RESEARCH ARTICLE

Independent and Additive Interaction Between Tumor Necrosis Factor β +252 Polymorphisms and Chronic Hepatitis B and C Virus Infection on Risk and Prognosis of Hepatocellular Carcinoma: a Case-Control Study

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Abstract

To assess the contribution of tumor necrosis factor (TNF)β +252 polymorphisms to risk and prognosis of hepatocellular carcinoma (HCC), we enrolled 150 pairs of sex- and age-matched patients with HCC, patients with cirrhosis alone, and unrelated healthy controls. TNFβ +252 genotypes were determined by polymerase chain reaction with restriction fragment length polymorphism. Multivariate analysis indicated that TNFβ G/G genotype [odds ratio (OR), 3.64; 95% CI, 1.49-8.91], hepatitis B surface antigen (OR, 16.38; 95% CI, 8.30-32.33), and antibodies to hepatitis C virus (HCV) (OR, 39.11; 95% CI, 14.83-103.14) were independent risk factors for HCC. There was an additive interaction between TNFβ G/G genotype and chronic hepatitis B virus (HBV)/HCV infection (synergy index=1.15). Multivariate analysis indicated that factors associated with TNFβ G/G genotype included cirrhosis with Child-Pugh C (OR, 4.06; 95% CI, 1.34-12.29), thrombocytopenia (OR, 6.55; 95% CI, 1.46-29.43), and higher serum α-fetoprotein concentration (OR, 2.53; 95% CI, 1.14-5.62). Patients with TNFβ G/G genotype had poor cumulative survival (p=0.005). Cox proportional hazard model indicated that TNFβ G/G genotype was a biomarker for poor HCC survival (hazard ratio, 1.70; 95% CI, 1.07-2.69). In conclusion, there are independent and additive effects between TNFβ G/G genotype and chronic HBV/HCV infection on risk for HCC. It is a biomarker for poor HCC survival. Carriage of this genotype correlates with disease severity and advanced hepatic fibrosis, which may contribute to a higher risk and poor survival of HCC. Chronic HBV/HCV infected subjects with this genotype should receive more intensive surveillance for early detection of HCC.

Keywords: Tumor necrosis factor β polymorphism - hepatocellular carcinoma - susceptibility - prognosis

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Introduction

Hepatocellular carcinoma (HCC) ranks the sixth most common cancer and the third most common cause of cancer death worldwide (Jemal et al., 2011). Hepatocarcinogenesis is a multistep process with a multifactorial etiology. (El-Serag, 2012; Forner et al., 2012; Gao et al., 2012; Su et al., 2013). Development of HCC is linked to environmental, dietary, life-style, and genetic factors. There is increasing evidence that HCC is inherently associated with up-regulation of cytokines (Haybaeck et al., 2009; Su et al., 2013).

A causal relationship between chronic hepatitis, hepatocellular damage, fibrosis, and hepatocarcinogenesis is well established (Stauffer et al., 2012; Dwyer et al., 2014). The well-known environmental risk factors for HCC include chronic infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV), and cirrhosis of any etiology (El-Serag, 2012; Forner et al., 2012; Yeo et al., 2013). Persistent hepatic inflammation is a hallmark of chronic HBV/HCV infection (El-Serag, 2012; Forner et al., 2012). The exact mechanisms driving hepatitis-induced HCC remain elusive. Among them, aberrant expression of cytotoxic cytokines is thought to be critically involved (Haybaeck et al., 2009; Su et al., 2013).

The proinflammatory cytokines tumor necrosis factor (TNF) α, TNFβ, and lymphotoxin (LT) β are members of the TNF superfamily (Aggarwal et al., 2012). TNFβ has a close structural homology and about 30% amino acid sequence identity to TNFα. It carries out most of

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the activities of TNFα. Both cytokines initiate similar (if not identical) biologic responses (Haybaeck et al., 2009; Aggarwal et al., 2012).

