Tumor Necrosis Factor-α Genotypes Are Associated with Hepatocellular Carcinoma Risk in Taiwanese Males, Smokers and Alcohol Drinkers

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Abstract. *Aim: Hepatocellular carcinoma (HCC), the fifth most common cancer worldwide, has high prevalence and mortality rates in Taiwan. Tumor necrosis factor-α (TNFα), an important proinflammatory cytokine, is involved in multiple physiological and pathogenic phenomena that lead to the destruction and dysregulation of tissues. The present study aimed to evaluate the contribution of TNFA genotype, together with cigarette smoking and alcohol drinking lifestyle to the risk of HCC. Materials and Methods: In this hospital-based case–control study, association of TNFA single-nucleotide polymorphisms −1031T/C, −863C/A, −857T/C, −308G/A and +489A/G, with HCC risk were examined in 298 patients with HCC and 889 age- and gender-matched healthy controls. Results: The percentages of AA, AG and GG TNFA −308G/A were 6.4%, 18.1% and 75.5% in the HCC patient group and 2.0%, 16.0% and 82.0% in the non-cancer control group, respectively. The AA and AG genotypes were associated with 3.42- and 1.23-fold higher odds of HCC than the GG genotype (95% confidence interval=1.76-6.63 and 0.87-1.74, respectively). No such significant difference was found for other polymorphic sites. We further stratified the populations by gender, cigarette smoking and alcohol drinking status to investigate their combined contributions with TNFA −308G/A genotype to HCC risk. The results showed that the AA and AG genotypes of TNFA −308G/A increased HCC susceptibility which was obvious among males, smokers, and alcohol drinkers, but not females, non-smokers, or non-drinkers (p=0.0003, 0.0003, 0.0014, 0.6127, 0.7442 and 0.3010, respectively). Conclusion: Our results suggest that the AA and AG polymorphism of TNFA −308G/A genotypes associated with HCC risk in Taiwan, particularly among males, smokers and alcohol drinkers.*

Liver cancer, the fifth rated cancer worldwide and the third most common cause of cancer mortality, is prevalent in countries of low- or middle-income (over 80%), especially in the Chinese Han population (about 50%) (1). Clinically, hepatocellular carcinoma (HCC) accounts for over 90% of cases and is characterized by frequent local recurrence but relatively fewer metastases than other types of cancer (2, 3). Well-known factors for HCC include chronic infection with hepatitis B and C viruses, alcohol consumption and alcohol-related cirrhosis, tobacco consumption, overweight, diabetes and contamination of cereal foodstuff with aflatoxin in selected countries (4-6). In spite of great advances in HCC diagnosis and treatment (7), approximately 70% of patients with HCC receiving curative therapies had local or extrahepatic tumor recurrence (8). Therefore, useful

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biomarkers for identifying high-risk populations, as well as novel early detection and prediction tools in addition to preventive care are urgently needed.

One of the proinflammatory cytokines, tumor necrosis factor (TNF)-α, exerts a variety of physiological and pathogenic effects on the activation of a cascade of inflammatory cytokines and tissue dysfunction and destruction (9, 10). TNFα has two receptors, TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2) and in liver physiology, TNF-R1 plays a predominant role. The functions of TNF can be pro-inflammatory *via* activating of NFκB-related pathways, or apoptotic *via* activating caspase-8 (11).

The studies on the contributions of genetic variations in TNFA to human diseases have focused on the polymorphisms in regulatory regions which directly control its transcription levels, and subsequently influence the circulating levels of TNFα and increase susceptibility to human diseases, such as cancer (12-14). Among the promoter polymorphic sites, the -308G/A polymorphism has been the most frequently studied *TNFA* genotype in human disease susceptibility (15). However, the results of studies in this area are inconsistent among different populations and diseases. For instance, it was found that the *TNFA* -308G/A polymorphism increased the risk of breast cancer, cervical cancer and gastric carcinoma (16-18). However, certain studies have reported that no statistically significant association existed between the TNFA -308G/A polymorphism and cancer risk (19, 20). Therefore, more information on other polymorphic sites and on more populations are beneficial for revealing the contribution of *TNFA* polymorphisms and their possible influence on transcription of the *TNFA* gene.

