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RESEARCH ARTICLE

TIMP-3 -1296 T>C and TIMP-4 -55 T>C gene polymorphisms play a role in the susceptibility of hepatocellular carcinoma among women

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Abstract The purpose of this study was to investigate genetic impact of *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* gene polymorphisms on the susceptibility and clinicopathological characteristics of hepatocellular carcinoma (HCC). A total of 759 subjects, including 530 healthy controls and 229 patients with hepatocellular carcinoma, were recruited in this study. Allelic discrimination of *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* polymorphisms was assessed with the ABI StepOne™ Real-Time PCR System. Among women group, individuals with *TC* or *CC* alleles of *TIMP-3 -1296 T>C* gene polymorphism protected against HCC (AOR=0.35, 95% confidence interval (CI)=0.12–0.97; p=0.04) compared to individuals with *TT* alleles, after adjusting for other confounders. Also, women with *TC* alleles and with *TC* or *CC* alleles of *TIMP-4 -55 T>C* polymorphisms had a 2.52-fold risk (95%CI=1.23–5.13; p=0.01) and 2.47-fold risk (95%CI=1.26–4.87; p=0.008) of developing HCC compared to

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individuals with *TT* alleles, after adjusting for other confounders. There was no synergistic effect between gene polymorphism and environmental risk factors, including tobacco and alcohol consumptions and clinical statuses of HCC as well as serum expression of liver-related clinicopathological markers. In conclusion, gene polymorphisms of *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* play a role in the susceptibility of HCC among Taiwan women.

Keywords *Tissue inhibitor of metalloproteinase-3* · *Tissue inhibitor of metalloproteinase-4* · Single nucleotide polymorphism · Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the malignant cancer-related deaths worldwide and is the third most common malignancy and the second leading cause of cancer-associated death in Taiwan [1]. The prognosis of HCC is destitute and only rarity of HCC patients are cured because cancer cells inevitably survive, which results in HCC recurrence or metastasis, and the therapeutic effect for inhibition of cancer cell growth also remains in straitened circumstances [2–5]. Therefore, detection of candidates who are susceptible to HCC for preventing this malignance is strongly recommended.

Tissue inhibitor of metalloproteinase (*TIMP*) family, including-1, 2, 3, and 4, are associated with the inhibition of matrix metalloproteinase (MMP), which contributes to the degradation of extracellular matrix (ECM) for tissue remodeling, cancer cell invasion and metastasis [6–8]. Among *TIMP* family, *TIMP-3* is suggested to induce apoptosis, cause cell cycle arrest, and inhibit MMP activity, angiogenesis, tumor growth, invasion, and migration of HCC cells [6, 7, 9–12]. The expression of *TIMP-3* are markedly suppressed in human HCC cancer cells [6, 11, 13] and the liver of mice with diet-induced hepatocarcinogenesis [10]. It was demonstrated that repeated injection of plasmid encoding *TIMP-3* gene can inhibit tumor growth of human HuHZ HCC cells xenografted into nude mice [9]. The expressions of *TIMP-3* and *TIMP-4* are significantly reduced in malignant prostate tissues compared to benign tissues [14] as well as significantly downregulated in lung tumors and lung cancer cell line, and suggested that hypermethylation in the promoter region of *TIMP-4* could be involved in the transcriptional repression of *TIMP-4* in lung cancer [15]. Overexpression of *TIMP-4* by transfecting of a full-length *TIMP-4* complementary DNA(cDNA) into MDA-MB-435 human breast cancer cells significantly inhibited MMP activity and cancer cell invasion as compared to non-transfected cells [16]. Besides, an animal study showed that *TIMP-4* positive tumors grew more slowly and revealed a less metastasis than *TIMP-4* negative tumors [16]. Also, both *TIMP-3* and *TIMP-4* were significantly increased in response to treatment with 20 μM and 40 μM of curcumin, a biphenyl compound in the herb *Curcumal longa* and possesses anticancer effect, in MDA-MB-231 human breast cancer cell line, and their overexpression correlated with downexpression of MMP-2 and MMP-9 in a concentration- and time-dependent manner [8]. We therefore hypothesized that both genes contribute to the regulation of HCC development and prognosis.

