Tumor Necrosis Factor-α 308.2 Polymorphism Is Associated with Advanced Hepatic Fibrosis and Higher Risk for Hepatocellular Carcinoma[1]

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Abstract

***BACKGROUND/AIMS:** Host genetic factor and hepatic fibrosis may predispose to risk for hepatocellular carcinoma (HCC). This study aimed to assess the association between tumor necrosis factor (TNF) α polymorphism and hepatic fibrosis, and risk for HCC.* ***METHODS:** One hundred eight pairs of gender-matched and age-matched patients with HCC and unrelated healthy controls were genotyped for TNF308.2 and TNF238.2 alleles with polymerase chain reaction and direct sequencing.* ***RESULTS:** The frequency of TNF308.1/TNF308.2 genotype in cases was higher than that in controls [odds ratio (OR) = 4.37]. Multivariate analysis indicated that TNF308.2 allele (OR = 3.23), hepatitis B surface antigen (OR = 17.17), and antibodies to hepatitis C virus (OR = 45.52) were independent risk factors for HCC. Surrogate markers for significant fibrosis implied that cases with the TNF308.2 allele have more advanced liver fibrosis. Moreover, multivariate analysis indicated that cirrhosis with Child-Pugh grade C, low serum albumin, and low platelet count were independent risk factors for carrying the TNF308.2 allele.* ***CONCLUSIONS:** TNF308.2 allele carriage and chronic hepatitis B virus/hepatitis C virus infection are independent risk factors for HCC. Carriage of the TNF308.2 allele correlates with disease severity and hepatic fibrosis, which may contribute to a higher risk for HCC.*

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fection with hepatitis B virus (HBV) and hepatitis C virus (HCV), and cirrhosis of any etiology are the major risk factors for HCC [1–8]. Overall, 75% to 80% of HCC cases are attributable to persistent viral infections with either HBV (50–55%) or HCV (25–30%) [1–8]. It is known that cytokines and cytotoxic T lymphocytes are among the predominant mechanisms of host defense against chronic HBV/HCV infection [9–11]. They can induce an inflammatory response that often leads to chronic liver injury [8–13].

Hepatocyte damage elicits an inflammatory response through activation of tissue macrophage Kupffer cells. These activated cells release an array of cytokines, including tumor necrosis factor (TNF) α, transforming growth factor-β, platelet-derived growth factor, and other factors that act on hepatic stellate cells that contribute to fibrogenesis [8,9,11,12]. Among antiviral cytokines, TNF-α plays a pivotal role in host immune response to HBV/HCV infection. Circulating TNF-α level increases during HBV [11–15] and HCV infection [11,16–19]. An increased TNF-α level correlates with the severity of hepatic inflammation, fibrosis, and tissue injury [11,15,16,20]. Persistent immune-mediated hepatic injury can initiate the process of fibrosis, cirrhosis, and, eventually, HCC [6,8–11].

The capacity for cytokine production in an individual has a major genetic component, and there exist striking differences

Introduction

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer in the world [1,2]. Hepatocarcinogenesis is a multistep process with a multifactorial etiology. Chronic in-

among individuals in terms of their ability to produce cytokines, which have been ascribed to single-nucleotide polymorphism (SNP) within the coding and regulatory regions of cytokine genes [21,22]. TNF-α expression is tightly controlled at the transcriptional and posttranscriptional levels [23,24]. Several biallelic polymorphisms have been described within the *TNF-α* gene. Of particular interest are two biallelic SNPs at the −238 or −308 position. These SNPs result in two allelic forms. The presence of guanine defines the common variant TNF1 allele, and the presence of adenine defines the less common variant TNF2 allele [24,25]. Both SNPs have been shown to influence TNF-α expression. TNF308.2 allele leads to an increased constitutive and inducible expression of TNF-α [23,24]. TNF308.2 SNP has been linked with several inflammatory, autoimmune, infectious, and malignant diseases [21,22]. The functional consequences for TNF238.2 allele are not yet clear. It also increases disease susceptibility [26].

