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ORIGINAL ARTICLE

The relevance of Tim-3 polymorphisms and F protein to the outcomes of HCV infection

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Abstract Hepatitis C virus (HCV) is one of the major causes of liver inflammation. The aim of this study was to investigate the associations of T-cell immunoglobulin and mucin domain-3 (Tim-3) polymorphisms and the alternate reading frame protein (F protein) with the outcomes of HCV infection. Three single-nucleotide polymorphisms (SNPs; rs10053538, rs12186731, and rs13170556) of Tim-3 were genotyped in this study, which included 203 healthy controls, 558 hepatitis C anti-F-positive patients, and 163 hepatitis C anti-F-negative patients. The results revealed that the rs12186731 CT and rs13170556 TC and CC genotypes were significantly less frequent in the anti-F-positive patients [odds ratio (OR) = 0.54, 95 % confidence interval (CI) = 0.35–0.83, $p = 0.005$; OR = 0.26, 95 % CI = 0.18–0.39, $p < 0.001$; and OR = 0.19, 95 % CI = 0.10–0.35, $p < 0.001$, respectively), and the rs13170556 TC genotype was more frequent in the chronic HCV (CHC) patients (OR = 1.70, 95 % CI = 1.20–2.40, $p = 0.002$). The combined analysis of the rs12186731 CT and rs13170556 TC/CC genotypes revealed a locus-dosage protective effect in the anti-F-positive patients (OR = 0.22, 95 % CI = 0.14–0.33, $p_{trend} < 0.001$). Stratified analyses revealed that the frequencies of the rs12186731 (CT + TT) genotypes were significantly lower in the older (OR = 0.31, 95 % CI = 0.15–0.65, $p = 0.002$) and female (OR = 0.30, 95 % CI = 0.17–0.52, $p < 0.001$) subgroups, and rs13170556 (TC + CC) genotypes exhibited the same effect in all subgroups (all $p < 0.001$) in the anti-F antibody generations. Moreover, the rs13170556 (TC + CC) genotypes were significantly more frequent in the younger (OR = 1.86, 95 % CI = 1.18–2.94, $p = 0.007$) and female (OR = 2.38, 95 % CI = 1.48–3.83, $p < 0.001$) subgroups of CHC patients. These findings suggest that the rs12186731 CT and rs13170556 TC/CC genotypes of Tim-3 provide potential protective effects with the F protein in the outcomes of HCV infection and that these effects are related to sex and age.

Introduction

More than 170 million people worldwide have been infected with hepatitis C virus (HCV) [1]. The majority of these patients fail to eliminate the virus and develop chronic liver

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diseases and the associated risk of severe liver damage, such as the damage resulting from hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [2]. HCV is a single-strand, positive-sense RNA virus that contains an open reading frame (ORF) and encodes structural (core, E1, and E2) and non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [3]. It is widely accepted that the outcomes of the infection may be influenced by the virus genetics, the host immune status, and the environment, and that the correlations between these factors are complex [4]. Although the mechanisms of T-cell dysfunction and exhaustion in chronically infected HCV patients are not well understood, the involvements of multiple immunoinhibitory receptors, such as programmed cell death-1 (PD-1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), and T-cell immunoglobulin and mucin domain-3 (Tim-3), have attracted increasing focus [5–7].

Tim-3 is a Th1-specific type I membrane protein that is rarely expressed on the surfaces of native T cells but is highly expressed on fully differentiated $CD4^+$ Th1 and $CD8^+$ Tc1 (cytotoxic) cells [8]. Tim-3 expression is increased on $CD4^+$ and $CD8^+$T cells in chronic HCV (CHC) infection, and Th1/Tc1 cytokine production is reduced [7]. Recently, Tim-3 was found to be a marker for regulatory T cells in human tumors [9] and to participate as a promoter in immunological tolerance via its interaction with its ligand, Galectin-9 [10]. Blocking the Tim-3–Tim-3 ligand interaction reinforces the ability to enhance T-cell proliferation and IFN-γ production [7]. As an immunosuppressive substance, Tim-3 has been extensively examined in relation to the immunity responses associated with hepatitis B virus (HBV), human immunodeficiency virus (HIV) infection, and HCC [11–13]. In chronic viral infections, HCV inhibits NF-κB-dependent miR-155 expression in NK cells, which, in turn, upregulates Tim-3 expression and leads to a feedback suppression of IFN-γ production [14]. High levels of Tim-3 expressed on activated NKs are associated with HCV infection, and this regulation might represent a target for the treatment of chronic viral infections [15].