TIMP-3 and *TIMP-4 genes* are separately located on chromosome 22q12.1 and 3p25 [15]. The promoter polymorphisms of *C* allele at the *−1296 T>C (rs9619311)* position of *TIMP-3* [17], and *C* allele at the *−55 T>C (rs3755724)* position of *TIMP-4* were respectively identified [18]. We suggested both *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* gene polymorphisms could alter the production or stabilization of functional proteins and influence the susceptibility of HCC [15]. However, the roles of these two gene polymorphisms on the risk of HCC are not investigated. The aim of this study was to estimate genetic impact of *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* gene polymorphisms on the susceptibility and clinicopathological characteristics of hepatocellular carcinoma among Taiwanese.

Materials and methods

Subjects and specimen collection

This was a hospital-based case–control study. A total of 229 patients with hepatocellular carcinoma diagnosed at Chung Shan Medical University Hospital, Taichung, Taiwan, were recruited as a case group between April 2006 and August 2013. The diagnoses of HCC were according to the characteristic criteria of the national guidelines for HCC [19], such as liver injury diagnosed by either histology or cytology irrespective of a-fetoprotein (AFP) titer where imaging data showed either one of following three cases: (1) one or more liver masses more than or equal to 2 cm in diameter via both computed tomography (CT) and magnetic resonance imaging (MRI), (2) one imaging data with early enhancement and a high level of AFP more than or equal to 400 ng/mL, and (3) one imaging data with early arterial phase contrast enhancement plus early venous phase contrast washout regardless of AFP level. A total of 530 healthy controls, who visited the Department of Family Medicine, Chung Shan Medical University Hospital, Taiwan for health examination, were selected based on no risks related to hepatocellular carcinoma and matched on demographic data of race, ethnic groups, and residential area. The whole blood specimens, collected from healthy controls and HCC patients, were placed in tubes containing EDTA and immediately centrifuged and stored at

−80 °C. Associated clinicopathological characteristics, such as HBsAg, anti-HCV, liver cirrhosis history, Child-Pugh grade, AFP, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and stage of HCC, were verified by chart review. Before commencing the study, approval was obtained from the Institutional Review Board of Chung Shan Medical University Hospital and informed written consent was obtained from each individual.

Genomic DNA extraction

Genomic DNA was extracted from whole blood samples collected from study subjects by QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacturer's instructions [20]. DNA was dissolved in TE buffer [10 mM Tris (PH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD_{260}. Final preparation was stored at −20 °C and used as templates in polymerase chain reaction (PCR) [21].

Real-time PCR

Allelic discrimination of the *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* gene polymorphisms was assessed with the ABI StepOne™ Real-Time PCR System (Applied Biosystems, Foster City , CA , USA) and analyzed using SDS vers. 3.0 software (Applied Biosystems), with the TaqMan assay. The FAM-primers used for analysis of TIMP-3 -1296 T>C (rs9619311) and TIMP-4 -55 T>C (rs3755724) genes polymorphisms were designed as FAM-5'- GAAGGG TGGAGCCCTGTC and FAM-5'- AGCTGCAGGAAGTG CTTTCAA, respectively. For each assay, appropriate controls (nontemplate and known genotype) were included in each typing run to monitor reagent contamination and as a quality control. The final volume for each reaction was 5 μL, containing 2.5 μL TaqMan Genotyping Master Mix, 0.125 μL TaqMan probe mix, and 10 ng genomic DNA. The real-time PCR included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To validate results from real-time PCR, around 5 % of assays were repeated and several cases of each genotype were confirmed by the DNA sequence analysis [22].

Statistical analysis

Hardy–Weinberg equilibrium was assessed using a goodness-of-fit χ^2 test for biallelic markers. The average is presented as the mean±SE. A Fisher's exact test was used to compare the differences of demographic characteristics distributions between healthy controls and patients with HCC, since the small sample size was present in some categorical variables. The one-way ANOVA test was used to detect the difference of continuous variables among three groups, and Scheffe correction was performed to check statistically significant difference between groups. The odds ratios (ORs) with their 95 % confidence intervals (CIs) of the association between genotype frequencies and hepatocellular carcinoma risk as well as clinical pathological characteristics were estimated by multiple logistic regression models after controlling for other covariates. A P value <0.05 was considered significant. The data were analyzed on SAS statistical software (Version 9.1, 2005; SAS Institute Inc., Cary, NC).