Fibrosis is the result of chronic liver injury regardless of etiology [11,27−35]. Serial liver biopsies are the current gold standard for evaluating the progression of fibrosis. However, the procedure is limited by its invasive nature, expense, morbidity, intraobserver and interobserver variabilities, and sampling errors [28]. To date, several routine laboratory tests, single or in combination, have been used as surrogate markers for predicting significant hepatic fibrosis. These tests include platelet count [28−33], cirrhosis discriminant score (CDS) [33], aspartate aminotransferase/platelet ratio index (APRI) [28,31,33,34], age−platelet index (AP index) [32,33,35], prothrombin time [35], and age [29,32,33,35]. These parameters confirm a positive correlation with liver fibrosis, which can be useful in predicting the progression of chronic liver disease. Among these, platelet count has been reported to demonstrate the strongest correlation with hepatic fibrosis and disease severity [30−33,35]. It has been demonstrated that low platelet count is predictive of the development of HCC [36,37]. This information suggests that advanced fibrosis may predispose to HCC.

Initially proposed to have anticarcinogenic effects, TNF-α has been shown later to be tumorigenic and to act as a tumor promoter [38,39]. We speculated that TNF SNP may increase the risk for HCC through enhanced hepatic fibrosis. We embarked on this case−control study to provide a more precise assessment of the hypothesis.

Patients and Methods

Study Population

One hundred eight consecutive newly diagnosed patients with HCC were enrolled as a case group. During the same study period, 108 unrelated healthy community residents who entered the hospital for health checkup were enrolled as a control group. Each healthy control was pair-matched by gender and age (± 5 years) to a patient with HCC. These subjects were hospitalized or had visited outpatient clinics at the Kaohsiung Medical University Hospital from January 2001 to December 2001.

Patients with HCC were eligible for the study if they were newly diagnosed by aspiration cytology or biopsy and were free from any known diseases with a genetic predisposition. The tumor was staged according to the tumor−node−metastasis system [40]. Cirrhosis was diagnosed with liver biopsy, abdominal sonography, and biochemical evidence of parenchymal damage plus endoscopic esophageal or gastric varices [41,42]. Patients with cirrhosis were classified into the three Child-Pugh grades based on their clinical status [43]. There was no space-occupying lesion in the liver in any healthy control, as evidenced by normal abdominal sonography. None of the controls had symptoms, signs, or biochemical evidence (including aminotransferase levels) of liver disease, known medical illness, or hereditary disorders at recruitment. All studied subjects were proved not to have other cancers at the initial screening examination. All study subjects were Han Chinese. Signed informed consent forms were obtained from all study subjects. The study was approved by the Investigation and Ethics Committee of the hospital.

DNA Extraction

Genomic DNA was isolated from EDTA preserved whole blood by standard protein K digestion and phenol−chloroform methods.

Serologic Examination

Hepatitis B surface antigen (HBsAg) and antibodies to hepatitis C virus (anti-HCV) were detected by Ausria-II and second-generation Abbott HCV EIA (Abbott Laboratories, North Chicago, IL), respectively. For anti-HCV, reactive specimens were retested. Only repeatedly reactive specimens were interpreted as anti-HCV−positive. Conventional liver function tests were measured by an autoanalyzer (Model 736; Hitachi, Tokyo, Japan). Peripheral blood platelet count and prothrombin time (expressed as international normalized ratio) were routinely determined in the clinical laboratory.