The alternate reading frame protein, also called F protein, has a 10-min half-life and is translated from the core encoding regions by ribosomal frame shifting [16]. HCV F protein exhibits the paradoxical effects of eliciting activation and apoptosis in human dendritic cells and stimulating T cells [17]. The Th1/Th2 cytokine response is well known to be correlated with the pathogenesis of HCV infection [18], and F protein stimulation of peripheral blood mononuclear cells (PBMCs) can influence the balance of Th1/Th2 cytokine responses [19]. Additionally, high levels of anti-F antibodies have been detected in the sera of HCC patients [20]. The significantly greater frequency of F protein in CHC patients compared with resolved patients indicates that F protein could be a risk factor in HCV infection [21].

Although multiple studies have documented the involvement of Tim-3 in viral infections and the participation of F protein in HCV persistence and disease progression, the interaction of Tim-3 with F protein in HCV infection remains unknown. Thus, we designed a case–control study to investigate the associations of Tim-3 polymorphisms and F protein with the outcomes of HCV infection among a high-risk Chinese population.

Materials and methods

Study subjects

A total of 924 subjects were included in our research, including 721 CHC patients and 203 healthy controls. All subjects were recruited from Danyang and Jurong (Jiangsu province, Southeast China) from June 2012 to December 2013. All individuals with co-infections with any other virus (such as HBV and HIV), those who suffered from other types of liver diseases (such as alcoholic, autoimmune, or metabolic liver diseases), those with other high-risk infection, and those who were treated with any antiviral medications during the trial were excluded. All subjects were categorized into three groups for the analysis. Group A included 558 anti-F-positive subjects with positive serum tests for anti-HCV antibody and anti-F antibody. Group B consisted of 163 anti-F-negative subjects with a positive serum anti-HCV antibody test and a negative anti-F antibody test. Group (A + B) included those diagnosed with HCV infection in addition to high levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) for more than half a year. Group C was composed of 203 healthy controls who did not test positive for any viral infection or liver disease.

A standardized questionnaire was administered by well-trained interviewers to collect information about demographics and environmental exposure histories to guarantee the quality of the data. Venous blood from all the subjects was collected for serological, virological, and immunological analyses, and the stage of liver fibrosis was detected by transient elastography (FibroScan®, Echosens, Paris, France) [22]. All of this study's protocols were approved by the ethics committee of Nanjing Medical University and the Human Investigation Committee of the Huadong Research Institute for Medicine and Biotechnics (Nanjing, Jiangsu, China).

Virological testing

Approximately 5–10 mL of venous blood was collected from each participant. The blood samples were isolated and stored at −80 °C for further extraction of the genomic DNA and the serum for packet detection. Viral RNA was extracted using Trizol reagent according to the manufacturer's instructions

(Trizol LS Reagent, Life Technologies, Rockville, MD, USA), and the HCV genotypes were tested with reverse transcription polymerase chain reaction (RT-PCR) with type-specific primers for the 5′ non-coding region (5′ NCR) [23]. Anti-HCV antibodies were identified with third-generation enzyme-linked immunosorbent assays (ELISAs) (KHB, Shanghai, China). Additionally, the HCV F protein was expressed in *Escherichia coli* and purified with a protein purification apparatus [24], and the anti-F antibodies were detected in all the patients' sera with indirect ELISA [25]. The demographic and clinical characteristics of all the subjects are summarized in Table 1.

Tim-3 SNPs selection

The Tim-3 single-nucleotide polymorphisms (SNPs) were selected based on the public HapMap SNP database (http://www.hapmap.org) and the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) using the criteria of a minor allele frequency (MAF) >5 % in the Chinese Han population, and the SNPs had to have been reported to be associated with viral infections, such as HIV and HBV [26, 27] or immune-related disorders [28]. Taken all the above factors into consideration, three SNPs, rs10053538, rs12186731, and rs13170556, were chosen for genotyping.