Results

In our recruited control group, the frequencies of genetic polymorphisms such as *TIMP-3 -1296 T>C* ($p>.05$; χ^2 value, 0.30) and *TIMP-4 -55 T>C* ($p>.05$; χ^2 value, 0.047) were in the Hardy–Weinberg equilibrium.

The demographical data and genetic distributions are shown in Table 1. There were significant differences of age and tobacco consumption between hepatocellular carcinoma patients and healthy controls. No association between gene polymorphisms and HCC was found. The adjusted odds ratios and 95 % confidence intervals were 0.83 (95%CI=0.52–1.33; $p=0.48$) and 0.29 (95%CI=0.03–2.70; $p=0.28$) to have HCC among individuals with *TC* and *CC* alleles compared to *TT* alleles of *TIMP-3 -1296 T>C*. People with *TC* and *CC* alleles of *TIMP-4 -55 T>C* genetic polymorphisms had 1.18 fold (95%CI=0.83–1.68; $p=0.35$) and 1.20 fold (95%CI=0.70–2.07; $p=0.50$) increased risk of developing HCC compared with those with *TT* homozygotes (Table 1).

The study also determined whether there was an interaction effect of gene-to-related-risk-factors on HCC susceptibility. The adjusted odds ratios and 95 % confidence intervals of genotypic frequencies and HCC susceptibility were estimated among persons with exposure to HCC-related risk factors, respectively. There was no significant association between genetic polymorphisms of *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* and HCC susceptibility among participants who had exposure to related environmental risk factors, including alcohol and tobacco consumption (Table 2). Also, there was no significant age-related association between genetic polymorphisms of *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* and HCC susceptibility (Table 3). However, among women group, subjects with *TC* or *CC* alleles of *TIMP-3 -1296 T>C* protected against HCC (AOR=0.35, 95%CI=0.12–0.97; $p=0.04$) compared to subjects with *TT* alleles, after adjusting for other confounders. Also, women with *TC* alleles and with *TC* or *CC* alleles of *TIMP-4 -55 T>C* had a 2.52-fold (95%CI= 1.23–5.13; $p=0.01$) and 2.47-fold (95%CI=1.26–4.87; $p=0.008$) of developing HCC compared to women with *TT* homozygotes, after adjusting for other confounders (Table 4).

Table 1 The distributions of demographical characteristics and gene polymorphisms in 530 healthy controls and 229 patients with hepatocellular carcinoma

Variable	Controls (n=530) (%)	Patients (n=229) (%)	AOR (95 % CI)	p value
Age (yrs)				
≤56	331 (62.5 %)	64 (27.9 %)	1.00	
>56	199 (37.5 %)	165 (72.1 %)	4.40 (3.06–6.31)	p<0.0001
Gender				
Female	92 (17.4 %)	68 (29.7 %)	1.00	
Male	438 (82.6 %)	161 (70.3 %)	0.75 (0.48–1.15)	p=0.18
Alcohol consumption				
No	321 (60.6 %)	148 (64.6 %)	1.00	
Yes	209 (39.4 %)	81 (35.4 %)	0.72 (0.50–1.04)	p=0.08
Tobacco consumption				
No	315 (59.4 %)	139 (60.7 %)	1.00	
es	215 (40.6 %)	90 (39.3 %)	1.48 (1.01–2.16)	p=0.04
TIMP-3				
TT	443 (83.6 %)	196 (85.6)	1.00	
TC	82 (15.5 %)	32 (14.0 %)	0.83 (0.52–1.33)	p=0.48
CC	5 (0.9 %)	1 (0.4 %)	0.29 (0.03–2.70)	p=0.28
TT	443 (83.6 %)	196 (85.6)	1.00	
TC or *CC*	87 (16.4 %)	33 (14.4 %)	0.79 (0.50–1.25)	p=0.32
TIMP-4				
TT	232 (43.8 %)	92 (40.2 %)	1.00	
TC	239 (45.1 %)	109 (47.6 %)	1.18 (0.83–1.68)	p=0.35
CC	59 (11.1 %)	28 (12.2 %)	1.20 (0.70–2.07)	p=0.50
TT	232 (43.8 %)	92 (40.2 %)	1.00	
TC or *CC*	298 (56.2 %)	137 (59.8 %)	1.18 (0.84–1.65)	p=0.31

