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ARTICLE

*TBX*21 polymorphisms are associated with virus persistence in hepatitis C virus infection patients from a high-risk Chinese population

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Abstract Hepatitis C virus (HCV) infection is a major cause of chronic liver disease and the varied outcomes of the infection depend on both viral and host factors. We have demonstrated that the HCV alternate reading frame protein (F protein) is related to Th1/Th2 bias which is involved in virus persistence in chronic hepatitis C (CHC) patients. The purpose of this study was to test the hypothesis that genetic variants of *TBX*21 (T cell specific T-box transcription factor) were associated with the outcomes of HCV infection and F protein generation. Three single nucleotide polymorphisms (SNPs) (rs17250932, rs2074190, rs4794067) in the *TBX*21 gene were genotyped in a case–control study in a cohort of a high-risk group, including 354 healthy controls and 747 CHC patients (190 anti-F protein antibody seronegative patients and 557 anti-F protein antibody seropositive patients). Results showed that the rs4794067 C allele in the *TBX*21 promoter was significantly more common in CHC patients (OR=1.335, 95 % CI=1.058–1.684, P=0.015), exceptionally in anti-F protein seropositive patients (OR=1.547, 95 % CI=1.140–2.101, P=0.005), compared with healthy controls. And the risk effect was also significantly high in patients with HCV 1b genotype and mild fibrosis (P=0.021, P=0.010, respectively). Compared with the most frequent haplotype TAT, haplotype analysis showed that the distribution of TAC was significantly different between the chronic HCV carrier group and the healthy group, and so was the anti-F antibody seronegativity group and the anti-F antibody seronegativity group (all P<0.001). Our results suggested that *TBX*21 variants may be involved in the etiology of this disease.

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Introduction

Hepatitis C virus (HCV) infection is one of the major causes of liver inflammation, and characterized by high risk of developing persistent virus survival and chronic liver disease [1]. It has been reported that more than 170 million people have been infected with this virus worldwide so far [2]. Recent data from WHO declared that around 75–85 % of newly infected people developed into chronic infection and 60–70 % of chronically infected people developed into chronic liver disease, including

cirrhosis or hepatocellular carcinoma (HCC) (http://www.who.int/mediacentre/factsheets/fs164/en/index.html).

Although the mechanisms of chronic HCV infection are not clarified clearly yet, it has been accepted that the outcomes of the infection may be influenced by virus variants, host genetic factors and environment, such as clinical treatment, source of infection, co-infected with other virus and so on [3, 4]. Because of the individual variability and highly different response to antiviral therapy, it was reasonable to postulate a genetic basis for this virus evading host immune surveillance and leading to chronic infection. Several association studies have revealed that the gene polymorphisms of human leukocyte antigen (HLA), interleukin-28(IL-28) and IL-18, are associated with viral persistence and disease progression [5–8].

Considering the factors of virus, the effects of HCV core protein have been studied, including pro- and anti-apoptotic effects, regulation of cell growth, and immune-regulatory properties [9–11]. In addition, a novel HCV protein, called alternate reading frame protein (F protein) and translated from the core encoding region by ribosomal frame shifting, has been reported challenging various activities attributed to core protein [12–14]. However, different from core protein, it seemed that the expression of F protein was related to the disease progression and individual variability. That was to say, the rate of anti-F antibodies positive was not 100 % and was increased along with the progression of hepatitis C virus infection [13, 14]. And we have gained an intriguing observation that decreased Th1 cytokine [IFN-gamma, (IFN-γ)] and increased Th2 cytokines (IL-2, IL-4) in anti-F protein antibody seropositive patients may not be a beneficial sign for the clearance of the virus [13, 14]. We suspected that F protein may be a novel product for the host to adapt to the immune environment and make a difference in Th1/Th2 cytokine balance.

T-bet, also known as *TBX*21, is a T cell specific T-box transcription factor and has been demonstrated to play essential roles in Th1/Th2 balance [15]. *TBX*21 is located in human chromosome 17q21.32 and can regulate helper T cell differentiated to Th1 cells through the promotion of Th1 cytokines and inhibition of Th2 cytokines [16]. Its single nucleotide polymorphisms (SNPs) have been shown to be associated with many diseases recently, such as rheumatoid arthritis (RA), asthma, and type 1 diabetes mellitus (DM-1) [17–20]. Recent data have shown that the SNPs of rs17250932 and rs4794067 were linked to the production of IL-4 and IFN-γ, and viral persistence in hepatitis B infection was also associated with the polymorphism of rs 4794067 in*TBX*21 [21, 22].