Genotyping assays

Genomic DNA was extracted from the peripheral blood with sodium dodecyl sulfate and protease K digestion followed by phenol–chloroform extraction and ethanol precipitation. The Tim-3 SNP genotyping was performed with an improved multiple ligase detection reaction (*iMLDR*), and the detection procedures were supported by Genesky Biotechnologies Inc. (Shanghai, China). The details included the following processes. A 10-μL PCR reaction including the following components was prepared for each sample: 1 μL of 10× PCR buffer including 15 mM Mg2+ (Takara, Japan), 1 μL of 0.2 mM dNTPs mixture, 1 U of HotStarTaq polymerase (Qiagen, Hilden, Germany), 1 μL of sample DNA (10 μM), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), and addition of RNase-free dH2O to reach a volume of 10 μL. The PCR reaction conditions included the following steps: 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 65 °C for 40 s, and 72 °C for 1.5 min; 24 cycles of 94 °C for 20 s, 59 °C for 30 s, and 72 °C for 1.5 min; and a final extension at 72 °C for 2 min, followed by storage at 4 °C until the next step. Ten-microliter samples of PCR products were purified with 5 U of shrimp alkaline phosphatase and 2 U of exonuclease I (Qiagen) at 37 °C for an hour, followed by inactivation at 75 °C for 15 min. Two allele-specific fluorescently labeled probes were used for the SNP detection within 1 μL of 10× binding buffer, 2 μL of multiplex PCR product, 0.25 μL of thermostable Taq DNA ligase (Takara), 0.4 μL of 5′ ligation primer (1 μM), 0.4 μL of 3′ ligation primer (2 μM), and 6 μL of RNase-free dH2O. The reaction was performed with 38 cycles of 94 °C for 1 min and 58 °C for 4 min, and the sample was subsequently maintained at 4 °C. The data were analyzed with GeneMapper Software v.4.1 (Applied Biosystems, Foster City, CA, USA). All of the genotyping was performed in a double-blinded fashion, and 100 % consistency was observed for a random 10 % of the experiments that were repeated. The sequences of the probes and primers for the selected SNPs are illustrated in Table 2.

Statistical analyses

The data were analyzed with SPSS 20.0 (version 20.0; SPSS Institute, Chicago, IL, USA). The distributions of the general demographic, clinical, and virological features and genotype frequencies among all subjects were evaluated with Student's t tests, χ^2 tests, one-way analysis of variance (ANOVA), or Kruskal–Wallis tests. The Hardy–Weinberg equilibriums (HWEs) were estimated with the χ^2 goodness-of-fit test among the controls for each SNP. The associations of the SNPs with the HCV infection risks and anti-F antibody states were estimated with logistic regression analysis models, as were the odds ratios (ORs) and 95 % confidence intervals (CIs), which were adjusted for age, sex, and HCV genotypes. The joint effects of the Tim-3 SNPs were assessed with respect to the number of putatively favorable genotypes. Statistical significance was set at $p < 0.05$. Bonferroni corrections were applied for multiple comparisons between different genotypes.

Results

General characteristics of the study subjects

A total of 924 samples (558 anti-F-positive subjects, 163 anti-F-negative subjects, and 203 healthy controls) were enrolled in this study, and the basic characteristics of these participants are presented in Table 1. Obviously, there were no significant differences in the age and sex distributions between the three groups (all p-values > 0.05), and the HCV RNA loads also exhibited no differences between the anti-F-positive group and the anti-F-negative group ($p = 0.271$). However, the levels of ALT/AST were found to be significantly different between the CHC cases and the healthy controls. Moreover, the HCV viral genotypes and the stages of liver fibrosis also exhibited differences between the two patient groups (all p-values < 0.001).