The adjusted odds ratios (AORs) with their 95 % confidence intervals were estimated by multiple logistic regression models after controlling for age, gender, alcohol, tobacco consumption, and genetic polymorphism

Table 2 Adjusted odds ratio (AOR) and 95 % confidence interval (CI) of interaction effect between genotypic frequencies and hepatocellular carcinoma related environmental risk factors

Variable	Controls	Patients	AOR (95 % CI)	p value
Among alcohol consumption (n=290)				
TIMP-3	Control (n=209) (%)	Case (n=81) (%)		
TT	179 (85.7 %)	69 (85.2 %)	1.00	
TC	28 (13.4 %)	12 (14.8 %)	1.00 (0.46–2.18)	p=0.99
CC	2 (0.9 %)	0 (0 %)	—	p=0.98
TC or *CC*	30 (14.3 %)	12 (14.8 %)	0.88 (0.41–1.90)	p=0.75
TIMP-4				
TT	93 (44.5 %)	34 (42.0 %)	1.00	
TC	86 (41.2 %)	36 (44.4 %)	0.95(0.52–1.71)	p=0.86
CC	30 (14.3 %)	11 (13.6 %)	0.82 (0.35–1.91)	p=0.64
TC or *CC*	116 (55.5 %)	47 (58.0 %)	0.99 (0.57–1.73)	p=0.99
Among tobacco consumption (n=305)				
TIMP-3	Control (n=90) (%)	Case (n=215) (%)		
TT	72 (80 %)	179 (83.3 %)	1.00	
TC	18 (20 %)	33 (15.3 %)	1.40 (0.67–2.91)	p=0.36
CC	0 (0 %)	3 (1.4 %)	—	p=0.98
TIMP-4				
TT	38 (42.2 %)	84 (39.1 %)	1.00	
TC	39 (43.3 %)	103 (47.9 %)	0.71 (0.39–1.31)	p=0.27
CC	13 (14.5 %)	28 (13.0 %)	0.69 (0.29–1.63)	p=0.40
TC or *CC*	52 (57.8 %)	131 (60.9 %)	0.79 (0.45–1.38)	p=0.41

The adjusted odds ratios (AORs) with their 95 % confidence intervals were estimated by multiple logistic regression models after controlling for age, gender, alcohol, tobacco consumption, and genetic polymorphism

Table 3 Adjusted odds ratio (AOR) and 95 % confidence intervals (CIs) of hepatocellular carcinoma associated with genotypic frequencies in different age groups

Variable	Controls	Patients	AOR (95 % CI)	p value
Age >56 years old (n=364)				
TIMP-3	Control (n=199) (%)	Case (n=165) (%)		
TT	163 (81.9 %)	141 (85.5 %)	1.00	
TC	32 (16.1 %)	24 (14.5 %)	0.82 (0.45–1.50)	p=0.52
CC	4 (2.0 %)	0 (0 %)	—	p=0.98
TC or *CC*	36 (18.1 %)	24 (14.5 %)	0.72 (0.40–1.30)	p=0.27
TIMP-4				
TT	91 (45.7 %)	66 (40.0 %)	1.00	
TC	85 (42.7 %)	79 (47.9 %)	1.19 (0.75–1.88)	p=0.42
CC	23 (11.6 %)	20 (12.1 %)	1.14 (0.56–2.33)	p=0.70
TC or *CC*	108 (54.3 %)	99 (60.0 %)	1.16 (0.75–1.78)	p=0.49
Age ≤56 years old (n=395)				
TIMP-3	Control (n=331) (%)	Case (n=64) (%)		
TT	280 (84.6 %)	55 (85.9 %)	1.00	
TC	50 (15.1 %)	8 (12.5 %)	0.89 (0.39–2.01)	p=0.78
CC	1 (0.3 %)	1 (1.6 %)	7.16 (0.43–119.0)	p=0.16
TC or *CC*	51 (15.4 %)	9 (14.1 %)	0.99 (0.45–2.16)	p=0.98
TIMP-4				
TT	141 (42.6 %)	26 (40.6 %)	1.00	
TC	154 (46.5 %)	30 (46.9 %)	1.19 (0.66–2.15)	p=0.55
CC	36 (10.9 %)	8 (12.5 %)	1.33 (0.54–3.26)	p=0.52
TC or *CC*	190 (57.4 %)	38 (59.4 %)	1.11 (0.64–1.93)	p=0.69

