* polymorphisms 509 C>T on the occurrence of HCC in patients chronically infected with HCV in a Chinese Han population. The results showed that HCC patients had a higher frequency of the *TGF-β1* -509 TT genotype distribution of

the *TGF-β1* -509 polymorphism and a lower frequency of the CC genotype. Serum TGF-β1 levels were significantly higher in patients with the TT genotype than in those with the CC genotype. In this study, we confirmed that the *TGF-β1* polymorphism 509 C>T is associated with the risk of HCC in HCV-infected patients.

Key words: Gene mutations; Single-nucleotide polymorphisms; TGF-β1 -509 CC genotype; Hepatocellular carcinoma; Hepatitis C virus; TGF-β1 -509 TT genotype

INTRODUCTION

Hepatocellular carcinoma (HCC) is ranked third among all cancers worldwide and is the second leading cause of cancer-related deaths in China (van den Bosch and Defreyne, 2012). Epidemiological evidence suggests that multiple-risk factors contribute to hepatocarcinogenesis, including genetic factors, environmental toxins, alcohol and drug abuse, autoimmune disorders, elevated hepatic iron levels, obesity, and hepatotropic viral infections (Sherman, 2010). Hepatitis C virus (HCV) is a small, enveloped, positive-sense single-stranded RNA virus that causes acute and often chronic hepatitis (Choo et al., 1989). Currently, an estimated 3% of the world's population, approximately 130-210 million people, is infected with HCV (Klibanov et al., 2011). Chronic HCV infection is a leading cause of progressive liver fibrosis and cirrhosis in up to 20% of patients; approximately 10-20% of cirrhotic patients may develop HCC within 5 years (Chiaramonte et al., 1999). Only a fraction of infected patients develop HCC during their lifetime, suggesting that the etiology of HCC is poorly understood (El-Serag, 2011). Several factors, such as viral kinetics, immune mechanisms, environmental factors, and genetic factors, may be responsible for the various HCV-related outcomes (Tolmane et al., 2012). Increasing evidence suggests that genetic factors contribute to HCC development.

Cytokines play a crucial role in the body's ability to fight viral infections such as hepatitis C; these molecules play a critical role in modulating the innate and adaptive immune systems and directly inhibit viral proliferation (Koziel, 1999). Genome-wide association studies have recently identified several single nucleotide polymorphisms in cytokine genes that are strongly associated with treatment outcome and spontaneous HCV clearance (Ge et al., 2009; Tanaka et al., 2009). Transforming growth factor-β (TGF-β) is a critical regulator of cell growth and differentiation, angiogenesis, extracellular matrix formation, immune response regulation, and cancer development and progression (Li et al., 2006; Clarke and Liu, 2008; Luwor et al., 2008). Changes in the secretion and levels of several cytokines, such as TGF-β, can cause dysregulation of the host immune response in chronic HCV patients (R-Viso et al., 2010; Kondo et al., 2011). Among 3 classical members of the TGF-β family, TGF-β1 is the most frequently up-regulated in tumors (Elliott and Blobe, 2005). The human gene encoding *TGF-β1* is located on chromosome 19q13, and variation among individuals in TGF-β1 production is thought to be under genetic control (Grainger et al., 1999). Several polymorphisms in the TGF-β1 gene have recently been identified, including +915 (Arg/Pro), +988 (C/A), -509 (C/T), -800 (G/A), and 2 others in the 10 and 25 codons of the first exon (Fujii et al., 1986). The most thoroughly studied 509 C>T polymorphism is located within a Yin-Yang1 consensus binding site (Pulleyn et al., 2001).

Identification of genetic factors related to the susceptibility to HCV-related HCC would facilitate studies aimed at understanding the complex process of hepatocarcinogenesis and improve the scientific basis for preventive interventions. Several studies have explored the association between *TGF-β1* polymorphisms and hepatocellular cancer risk. However, the results have been controversial (Xiang et al., 2012). Therefore, we analyzed the effect of the *TGF-β1* polymorphism 509 C>T on the occurrence of HCC in patients chronically infected with HCV in a Chinese Han population.

