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TNFα promoter polymorphism is a risk factor for susceptibility in hepatocellular carcinoma in Korean population

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ABSTRACT

Background: The underlying genetic factors for the development and progression of hepatocellular carcinoma (HCC) are largely unknown. *TNFα* is a well characterized inflammatory mediator and is implicated in the development of HCC. We investigated *TNFα* polymorphisms for association with HCC.
Methods: The study population consisted of 227 HCC patients and 365 age and sex matched Korean controls. *TNFα* polymorphisms (G-238A, C-857T, and C-863A) were genotyped using pyrosequencing analysis. TNFα levels in patients with HCC were determined by enzyme linked immunosorbent assay (ELISA). Logistic regression analysis was used to determine the association with HCC and haplotype was calculated using EH program.
Results: Of three *TNFα* polymorphisms investigated in our study, C-863A did not correlate with HCC. However, both G-238A and C-857T were found to be significantly associated with HCC. *TNFα* −238A allele was more frequent in HCC patients than in control [$P=0.012$; odds ratio (OR), 1.89; 95% confidence interval (CI), 1.14–3.13]. *TNFα* −857T was significantly associated with HCC patients ($P=0.001$; OR, 1.63; 95% CI, 1.21–2.19). Haplotype analysis revealed that the GTC haplotype (G-238A, C-857T, C-863A) was a risk marker for HCC ($P=0.0021$). Serum TNFα level was significantly increased in HCC patients with CT + TT genotype for *TNFα* −857 ($P=0.018$).
Conclusion: Our data imply that *TNFα* G-238A and C-857T, not C-863A, polymorphisms may confer different susceptibilities to the development of HCC with *TNFα* −238A and −857T alleles playing as risk factors.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the third most common cancer in men and sixth in women in Korea. The geographic distribution of HCC is markedly different throughout the world. More than 80% of HCC occur in Asia, especially southeastern and Far East Asia where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic. The incidence of HCC in the Western world, however, is gradually increasing in accordance with the increasing incidence of HCV and now ranks as the fifth most common cancer in the world [1,2].

Approximately 70–80% of HCC in humans are linked to persistent viral infections with either HBV or HCV [3–6]. Prolonged exposure to HBV or HBC elicits the cellular immune response through activation of tissue macrophage Kupffer cells, which in turn produce an array of cytokines [7,8]. Of particular importance is tumor necrosis α (TNFα), which plays an important role in the immune pathogenesis of HCC. An increased TNFα level has been demonstrated in HBV and HCV infections [9–11] and is associated with the severity of hepatic inflammation, fibrosis, and tissue injury [12,13].

There exist individual differences in the production of TNFα which is tightly controlled at the transcriptional and post-transcriptional levels [14,15]. Also inflammatory response causes accumulated chromosomal abnormalities and altered gene expressions, which eventually leads to the development of HCC [16]. HCC is not exception to the well known fact that family history is a risk factor for the development of cancer. Thus it is quite tempting to speculate that as yet unraveled genetic factors play a significant role in the development or pathogenesis of HCC.

2. Patients and methods

2.1. Study population

Two hundred and thirty newly diagnosed patients with HCC (191 males and 39 females) were enrolled at Dongsan Medical Center. Each patient was diagnosed by aspiration cytology or biopsy and the tumor was staged according to the tumor-node-

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Table 1
Demographics and clinical variables of hepatocellular carcinoma patients ($N=227$).

Sex (male:female)	189:38
Age (mean value, SD)	57.7 ± 10.5
Type	
Diffuse	12
Massive	65
Nodular	150
Stage	
I and II	82
III, IV-A, and IV-B	145
Metastasis	
Present	24
Absent	203
Portal vein involvement	
Positive	80
Negative	147
Size	
<5 cm	126
≥5 cm	101
Cause	
Alcohol	35
Hepatitis B and C	188
Unidentified	4

metastasis system [17]. Tumor metastasis was diagnosed by computed tomography and portal vein involvement was determined by angiography. Control subjects consisted of 313 males and 64 females (mean age, 56.0 ± 9.2 y), who had visited outpatient clinics for their routine health checkups from September to December, 2004. Controls showed no clinical evidence of space-occupying lesion in the liver and no symptoms, signs, or biochemical evidence of liver disease, or other medical illness. All study subjects were Koreans. Written informed consent was obtained from each subject (Table 1). This study was approved by the ethics review committee of the Institutional Review Board, College of Medicine, Keimyung University.

2.2. Genomic DNA extraction and polymerase chain reaction (PCR)

Peripheral blood samples for DNA extraction from all subjects were collected in EDTA (ethylenediaminetetraacetic acid) tube. Genomic DNA was extracted from the white blood cells with the use of a DNA isolation kit for mammalian blood (Macherey-Nagel GmbH & Co.—Germany). The polymorphic regions for *TNFα* C-857T and *TNFα* C-863A were amplified by PCR (Gene-Amp PCR System 9600; Perkin-Elmer) with the primers (sense, 5′-CTC TGA GGA ATG GGT TAC AG-3′; antisense, 5′-CTC TAC ATG GCC CTG TCT T-3′). A 110 bp fragment of the *TNFα* was amplified by PCR in a 25 μl reaction volume containing standard reaction buffer [1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.1% Triton-X 100, 0.01% (v/v) stabilizer and 10 mmol/l Tris–HCl, pH 9.0] using 5 μmol/l of each primer, 0.5 U *Taq* DNA polymerase (HT Biotechnology Ltd.—UK), 25 μmol/l of each dNTP, and 25 ng of extracted genomic DNA as a template with 30 cycles at 95 °C for 30 s, 62 °C for 45 s, and 72 °C for 30 s. The 110 bp amplicon was checked for size and purity by 3% agarose gel electrophoresis. *TNFα* G-238A containing polymorphic region was also amplified by PCR with the primers (sense, 5′-GCA TCC TGT CTG GAA GTT AG-3′; antisense, 5′-ACA CAA GCA TCA AGG ATA CC-3′).