The adjusted odds ratios (AORs) with their 95 % confidence intervals were estimated by multiple logistic regression models after controlling for age, gender, alcohol, tobacco consumption, and genetic polymorphism

Table 4 Adjusted odds ratio (AOR) and 95 % confidence intervals (CIs) of hepatocellular carcinoma associated with genotypic frequencies in different gender groups

Variable	Controls	Patients	AOR (95 % CI)	p value
Male (n=599)				
TIMP 3	Control (n=438) (%)	Case (n=161) (%)		
TT	367 (83.8 %)	134 (83.2 %)	1.00	
TC	68 (15.5 %)	26 (16.2 %)	0.96 (0.56–1.63)	p=0.89
CC	3 (0.7 %)	1 (0.6 %)	0.65 (0.05–7.32)	p=0.72
TC or *CC*	71 (16.2 %)	27 (16.8 %)	0.94 (0.56–1.59)	p=0.84
TIMP-4				
TT	177 (40.4 %)	68 (42.2 %)	1.00	
TC	211 (48.2 %)	72 (44.7 %)	0.88(0.58–1.33)	p=0.56
CC	50 (11.4 %)	21 (13.1 %)	1.04 (0.56–1.96)	p=0.88
TC or *CC*	261 (59.6 %)	93 (57.8 %)	0.87 (0.59–1.29)	p=0.50
Female (n=160)				
TIMP 3	Control (n=92) (%)	Case (n=68) (%)		
TT	76 (82.6 %)	62 (91.2 %)	1.00	
TC	14 (15.2 %)	6 (8.8 %)	0.40 (0.14–1.15)	p=0.09
CC	2 (2.2 %)	0 (0 %)	—	p=0.98
TC or *CC*	16 (17.4 %)	6 (8.8 %)	*0.35 (0.12–0.97)*	*p= 0.04*
TIMP-4				
TT	55 (59.8 %)	24 (35.3 %)	1.00	
TC	28 (30.4 %)	37 (54.4 %)	*2.52 (1.23–5.13)*	*p= 0.01*
CC	9 (9.8 %)	7 (10.3 %)	2.29 (0.69–7.55)	p=0.17
TC or *CC*	37 (40.2 %)	44 (64.7 %)	*2.47 (1.26–4.87)*	*p= 0.008*

Italic refer p value <0.05 is statistically significant.

The adjusted odds ratios (AORs) with their 95 % confidence intervals were estimated by multiple logistic regression models after controlling for age, gender, alcohol, tobacco consumption, and genetic polymorphism.

The relationship between gene polymorphisms of *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* and clinical pathological characteristics are shown in Table 5 and Table 6, respectively. There was no significantly association between gene polymorphisms and clinicopathological status of hepatocellular carcinoma.

Furthermore, we estimated the relations between genotypic frequencies and serum levels of liver-related clinicopathological markers such as alpha-fetoprotein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), as well as the ratio of AST to ALT among HCC patients. Also, there was no significantly association between liver-related clinicopathological markers and genotypic distribution of *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* (Table 7).

Discussion

In this study, we firstly provide novel information of the impacts of *TIMP-3 -1296 T>C (rs9619311)* and *TIMP-4 -55 T>C (rs3755724)* gene polymorphisms on the susceptibility of hepatocellular carcinoma among women.