Table 1 Demographic and clinical characteristics of all participants (anti-F-positive subjects, anti-F-negative subjects, and healthy controls)

Variables	Group A (%), $n=558$	Group B (%), $n=163$	Group C (%), $n=203$	p-Value
Age (mean ± SD)	57.82 ± 6.31	57.21 ± 6.83	57.33 ± 6.23	0.433[a]
Sex				0.355[b]
Females	238 (42.7)	72 (44.2)	91 (44.8)	
Males	320 (57.3)	91 (55.8)	112 (55.2)	
ALT (IU/L)	74.78 ± 38.48	61.54 ± 34.99	24.61 ± 9.62	<0.001[c]
AST (IU/L)	71.5 ± 32.31	61.79 ± 33.02	27.39 ± 10.1	<0.001[c]
HCV RNA($\times 10^6$ copies/mL)	3.09 ± 1.31	3.18 ± 1.22		0.271[c]
HCV genotype				<0.001[b]
1b	471 (84.4)	119 (73)	–	
Non-1	87 (15.6)	44 (27)	–	
Stage of liver fibrosis				<0.001[b]
F0	65 (11.6)	25 (15.4)	–	
F1	235 (42.1)	78 (47.9)	–	
F2	111 (19.9)	33 (20.2)	–	
F3	97 (17.4)	17 (10.4)	–	
F4	50 (9)	10 (6.1)	–	

Group A: anti-F-positive HCV patients; Group B: anti-F-negative HCV patients; Group C: healthy control; Group (A + B): HCV-infected individuals

ALT: alanine aminotransferase; AST: aspartate aminotransferase

1b: genotype 1b and 1b mixed; Non-1b: genotypes 1a, 2, 3, and others

Stage of liver fibrosis: liver fibrosis in patients was diagnosed with transient elastography; F0: no fibrosis; F1: mild fibrosis; F2: moderate fibrosis; F3: severe fibrosis; F4: cirrhosis

[a] p-Value of one-way ANOVA among three groups

[b] p-Value of χ^2 test among three/two groups

[c] p-Value of Kruskal–Wallis test or Mann–Whitney U test among three/two groups

Associations of the Tim-3 polymorphisms with anti-F antibody generation in CHC infection

As displayed in Table 3, the observed genotype frequencies among the controls were in agreement with HWE ($p=0.148$ for rs10053538, $\chi^2=2.09$; $p=0.102$ for rs12186731, $\chi^2=2.67$; and $p=0.318$ for rs13170556, $\chi^2=0.997$). The logistic regression analyses revealed that the frequency of the rs12186731 CT genotype in the anti-F-positive subjects was significantly different (for the CT genotype: OR = 0.54, 95 % CI = 0.35–0.83, $p=0.005$; additive model: OR = 0.50, 95 % CI = 0.33–0.76, $p=0.001$) than that among the anti-F-negative subjects. The carriers of the rs13170556 TC and CC genotypes were differently distributed in the different anti-F generations (for the TC genotype: OR = 0.26, 95 % CI = 0.18–0.39, $p<0.001$; for the CC genotype: OR = 0.19, 95 % CI = 0.10–0.35, $p<0.001$). Moreover, the frequency of the rs13170556 genotype was significantly different between the CHC patients and the healthy controls (for the TC genotype: OR = 1.70, 95 % CI = 1.20–2.40, $p=0.002$; additive model: OR = 1.77, 95 % CI = 1.27–2.45, $p=0.001$). However, no significant correlations with the distribution of the rs10053538 genotype were observed in the overall analyses (all p-values > 0.017).

Joint effect analysis of rs12186731 and rs13170556 with the anti-F antibody states of the CHC infection patients

Because two SNPs (rs12186731 and rs13170556) were found to be associated with significant differences in the anti-F antibody generations, we evaluated the combined effect of these SNPs according to the number of putatively favorable genotypes (rs12186731 CT and rs13170556 TC and CC) in the different anti-F antibody states. The results are presented in Table 4.

A locus-dosage effect was observed in the number of favorable genotypes; the proportions of carriers of one and/or two favorable genotypes significantly differed between the anti-F-positive patients and the anti-F-negative patients (OR = 0.22, 95 % CI = 0.14–0.33, $p<0.001$).