The aim of our study was to further determine whether virus susceptibility, disease improvement, and F protein generation are associated with *TBX*21 gene polymorphisms in Chinese Han people. Three SNPs (rs17250932, rs2074190, rs4794067) in *TBX*21 gene were systematically selected and genotyped in 190 anti-F protein negative cases, 557 anti-F protein positive subjects and 354 healthy controls. By assessing the frequencies of the SNPs mentioned above, this was the first study to explicate the possible mechanisms of T-bet in the progression of chronic HCV infection.

Materials and methods

Study subjects

A total of 1,101 subjects were enrolled in our study, including 747 chronic HCV infection cases and 354 healthy controls. All subjects were recruited from Jurong and Danyang, Jiangsu Province, in southern China from June 2012 to December 2012 and had blood histories from the past 20–30 years. Patients co-infected with any other virus (such as HBV, HIV), with other types of liver diseases (such as autoimmune, alcoholic or metabolic liver diseases), with other high-risk infection behavior (which was a small population such as drug use or hemodialysis) or previous interferon and/or ribavirin therapy were excluded. All subjects were categorized into three groups for analysis. The healthy control group (group A) included 354 subjects who were tested anti-HCV seronegative. The patients were diagnosed with chronic HCV infection which was confirmed anti-HCV seropositive, HCV RNA seropositive, elevated alanine animotransferase (ALT) levels for more than two years. In addition, the HCV F protein was expressed and purified in our term and the anti-F protein antibodies were detected in all patients' serum using indirect enzyme linked immunosorbent assay (ELISA) according to our previous report [13]. Subsequently, all patients were assigned into two groups: anti-F negative (group B) and anti-F positive (group C) according to the presence of anti-F antibodies.

All subjects provided informed consent to participate in this study and were interviewed by well-trained interviewers. The standardized questionnaire included information about demographic data and environmental exposure history. Subjects' venous blood was collected for serological, virological, and immunological analyses, and the stage of liver fibrosis was measured by transient elastography (FibroScan®, Echosens, Paris, France) [23]. This study was approved by the ethics committee of Nanjing Medical University and ethics approval was obtained from the Human Investigation Committee of the Huadong Research Institute for Medicine and Biotechnics (Nanjing, China).

Virological tests

An approximately 5 mL venous blood sample was collected from each subject, and blood cells were isolated and stored at −80 °C for further extraction of genomic DNA. Viral RNA in patients' sera was extracted using the Trizol reagent according to the manufacturer's suggested protocol (Trizol LS Reagent, Life Technologies, Rockville, MD, USA) and the HCV

Table 1 SNPs that meet the selection inclusion

rs-number	Position	SNPs	MAF	Region
rs17250932	45,809,307	C/T	0.066 (1000 g[a])	5′Flanking
rs2074190	45,811,210	G/A	0.119	exon1
rs4794067	45,808,828	C/T	0.135	5′Flanking

[a] The MAF data of this site did not represent in Hapmap database, and related information about MAF was shown in the 1000 Genomes Project

SNPs single nucleotide polymorphisms, *MAF* minor allele frequency

genotypes were determined by reverse transcription RNA into cDNA and polymerase chain reaction (PCR) with type-specific primers in the 5′ non-coding region (5′NCR) [24]. Anti-HCV antibodies were tested by the third generation HCV enzyme immunoassays (KHB, Shanghai, China). Anti-F protein antibodies were tested as described above.

SNPs selection

We searched the SNPs from the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) and public HapMap SNP database (http://www.hapmap.org). SNPs meeting the following conditions were prior to be brought into our study: minor allele frequency (MAF) >5 % in Chinese Han population; reported to be associated with other diseases, especially liver or immune related disorders; no association with HCV infection in previous researches [17–22]. Given the genetic variation scanning for the promoter and/or exons of human *TBX*21 gene and studies about disease susceptibility, three SNPs were represented in Table 1.