TIMP-3 and TIMP-4 are associated with the inhibition of MMPs activity, tumor cell proliferation, invasion, and migration; and their expression are downregulated in several cancer cells; and therefore, their genetic polymorphisms could affect the process of carcinogenesis [6–16]. Only few studies investigated the effect of *TIMP-3 -1296 T>C* gene polymorphism on cancer susceptibility [23–25], but no study estimated the association between *TIMP-4 -55 T>C* genetic polymorphism and cancer risk. Lei et al. recruited 959 patients with breast cancer and 952 healthy control to investigate the association between polymorphisms and breast cancer susceptibility and found the *C* allele carriers of the *TIMP-3 -1296 T>C* SNP slightly increased the susceptibility to have breast cancer among Swedish (OR=1.25, 95%CI=1.05–1.50) [23]. However, Peterson et al. found no association between *TIMP-3 -1296 T>C* gene polymorphism and breast cancer risk and survival among enrolled 1,062 patients with breast cancer and 1,069 healthy control [24]. Also, there was no significantly different distribution of *TIMP-3 -1296 T>C* polymorphism between 241 patients with bladder cancer and 199 healthy controls [25].

To the best of our knowledge, both *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* genetic polymorphisms have not been

Table 5 Adjusted odds ratio (AOR) and 95 % confidence intervals (CI) of clinical statuses and *TIMP-3* genotype frequencies in hepatocellular carcinoma patients (*n*=229)

Variable	*n* (%)	*n* (%)	AOR (95 % CI)	*p* value
Clinical stage	*TT* (*n*=196)	*TC* or *CC* (*n*=33)		
Stage I/II	124 (63.3 %)	25 (75.8 %)	1.00	
Stage III/IV	72 (36.7 %)	8 (24.2 %)	1.32 (0.03–47.10)	*p*=0.87
Tumor size				
≦ T2	127 (64.8 %)	25 (75.8 %)	1.00	
> T2	69 (35.2 %)	8 (24.2 %)	0.34 (0.01–12.48)	*p*=0.56
Lymph node metastasis				
No	187(95.4 %)	33 (100 %)	1.00	
Yes	9 (4.6 %)	0 (0 %)	—	*p*=0.98
Distant metastasis				
No	185 (94.4 %)	33 (100 %)	1.00	
Yes	11 (5.6 %)	0 (0 %)	—	*p*=0.98
Child-Pugh grade				
A	142 (72.5 %)	26 (78.8 %)	1.00	
B or C	54 (27.5 %)	7 (21.2 %)	0.79(0.30–2.05)	*p*=0.63
HBsAg				
Negative	113 (57.6 %)	21 (63.6 %)	1.00	
Positive	83 (42.4 %)	12 (36.4 %)	0.56 (0.20–1.50)	*p*=0.24
Anti-HCV				
Negative	98 (50 %)	19 (57.6 %)	1.00	
Positive	98 (50 %)	14 (42.4 %)	0.51 (0.19–1.31)	*p*=0.16
Liver cirrhosis				
Negative	44 (22.5 %)	12 (36.4 %)	1.00	
Positive	152 (77.5 %)	21 (63.6 %)	0.56 (0.23–1.36)	*p*=0.20

The adjusted odds ratios (AORs) with their 95 % CI were estimated by multiple logistic regression models, after controlling for age, gender, alcohol, tobacco consumption, HBsAg, anti-HCV status, liver cirrhosis disease history, Child-Pugh grade, tumor size, lymph node metastasis, distant metastasis, and clinical stage for each estimated variable. > T2: multiple tumor more than 5 cm or tumor involving a major branch of the portal or hepatic vein(s)

Table 6 Adjusted odds ratio (AOR) and 95 % confidence intervals (CI) of clinical statuses and *TIMP-4* genotype frequencies in hepatocellular carcinoma patients (n=229)