Stratified analyses of the three SNPs in Tim-3

To investigate the deep associations of the three SNPs (i.e., rs10053538, rs12186731, and rs13170556) with the anti-F antibody states of the HCV infection patients, we performed stratified analyses involving several subgroups. The results are presented in Table 5. The frequencies of the rs12186731

Table 2 Probes and primers of investigated Tim-3 single-nucleotide polymorphisms (SNPs) for genotyping assays

SNPs	MAF	*iMLDR* probe/primers sequences
rs10053538 C → A	0.066 (1000 g[a])	Probe-C: NED-TCTCTCGGGTCAATTCGTCCTTGGGTGGATCGCCTGAGGACG
		Probe-A: PET-TGTTCGTGGGCCGGATTAGTGGGTGGATCGCCTGAGGACT
		Pc: GGAGTTCAAGAACAGCCTGACCATTTTTTTTTT
		Forward primer: TGGAGTTTCGCTCTTTTTGTCCA
		Reverse primer: ATGTGCCTTTCGGCAAATCAAT
rs12186731 C → T	0.167	Probe-C: NED-TCTCTCGGGTCAATTCGTCCTTCTCCAGCCTGGGCGACACAG
		Probe-T: PET-TGTTCGTGGGCCGGATTAGTCTCCAGCCTGGGCGACACAA
		Pc: CAAGACTCTGTCTCAAAACAAAAACAAATTTTTTTTT
		Forward primer: CAGCATGAACTTGGTCAAGGCTCT
		Reverse primer: TGTGCGCTCTTAGTCCCAGCTA
rs13170556 T → C	0.172	Probe-T: PET-TGTTCGTGGGCCGGATTAGTCAACATCACAGGATGGCTGAGTT
		Probe-C: NED-TCTCTCGGGTCAATTCGTCCTTCAACATCACAGGATGGCTGAATC
		Pc: CAAATCACCTTTGATCTGTCACCTTGTTTTTTTTT
		Forward primer: TGGTTGCTCCAGAGTCCCGTAG
		Reverse primer: CCCACAACACAGAAGGGCACAT

iMLDR: improved multiple ligase detection reaction

1000 g[a] Minor allele frequencies from HapMap of Han Chinese in Beijing, China (CHB) or Human Genome Project

Pc: ligation primer

MAF: minor allele frequency

Table 3 Distributions of Tim-3 genotypes among the anti-F-positive, anti-F-negative, and control groups

Genotype	Group A (%), $n = 558$	Group B (%), $n = 163$	Group C (%), $n = 203$	Group (A + B)/Group C		Group A/Group B	
				OR (95 % CI)*	*p*-Value*	OR (95 % CI)*	*p*-Value*
rs10053538							
CC	487 (87.2)	140 (85.9)	172 (84.7)	–	–	–	–
CA	64 (11.5)	18 (11)	29 (14.3)	0.78 (0.49–1.22)	0.273	1.02 (0.58–1.77)	0.954
AA	7 (1.3)	5 (3.1)	2 (1.0)	1.67 (0.37–7.53)	0.507	0.42 (0.13–1.33)	0.139
Additive model				0.83 (0.54–1.29)	0.413	0.89 (0.53–1.47)	0.642
Dominant model				1.72 (0.38–7.77)	0.479	0.41 (0.13–1.33)	0.138
rs12186731							
CC	473 (84.8)	120 (73.6)	177 (87.2)	–	–	–	–
CT	82 (14.7)	39 (23.9)	21 (10.3)	1.73 (1.01–2.83)	0.301	**0.54 (0.35–0.83)**	**0.005**
TT	3 (0.5)	4 (2.5)	5 (2.5)	0.41 (0.13–1.30)	0.128	0.18 (0.04–0.81)	0.026
Additive model				1.47 (0.94–2.32)	0.094	**0.50 (0.33–0.76)**	**0.001**
Dominant model				0.38 (0.12–1.20)	0.100	0.20 (0.04–0.91)	0.037
rs13170556							
TT	339 (60.8)	46 (28.2)	136 (67)	–	–	–	–
TC	186 (33.3)	94 (57.7)	58 (28.6)	**1.70 (1.20–2.40)**	**0.002**	**0.26 (0.18–0.39)**	**<0.001**
CC	33 (5.9)	23 (14.1)	9 (4.4)	2.19 (1.05–4.54)	0.036	**0.19 (0.10–0.35)**	**<0.001**
Additive model				**1.77 (1.27–2.45)**	**0.001**	**0.25 (0.17–0.37)**	**<0.001**
Dominant model				1.80 (0.88–3.71)	0.110	**0.38 (0.21–0.66)**	**0.001**

Group A: anti-F-positive patient; Group B: anti-F-negative patients; Group C: healthy controls; Group (A + B): infected individuals; OR: odds ratio; 95 % CI: 95 % confidence interval