Genotyping assays

Genomic DNA was extracted from peripheral blood by sodium dodecyl sulfate lysis and protease K digestion, followed by phenol-chloroform extraction and ethanol precipitation. Genotyping of SNPs was performed using PCR-ligase detection reaction (TaqMan assay) on an ABI Prism 377 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the technical support was from Genesky Biotechnologies Inc., Shanghai, China. Briefly, a 10-ul PCR reaction was prepared for each sample, containing 1×GC-I buffer (Takara, Japan), 3.0 mM Mg2+, 0.3 mM dNTPs, 1 U HotStarTaq polymerase (Qiagen, Hilden, Germany), 1 μL of sample DNA and 1 μL of each primer. The PCR program was described as followed: 95 °C for 2 min; 11 cycles of 94 °C for 20 s, 65 °C for 40 s, 72 °C for 1.5 min; 24 cycles of 94 °C for 20 s, 59 °C for 30 s, 72 °C for 1.5 min; and a final extension at 72 °C for 2 min. PCR products were treated with 5U of Shrimp Alkaline Phosphatase and 2U of Exonuclease I (Qiagen) and inactivation for 5 min. For ligase detection reaction, two allele-specific probes and one fluorescently labeled probe were used and this reaction was carried out in 1 μL of 10 × binding buffer, 0.25 μL of thermostable Taq DNA ligase (Takara), 0.4 μL of 1 μM 5′ ligation primer, 0.4 μL of 2 μM 3′ ligation primer, 2 μL of multiplex PCR product, and 6 μL of double-distilled H2O for 38 cycles of 94 °C for 1 min, 58 °C for 4 min and maintained at 4 °C. The products were denatured at 95 °C for 5 min before loaded on a ABI3730XL sequencer. Data were analyzed using GeneMapper Software v.4.0 (Applied Biosystems). The information of probes and primers for the selected SNPs was listed in Table 2. All the genotyping was performed in a blinded fashion without

Table 2 Probes and primers for analysis of *TBX21* polymorphisms

Position	SNPs	iMLDR primers/probe
−45809307T→C	rs17250932	Forward primer: GGGAGAACTGGGGAGGACTGGG
		Reverse primer: AGGATTAACATTTCAGTGAACACCCTCTGA
		Probe-T:TACGGTTATTCGGGCTCCTGTATGTGTAGTGTTTATATAAATTATATATTTGTGGAT
		Probe-C:TTCCGCGTTCGGACTGATATATGTGTAGTGTTTATATAAATTATATATTTGTGAAC
		Probe-P:GTGTGCAAAAGACAGAGACAGAGAgtatataa
−45811210A→G	rs2074190	Forward primer: GGACGCCGAGGGCTACCAG
		Reverse primer: GAAGCCAAAGCAGTGAGGGACA
		Probe-G:TCTCTCGGGTCAATTCGTCCTTACTACGCGCTACCCGCGTGG
		Probe-A:TGTTCGTGGGCCGGATTAGTACTACGCGCTACCCGCGTGA
		Probe-P:CTGGAGGTGTCGGGGAAACTTTTTTTT
−45808828T→C	rs4794067	Forward primer: GCTGCCCCACTTTGAACATCAG
		Reverse primer: GGAGGCTTGGGGGAGAGAGAAT
		Probe-T:TACGGTTATTCGGGCTCCTGTCCTGTTCCTTAAGGTACGGAGAAATCGT
		Probe-C:TTCCGCGTTCGGACTGATATCCTGTTCCTTAAGGTACGGAGAAATTGC
		Probe-P:GGGTAAGGTGTTGRGGAGGATTTTTTTTTT

iMLDR improved multiple ligase detection reaction

Table 3 Distributions of selected variables in healthy controls and HCV infection patients