MATERIAL AND METHODS

Design and study population

A total of 393 patients with chronic HCV genotype 1 infection from the Affiliated People's Hospital, Jiangsu University, were enrolled in this study, including 159 patients with HCV-associated HCC and 234 patients without HCC. In addition, 375 age- and gender-matched healthy volunteers with no evidence of recent or remote HCV infection were enrolled in this study. All subjects were of Chinese Han descent. The following clinical characteristics of HCC patients were obtained at the time of whole blood collection: tumor size, presence of metastasis, and portal vein thrombosis. The diagnosis of HCC was confirmed by using several imaging techniques, including abdominal ultrasound and triphasic abdominal computed tomography with contrast and magnetic resonance imaging, according to the newly established diagnostic criteria (EASL 2011 and AASLD 2010 guidelines). Informed consent was obtained from all research participants. The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Ethics Committees of the Affiliated People's Hospital, Jiangsu University.

Biochemical analyses

In all subjects, between 8:00 and 9:00 am, peripheral venous blood was collected in BD-Vacutainer tubes (sodium heparin) after an overnight fast of 12 h. The markers of hepatic function, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total protein, total bilirubin, and creatinine, were measured in serum using routine enzymatic methods with a Hitachi 7050 automatic analyzer (Hitachi Corp., Tokyo, Japan). RNA was extracted from serum samples following manufacturer instructions (QIAamp Viral RNA Mini Kit from Qiagen GmbH, Hilden, Germany). In addition, quantitative detection of human HCV RNA in serum was performed by conducting an Amplicor-HCV monitor assay (Roche Molecular Diagnostics, Pleasanton, CA, USA).

Measurement of serum TGF-β1

Serum concentrations of TGF-β1 were assessed using a commercial enzyme-linked immunosorbent assay kit for TGF-β1 with a detection limit of 7 pg/mL (Quantikine® Human TGF-β1 Immunoassay; R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions.

Genotyping *TGF-β1* polymorphisms 509 C>T

Genomic DNA was extracted from ethylenediaminetetraacetic acid-anticoagulated

whole blood using a spin column method according to the manufacturer protocol (QIAamp Blood Kit, Qiagen). Primers and conditions for polymerase chain reaction (PCR) analysis were as described previously (Ohtsuka et al., 2002). The optimized reaction conditions consisted of 40 ng genomic DNA in a reaction volume of 30 μL containing 0.16 μM of each primer, 30 μM of each dNTP, 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, and 0.5 U Taq DNA polymerase (Takara, Shiga, Japan). Amplification was carried out for 35 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and a 10-min extension at 72°C. The 808-bp PCR product was digested with *Sau*I endonuclease (Fermentas, Vilnius, Lithuania). The *TGF-β1* -509 CC genotype showed 2 fragments of 617 and 191 bp, while the *TGF-β1* -509 TT genotype appeared as a single band of 808 bp. The presence of all 3 fragments (808, 617, and 191 bp) defined *TGF-β1* -509 CT genotype individuals.

Statistical analysis

The results for continuous variables are reported as means ± standard deviations (SD). Hardy-Weinberg equilibrium analysis was applied to *TGF-β1* alleles in each group. The SPSS12.0 statistical software was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). The χ^2 test was used to compare age, gender, smoking status, drinking status, and genotype and allele frequencies between groups. The statistical difference in genotype distribution and allele frequencies in both control and case subjects was assessed by using the χ^2 test or the Fisher exact test. Odds ratios (ORs) and confidence intervals (CIs) were calculated. All analyses were two-sided and $P < 0.05$ was considered to be statistically significant.

RESULTS

General characteristics of the subjects

A total of 393 local ethnic Chinese Han subjects with chronic HCV (234 HCV without HCC + 159 HCV with HCC) and 375 healthy volunteers were enrolled in our study. The demographic characteristics of cases and healthy volunteers are summarized in Table 1. No significant differences were found in age and gender distributions between the cases and controls (P values were 0.526 and 0.632, respectively). The smoking rates of the 2 groups were also similar (P = 0.829). However, the drinking rates were significantly higher among cases compared to controls (P = 0.015). ALT, AST, total bilirubin, and creatinine levels were significantly higher among the cases compared to controls (P < 0.05). However, albumin and total protein levels were significantly lower among cases compared to controls (P < 0.05). In addition, patients with HCC showed higher levels of total bilirubin, creatinine, ALT, and AST compared to patients without HCC.

Clinical characteristics of patients with HCC

With regard to the clinical characteristics of the HCC patients, 41 patients (25.8%) had portal vein thrombosis and 17 patients (10.7%) had lymph node metastasis (Table 2). Tumor diameter was <5 cm in 78 patients (49.1%).