In this study, we aimed to reveal the frequencies of genotypes of −1031T/C, −863C/A, −857T/C, −308G/A and +489A/G polymorphisms of *TNFA*, focusing on the combined effects of *TNFA* genotypes with factors such as smoking and drinking status on HCC susceptibility among Taiwanese people.

Materials and Methods

Investigated population and sample collection. Two hundred and ninety-eight patients diagnosed with HCC were recruited at the Departments of Surgery at the China Medical University Hospital, Taiwan, during 2004-2010. Three-times as many non-cancer healthy volunteers, as controls, were selected by matching for age and gender after initial random sampling from the Health Examination Cohort of China Medical University Hospital. The exclusion criteria of the controls included previous malignancy, metastasized cancer from other or unknown origin, and any genetic or familial diseases. The included control population was 894 individuals. Each patient and non-cancerous healthy person completed a self-administered questionnaire and provided their peripheral blood samples. Our study was approved by the Institutional Review Board of the China Medical University Hospital (DMR103-IRB-094) and written-informed consent was obtained from all participants. Those who have the habit of consuming cigarette and alcohol for at least half year were defined as ever smokers and drinkers.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) genotyping conditions. Genomic DNA was extracted from peripheral blood leucocytes using the QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and stored as previously published (21-23). The paired primers and restriction enzymes applied for TNFA genotyping are summarized in Table I. The PCR conditions were one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s, and a final extension at 72˚C for 10 min. After the PCR process, 10 μl of product was loaded into a 3% agarose gel containing ethidium bromide for electrophoresis. The genotype analysis was performed by two researchers independently and blindly. During this process, five of the control samples were excluded since their PCR products were not sufficient for clear genotyping recognition after PCR-RFLP.

Statistical analyses. Finally, 889 of the controls and 298 cases with genotypic and clinical data were analyzed and the data are shown in Tables III-VI. Pearson's Chi-square and Fisher's exact (when one or more cells were less than five) tests were used to compare the distribution of the genotypes between case and control groups. Differences between data were recognized as significant when the statistical *p*-value was less than 0.05. The HCC risk associated with the genotypes was estimated as an odds ratio (ORs) and 95% confidence intervals (CIs) by logistic regression.

Results

The characteristics for the investigated 298 patients with HCC and 889 non-cancer controls are summarized in Table II. Since we applied frequency matching methodology to select the non-cancer healthy controls, the distributions of age and gender were comparable among the cases and the controls (Table II). Regarding personal behavior, the HCC cases had a significantly higher percentage of smokers (75.2% *vs*. 65.1%, p=0.0017) and drinkers (69.1% *vs*. 58.3%, p=0.0011) than the non-cancerous controls (Table II).

The distributions of *TNFA* −1031T/C, −863C/A, −857T/C, −308G/A, +489A/G genotypes together with their frequencies among the controls and the patients with HCC are shown in Table III. After the statistical analysis, it was found that the genotypic frequencies of −1031T/C, −863C/A, −857T/C and +489A/G were not significantly different between the control and patient groups. For the −308G/A, the GG, AG and AA genotypic frequencies were 82.0%, 16.0% and 2.0% for the controls and 75.5%, 18.1% and 6.4% for the patients, respectively (Table III). The *p*-value for trend was 0.0008, and *p*-values for AG *versus* GG, AA *versus* GG, AG+AA *versus* GG and AA *versus* GG+AG at −308G/A were 0.2358, 0.0003, 0.0187 and 0.0007, respectively (Table III). The ORs for AG *versus* GG, AA *versus* GG, AG+AA *versus* GG and AA *versus* GG+AG at −308G/A were 1.23 (95% CI=0.87-1.74), 3.42 (95%