Polymorphism Genotyping

For TNF308 and TNF238 genotyping, we modified the methods described previously [26]. Briefly, a 328-bp fragment spanning positions −396 to −69 of the 5′-untranslated region of the *TNF-α* gene was amplified using primers TNF396 (5′-TTCCTGCATCCGTCTGGAA-3′) and TNF69 (5′-CAGCGGAAAACTCCTTGGT-3′). Amplification was performed in a thermocycler (GeneAmp 9700; Perkin Elmer, Norwalk, CT) with 100 ng of genomic DNA, 25 pM of each primer, 200 μM total dNTP, 1.5 mM MgCl₂, standard polymerase chain reaction (PCR) buffer, and 2 U of *Taq* polymerase (Perkin Elmer). The following cycling conditions were used: 60 seconds at 94°C, 60 seconds at 55°C, and 60 seconds at 72°C for 25 cycles. For SNP determination, direct sequencing of the entire 328-bp fragment was performed. The PCR product and either sense or antisense PCR primer were determined, and the Big Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystems, Foster City, CA) was performed, followed by detection on an ABI Prism DNA sequencer (PE Applied Biosystems). This

led to the identification of heterozygous and homozygous individuals for each of the four polymorphisms (TNF238.1, TNF238.2, TNF308.1, and TNF308.2 alleles).

Surrogate Markers for Hepatic Fibrosis

From routine laboratory data, surrogate markers associated with fibrosis, including platelet count, AP index, CDS, and APRI, were calculated exactly as originally described [31–33]. The selected cutoff values for significant fibrosis (AP index $\geq$ 6; CDS $\geq$ 8; APRI $\geq$ 1.5; platelet count < 150×10^9 l^{-1}) were adapted from Lackner et al. [33].

Statistical Analysis

Continuous variables were expressed as medians (with ranges in parentheses), whereas categorical variables were expressed as numbers (percentages). The difference between the medians of continuous variables was compared using Mann-Whitney *U* test. Categorical variables were compared using chi-square test with Yates correction or Fisher's exact test, where appropriate. Mantel extension test for trend was used to examine the dose–response relationship for risk estimates of various combinations of risk factors. Odds ratio (OR) with 95% confidence interval (95% CI) was used to estimate causal relations between risk factors and exposure. Conditional logistic regression analysis was used for multivariate analysis. Unconditional stepwise logistic regression analysis was used to estimate risk factors for carrying the TNF308.2 allele in HCC patients. Adjusted OR and 95% CI were derived from logistic regression coefficients to provide an estimate of the statistical association between a given variable and the disease (HCC), with the other variables held constant. Two-tailed *P* values and 95% CI were given where appropriate. An α of .05 was used as an indicator of statistical significance. In the genotyping of TNF-α, the *P* value was corrected for the number of comparisons at each locus (P_c) according to Bonferroni correction. In each group, the distribution of TNF-α genotypes was tested for deviation from the Hardy-Weinberg equilibrium using goodness-of-fit chi-square test.

Results

Demographic Information of Cases and Controls

The demographic characteristics of the subjects studied are given in Table 1. There was no statistical difference in gender distribution and median age between cases and controls. At least one marker of HBsAg or anti-HCV was found in 89.81% (97 of 108) of patients with HCC. Cirrhosis was found in 91 patients (84.25%). The frequency distributions of Child-Pugh grades A, B, and C in cirrhotic HCC were 43.96%, 39.56%, and 16.48%, respectively.

Polymorphisms in Patients and Controls

The alleles at the TNF-α −238 and −308 positions in studied subjects were in accordance with the Hardy-Weinberg equilibrium (data not shown). Compared to healthy controls, patients with HCC had a lower frequency of TNF308.1/

Table 1. Basic Characteristics of the Subjects Studied.

Parameters	Cases (*n* = 108)	Controls (*n* = 108)
Gender (male:female)	77:31	77:31
Age in years [median (range)]	52 (32–84)	51 (30–84)
HBsAg/anti-HCV		
Negative/negative	11	79
Negative/positive	27	5
Positive/negative	58	24
Positive/positive	12	0
Cirrhosis	91	–
Child-Pugh grade		
A	40	–
B	36	–
C	15	–
Tumor–node–metastasis stage of HCC		
I	12	–
II	47	–
III (A–C)	45	–
IV	4	–

TNF308.1 genotype (74.07% *vs* 92.59%; P_c = .0015) and a higher frequency of TNF308.1/TNF308.2 genotype (25.00% *vs* 7.41%; P_c = .0024). Homozygous TNF308.2/TNF308.2 genotype was found in one HCC patient and in none of controls. Putting together, the frequency of genotypes containing the TNF308.2 allele in patients (25.93%; 28 of 108) was higher than that in controls (7.41%; 8 of 108; P_c = .0015). The frequency of the TNF308.2 allele in HCC patients (13.43%; 29 of 216) was significantly higher than that (3.70%; 8 of 216) in controls (P_c = .001).