2.3. Sample preparation for pyrosequencing reactions

The antisense primer was biotinylated at the 5′ end to allow immobilization onto streptavidin sepharose beads (Streptavidin Sepharose HP; Amersham Pharmacia Biotech) according to the manufacturer's protocol (Pyrosequencing Advanced Biotechnologies, Uppsala, Sweden). Pyrosequencing of 20 μl of the PCR product immobilized onto the beads was performed using the sequencing primers (C-857T and C-863A, 5′-CAT GGC CCT GTC TTC-3′; G-238A, 5′-CCA TCC TCC CTG CT-3′), and SNP Reagent kits (Pyrosequencing Advanced Biotechnologies, Uppsala, Sweden) according to the manufacturer's instructions. SNP genotype analysis was performed using the SNP software in a PSQ 96 system (Pyrosequencing Advanced Biotechnologies, Uppsala, Sweden).

2.4. Enzyme linked immunosorbent (ELISA)

Blood was collected in standard cubes without anticoagulant, and was immediately centrifuged for 20 min, at 3000 rpm. Serum was stored at −20 °C until the time of final measurements. Serum levels of *TNFα* were measured using specific ELISA kits (Enzyme Amplified Sensitivity Immunoassay, Biosource, Belgium, Europe: Human *TNFα*), according to manufacturer's instructions.

2.5. Statistical analysis

The frequencies of distribution polymorphisms and control subjects were compared using the chi-square (χ^2) test. The odds ratios (OR) and 95% confidence internals (CI) were calculated to quantify the association between HCC and *TNFα* G-238A, *TNFα* C-857T and *TNFα* C-863A polymorphisms at the 5% level of significance. The SAS statistical package (release 8.02; SAS Institute Inc) was used. Haplotype was calculated using the EH program.

3. Results

The demographic characteristics of the study subjects are given in Table 1. HCC was more predominantly found in male than in female subjects (189 *vs* 38). Of 227 HCC cases, HBsAg or anti-HCV was found in 188 patients (82.8%).

The alleles at the *TNFα* −238, −857, and −863 positions in all study subjects were in accordance with the Hardy–Weinberg equilibrium (data not shown). The genotype distributions and allele frequencies of *TNFα* G-238A, C-857T, and C-863A are shown in Table 2. A variant homozygous of −238 polymorphism was found in one control and in none of HCC patients. Higher frequency of −238A allele was found in HCC patients (4.1% *vs* 7.5%; $P=0.012$). Genotype distribution of C-857T polymorphism was found to be strongly associated with HCC ($P=0.002$). Allelic frequencies of C-857T polymorphism were also significantly associated with HCC ($P=0.001$). No significant difference was observed in the genotype and allele frequencies of C-863A polymorphism between patients and controls.

The genotype distributions and allele frequencies of *TNFα* G-238A, C-857T, and C-863A in HCC with HBV/HCV and HCC without HBV/HCV were also evaluated (Table 3). −238G allele was more frequently found in HCC patents with HBV/HCV (93.9% *vs* 85.7%; $P=0.017$). No significant difference was observed in the genotype and allele frequencies of C-857T and C-863A polymorphisms between HCC with HBV/HCV and HCC without HBV/HCV.

The genotype distributions of *TNFα* G-238A, C-857T, and C-863A in HCC patients with different clinical characteristics (type, metastasis, portal vein involvement, stage, cause, and size) are shown in Table 4.

Table 2
TNFα polymorphisms: genotype and allele frequencies in Korean control individuals and the patients with hepatocellular carcinoma (HCC).

SNP	*TNFα* genotype frequencies				*TNFα* allele frequencies				
		Control (%)	HCC (%)	[a]*P*		Control (%)	HCC (%)	[a]*P*	OR (95% CI)
G-238A	GG	336 (92.1)	193 (85.0)	**0.014**	G	700 (95.9)	420 (92.5)	**0.012**	1.89 (1.14–3.13)
	GA	28 (7.7)	34 (15.0)						
	AA	1 (0.3)	0 (0.0)		A	30 (4.1)	34 (7.5)		
C-857T	CC	258 (70.7)	137 (60.4)	**0.002**	C	616 (84.4)	349 (76.9)	**0.001**	1.63 (1.21–2.19)
	CT	100 (27.4)	75 (33.0)						
	TT	7 (1.9)	15 (6.6)		T	114 (15.6)	105 (23.1)		
C-863A	CC	252 (69.0)	151 (66.5)	NS	C	605 (82.9)	372 (81.9)	NS	1.07 (0.79–1.45)
	CA	101 (27.7)	70 (30.8)						
	AA	12 (3.3)	6 (2.6)		A	125 (17.1)	82 (18.1)		

P values <0.05 are written in bold text.
[a] Calculated using the χ^2 test.