Variable	n (%)	n (%)	AOR (95 % CI)	p value
Clinical Stage	*TT* (n=92)	*TC* or *CC* (n=137)		
Stage I/II	60 (65.2 %)	89 (65 %)	1.00	
Stage III/IV	32 (34.8 %)	48 (35 %)	0.55 (0.08–3.83)	p=0.55
Tumor size				
≦ T2	61 (66.3 %)	91 (66.4 %)	1.00	
> T2	31 (33.7 %)	46 (33.6 %)	1.68 (0.25–11.23)	p=0.59
Lymph node metastasis				
No	91 (98.9 %)	129 (94.2 %)	1.00	
Yes	1 (1.1 %)	8 (5.8 %)	9.04 (0.88–92.28)	p=0.06
Distant metastasis				
No	87 (94.6 %)	131 (95.6 %)	1.00	
Yes	5 (5.4 %)	6 (4.4 %)	0.56 (0.13–2.42)	p=0.44
Child-Pugh grade				
A	67 (72.8 %)	101 (73.7 %)	1.00	
B or C	25 (27.2)	36 (26.3 %)	0.97(0.52–1.82)	p=0.93
HBsAg				
Negative	56 (60.9 %)	78 (56.9 %)	1.00	
Positive	36 (39.1 %)	59 (43.1 %)	1.24 (0.61–2.52)	p=0.54
Anti-HCV				
Negative	45 (48.9 %)	72 (52.5 %)	1.00	
Positive	47 (51.1 %)	65 (47.5 %)	0.90 (0.45–1.78)	p=0.77
Liver cirrhosis				
Negative	21 (22.8 %)	35 (25.5 %)	1.00	
Positive	71 (77.2 %)	102 (74.5 %)	0.92 (0.46–1.80)	p=0.81

The adjusted odds ratios (AORs) with their 95 % CI were estimated by multiple logistic regression models, after controlling for age, gender, alcohol, tobacco consumption, HBsAg, anti-HCV status, liver cirrhosis disease history, Child-Pugh grade, tumor size, lymph node metastasis, distant metastasis, and clinical stage for each estimated variable. > T2: multiple tumor more than 5 cm or tumor involving a major branch of the portal or hepatic vein(s)

studied in HCC. Our current study was designed to estimate the relationship between both gene polymorphisms and HCC susceptibility, and we firstly found that *C* allele of *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* genetic variants significantly associated with the susceptibility of HCC among women but not in male people. In female population, individuals with *TC* or *CC* alleles of *TIMP-3 -1296 T>C* gene polymorphism significantly protected against HCC (AOR=0.35, 95%CI= 0.12–0.97; p=0.04) compared to individuals with *TT* alleles, after adjusting for other confounders. Moreover, women with *TC* alleles and with *TC* or *CC* alleles of *TIMP-4 -55 T>C* polymorphisms had a 2.52-fold risk (95%CI=1.23–5.13; p= 0.01) and 2.47-fold risk (95%CI=1.26–4.87; p=0.008) of developing HCC compared to individuals with *TT* alleles,

Table 7 The expression of clinicopathological markers in different *TIMP-3* and *TIMP-4* genotype frequencies of hepatocellular carcinoma patients (n=229)

Genotypic frequencies				
	Mean±SE *TT* (n=196)	Mean±SE *TC* (n=32)	Mean±SE *CC* (n=1)	*P* value
TIMP-3				
Alpha-fetoprotein (ng/ml)	4,094.58±1201.67	2,133.55±1488.68	229.25±0	p=0.79
AST (IU/I)	175.42±26.27	153.03±29.35	20.0±0	p=0.85
ALT (IU/I)	144.85±20.31	148.29±31.45	16.0±0	p=0.89
AST/ALT	1.50±0.09	1.41±0.15	1.25±0	p=0.90
TIMP-4				
	TT (n=92)	*TC* (n=109)	*CC* (n=28)	
Alpha-fetoprotein (ng/ml)	3,169.52±1299.16	4,955.17±1891.87	1,404.72±1091.23	p=0.50
AST (IU/I)	121.81±22.09	208.46±40.94	191.82±63.77	p=0.19
ALT (IU/I)	128.38±26.98	161.09±28.16	135.07±41.21	p=0.68
AST/ALT	1.38±0.13	1.57±0.12	1.53±0.18	p=0.56