There was no significant difference in the frequency of TNF238.1/TNF238.2 genotype between patients (5.56%) and controls (1.85%). There was no homozygous TNF238.2/TNF238.2 genotype in the subjects studied. The TNF238.2 allele was found in 2.78% (6 of 216) of patients and in 0.93% (2 of 216) of controls.

Independent Risk Factors for HCC, By Univariate and Multivariate Analyses

Using healthy controls as a reference group, univariate analysis indicated that TNF308.2 allele (*P* = .0015), HBsAg positivity (*P* = .0001), and anti-HCV positivity (*P* = .0001) were significant risk factors for HCC (Table 2), whereas male gender, older age (> 50 years), and TNF238.2 allele were not. Multivariate analysis indicated that TNF308.2 allele (OR = 3.23; *P* = .032), HBsAg positivity (OR = 17.47; *P* = .0001), and anti-HCV positivity (OR = 45.52; *P* = .0001) were independent risk factors for HCC (Table 2).

Clinical Characteristics in HCC Patients, By TNF308.2 Allele Status

As shown in Table 3, all TNF308.2 alleles were found in patients with cirrhosis (*P* = .003; Fisher's exact test). The frequency of carrying the TNF308.2 allele in patients with Child-Pugh grade A was lower than that in patients with Child-Pugh grade B (*P* = .032) or in patients with Child-Pugh grade C (*P* = .0002). Moreover, the higher is the Child-Pugh grade, the higher is the frequency of carrying the TNF308.2 allele ($P_{\text{for trend}}$ = .0001). Note that the frequency of carrying the TNF308.2 allele in patients with anti-HCV alone was higher

Table 2. Risk for HCC, By Univariate and Multivariate Analyses.

Risk Factors	Cases (n = 108)	Controls (n = 108)	OR (95% CI)	Adjusted OR* (95% CI)
TNF308.2 allele				
Present	28	8	4.37 (1.78–11.08)	3.23 (1.10–9.44)
Absent	80	100	1.0	1.0
HBsAg				
Positive	70	24	6.44 (3.39–12.34)	17.47 (7.82–39.02)
Negative	38	84	1.0	1.0
Anti-HCV				
Positive	39	5	11.64 (4.11–35.49)	45.52 (13.23–156.65)
Negative	69	103	1.0	1.0
TNF238.2 allele				
Present	6	2	3.11 (0.55–24.51)	–
Absent	102	106	1.0	

*Adjusted for sex, age > 50 years, HBsAg, anti-HCV, and TNF308.2 allele by conditional logistic regression analysis.

than that in patients with HBsAg alone (P = .027). All patients carrying the TNF308.2 allele had increased serum alanine aminotransferase (ALT) levels above the upper limit of normal (P = .003; Fisher's exact test). There was no significant difference with regard to gender, older age (> 50 years), and increased serum levels of α-fetoprotein and aspartate aminotransferase (Table 3). Among conventional liver function tests, the median serum albumin level in patients with the TNF308.2 allele (3.1 g/dl; range, 2.1–4.1 g/dl) was significantly lower than that in those without (3.7 g/dl; range, 2.6–4.4 g/dl; P = .0001). The serum ALT level in the former (median = 94 IU/l; range, 44–577 IU/l) was significantly higher than that in the latter (median = 66 IU/l; range, 14–718 IU/l; P = .025). There was no significant difference with regard to

Table 3. TNF308.2 Allele in Relation to Clinical Parameters in HCC Patients.