Table 1. Demographic characteristics of the study subjects.

Variable	Controls (N = 375) [N (%)]	Patients without HCC (N = 234) [N (%)]	P	Patients with HCC (N = 159) [N (%)]	P
Age (years)					
≤50	221 (58.9)	129 (55.1)	0.356	89 (56.0)	0.526
>50	154 (41.1)	105 (44.9)		70 (44.0)	
Gender					
Male	281 (74.9)	180 (76.9)	0.578	116 (73.0)	0.632
Female	94 (25.1)	54 (23.1)		43 (27.0)	
Smoking status					
Ever	244 (65.1)	145 (62.0)	0.438	105 (66.0)	0.829
Never	131 (34.9)	89 (38.0)		54 (34.0)	
Drinking status					
Ever	169 (45.1)	133 (56.8)	0.005	90 (57.9)	0.015
Never	206 (54.9)	101 (43.2)		69 (42.1)	
ALT (U/L)	27.8 ± 8.5	50.2 ± 17.3	<0.05	67.7 ± 20.9	<0.05*#
AST (U/L)	28.9 ± 10.2	66. 2 ± 25.7	<0.05	153.4 ± 68.8	<0.05*#
Albumin (g/dL)	3.9 ± 1.1	2.2 ± 0.5	<0.05	2.1 ± 0.7	<0.05*
Total protein (g/dL)	7.6 ± 0.8	7.1 ± 0.7	<0.05	7.2 ± 0.9	<0.05*
Total bilirubin (μM)	0.75 ± 0.3	2.7 ± 0.7	<0.05	4.5 ± 1.4	<0.05*#
Creatinine (mg/dL)	0.9 ± 0.6	1.3 ± 0.5	<0.05	1.5 ± 0.7	<0.05*#

*$P < 0.05$ compared with the control group. #$P < 0.05$ compared with HCV patients without HCC.

Table 2. Clinical characteristics of patients with HCC.

	HCV patients with HCC (N = 159)
Metastasis	
Present	17
Absent	142
Portal vein thrombosis	
Positive	41
Negative	118
Tumor size	
<5 cm	78
≥5 cm	81

Association between *TGF-β1* gene polymorphism 509 C>T and HCC

The genotype at locus rs1800469 was identified in patients and healthy volunteers. Allele and genotype distributions are shown in Table 3. The genotype distributions were in Hardy-Weinberg equilibrium in each studied group. The CT genotype at position -509 of the *TGF-β1* gene prevailed in all 3 groups (healthy controls, 42.9%; HCV patients without HCC, 43.2%; HCV patients with HCC, 42.1%). Compared to healthy controls, HCC patients showed a lower frequency of the CC genotype of the *TGF-β1* -509 polymorphism and a higher frequency of the TT genotype. No significant difference was observed in the distribution of either genotype or allelic frequency in controls and HCC patients without HCC. The risk of HCC was significantly higher among HCC patients carrying the TT genotype than in patients carrying the CC genotype [OR (95%CI) = 1.820 (1.051-3.153), P = 0.036] or carrying at least 1 T allele at position -509 compared to carrying the C allele [OR = 1.383 (1.037-1.844), P = 0.028].

Effect of *TGF-β1* gene polymorphism 509 C>T on serum levels of TGF-β1

We assessed the serum levels of TGF-β1 in HCV patients with or without HCC and

healthy subjects. As shown in Table 4, the TGF-β1 serum level was significantly higher in subjects with the TT genotype than in those with the CC genotype in all groups.

Table 3. Distribution of *TGF-β1* genotypes in patients and the control group.

	Healthy control (N = 375) [N (%)]	HCV patients without HCC (N = 234) [N (%)]	OR (95%CI)	P	HCV patients with HCC (N = 159) [N (%)]	OR (95%CI)	P
TGF-β1 -509							
CC	143 (38.1)	91 (38.9)			50 (25.6)		
CT	161 (42.9)	101 (43.2)	0.986 (0.686-1.416)*	0.938	67 (42.1)	1.190 (0.774-1.830)*	0.427
						1.207 (0.760-1.918)#	0.425
TT	71 (18.9)	42 (17.9)	0.930 (0.585-1.477)*	0.757	42 (26.4)	1.692 (1.027-2.787)*	0.038
						1.820 (1.051-3.153)#	0.036
C allele	447	283			167		
T allele	303	185	0.964 (0.762-1.221)*	0.763	151	1.334 (1.024-1.737)*	0.036
						1.383 (1.037-1.844)#	0.028

*Compared with the control group. #Compared with HCV patients without HCC.