Chronic HBV/HCV infection can induce an inflammatory response that often lead to chronic liver injury which may activate lymphocytes (T cells, B cells and natural killer cells) to release TNFβ (Haybaeck et al., 2009; Aggarwal et al., 2012). It is an important mediator of hepatic fibrogenesis (Aggarwal et al., 2012). TNFβ can trigger TNF receptor (TNFR) 1 and TNFR2, inducing the classical and alternative nuclear factor - kB (NF-kB) signaling pathways, resulting in hepatic fibrogenesis (Haybaeck et al., 2009; Luedde and Schwabe, 2011; Aggarwal et al., 2012).

TNFβ has been implicated in the pathogenesis of acute and chronic HBV/HCV infection (Goyal et al., 2004; Tsuchiya et al., 2004; Suneetha et al., 2006). Recently, TNFβ, LTβ and LTβ receptor are shown to be upregulated in HBV- or HCV-induced hepatitis and HCC (Haybaeck et al., 2009). Sustained LT signaling represents a pathway involved in hepatitis-induced HCC (Haybaeck et al., 2009; Dwyer et al., 2014).

Single nucleotide polymorphism (SNP) of cytokine genes may affect the amount of cytokine expression (Haybaeck et al., 2009). Genetic variations in different individuals may alter the function of cytokine proteins, influencing the risk (Cheng et al., 2013; Liu et al., 2014; Wang et al., 2014; Zhang et al., 2014) and clinical outcomes of HCC (Pan et al., 2014).

There is a biallelic NCo I polymorphism in the coding region, at position +252 within the first intron, of the TNFβ gene. This polymorphism results in two allelic forms. The presence of A (adenine) defines the common TNFβ allele (10.5 kb), and the presence of G (guanine) defines the less common variant TNFβ allele (5.5 kb) (Messer et al., 1991). The latter has been linked to increased production of both TNFβ (Messer et al., 1991; Menges et al., 2008) and TNFα (Menges et al., 2008), respectively. The TNFβ allele has been linked with several inflammatory, autoimmune, infectious, and malignant diseases (Messer et al., 1991; Goyal et al., 2004; Tsuchiya et al., 2004; Suneetha et al., 2006; Menges et al., 2008; Haybaeck et al., 2009).

Hepatic fibrosis is a deleterious consequence of ongoing hepatic inflammation regardless of etiology. Growing evidence indicates that it is a pivotal and necessary stage to HCC (Tsai et al., 2004; Jeng et al., 2007; 2014; Haybaeck et al., 2009). The risk for HCC increased with severity of liver injury and adverse fibrosis or cirrhosis (El-Serag, 2012; Forner et al., 2012).

Cytokines are central in determining whether immune responses in the tumor microenvironment promote or inhibit cancer, or participate in tumor growth, invasion, and remote metastasis (Okamoto et al., 2010). It is known that functional genetic polymorphisms in cytokines are associated with the prognosis of various cancers (Du et al., 2010; Okamoto et al., 2010; Li et al., 2012; Lech-Maranda et al., 2013; Pan et al., 2014)

Recently, cytokine polymorphisms in TNF superfamily has been reported to be associated with susceptibility (Jeng et al., 2007, 2009; Cheng et al., 2013; Tian et al., 2014) and outcome of HCC (Pan et al., 2014). There is no information available on the association between TNFβ polymorphism and risk and prognosis of HCC. As TNFβ is an important mediator of hepatic fibrogenesis, we speculated that TNFβ +252 polymorphism might be a biomarker for susceptibility and prognosis of HCC. This case–control study was conducted to prove this hypothesis.

Materials and Methods

Study population

The study population included 150 consecutive patients with HCC, 150 consecutive patients with cirrhosis alone, and 150 unrelated healthy community residents who entered the hospital for health check-up. Each subject studied was pair-matched by sex and age (±5 year). These subjects were hospitalized or had visited outpatient clinics at Kaohsiung Medical University Hospital from January 2004 to December 2005.