Parameters	Group	n	With TNF308.2 Allele [n (%)]	P
Gender	Male	77	17 (22.07)	NS
	Female	31	11 (35.48)	
Age (years)	≤ 50	44	9 (20.45)	NS
	> 50	64	19 (29.68)	
Cirrhosis	Yes	91	28 (30.76)	.003
	No	17	0 (0.00)	
Child-Pugh	A*	40	5 (12.50)[†,‡]	.0001
	B*	36	13 (36.11)[†]	
	C*	15	10 (66.67)[‡]	
HBsAg/anti-HCV	Negative/negative	11	3 (27.27)	NS
	Negative/positive	27	12 (44.44)[§]	
	Positive/negative	58	11 (18.96)[§]	
	Positive/positive	12	2 (16.67)	
α-Fetoprotein (ng/ml)	≤ 20	38	11 (28.94)	NS
	> 20	70	17 (24.28)	
AST (IU/l)	≤ 40 (ULN)	16	3 (18.75)	NS
	> 40	92	25 (27.17)	
ALT (IU/l)	≤ 40 (ULN)	19	0 (0.0)	.003
	> 40	89	28 (31.46)	

NS, not significant; *ULN*, upper limit of normal.
*$P_{\text{for trend}}$ = .0001 (Mantel extension test for trend).
[†]OR = 3.95; 95% CI = 1.10–14.89; P = .032.
[‡]OR = 14.00; 95% CI = 2.80–78.00; P = .0002.
[§]OR = 3.41; 95% CI = 1.12–10.56; P = .027.

serum levels of bilirubin, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase (data not shown).

Surrogate Markers for Hepatic Fibrosis in HCC Patients, By TNF308.2 Allele Status

As shown in Table 4, TNF308.2 allele carriers tended to have lower peripheral blood platelet count (P = .0001), worse prothrombin time (P = .001), and higher levels of AP index (P = .001), APRI (P = .004), and CDS (P = .0001). Using cut-off values for significant fibrosis defined by Lackner et al. [33], the frequency of thrombocytopenia in patients with the TNF308.2 allele was higher than that in patients not carrying the TNF308.2 allele (P = .0003). Moreover, the frequencies of increased AP index, APRI, and CDS values in patients with the TNF308.2 allele were greater than those in patients without them (P = .010, P = .003, and P = .0001, respectively). Multivariate analysis indicated that low platelet count (P = .012), lower serum albumin level (P = .002), and cirrhosis with Child-Pugh grade C (P = .039) were independent risk factors for carrying the TNF308.2 allele (Table 5).

Discussion

Our results indicated that TNF308.2 SNP is an independent risk factor for HCC (Table 2). Moreover, TNF308.2 SNP is associated with significant hepatic fibrosis (Table 4) and more severe liver damage (Tables 3 and 5), which may predispose to HCC development. This study demonstrated that host genetic factor (TNF308.2 SNP) plays an important role in hepatic carcinogenesis.

It has been found that circulating TNF-α levels were elevated in patients with HCC [44,45]. In the current study, there was a strong association between TNF308.2 SNP and risk for HCC (Table 2). Although we did not measure circulating TNF-α levels, TNF308.2 SNP has already been shown to increase the constitutive and inducible expression of TNF-α protein [23,24], possibly caused by the differential binding of a nuclear protein to the TNF308.2 allele [24]. It is reasonable to speculate that the high circulating TNF-α levels found in patients with HCC may be attributed to TNF308.2 SNP. Therefore, this polymorphism could be a causal predisposing factor for HCC.

Table 4. Surrogate Markers for Hepatic Fibrosis in HCC Patients, By TNF308.2 Allele Status.