For multiple comparisons among genotypes, we applied the Bonferroni correction and the *p*-value was adjusted to 0.017 (0.05/3)

Data in **bold** indicate statistically significant values

*Logistic regression model, adjusted by age, sex, and/or viral genotypes

Table 4 Joint effect analysis of rs12186731 and rs13170556 genotypes in the anti-F-positive and anti-F-negative groups

Number of favorable factors	Group A (%), $n = 558$	Group B (%), $n = 163$	Group A/Group B	
			OR (95 % CI)*	p-Value*
0	294 (52.7)	32 (19.6)	–	–
1	230 (41.2)	106 (65)	**0.24 (0.15–0.36)**	**<0.001**
2	34 (6.1)	25 (15.4)	**0.14 (0.08–0.27)**	**<0.001**
Trend				
0	294 (52.7)	32 (19.6)	–	–
1–2	264 (47.3)	131 (80.4)	**0.22(0.14–0.33)**	**<0.001**

Group A: anti-F-positive patients; Group B: anti-F-negative patients

0: rs12186731 CC and rs13170556 TT, rs12186731 TT and rs13170556 TT

1: rs12186731 CT and rs13170556 TT, rs12186731 CC and rs13170556 TC, rs12186731 CC and rs13170556 CC, rs12186731 TT and rs13170556 TC, rs12186731 TT and rs13170556 CC

2: rs12186731 CT and rs13170556 CT, rs12186731 CT and rs13170556 CC

Data in **bold** indicate statistically significant values

*Logistic regression model, adjusted by age, sex, and/or viral genotypes

(CT + CC) genotypes were significantly different in the anti-F-positive subjects in the older (age > 58 years, OR = 0.31, 95 % CI = 0.15–0.65, $p = 0.002$) and female (OR = 0.30, 95 % CI = 0.17–0.52, $p < 0.001$) subgroups compared with the frequencies in the anti-F-negative subjects. Furthermore, the rs13170556 (TC + TT) genotypes were significantly associated with differences in CHC infection in the younger (age ≤ 58 years, OR = 1.86, 95 % CI = 1.18–2.94, $p = 0.007$) and female (OR = 2.38, 95 % CI = 1.48–3.83, $p < 0.001$) subgroups. Moreover, the data also revealed that the frequencies of the rs13170556 (TC + CC) genotypes were different between the different anti-F antibody generations in all the subgroups (all p-values < 0.001). However, there were still no significant associations of the rs10053538 genotype with HCV infection susceptibility and/or persistence in the overall analysis (all p-values > 0.017).

Discussion

Previous studies have revealed that patients with HCV infections develop specific humoral and cellular responses against the F protein [29], which is an additional protein synthesized by the core coding sequence frame [16]. The mechanisms that regulate T cells in HCV infection have been widely proven [30–32]. The high levels of Tim-3 on the surfaces of activated NK cells in CHC patients have provided a better understanding of the Tim-3 pathway [15], including the fact that Tim-3 might be a marker of NK cell differentiation [33]. The induction of its ligand, Galectin-9, by monocytes, macrophages, and Kupffer cells can modulate the innate and adaptive immune responses and provide a potential novel immunotherapeutic target in HCV infection [34, 35]. However, the interaction between Tim-3 and F protein in CHC infection remains unclear, especially in terms of the relevance of genetic variations.

In line with several observations, the blocking of Galectin-9 signals to Tim-3-expressing T cells results in improved immune responses [36], and Tim-3 genetic variations have been found to be associated with an increased susceptibility to osteoarthritis (OA), possibly due to an upregulation of INF-γ expression by $CD4^+$ T cells [28]. As described in detail, rs10053538 (-1516G/T), which is located in the promoter, has been found to be related to an increased risk for the distant metastasis of gastric cancer [37]. The genetic variants of Tim-3 exert important influences on the progression of HBV infection patients, with specific Tim-3 polymorphisms among those infected with HBV that might be potential candidates for HCC and HBsAg seroclearance [38], which indicates that Tim-3 polymorphisms may affect the disease susceptibility and HCC traits associated with chronic HBV infection. However, there are no reports about the associations of any SNPs with HCV. Therefore, we performed the present study to provide novel information about Tim-3 polymorphisms in CHC infection patients.