Patients with HCC were eligible for the study if they were newly diagnosed by aspiration cytology or biopsy. HCC was staged according to the tumor-node-metastasis (TNM) classification (Greene et al., 2002). Cirrhosis was diagnosed by liver biopsy, abdominal sonography, biochemical evidence of parenchymal damage plus endoscopic esophageal or gastric varices (Tsai et al., 1993). Patients with cirrhosis were classified into the 3 Child-Pugh grades based on their clinical status. There was no space-occupying lesion in the liver in any healthy control or patients with cirrhosis alone, as evidenced by normal abdominal sonography. None of the controls had symptom, sign, or biochemical evidence (including aminotransferase levels) of liver disease at recruitment. All study subjects were Han Chinese. Signed informed consent was 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 a standard proteinase 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 the second or third 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 autoanalyzer (Hitachi, Model 736, Tokyo, Japan).

Polymorphism genotyping

The genotypes of TNFβ +252 (rs909253) were determined by polymerase chain reaction (PCR) with restriction fragment length polymorphism. We followed the methods described previously (Tsuchiya et al., 2004). A 782-bp fragment of genomic DNA containing the polymorphic Nco I restriction site was amplified. The primers used were 5'-CCG TGC TTC GTG CTT TGG

ACT A-3' (forward) and 5'- AGA GGG GTG GTA GCT TGG GTT C-3' (reverse), respectively. Amplification was performed in a thermocycler (GeneAmp 9700, Perkin Elmer, Norwalk, Connecticut) with 50 ul of PCR reaction mixture consisting of 500 ng of genomic DNA, 10 pM of each primer, 200 uM total dNTP, 2 mM $MgCl_2$, stand PCR buffer and 2 U Tag polymerase (Perkin Elmer, Norwalk, Connecticut). The following cycling conditions were used: 5 minutes at 95°C, 30 seconds at 94°C for 31 cycles, 150 seconds at 61°C, 30 seconds at 72°C and 10 minutes at 72°C. Subsequent to the amplification, 10 ul of the PCR product was digested with 5 units of Nco I at 37°C for 16 h on a 2% agarose gel and stained with ethidium bromide. The variant G allele contains an Nco I site and is digested into 586-bp and 196-bp fragments. Nco I does not cleave the A allele (782-bp). The heterozygous genotype (A/G) includes the presence of all three fragments.

Statistical analysis

The distribution of TNFβ genotypes in subjects studied was tested for deviation from the Hardy-Weinberg equilibrium using a goodness-of-fit χ^2 test.

The following statistical analyses were performed using the SPSS19.0 statistical package (IBM Co., Armonk, NY, USA). The difference between medians of continuous variables was analyzed with the Mann-Whitney U test. Categorical variables were compared with the χ^2 test with Yates' correction or Fisher's exact test where appropriate. Odds ratio (OR) with 95% confidence interval (95%CI) was used to estimate causal relations between risk factors and exposure. The conditional logistic regression analysis was used for multivariate analysis. Unconditional stepwise logistic regression analysis was used for estimating factors associated with TNFβ G/G genotype 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.

The additive model was used to assess the interactive effects among risk factors through logistic regression analysis and calculation of synergy Index (SI) as previously described (Rothman, 1986). By crossing TNFβ G/G genotype and chronic HBV/HCV infection, dummy variables of four categories were obtained (two for the presence of each risk factor in the absence of other, one indicating the presence of joint risk factors, and one for unexposed to either risk factor. The latter was used as the reference category in the regression model).

The cumulative survival of HCC was defined as the time from the date of diagnosis to death or to the last contact. The end of observation period is at end of December 2012. We used the Kaplan-Meier method and log-rank test to compute cumulative survival rate. Multivariate analysis with the Cox proportional hazards model was used to evaluate the independent roles of factors related to survival. Two-tailed P values and 95%CI were given where appropriate. An alpha of 0.05 was used as the indicator of statistical significance.