Table I. *Summary of the rs numbers, primers, amplicon lengths before and after enzyme digestion, restriction enzymes for all the TNFA polymorphisms investigated.*

rs Number	Primer sequence	Restriction enzyme	Amplicon length (bp)	Genotypes and enzymatic fragment sizes (bp)
−1031 (T/C) (rs1799964)	F: 5'-TATGTGATGGACTCACCAGG-3' R: 5'-CCTCTACATGGCCCTGTCTT-3'	Bbs I	264 C: 193 + 71	T: 264
−863 (C/A) (rs1800630)	F: 5'-GGCTCTGAGGAATGGGTTAC-3' R: 5'-CTACATGGCCCTGTCTTCGT-3'	Direct sequencing		
−857 (T/C) (rs1799724)	F: 5'-GGCTCTGAGGAATGGGTTAC-3' R: 5'-CCTCTACATGGCCCTGTCTA-3'	Direct sequencing		
−308 (G/A) (rs1800629)	F: 5'-AGGCAATAGGTTTTGAGGGC-3' R: 5'-ACACTCCCCATCCTCCCGGC-3'	Nco I	117	A: 117 G: 97 + 20
+489 (A/G) (rs1800610)	F: 5'-CCACATCT GTCTCCATATCT-3' R: 5'-CGCAAGAGAGGGAGAGAGAT-3'	HpyCH4 IV	249	A: 249 G: 178 + 71

Table II. *Distributions of selected demographic data of the 298 patients with hepatocellular carcinoma and the 889 matched controls.*

Characteristic	Controls (n=889)			Patients (n=298)			*p*-Value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			55.4 (4.9)			52.3 (4.5)	0.7418
Gender							
Male	636	71.5%		213	71.5%		
Female	253	28.5%		85	28.5%		0.9830
Smoking status							
Ever smokers	579	65.1%		224	75.2%		
Non-smokers	310	34.9%		74	24.8%		0.0017*
Drinking status							
Ever drinkers	518	58.3%		206	69.1%		
Non-drinkers	371	41.7%		92	30.9%		0.0011*

[a]Based on Chi-square test. *$p<0.05$.

CI=1.76-6.63), 1.48 (95% CI=1.08-2.02) and 3.42 (95% CI=1.77-6.61), respectively. Statistically speaking, the heterozygous AG and homozygous AA genotypes of *TNFA* −308G/A contributed to increased risk of HCC.

According to the records during the past decade, there were more males than females with HCC in Taiwan. We were interested in the genotypic contribution of *TNFA* −308G/A to gender difference in HCC susceptibility. After stratification by gender, the results showed that the genotypes of *TNFA* −308G/A were differently distributed among males (p=0.0003) but not females (p=0.6127) (Table IV).

The interaction of the genotype of *TNFA* −308G/A with cigarette smoking and alcohol drinking of the participants was also analyzed since HCC has been reported to be related smoking and alcohol consumption. The results in Table V show that the genotypic distribution of the variant *TNFA* −308G/A genotypes was differently distributed between HCC and control groups who were ever smokers (p=0.0003), but not for those who were non-smokers (p=0.7442) (Table V). Similarly, the results in Table VI showed that the genotypic distribution of the variant *TNFA* −308G/A genotypes was different between HCC and control groups who were ever drinkers (p=0.0014), but not different among the non-drinkers (p=0.3010) (Table VI). Overall, it seems that there exists an interaction between genetic (*TNFA* −308G/A genotype) and lifestyle (smoking and alcohol drinking behaviors) factors for HCC susceptibility.

Discussion

Liver cancer is a complex disease of uncertain etiological carcinogenesis. In recent years, the genomic contribution to liver cancer susceptibility in Taiwan has been investigated (24-27). *TNFA* gene is located in the class III region of

Table III. *Distribution of TNFA genotypes among the 298 patients with hepatocellular carcinoma and the 889 matched controls.*