Variables	Group A (%)	Group B (%)	Group C (%)	*P*
N	354	190	557	
Age [median (P25, P75)]	56 (49, 64)	56 (49, 61)	57 (50, 62)	0.065[a]
Sex				0.264[b]
Females	238 (67.2)	140 (73.7)	394 (70.7)	
Males	116 (32.8)	50 (26.3)	163 (29.3)	
ALT [median (P25, P75)]	21 (19, 23)	32.5 (20, 49)	31 (18, 49)	<0.001[a]
AST [median (P25, P75)]	23 (20, 27)	33.5 (25,46)	31 (24, 45)	<0.001[a]
ALB [median (P25, P75)]	47.0 (44.7, 47.8)	48.1 (46.4, 50.0)	48.7 (46.9, 49.8)	<0.001[a]
HCV RNA ($\times 10^6$ copies/mL)	–	2.78 (1.56, 3.43)	2.89 (1.89, 3.43)	0.192[a]
HCV genotype				<0.001[b]
1b	–	136 (71.2)	420 (75.4)	
Non-1	–	54 (28.4)	137 (24.6)	
Education level				<0.001[b]
primary school and below	234 (66.1)	121 (63.7)	376 (67.5)	
Junior	98 (27.7)	58 (30.5)	159 (28.5)	
Senior	20 (5.6)	11 (5.8)	22 (4.0)	
College	2 (0.6)	0	0	
Stage of liver fibrosis				<0.001[b]
F0	–	22 (11.6)	67 (12.0)	
F1	–	97 (50.1)	243 (43.6)	
F2	–	36 (18.9)	101 (18.1)	
F3	–	18 (9.5)	83 (14.9)	
F4	–	17 (8.9)	63 (11.3)	

Group A: healthy controls; Group B: anti-F negative subjects; Group C: anti-F positive subjects

HCV hepatitis C virus, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALB* albumin

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

Stage of liver fibrosis: Diagnosis methods for assessing liver fibrosis in patients were according with imaging examination; F0: no fibrosis; F1: mild fibrosis; F2: moderate fibrosis; F3: severe fibrosis; F4: cirrhosis

[a] Kruskal-Wallis test

[b] χ^2-test

knowledge of the patients' clinical data. The accordance rate of each SNP was 100 % for the repeated experiments of 10 % random samples.

Statistical analysis

Statistical analysis was carried out using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). The distributions of general demographic, clinical and virological features were evaluated using the Student's *t* test, χ^2-test, Kruskal-Wallis test. Allele frequencies for each SNP were determined by gene counting, and Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit χ^2-test. The association between SNPs and chronic HCV infection risk and anti-F antibody generation was determined using logistic regression analysis by computing the odds ratios (ORs) and 95 % confidence intervals (CIs), and *P*-value less than 0.05 was considered statistically significant. A linear regression model was used to analyse the relevance between HCV RNA load and stage of liver fibrosis. *TBX*21 haplotypes were assigned by the PHASE program.

Results

Samples' characteristics

The selected characteristics of 354 healthy controls, 190 anti-F seronegative patients and 557 anti-F seropositive patients were summarized in Table 3. Significant differences were observed in the distribution of ALT/AST levels between the healthy group and patients, and HCV genotype as well as stages of liver fibrosis also showed differences between the two patients' groups (all $P<0.001$). Also, subjects showed no differences in the distribution of age and sex among three groups in our study (all $P>0.05$). HCV RNA loads also showed no differences in the distribution between anti-F

Table 4 *TBX21* genotype distribution in healthy controls and chronic HCV patients

Genotype/ allele	Group A (%) (n=354)	Group B (%) (n=190)	Group C (%) (n=557)	Group C/ Group B		Group (B + C)/ Group A	
				OR (95%CI)[a]	p [a]	OR (95%CI)[a]	p [a]
rs17250932							
TT	298 (84.2)	158 (83.2)	471 (84.6)				
TC	51 (14.4)	31 (16.3)	79 (14.2)				
CC	5 (1.4)	1 (0.5)	7 (1.2)				
Additive model				0.990 (0.658, 1.491)	0.962	0.965 (0.707, 1.317)	0.822
Dominant model				0.938 (0.600, 1.467)	0.779	0.979 (0.691, 1.387)	0.906
T/ C	647 (91.4)/ 61 (8.6)	347 (91.3)/ 33 (8.7)	1021 (91.7)/ 93 (8.3)	0.990 (0.652, 1.502)	0.962	0.961 (0.698, 1.325)	0.810
rs2074190							
AA	280 (79.1)	152 (80.0)	436 (78.3)				
AG	69 (19.5)	36 (18.9)	115 (20.6)				
GG	5 (1.4)	2 (1.1)	6 (1.1)				
Additive model				1.121 (0.764, 1.643)	0.560	1.004 (0.755, 1.336)	0.977
Dominant model				1.142 (0.757, 1.724)	0.527	1.022 (0.749, 1.396)	0.890
A/ G	629 (88.8)/ 79 (11.2)	340 (89.5)/ 40 (10.5)	987 (88.6)/ 127 (11.4)	1.117 (0.766, 1.630)	0.565	1.003 (0.754, 1.333)	0.984
rs4794067							
TT	271 (76.6)	149 (78.4)	399 (71.6)				
CT	76 (21.5)	37 (19.5)	102 (18.3)				
CC	7 (1.9)	4 (2.1)	56 (10.1)				
Additive model				**1.547 (1.140, 2.101)**	**0.005**	**1.335 (1.058, 1.684)**	**0.015**
Dominant model				1.460 (0.985, 2.163)	0.059	1.180 (0.878, 1.585)	0.273
T/ C	618 (87.3)/ 90 (12.7)	335 (88.2)/ 45 (11.8)	900 (80.8)/ 214 (19.2)	**1.775 (1.256, 2.510)**	**0.001**	**1.436 (1.109, 1.861)**	**0.006**