Results

Demographic profile of cases and controls

Details of the demographic characteristics of subjects studied were given in Table 1. At least one marker of HBsAg or anti-HCV was found in around 90.0% of patients with HCC or those with cirrhosis alone. Cirrhosis was found in 90.7 % (136 of 150) of patients with HCC.

Polymorphisms and alleles of the TNFβ +252 in patients and controls

TNFβ +252 genotypes in all subjects studied were in accordance with the Hardy-Weinberg equilibrium (data not shown). Genotype and allelic frequencies were presented in Table 2. The frequency of the variant TNFβ G/G genotype (26.0%) in patients with HCC were higher than that in patients with cirrhosis alone (14.7%, p=0.022) or healthy control (6.7%, p=0.0001). The frequency of the variant TNFβ G/G genotype in patients with cirrhosis alone was higher than that in healthy controls (p=0.040). On the contrary, the frequency of TNFβ A/A genotype in healthy controls was higher than that in patient group (each p=0.0001; Table 2).

The frequency of variant G allele in HCC patients (47.7%) was higher than that in patients with cirrhosis alone (37.3%; p=0.013) or healthy control (20.7%; p=0.0001). The prevalence of G allele in patients with cirrhosis was also higher than that in healthy controls (p=0.0001).

Table 1. Basic Characteristics of the Subjects Studied

Parameters		HCC (n=150)	Cirrhosis (n=150)	Healthy Controls (n=150)	p^a
Gender (M:F)		115:35	115: 35	115:35	NS
Age (median (ranges)) (yrs)		59 (37-74)	57 (37-73)	58 (35-73)	NS
HBsAg/anti-HCV	Negative/negative	15	16	110	0.0001
	Negative/positive	37	46	7	
	Positive/negative	84	83	33	
	Positive/positive	14	5	0	
	Cirrhosis	136	150	-	
Child-Pugh grade	A	70	77	-	
	B	42	51	-	
	C	24	22	-	
Tumor stage (I/II/III/IV)		25/36/59/30	-		

*anti-HCV, antibodies to hepatitis C virus; HBsAg, hepatitis B surface antigen; NS, nonsignificant; [a] Continuous variables and category variables were analyzed by Mann-Whitney U test and χ^2 test with Yates' correction, respectively

Independent risk factors for HCC by univariate and multivariate analyses

Using healthy control as a reference group, univariate analysis indicated that TNFβ G/G genotype, HBsAg-positivity, and anti-HCV-positivity were associated with the presence of HCC (Table 3). Multivariate analysis indicated that TNFβ G/G genotype (OR=3.64; 95%CI, 1.49-8.91), HBsAg-positivity (OR=16.38; 95%CI, 8.30-32.33), and anti-HCV-positivity (OR=39.11; 95%CI, 14.83-103.14) were independent risk factors for the presence of HCC (Table 3).

Interaction between TNFβ G/G genotype and chronic hepatitis B/hepatitis C virus infection on risk of HCC

As shown in Table 4, using subjects without carrying TNFβ G/G genotype and without chronic HBV/HCV infection as a reference, the risk for HCC increased in subjects with TNFβ G/G genotype alone (OR= 12.97; 95%CI, 2.57-65.57) or subjects with either chronic HBV/HCV infection alone (OR=29.48; 95%CI, 14.14-61.46). The highest OR was found in subjects with either chronic HBV/HCV infection who harbored TNFβ G/G genotype (OR=48.64; 95%CI, 17.51-135.09). Calculation of synergy index (SI) showed that there was an additive interaction between chronic HBV/HCV infection and carriage of TNFβ G/G genotype (SI =1.15; Table 4). However, there was no multiplicative interaction among them on multivariate analysis (data not shown).