The one-way ANOVA test was used to detect the difference of continuous variables among three groups, and Scheffe correction was performed to check statistically significant difference between groups

after adjusting for other confounders. It has been reported that promoter hypomethylation at −699, −502, −880, and –928 bp upstream of the transcription start point of *TIMP-3* significantly increased the gene expression of *TIMP-3* [26]; however, hypermethylation of *TIMP-3* resulted in decreased expression of *TIMP-3* and was associated with the risk of glioblastoma and pancreatic endocrine tumors [27, 28]. It is believed that women have a lower incidence of HCC than men partly because of protective effect of estrogen [29, 30]. It was found that postmenopausal hormone replacement therapy could be a protective factor from HCC [29]. Estrogen receptor-α promoter hypermethylation and reduced gene expression were found in HCC samples [30, 31], and the decreased expression was significantly related to stimulate hepatoma cell proliferation [30], high liver damage score, pathological invasion of the intrahepatic portal vein as well as the size of tumor [31]. Celebiler Cavusoglu et al. collected 62 invasive breast tumors to estimate breast cancer-related gene expression, they found the level of *TIMP-3* was positively correlated with that of estrogen receptor-α (p=0.0001) [32]. Lei et al. found that breast cancer patients who were both *TT* homozygotes at position *−1296* in *TIMP-3* tended to have both estrogen and progesterone receptor negative tumors (OR=1.81, 95 % CI= 1.03–3.21 and OR=2.10, 95%CI=1.18–3.86, respectively) [23]. Moreover, it was speculated that estrogen regulated the expression of *TIMP-4* by binding to the *TIMP-4* promoter region and downregulation of *TIMP-4* mRNA followed downregulation of estrogen receptor-α [33, 34]. We suggested that women with *TC* or *CC* alleles of *TIMP-3 -1296 T>C* gene polymorphism could tend to promote the level of estrogen receptor or advantage of a high level expression of *TIMP-3* that maybe modulated by estrogen receptor-α, and lead to their protective effect to against HCC. In addition, both estrogen and estrogen receptor-α gene expressions make a profit of high level gene expression of *TIMP-4* among women, which benefit to women from HCC; unfortunately, female individuals with *TC* alleles and with *TC* or *CC* alleles of *TIMP-4 -55 T>C* polymorphisms could limit the expression of *TIMP-4* which upregulated by estrogen and estrogen receptor-α, and subsequently increased their risk to have HCC. Our novel findings promote us to pay attention to different cancer-related mechanism and preventive strategy between female and male population and offer valuable information for further investigation.

It was reported that gene expressions of *TIMP-3* and *TIMP-4* were increased in heart tissue among alcohol-exposed rats [35]. Genetic polymorphisms, including *CCL5-28, CCL5-403*, and *CCR5*, and their synergic effects with alcohol and tobacco consumption have been found to increase HCC risk [36]. Moreover, phase II enzymes, including *glutathione s-transferase theta* (*GSTT1)* and *glutathione s-transferases P1* (*GSTP1*), gene polymorphisms show increased age-related susceptibility to HCC [37, 38]. Also, the level of *TIMP-3* protein in HBV-derived hepatomal cells was significantly lower than that in non-HBV-derived hepatoma cells and human normal liver hepatocyte cell line [6]. We therefore estimated interaction effect of both *TIMP-3 -1296 T>C* and *TIMP-4 -55 T>C* genetic polymorphisms and HCC-related environmental risk factor, such as alcohol and tobacco consumptions, as well as age-related susceptibility on HCC risk. Beside, the relationships between genetic polymorphisms and clinical statues and serum expression of clinicopathological markers among HCC patients were estimated. There was no synergistic effect between gene polymorphism and environmental risk factors and age-related susceptibility to HCC. Also, no association between these two genetic polymorphism and clinical statuses as well as clinicopathological markers was found. We hypothesized that these two genetic variants might not influence the progression of hepatocellular carcinoma and the contribution of these two gene polymorphisms on the susceptibility of HCC among women could be through the estrogen-related mechanism instead of affecting liver injury-related clinicopathological characteristics.

In conclusion, both *TIMP-3 -1296 T>C* (*rs9619311*) and *TIMP-4 -55 T>C* (*rs3755724*) gene polymorphisms are important factors for the susceptibility of hepatocellular carcinoma among Taiwan women but they might not influence the clinical pathological progression of HCC, and the contribution of these two gene polymorphisms on the susceptibility of HCC among women could be through estrogen-related mechanism.

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Conflicts of Interest None.

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