Group A: healthy controls; Group B: anti-F negative subjects; Group C: anti-F positive subjects

Group (B + C): Infected individuals

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

Bold text indicates statistically significant values

[a] Logistic regression model, adjusted by age, sex and/or viral genotypes

seronegative subjects and anti-F seropositive subjects (P=0.192). In this study, the rate of anti-F antibodies positive was 74.6 % (557/747) and was consistent with our previous research and others reports (data not shown) [13, 14, 25].

Association analysis of *TBX*21 genetic polymorphisms with chronic HCV and anti-f antibody generation

The genotype distributions of rs17250932, rs2074190 and rs4794067 in *TBX*21 among three groups are presented in Table 4. The observed genotype frequencies among the control subjects were in agreement with the Hardy-Weinberg equilibrium (rs17250932: χ^2=2.56, P=0.11; rs2074190: χ^2=0.10, P=0.75; rs4794067: χ^2=0.38, P=0.54). As shown in Table 4, there was a significantly increased frequency of rs4794067 C allele in chronic HCV carrier subjects (OR=1.335, 95 % CI=1.058–1.684, P=0.015), compared to those in healthy controls. In addition, the frequency of rs4794067 C allele was higher exceptionally in anti-F positive subjects compared to those in anti-F negative subjects (OR=1.547, 95 % CI=1.140–2.101, P=0.005). However, no significant associations between the other two SNPs and HCV infection susceptibility and/or persistence were observed in the overall analysis (all P> 0.05).

Stratified analysis of the SNPs in *TBX*21 and the risk of persistent HCV infection

To determine the subgroup differences in the association of genetic polymorphisms (rs17250932, rs2074190 and rs4794067) with HCV infection or anti-F antibody generation, we performed a stratified analysis by disease-related characteristics (Tables 5 and 6). As shown, the CC genotype frequency of rs4794067 in anti-F seropositive subjects was significantly more common among subgroups of HCV 1b genotype, mild fibrosis than anti-F seronegative subjects (P=0.021, P=0.010, respectively), and the rs2074190 G allele showed increased frequency in the HCV non-1b genotype subgroup between the two groups (P=0.029). Furthermore, patients carrying the rs4794067 C allele might be related to an increased

Table 5 Stratified analysis for HCV genotype and stage of liver fibrosis on association between *TBX*21 rs17250932, rs2074190 and rs4794067 polymorphisms between anti-F negative subjects and anti-F positive subjects