Characteristics in HCC patients by status of TNFβ G/G genotype

The frequency of TNFβ G/G genotype in patients with cirrhosis was higher than that in patients without (28.7% vs. 0%, p=0.021; Fisher's exact test; Table 5). Among patients with cirrhosis, the frequency of carrying this SNP in patients with Child-Pugh A (14.3%) was lower than that in patients with Child-Pugh B (35.7%; p=0.016) or patients with Child-Pugh C (58.3%; p=0.0001; Table 5). In addition, there was a positive linear trend in the frequency of the variant SNP from Child-Pugh A to grade B and grade C ($p_{\text{for trend}}$ =0.005). The prevalence of carrying TNFβ G/G genotype in patients with higher AFP (>400 ng/ml) was higher than that in patients with lower AFP (37.5% vs.19.1%; p=0.022). Patients with thrombocytopenia (platelet count <150 x10^9/L) had higher frequency of this SNP (31.9%) than those without (5.9%; p =0.002). There was no significant difference with regard to sex, age > 50 years, TNM staging, patients with anticancer therapy, patients with abnormal serum aminotransferase concentration (data not shown). Multivariate analysis indicated that cirrhosis with Child-Pugh C (OR=5.20, 95%CI, 1.97-13.75), thrombocytopenia (OR= 5.06, 95%CI, 1.34-19.08), and serum AFP concentration >400 ng/ml (OR=2.33, 95%CI, 1.04- 5.21) were independent factors for harboring TNFβ G/G genotype (Table 6).

TNFβ G/G genotype as a biomarker for poor survival in patients with HCC

The median survival in 39 patients with TNFβ G/G genotype (1.62 year; 95%CI, 1.03-2.21 year) was shorter than that in 111 patients without (2.14 year; 95%CI, 1.58-2.70 year) (p=0.005, Kaplan-Meier method with log-rank test; Figure 1). Multivariate analysis with the Cox proportional model indicated that the TNFβ G/G genotype (hazard rate, 1.74; 95%CI, 1.11-2.73; p=0.015), cirrhosis with Child-Pugh C (hazard rate, 2.59; 95%CI, 1.47-4.55; p=0.001), and higher TNM stage (stage III and IV) (hazard rate, 4.55; 95%CI, 2.78-7.44; p=0.0001) were independent factors for poor HCC survival, whereas anti-cancer therapy (hazard rate, 0.07; 95%CI, 0.04-

Table 2. Distribution of TNFβ Genotypes in Patients and Controls

Genotype/ Variant allele	HCC (n=150) n (%)	LC (n=150) n (%)	Controls (n=150) n (%)	p^a	p^b	p^c
AA	46 (30.7)	60 (40.0)	98 (65.3)	NS	0.0001	0.0001
AG	65 (43.3)	68 (45.3)	42 (28.0)	NS	0.008	0.003
GG	39 (26.0)	22 (14.7)	10 (6.7)	0.022	0.0001	0.040
G allele	143 (47.7)	112 (37.3)	62 (20.7)	0.013	0.0001	0.0001

*HCC, hepatocellular carcinoma; LC, Liver cirrhosis; NS, Nonsignant'; TNF β, tumor necrosis factor β; [a]HCC *vs* LC; [b]HCC *vs* Control; [c]LC *vs* Control

Table 3. Risk for HCC by Univariate and Multivariate Analyses

Risk factors	HCC (n=150) n (%)	Controls (n=150) n (%)	OR (95% CI)	Adjusted OR [a] (95% CI)
TNFβ GG				
Present	39 (26.0)	10 (6.7)	4.92 (2.35-10.29)	3.64 (1.49-8.91)
Absent	111 (74.0)	140 (93.3)	1.0	1.0
HBsAg				
Positive	98 (62.3)	33 (22.0)	6.68 (3.88-11.55)	16.38 (8.30-32.33)
Negative	52 (37.7)	117 (78.0)	1.0	1.0
Anti-HCV				
Positive	51 (34.0)	7 (4.67)	10.52 (4.59-24.14)	39.10 (14.83- 103.14)
Negative	99 (66.0)	143 (95.33)	1.0	1.0

*anti-HCV, antibodies to hepatitis C virus; CI, confidence interval; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; OR, odds ratio; TNF β, tumor necrosis factor β; [a] Adjusted for HBsAg, and anti-HCV by logistic regression analysis.