According to our results, the distribution analyses revealed that the rs13170556 TC genotype was more frequent among the patients than the healthy controls; thus, this genotype may play a predisposing role in CHC infection. Additionally, the results confirmed the correlations of the Tim-3 SNPs and F protein with HCV infection in the Chinese Han population. The frequencies of the rs12186731 CT and rs13170556 (TC + CC) genotypes were significantly lower in the anti-F-positive subjects than in the anti-F-negative subjects, which indicated that some Tim-3 genotypes may be associated with an antagonistic effect of F protein on the regulation of HCV infections.

Table 5 Stratified analyses of Tim-3 rs10053538, rs12186731, and rs13170556 polymorphisms with age and sex among the anti-F-positive, anti-F-negative, and control groups

SNPs	Allele	Subgroups	Group A (%) $n = 558$		Group B (%) $n = 163$		Group C (%) $n = 203$		Group (A + B)/Group C		Group A/Group B	
									OR (95 % CI)*	*p*-Value*	OR (95 % CI)*	*p*-Value*
rs10053538	C/A		CC	CA + AA	CC	CA + AA	CC	CA + AA				
		Age										
		≤58 years	219 (86.6)	34 (13.4)	79 (87.8)	11 (12.2)	95 (84.1)	18 (15.9)	0.80 (0.44–1.44)	0.450	1.11 (0.54–2.31)	0.773
		>58 years	268 (87.9)	37 (12.1)	61 (83.6)	12 (16.4)	77 (85.6)	13 (14.4)	0.87 (0.45–1.68)	0.672	1.01 (0.50–2.33)	0.854
		Sex										
		Male	208 (87.4)	30 (12.6)	63 (87.5)	9 (12.5)	77 (84.6)	14 (15.4)	0.78 (0.39–1.56)	0.476	1.02 (0.46–2.30)	0.955
		Female	279 (87.1)	41 (12.9)	77 (84.6)	14 (15.4)	95 (84.8)	17 (15.2)	0.94 (0.51–1.74)	0.848	0.81 (0.42–1.56)	0.524
rs12186731	C/T		CC	CT + TT	CC	CT + TT	CC	CT + TT				
		Age										
		≤58 years	211 (83.4)	42 (16.6)	69 (76.6)	21 (23.3)	100 (88.5)	15 (11.5)	1.73 (0.91–3.28)	0.094	0.67 (0.37–1.21)	0.185
		>58 years	262 (85.9)	43 (14.1)	51 (69.9)	22 (30.1)	77 (85.6)	15 (14.4)	0.86 (0.54–1.37)	0.536	**0.31 (0.15–0.65)**	**0.002**
		Sex										
		Male	192 (80.7)	46 (19.3)	58 (80.6)	12 (19.4)	79 (86.8)	12 (13.2)	1.36 (0.68–2.73)	0.388	1.02 (0.52–2.01)	0.949
		Female	281 (87.8)	39 (12.2)	62 (68.1)	29 (32.1)	98 (87.5)	14 (12.5)	1.12 (0.59–2.14)	0.733	**0.30 (0.17–0.52)**	**<0.001**
rs13170556	T/C		TT	TC + CC	TT	TC + CC	TT	TC + CC				
		Age										
		≤58 years	163 (64.4)	90 (35.5)	24 (26.7)	66 (74.3)	78 (69)	35 (31)	**1.86 (1.18–2.94)**	**0.007**	**0.21 (0.12–0.35)**	**<0.001**
		>58 years	176 (57.7)	129 (42.3)	22 (30.1)	51 (69.9)	58 (64.9)	32 (35.6)	1.64 (1.02–2.64)	0.042	**0.24 (0.13–0.45)**	**<0.001**
		Sex										
		Male	141 (59.2)	97 (40.8)	22 (30.6)	50 (69.4)	56 (61.5)	35 (38.5)	1.56 (0.94–2.57)	0.084	**0.31 (0.18–0.55)**	**<0.001**
		Female	198 (61.9)	122 (38.1)	24 (26.4)	67 (73.6)	80 (71.4)	32 (28.6)	**2.38 (1.48–3.83)**	**<0.001**	**0.22 (0.13–0.36)**	**<0.001**