SNPs	Allele	Subgroups		Group B (%)[a] (n=190)	Group C (%)[a] (n=557)	Group C/ Group B	
						OR(95 % CI)	P[b]
rs17250932	T/C	HCV genotype	1b	114(83.8)/21(15.5)/1(0.7)	361(86.0)/55(13.0)/4(1.0)	0.895(0.548,1.462)	0.658
			Non-1b	44(81.5)/10(18.5)/0(0.0)	110(80.3)/24(17.5)/3(2.2)	1.181(0.555,2.510)	0.666
		Stage of liver fibrosis	F0	18(81.8)/4(18.2)/0(0.0)	57(85.1)/9(13.4)/1(1.5)	0.963(0.297,3.125)	0.950
			F1	83(85.6)/14(14.4)/0(0.0)	212(87.2)/27(11.1)/4(1.7)	1.025(0.550,1.908)	0.939
			F2	30(83.3)/6(16.7)/0(0.0)	82(81.2)/18(17.8)/1(1.0)	1.232(0.469,3.237)	0.672
			F3	12(66.7)/5(27.7)/1(5.6)	69(83.1)/13(15.7)/1(1.2)	0.426(0.160,1.138)	0.089
			F4	15(88.2)/2(11.8)/0(0.0)	51(81.0)/12(19.0)/0(0.0)	1.589(0.313,8.074)	0.577
rs2074190	A/G	HCV genotype	1b	104(76.5)/30(22.0)/2(1.5)	335(79.8)/81(19.3)/4(0.9)	0.845(0.551,1.298)	0.442
			Non-1b	48(88.9)/6(11.1)/0(0.0)	101(73.7)/34(24.8)/2(1.5)	**2.806(1.113,7.076)**	**0.029**
		Stage of liver fibrosis	F0	17(77.3)/5(22.7)/0(0.0)	53(79.1)/12(17.9)/2(3.0)	1.029(0.369,2.872)	0.956
			F1	79(81.5)/17(17.5)/1(1.0)	193(79.4)/47(19.4)/3(1.2)	1.173(0.672,2.047)	0.575
			F2	27(75.0)/9(25.0)/0(0.0)	77(76.2)/24(23.8)/0(0.0)	0.926(0.380,2.257)	0.866
			F3	13(72.2)/4(22.2)/1(5.6)	66(79.5)/16(19.3)/1(1.2)	0.598(0.217,1.647)	0.320
			F4	16(94.1)/1(5.9)/0(0.0)	47(74.6)/16(25.4)/0(0.0)	5.585(0.675,46.241)	0.111
rs4794067	T/C	HCV genotype	1b	115(78.8)/28(19.2)/3(2.0)	305(71.9)/71(16.8)/48(11.3)	**1.500(1.063,2.116)**	**0.021**
			Non-1b	44(81.5)/9(16.7)/1(1.6)	98(71.5)/31(22.6)/8(5.9)	1.705(0.876,3.321)	0.116
		Stage of liver fibrosis	F0	18(81.8)/4(18.2)/0(0.0)	46(68.7)/15(22.4)/6(8.9)	2.210(0.795,6.145)	0.129
			F1	79(81.4)/18(18.6)/0(0.0)	177(72.9)/37(15.2)/29(11.9)	**1.861(1.162,2.981)**	**0.010**
			F2	26(72.2)/8(22.2)/2(5.6)	70(69.3)/21(10.8)/10(9.9)	1.213(0.646,2.275)	0.548
			F3	11(61.1)/6(33.3)/1(5.5)	63(75.9)/13(15.7)/7(8.4)	0.756(0.348,1.640)	0.479
			F4	15(88.2)/1(5.9)/1(5.9)	43(68.3)/16(25.4)/4(6.3)	2.413(0.759,7.670)	0.136

Group B: anti-F negative subjects; Group C: anti-F positive subjects

Bold text indicates statistically significant values

[a] Major homozygote/heterozygote/rare homozygote

[b] Derived from additive model, adjusted by age and sex, likely viral genotypes, and /or stage of liver fibrosis (the stratified factor in each stratum was excluded)

risk of anti-F antibody generation in the mild fibrosis subgroup and HCV 1b genotype subgroup (OR=1.861, 95 % CI=1.162–2.981; OR=1.500, 95 % CI=1.063–2.116, respectively). And linear regression modeling also suggested the HCV RNA loads were positively correlated with the stage of liver fibrosis (r=0.821, t=39.247, P<0.001) (data not shown). But no evidence showed significant associations between the genotypes of rs17250932 and the risk of disease.

Table 6 shows that the carriage of the C allele in the rs4794067 gene locus appeared to be associated with an increasing risk of persistent HCV infection in a younger subgroup (≤56 years, OR=1.423, 95%CI=1.028–1.970, P=0.034) and female subgroup (OR=1.374, 95 % CI=1.032–1.831, P=0.030). In addition, the results also revealed that the rs4794067 C allele could increase the risk of anti-F antibody generation in an older subgroup (>56 years, OR=1.858, 95%CI=1.151–2.998, P=0.011) and a male subgroup (OR=2.139, 95%CI=1.084–4.220, P=0.028).