Table 4. Interaction Between TNFβ Genotype and Chronic HBV /HCV Infection on Risk for Hepatocellular Carcinoma

TNFβ GG	HBsAg / anti-HCV	β	SE	p	OR (95% CI)	Synergy index [a]
Absent	Both negative				1.0	1.15
Present	Both negative	2.56	0.83	0.002	12.97 (2.57-65.57)	
Absent	Either positive	3.38	0.38	0.0001	29.48 (14.14-61.46)	
Present	Either positive	3.88	0.52	0.0001	48.64 (17.51-135.09)	

*anti-HCV, antibodies to hepatitis C virus; β, coefficient; CI, confidence interval; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC., hepatocellular carcinoma; OR, odds ratio.; TNF β, tumor necrosis factor β ; [a]Synergy Index = (OR11-1)/(OR01 + OR10 -2), where OR11 is odds ratio of the joint effect of 2 risk factors; OR01 and OR10 are OR of each risk factor in the absence of the other (Rothman, 1986)

Table 5. TNFb G/G Genotype in Relation to Clinical Parameters in HCC Patients

Parameters	Group	n	With TNFβ G/G n (%)	OR (95% CI)	p^a
Gender	Male	115	31 (27.0)	1.25 (0.51-3.03)	NS
	Female	35	8 (22.9)	1.0	
Age (yr)	>50	121	30 (24.8)	0.73 (0.30-1.78)	NS
	≤50	29	9 (31.0)	1.0	
Cirrhosis	Yes	136	39 (28.7)	1.40 (1.26-1.56)	0.021
	No	14	0 (0.0)	1.0	
Child-Pugh grade	A[b]	70	10 (14.3)	1.0	0.001
	B[b]	42	15 (35.7)	3.33 (1.21-9.27)	0.016
	C[b]	24	14 (58.3)	8.40 (2.62-27.85)	0.0001
HBsAg	positive	98	27 (27.6)	1.27 (0.58-2.77)	NS
	negative	52	12 (23.1)	1.0	
Anti-HCV	positive	51	8 (15.7)	0.41 (0.17-0.97)	NS
	negative	99	31 (31.3)	1.0	
AFP (ng/ml)	>400	56	21 (37.5)	2.53 (1.20-5.34)	0.022
	≤400	94	18 (19.1)	1.0	
AST (IU/ML)	>40	143	37 (25.9)	0.87 (0.16-4.69)	NS
	≤40 (ULN)	7	2 (28.6)	1.0	
ALT (IU/ML)	>40	133	38 (28.6)	6.40 (0.82-49.96)	NS
	≤40 (ULN)	17	1 (5.9)	1.0	
Platelet	≤150	116	37 (31.9)	7.49 (1.70-32.95)	0.002
($x10^9$/L)	>150	34	2 (5.9)	1.0	

*AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartic aminotransferase; CI, confidence interval; HCC, hepatocellular carcinoma; NS, nonsignificant; OD, odds ratio; TNFβ, tumor necrosis factor β; [a]χ^2 test with Yates' correction or Fisher's exact test where appropriate; [b]$p_{\text{for trend}}$=0.005 (Mantel-extension test for trend)

Table 6. Multivariate Analysis of Factors Associated with Harboring TNFβ G/G Genotype in Patients with HCC[a]

Variables	β	SE	*p* value	OR (95%CI)
Cirrhosis with Child-Pugh C	1.65	0.50	0.003	5.20 (1.97-13.75)
Thrombocytopenia	1.62	0.68	0.017	5.06 (1.34-19.08)
AFP > 400 ng/ml	0.84	0.41	0.040	2.33 (1.04-5.21)