Parameter	TNF308.2 Allele		P
	Present (n = 28)	Absent (n = 80)	
Platelet ($\times 10^9 \, l^{-1}$)	79 (28–124)	137 (40–390)	.0001
Prothrombin time (international normalized ratio)	1.2 (1.0–1.5)	1.1 (1.0–1.3)	.001
AP index	8 (6–9)	7 (2–8)	.001
APRI	2.8 (0.7–20.6)	1.6 (0.3–21.6)	.004
CDS	8 (5–18)	6 (0–8)	.0001
Thrombocytopenia* (%)	100.0	62.50	.0003
AP index ≥ 6* (%)	100.0	76.25	.010
APRI ≥ 1.5* (%)	82.14	55.00	.003
CDS ≥ 8* (%)	50.00	2.50	.0001

*Cutoff values for significant fibrosis were adapted from Lackner et al. [33].

Table 5. Multivariate Analysis of Risk Factors for Carrying the TNF308.2 Allele in Patients with HCC.

Variables	β	SE	P	OR (95% CI)
Platelet count	−0.53	0.21	.012	0.58 (0.39−0.88)
Albumin	−4.11	1.33	.002	0.02 (0.001−0.224)
Child-Pugh grade C	2.83	1.36	.039	16.96 (1.16−248.29)

Unconditional stepwise logistic regression analysis—dependent variable: presence of TNF308.2 allele; independent variables: male gender, age > 50 years, Child-Pugh grade, platelet count, albumin, AST, ALT, and prothrombin time.

Chronic HBV/HCV infection has well been established as an independent risk factor for HCC [1−4,6−8]. Besides oncogenic viruses, chronic HBV/HCV infection may lead to persistent hepatocyte necroinflammation and hepatic fibrosis [6,8,11]. Both inflammatory by-products caused by HBV/HCV infection [13,46,47] and TNF-α derived from activated Kupffer cells [48] produce oxygen-derived free radicals and other reactive oxygen species. These compounds are important mediators of hepatic fibrogenesis in liver injury [27]. Moreover, TNF-α plays an important role in hepatic fibrogenesis and progression of fibrosis in chronic liver disease [11,20,27,48−50]. TNF-α may also induce the production of other fibrogenic factors, such as tumor growth factor-β, interleukin (IL) 1, and IL-6 [51]. Moreover, both univariate (Table 4) and multivariate (Table 5) analyses indicated that among 50% to 100% of our patients with TNF308.2 SNP had significant hepatic fibrosis. Our results are in consistency with a previous observation that TNF-α SNP is associated with advanced hepatic fibrosis [49,50,52], which may contribute to HCC [4,6,8,9,11,27,53,54].

HCC commonly develops in the background of chronic hepatitis and/or cirrhosis, which, in part, is mediated by cytokines [9]. As shown in Table 1, 84.26% of our HCC patients had underlying cirrhosis. Serum ALT level is a marker of hepatic necroinflammation in patients with chronic liver disease [5]. Sustained abnormal ALT levels in cirrhotic patients are associated with a higher risk for HCC [3,5]. All patients with the TNF308.2 allele had cirrhosis (Table 3). Our data indicated that HCC patients with TNF308.2 SNP had more severe liver damage. In addition, TNF-α may stimulate the release of other inflammatory cytokines (IL-1, IL-6, IL-8, and IL-10) that can cause or aggravate liver damage [9,22,23]. Hence, TNF308.2 SNP may aggravate persistent liver inflammation and hepatic injury. Taking all information together, it is obvious that hepatic fibrosis is a pivotal and necessary stage to cirrhosis [11,13,20,27,48,53] and/or HCC [4,6,8,27,37,51,53,54]. TNF308.2 SNP may accelerate the progression of cirrhosis to HCC owing to its accelerated progression of hepatic necroinflammation and fibrosis [20,27,48,50].

The current study demonstrated that TNF308.2 SNP is an independent risk factor for HCC. Our results are consistent with those previously reported [55], but quite different from another study [56]. However, one of the shortcomings of this study is its rather small sample size; therefore, the results should be confirmed in a larger series, as well as in patients of different ethic origins. In conclusion, HCC patients with the TNF308.2 allele had more severe hepatic fibrosis and liver damage. TNF308.2 SNP correlates with more severe hepatic fibrosis, which contributes to a higher risk for HCC.

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