Haplotype analysis among the three groups

To further evaluate the combined effect of three *TBX*21 polymorphisms (order of SNPs comprising haplotype: rs17250932T/C, rs2074190A/G and rs4794067T/C) on the risk of HCV persistent infection and F protein generation, we performed the haplotype analysis after measurement of pairwise linkage disequilibrium (LD) which was determined using the CubeX software (http://www.oege.org /software / cubex/) to calculate D' (standardized linkage disequilibrium coefficient) and r^2 (correlation coefficient) [26]. *TBX*21 rs17250932, rs2074190 and rs4794067 polymorphisms were found weak LD to each other: rs17250932/rs2074190 (D'=0.83, r^2=0.40), rs17250932/ rs4794067 (D'=0.90, r^2=0.53), rs2074190/ rs4794067 (D'=0.91, r^2=0.56). A total of eight haplotypes were derived from the observed genotypes and the haplotype frequencies are shown in Table 7. Compared with the most frequent TAT haplotype, TAC haplotype distribution was significantly different between the chronic HCV carrier group and the healthy group, as well as the anti-F seronegative

Table 6 Stratified analysis for age and sex on association between *TBX*21 rs17250932, rs2074190 and rs4794067 polymorphisms among controls and case

SNPs	Allele	Subgroups	Group A (%)[a] (*n*=354)	Group B (%)[a] (*n*=190)	Group C (%)[a] (*n*=557)	Group C/ Group B		Group (B + C) / Group A	
						OR(95 % CI)	P^b	OR(95 % CI)	P^b
rs17250932	T/C	Age							
		≤56	154(84.6)/25(13.7)/3(1.6)	81(82.7)/16(16.3)/1(1.0)	225(82.7)/44(16.2)/3(1.1)	1.037(0.592,1.816)	0.899	1.077(0.698,1.661)	0.737
		>56	144(83.7)/26(15.1)/2(1.2)	77(83.7)/15(16.3)/0(0.0)	246(86.3)/35(12.3)/4(1.4)	0.931(0.512,1.692)	0.814	0.881(0.563,1,378)	0.579
		Sex							
		Females	200(84.0)/36(15.1)/2(0.8)	113(80.7)/26(18.6)/1(0.7)	331(84.0)/58(14.7)/5(1.3)	0.894(0.565,1.415)	0.633	1.052(0.719,1.540)	0.794
		Males	98(84.5)/15(12.9)/3(2.6)	45(90.0)/5(10.0)/0(0.0)	140(85.9)/21(12.9)/2(1.2)	1.490(0.564,3.934)	0.421	0.786(0.454,1.361)	0.390
rs2074190	A/G	Age							
		≤56	149(81.9)/31(17.0)/2(1.1)	75(76.5)/22(22.5)/1(1.0)	205(75.4)/64(23.5)/3(1.1)	1.049(0.631,1.745)	0.854	1.377(0.908,2.089)	0.132
		>56	131(76.2)/38(22.1)/3(1.7)	77(83.7)/14(15.2)/1(1.1)	231(81.1)/51(17.9)/3(1.0)	1.201(0.670,2.151)	0.538	0.758(0.508,1.130)	0.174
		Sex							
		Females	188(79.0)/50(21.0)/0(0.0)	111(79.3)/27(19.3)/2(1.4)	316(80.2)/74(18.8)/4(1.0)	0.978(0.628,1.525)	0.923	0.989(0.690,1.417)	0.952
		Males	92(79.3)/19(16.4)/5(4.3)	41(82.0)/9(18.0)/0(0.0)	120(73.6)/41(25.2)/2(1.2)	1.635(0.752,3.557)	0.215	1.016(0.634,1.628)	0.947
rs4794067	T/C	Age							
		≤56	143(78.6)/33(18.1)/6(3.3)	74(75.5)/20(20.4)/4(4.1)	192(70.6)/54(19.9)/26(9.6)	1.327(0.888,1.982)	0.167	**1.423(1.028,1.970)**	**0.034**
		>56	128(74.4)/43(25.0)/1(0.6)	75(81.5)/17(18.5)/0(0.0)	207(72.6)/48(16.9)/30(10.5)	**1.858(1.151,2.998)**	**0.011**	1.255(0.901,1.750)	0.180
		Sex							
		Female	182(76.5)/53(22.3)/3(1.3)	106(75.7)/31(22.1)/3(2.2)	283(71.8)/73(18.5)/38(9.7)	1.388(0.979,1.968)	0.065	**1.374(1.032,1.831)**	**0.030**
		Male	89(76.7)/23(19.8)/4(3.5)	43(86.0)/6(12.0)/1(2.0)	116(71.2)/29(17.8)/18(11.0)	**2.139(1.084,4.220)**	**0.028**	1.248(0.840,1.855)	0.272