AFP, α-fetoprotein; β, coefficient value; CI, confidence Interval; HCC, hepatocellular carcinoma; OR, odds ratio; SE, standard error; TNFβ, tumor necrosis factor β; [a]Unconditional stepwise logistic regression analysis: Dependent variable: presence of TNFβ G/G genotype Independent variables: male gender, age >50 years, Cirrhosis with Child-Pugh C, thrombocytopenia (platelet count <150 $x10^9$/L), and serum AFP >400 ng/ml

Table 7. Factors Associated with Cumulative Survival in Patients with HCC by COX Proportional Hazard Regression Analysis

Variables	β	SE	*p*	Hazard ratio (95% CI)
TNFβ G/G	0.56	0.23	0.015	1.74 (1.11-2.73)
Cirrhosis with Child-Pugh C	0.95	0.29	0.001	2.59 (1.47-4.55)
TNM (stage III and IV)	1.51	0.25	0.0001	4.55 (2.78-7.44)
Anticancer therapy	-2.64	0.32	0.0001	0.07 (0.04-0.13)
Age > 50 years	-0.68	0.24	0.005	0.51 (0.31-0.81)

β, coefficient, CI, confidence interval; HCC, hepatocellular carcinoma; SE, standard error; TNFβ, tumor necrosis factor β; TNM tumor, node, metastasis

0.13; *p*=0.001) and older age (hazard rate, 0.51; 95%CI, 0.31-0.81; *p*=0.005) were protective to patients' survival (Table 7).

Discussion

Using a formal epidemiologic approach, we demonstrated that there was an independent and additive interactions between the variant TNFβ G/G genotype and chronic HBV/HCV infection on presence for HCC. The TNFβ G/G genotype was a biomarker for poor survival

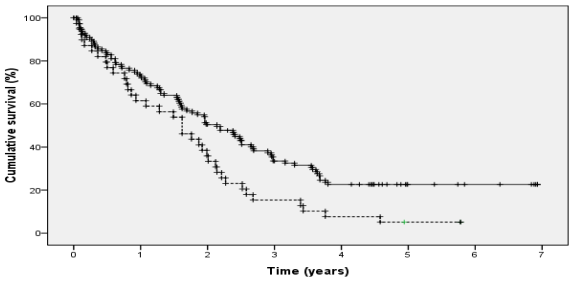

Figure 1. Cumulative Survival Curves by Status of the TNFβ G/G Genotype. The median survival in 39 patients with TNFβ G/G genotype (1.62 year; 95%CI, 1.03-2.21 year) was shorter than that in 111 patients without (2.14 year; 95%CI, 1.58-2.70 year) (*p*=0.005, Kaplan-Meier method with log-rank test)

of HCC. Moreover, this SNP was correlated with more severe liver damage and advanced hepatic fibrosis, which may contribute to higher risk and poor prognosis of HCC.

So far, the pathogenic mechanisms between the variant TNFβ +252 G/G genotype and risk for HCC remain largely unknown. Aberrant expression of cytokines is thought to be critically involved (Haybaeck et al., 2009; Aggarwal et al., 2012; Nahon et al., 2012). TNFβ is oncogenic and acts as a tumor promoter (Aggarwal et al., 2012). There are mRNA up-regulations of TNFα, TNFβ, and TNF receptor1 in HBV/HCV-related chronic hepatitis and HCC (Haybaeck et al., 2009). The TNFβ G/G SNP has been linked to increased production of TNFβ (Messer et al., 1991; Menges et al., 2008) and TNFα (Menges et al., 2008). As TNFβ carries out most of the activities of TNFα, this SNP may increase TNFα activity which was correlated with severity of hepatic inflammation, tissue injury and hepatic fibrosis (Jeng et al., 2007; 2009). Through TNFR1 signaling, TNFβ may activate the key inflammatory transcriptional regulator factor NFκB (Haybaeck et al.,