Group A: anti-F-positive patients; Group B: anti-F-negative patients; Group C: healthy controls; Group (A + B): infected individuals; OR: odds ratio; 95 % CI: 95 % confidence interval

For multiple comparisons among genotypes, we applied the Bonferroni correction and the *p*-value was adjusted to 0.017 (0.05/3)

Data in **bold** indicate statistically significant values

*Logistic regression model, adjusted by age, sex, and/or viral genotypes

Moreover, the locus-dosage findings indicated that this effect was highly significant. There is no doubt that F protein increases the risk of viral responses in HCV infection [17, 19–21], and elevated circulating levels of Tim-3 have been reported in HCV-infected individuals [36], which indicates that Tim-3 could play a dangerous role in the outcomes of HCV infection [13, 15]. Tim-3 gene polymorphisms and F protein were found to exert an inhibitory effect in the analysis of our sample, as indicated by the finding that the balance of Th1/Th2 was disrupted by F protein. Tim-3 may contribute to the HCV-associated bias in the Th1/Th2 responses. Because all the detected samples were from the PBMCs, additional clinical in vivo research is needed to explore the underlying mechanisms.

Additionally, the stratified analyses of our sample suggested significantly increased frequencies of the rs12186731 (CT + TT) genotypes in the older and female subgroups, whereas the rs13170556 (TC + CC) genotypes were less frequent in all the subgroups of the anti-F-positive subjects compared with the anti-F-negative subject subgroups. Moreover, the rs13170556 (TC + CC) genotype frequencies indicated that the susceptibilities to HCV infection were significantly higher in the younger and female subgroups; thus, there was a strong correlation between sex and age. Sex differences have been identified as a barrier that needs to be overcome when managing HCV infections in patients of different ages [39]. Sex differences influence fibrosis progression and the likelihood of initiating HCV antiviral therapy, and are associated with the outcomes of treatment [40]. Therefore, the infections in different age and gender groups may necessitate different treatments.

No significant correlations of the rs10053538 genotypes with HCV infection were observed. However, a previous report declared that a Tim-3 (rs10053538, -1516G/T) polymorphism was found to be associated with some traits of HCC, including tumor grade and lymph node metastasis, which were more frequent in HBV-infected patients with the GT and TT genotypes [27]; however, little difference was observed in HCV expression. Although Tim-3 gene polymorphisms result in different performances in different viral infections, they are still considered to be potential risk factors in HCV infections.

The functional understanding of the polymorphisms in the Tim-3 gene is, thus far, incomplete. The Tim-3 SNPs in the promoter region do not have functional effects in vitro and have no associations with allergic diseases [41]. Regarding viral infections, many uncontrollable factors affect the expression results and may increase the difficulty of experimentally studying a single variable. Based on a fundamental reason, we have proposed a hypothesis to explain the interactions of Tim-3 genotypes with F protein in CHC infection.

These findings demonstrated that the rs12186731 CT and rs13170556 (TC + CC) genotypes were associated with protective effects in HCV infections in anti-F antibody generations that were mediated by age and gender-related regulation. Regarding the rs13170556 genotype, in our study, a potentially increased risk of infection was observed among the HCV patients with this genotype, and this risk was also associated with age and gender-related regulations, as indicated by the stratified analyses. However, this study is still limited by the sample size of the population and the small number of polymorphisms examined. The selection of SNPs might have been insufficient to detect the effects of the Tim-3 gene on susceptibility. Due to the imperfections of the geographical area and the uniformity of the ethnicities of our population, a replicate study with an independent cohort is still needed to confirm these observations.

Conclusions

In conclusion, our study is the first to demonstrate that the rs12186731 and rs13170556 genotypes of T-cell immunoglobulin and mucin domain-3 (Tim-3), in addition to the alternate reading frame protein (F protein), were associated with the outcomes of hepatitis C virus (HCV) infections in a Chinese population. All these analyses of Tim-3 may be useful in the design of immunotherapeutic strategies that may resolve T cell immune responses and complement available antiviral therapies by blocking the inhibitory signaling pathways. The interactions between Tim-3 polymorphisms and F protein in HCV infection may provide a specific new target for the treatment and prevention of HCV infections.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in the studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed content Informed consent was obtained from all individual participants included in this study.

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