Genotype	Controls		Patients		OR (95% CI)[a]	*p*-Value[b]
	n	%	n	%		
−1031T/C rs1799964						
TT	531	59.7%	177	59.4%	1.00 (reference)	
CT	319	35.9%	103	34.6%	0.97 (0.73-1.28)	0.8312
CC	39	4.4%	18	6.0%	1.38 (0.77-2.48)	0.2718
p-Value for trend						0.5006
(CT+CC) *vs*. TT					1.01 (0.78-1.32)	0.9456
CC *vs*. (TT+CT)					1.40 (0.79-2.49)	0.2728
−863C/A rs1800630						
CC	620	69.7%	198	66.4%	1.00 (reference)	
AC	231	26.0%	81	27.2%	1.10 (0.81-1.48)	0.5380
AA	38	4.3%	19	6.4%	1.57 (0.88-2.78)	0.1520
p-Value for trend						0.2828
(AC+AA) *vs*. CC					1.16 (0.88-1.54)	0.3114
AA *vs*. (CC+AC)					1.53 (0.87-2.69)	0.1585
−857T/C rs1799724						
CC	622	70.0%	203	68.1%	1.00 (reference)	
CT	228	25.6%	79	26.5%	1.06 (0.79-1.43)	0.6977
TT	39	4.4%	16	5.4%	1.26 (0.69-2.30)	0.5191
p-Value for trend						0.7268
(CT+TT) *vs*. CC					1.09 (0.82-1.45)	0.5613
TT *vs*. (CC+CT)					1.24 (0.68-2.25)	0.5241
−308G/A rs1800629						
GG	729	82.0%	225	75.5%	1.00 (reference)	
AG	142	16.0%	54	18.1%	1.23 (0.87-1.74)	0.2358
AA	18	2.0%	19	6.4%	3.42 (1.76-6.63)	0.0003*
p-Value for trend						0.0008*
(AG+AA) *vs*. GG					1.48 (1.08-2.02)	0.0187*
AA *vs*. (GG+AG)					3.42 (1.77-6.61)	0.0007*
+489A/G rs1800610						
GG	574	64.6%	198	66.4%	1.00 (reference)	
AG	278	31.3%	89	29.9%	0.93 (0.70-1.24)	0.6614
AA	37	4.1%	11	3.7%	0.86 (0.43-1.72)	0.7361
p-Value for trend						0.8246
(AG+AA) *vs*. GG					0.92 (0.70-1.21)	0.9450
AA *vs*. (GG+AG)					0.88 (0.44-1.75)	0.8653

[a]Odds ratio (ORs) and 95% confidence intervals (CIs). [b]Based on Chi-square test.* Significant at $p<0.05$.

human major histocompatibility complex (MHC) on chromosome 6p21 (28). Its encoded protein is an important inflammatory factor that acts as a master switch in the interaction between extracellular inflammation stimulation and intracellular signal transduction during carcinogenesis. A wide variety of evidence has pointed to a critical role of TNFα in tumor proliferation, migration, invasion and angiogenesis. Among the studied SNPs of *TNFA*, −1031T/C, −863C/A, −857T/C, −308G/A and +489A/G, the −308G/A is the one most commonly studied. A few studies have shown conflicting evidence for and against association of HCC with *TNFA* −308G/A among different populations (13, 29-32). Several of the studies found that the A allele was associated with increased risk in Turkish (13), Shandong (30) and Shanghai (32) people; while others found no association in Beijing and Italian people (29, 31). A recent meta-analysis of the *TNFA* −308G/A promoter polymorphism in HCC showed evidence of association for the A allele among Asians (33). In accordance with this, our results strongly suggest that the A allele and genotypic frequencies of the polymorphism at −308G/A were significantly different in Taiwanese HCC patients and controls. The functional analysis of polymorphism in the promoter region of *TNFA* −308G/A position also yielded conflicting observations. Some studies have suggested that the protein encoded by A allele of *TNFA* −308G/A has higher transcriptional activity, whereas other studies showed that this polymorphism appeared not to influence TNFα

Table IV. *Distribution of TNFA genotypes among patients with hepatocellular carcinoma after stratification by gender.*

Variable	−308G/A rs1800629 genotype			
	GG (%)	AG (%)	AA (%)	*p*-Value[a]
Males				
Controls	523 (82.2%)	102 (16.1%)	11 (1.7%)	
Cases	159 (74.7%)	39 (18.3%)	15 (7.0%)	0.0003*
Females				
Controls	206 (81.4%)	40 (15.8%)	7 (2.8%)	
Cases	66 (77.7%)	15 (17.6%)	4 (4.7%)	0.6127

[a]Based on Chi-square test or Fisher's exact text (when cell less than 5).*Significant at $p<0.05$.