Table 4. Effect of *TGF-β1* gene polymorphisms on TGF-β1 serum levels (ng/mL).

	TGF-β1		
	CC	CT	TT
Control	3.3 ± 1.0	3.6 ± 1.2	4.0 ± 1.5
HCV patients without HCC	15.2 ± 3.1	16.5 ± 3.7	17.1 ± 4.1
HCV patients with HCC	20.3 ± 3.3	21.8 ± 3.7	23.1 ± 4.4

DISCUSSION

Chronic HCV infection is one of the most common infectious diseases, with 170 million people estimated to be infected with HCV worldwide (Kim et al., 2013). HCV infection leads to high morbidity and mortality due to the development of liver cirrhosis and HCC. The long-term hepatic impact of HCV infection is highly variable, from minimal changes to chronic hepatitis, extensive fibrosis, cirrhosis, and HCC. Previous studies over the past few years have suggested that host genetic factors affect the course of various viral infections, including cases of HCV infection (Bengsch et al., 2009). Because cytokines regulate the immune response, polymorphisms in cytokine genes or variations in their expression may affect an individual's susceptibility to infectious diseases (Murtaugh and Foss, 2002). Numerous candidate genes and their allelic variations have been analyzed as potential biomarkers affecting the natural course of chronic HCV (Weatherall et al., 1997).

TGF-β1 acts as a growth inhibitor in normal cells, whereas in tumor cells, it loses the ability to mediate growth inhibition and instead promotes tumor progression by enhancing the migration, invasion, and survival of tumor cells. Overexpression of TGF-β1 in tumor cells provides additional oncogenic activities by circumventing the host immune surveillance (Oh et al., 2013). In liver diseases, the persistence of chronic inflammation, as observed in chronic viral hepatitis, plays a major role in determining the shift in the TGF-β1 signaling pathway from tumor suppression to increasing the risk of HCC (Lu et al., 2006).

In this study, we investigated the association between one single nucleotide polymorphism, rs1800469, in the *TGF-β1* gene and the susceptibility to HCC in a Chinese Han popu-

lation infected with HCV. We found that the frequency of the TT genotype was significantly increased in HCC patients. These findings support the results of Falleti et al. (2008), who detected significant genotypic differentiations between controls and cirrhotic patients, but not between cirrhotic patients with and without HCC. In our study, we found that the risk of HCC was significantly higher among subjects with HCV infection carrying the TT genotype than patients carrying the CC genotype. By contrast, Qi et al. (2009) reported that the *TGF-β1* -509 CC genotype was associated with hepatitis B virus-related HCC in a Chinese population. In another study, Wang et al. (2005) reported that the C allele of *TGF-β1* -509, but not the T allele, might play an important role in the progression of liver cirrhosis. The differences in these results may have been caused by different clinical characteristics and the different ethnic groups studied. Shi et al. (2012) found that the *TGF-β1* -509 T allele did not influence the risk of developing HCC. Differences between their study and our findings may have resulted from the experimental designs. The sample size of their study was relatively small (73 HCC patients and 117 cancer-free healthy subjects). In addition, they did not determine whether patients were infected with hepatitis B virus or HCV. All HCC patients in our study were infected with HCV.

In our study, subjects carrying the T allele at position -509 were associated with higher serum concentrations of TGF-β1 in the patient and control groups, suggesting that the T allele modulates serum concentrations of TGF-β1. These results agreed with those of Radwan et al. (2012), who reported that the presence of the T allele at position -509 was associated with higher concentrations of TGF-β1. The opposite results were obtained in a study conducted by Qi et al. (2009), which suggested that the presence of a C allele at position -509 of the *TGF-β1* gene may lead to higher plasma TGF-β1 levels in HCC patients with chronic hepatitis B virus infection in a Chinese population.

In conclusion, the *TGF-β1* -509 gene polymorphism is associated with the risk of HCC in patients with chronic HCV infection in a Chinese Han population. Further studies showed that this effect could be due to the modification of serum TGF-β1 levels. Genetic testing of *TGF-β1* -509 genes may be useful for identifying high-risk individuals such as subjects with HCV infection, and the results may encourage the higher risk population to receive medical examinations frequently for early detection of HCC.

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