2009; Luedde and Schwabe, 2011; Aggarwal et al., 2012). Several lines of evidence indicate that NFκB pathway plays a pivotal role on hepatic inflammation, fibrosis, and HCC development (Haybaeck et al., 2009; Luedde and Schwabe, 2011; Dwyer et al., 2014). Hence, the TNFβ G/G SNP may aggravate persistent liver inflammation, hepatic injury, and progression of fibrosis in chronic liver disease. Moreover, our data indicated that HCC patients with higher serum AFP level, low platelet count and cirrhosis with Child-Pugh C were independent factors for harboring the TNFβ SNP (Table 5 and Table 6). Earlier study indicates an association between elevated serum AFP level and hepatic fibrosis (Bruce et al., 2008). Platelet count is reported to demonstrate the strongest correlation with hepatic fibrosis, portal hypertension, disease severity, and as a predictor of HCC (Lu et al., 2006). In light of these findings, patients with the TNFβ G/G genotype correlated with more severe liver disease and advanced fibrosis. This SNP may accelerate progression of hepatic fibrosis and liver injury, which could worsen chronic HBV/HCV-related liver disease and lead to earlier development of cirrhosis, resulting in a higher risk for HCC (Haybaeck et al., 2009). Moreover, fibrosis and its end-point cirrhosis are the main causes of morbidity and mortality in chronic liver disease (Luedde and Schwabe, 2011; El-Serag, 2012). It is considered as a useful indicator for poor prognosis in HCC (Luedde and Schwabe, 2011; Forner et al., 2012). This fact could explain that the TNFβ G/G genotype as a biomarker for poor HCC survival (Figure 1; Table 7). Accordingly, the TNFβ G/G genotype could be a causal predisposing factor for higher risk and poor survival of HCC through advanced fibrosis.

Regardless of etiology, chronic inflammation produces oxygen-derived free radicals and other reactive oxygen or nitrogen species (Choi and Ou, 2006; Schwabe and Brenner, 2006; Luedde and Schwabe, 2011). These compounds have been implicated as important mediators of hepatic fibrogenesis. They can be found in the inflammatory byproducts derived from chronic HBV/HCV infection (Choi and Ou, 2006; Schwabe and Brenner, 2006), TNFα derived from the the TNFβ G/G genotype (Menges et al., 2008) or activated Kupffer cells (Schwabe and Brenner, 2006). These compounds may cause oxidative DNA damage, which increase the risk for genomic alterations causing hepatic mutagenesis and carcinogenesis (Schwabe and Brenner, 2006; Luedde and Schwabe, 2011). These observations may explain, at least in part, the additive interaction between the TNFβ G/G SNP and chronic HBV/HCV interaction on risk for HCC (Table 4).

The principal strengths of the current study are the compelling associations identified. Genetic testing of the TNFβ +252 SNP may be useful in detecting high-risk individuals for HCC, particular in HCC endemic area such as Asia Pacific region (Bridges et al., 2011). However, this study carries some weaknesses. First, this is a hospital-based and not a population-based study, potential sources of bias caused by errors in determination of the study exposures or in ascertainment of study subjects may exist. Second, the power of our molecular epidemiologic analysis is limited by the relative small sample size. Further study on larger, independent groups of cancer patients and unrelated healthy controls should be undertaken to test and possibly extend our conclusions. Third, this study was performed in Han Chinese; therefore, the observed finding may not be generalizable to other populations. Hence, the results should be confirmed in a larger series as well as in patients of different ethic origin.

In conclusion, there are independent and additive interactive effects between the TNFβ G/G genotype and chronic HBV/HCV infection on risk for HCC. It is a biomarker for poor HCC survival. Carriage of this genotype correlated with disease severity and advanced hepatic fibrosis, which may contribute to a higher risk and poor survival of HCC. Chronic HBV/HCV infected subjects with this variant genotype should receive more intensive surveillance for early detection of HCC.

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