Table V. *Distribution of TNFA genotypes among patients with hepatocellular carcinoma after stratification by personal smoking habit.*

Variable	−308G/A rs1800629 genotype			
	GG (%)	AG (%)	AA (%)	*p*-Value[a]
Smokers				
Controls	477 (82.4%)	92 (15.9%)	10 (1.7%)	
Cases	167 (74.6%)	41 (18.3%)	16 (7.1%)	0.0003*
Non-smokers				
Controls	252 (81.3%)	50 (16.1%)	8 (2.6%)	
Cases	58 (78.4%)	13 (17.6%)	3 (4.0%)	0.7442

[a]Based on Chi-square test or Fisher's exact text (when cell less than 5). *Significant at $p<0.05$.

Table VI. *Distribution of TNFA genotypes among patients with hepatocellular carcinoma after stratification by personal alcohol drinking habit.*

Variable	−308G/A rs1800629 genotype			
	GG (%)	AG (%)	AA (%)	*p*-Value[a]
Drinkers				
Controls	424 (81.9%)	84 (16.2%)	10 (1.9%)	
Cases	155 (75.2%)	36 (17.5%)	15 (7.3%)	0.0014*
Non-drinkers				
Controls	305 (82.2%)	58 (15.6%)	8 (2.2%)	
Cases	70 (76.1%)	18 (19.6%)	4 (4.3%)	0.3010

[a]Based on Chi-square (drinkers) and Fisher's exact text (non-drinkers). *Significant $p<0.05$.

production (34, 35). The genotypic-phenotypic correlation between the *TNFA* −308G/A genotype and circulation concentration of TNFα remains to be characterized.

In the present study, we improved our analytical power *via* enrolling a larger population of controls than our previous genotypic investigations in HCC (25-27). We have also further examined the interaction between genetic and lifestyle factors. In Table II, cigarette smoking and alcohol drinking lifestyles were found to be risk factors for HCC (Table II). After finding that the AG and AA genotypes of *TNFA* −308G/A were associated with HCC risk in the whole population (Table III), we aimed at revealing the contribution of *TNFA* −308G/A genotypes to Taiwanese in different groups by gender, and smoking and alcohol drinking behaviors. After stratifying both the controls and cases according to their gender, it was found that AG and AA genotypes of *TNFA* −308G/A were associated with increased risk of HCC for Taiwanese males (Table IV). For the females, the frequency of wild-type GG genotype was lower in the case group (77.7%) than the control group (81.4%), but the difference did not reach the statistically significant level ($p=0.6127$) (Table IV). As liver cancer is recognized to be one of the tobacco- and alcohol-related cancer types (6, 36), the interactions of *TNFA* −308G/A genotypes with smoking and drinking habits on HCC risk in Taiwanese were also of interest. For the smoking status, we found that AG and AA genotypes of *TNFA* −308G/A were associated with increased risk of HCC for the smokers but not the non-smokers (Table V). Similarly as for the alcohol drinking status, the association between *TNFA* −308G/A genotypes with HCC risk was obvious among ever drinkers but not non-drinkers (Table VI). Overall, males carrying variant A allele at *TNFA* −308G/A site may have higher HCC risk than those carrying wild-type G allele, especially for smoking- and alcohol-induced HCC development.

In conclusion, our findings suggest that the A allele of *TNFA* −308G/A is associated with increased HCC risk, especially among those who are male, smokers and alcohol drinkers in Taiwan. Further prospective studies in different ethnic populations will be necessary to confirm our findings and to elucidate the underlying genomic–lifestyle interactions for the carcinogenesis of HCC.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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