Group A: healthy controls; Group B: anti-F negative subjects; Group C: anti-F positive subjects. Group (B + C): infected individuals

Bold text indicates statistically significant values

[a] Major homozygote/heterozygote/rare homozygote [b] Derived from additive model, adjusted by age or sex, and/or viral genotypes

Table 7 Distribution of the estimated haplotype frequencies for *TBX21* polymorphisms in controls and case

Haplotype[a]	Group A (2n=708)(%)	Group B (2n=380)(%)	Group C (2n=1114) (%)	Group C/ Group B		Group (B + C) / Group A	
				OR(95 % CI)[b]	P^b	OR(95 % CI)[b]	P^b
TAT	604(85.3)	324(85.2)	869(78.0)	–	–	–	–
CGC	43(6.1)	25(6.6)	71(6.4)	1.084(0.674,1.744)	0.738	1.123(0.773,1.632)	0.541
CGT	3(0.4)	0(0.0)	5(0.4)	–	0.999	0.883(0.210,3.724)	0.866
CAC	12(1.7)	6(1.6)	11(1.0)	0.716(0.261,1.964)	0.516	0.676(0.320,1.429)	0.306
CAT	2(0.3)	2(0.5)	5(0.4)	0.939(0.181,4.882)	0.940	1.726(0.357,8.346)	0.497
TGC	22(3.1)	6(1.6)	28(2.5)	1.740(0.712,4.251)	0.224	0.795(0.460,1.374)	0.412
TGT	10(1.4)	9(2.4)	22(2.0)	0.931(0.423,2.051)	0.859	1.579(0.768,3.247)	0.214
TAC	12(1.7)	8(2.1)	103(9.3)	**4.708(2.264,9.789)**	**<0.001**	**4.728(2.584,8.651)**	**<0.001**

Group A: healthy controls; Group B: anti-F negative subjects; Group C: anti-F positive subjects; Group (B + C): infected individuals

Bold text indicates statistically significant values

[a] Order of single nucleotide polymorphisms comprising the *TBX*21 haplotypes: rs17250932T/C, rs2074190A/G and rs4794067T/C

[b] Logistic regression model, adjusted with covariates (including age, sex and/or viral genotypes)

group and anti-F seropositive group (all $P<0.001$). There was a significantly increased frequency of TAC haplotype in the chronic HCV infection subjects, exceptionally in anti-F seropositive subjects (OR=4.708, 95%CI=2.264–9.789; OR=4.728, 95%CI=2.584–8.651, respectively).

Discussion

HCV is a single-stranded (ss) RNA virus and the genome (~9.6 kb) includes a long open reading frame (ORF) encoding a polyprotein of approximately 3,000 amino acids which is processed by viral and cellular proteases into structural (core, E1, and E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [27]. Apart from these proteins, a novel HCV protein, called alternate F protein, has been previously reported [12]. As a ribosomal frameshift protein of HCV core, it expresses in all HCV genotypes with ribosomal frame shift occurring at codons 11–161 in HCV 1a genotype and 42–141 in HCV 1b genotype [12, 28, 29]. However, the expression of F protein showed high sequence diversity and individual variability with a very short half-life [13]. So we detected the anti-F antibody instead of the F protein itself in our study.

Persistent HCV infection is characterized by invalid adaptive immune response which included inefficient $CD4^+$T cell early and weak $CD8^+$cytotoxic T-lymphocyte (CTL) response subsequently [1, 2, 30]. Apart from CTL, the Th1-type cytokine IFN-γ may also exert an inhibition effect on HCV replication in chronically infected patients [30]. We have demonstrated that IFN-γ declined while IL-4 elevated in anti-F protein seropositive HCV patients and F protein stimulated groups [14]. In addition, HCV F could induce IL-6 secretion in HCC patients [13]. Our previous results also demonstrated that the rate of anti-F protein seropositivity was increased along with the progression of the disease [13]. All these data implied that the expression of the F protein during HCV infection plays an important role in the development of persistent infection. And F protein generation may be advantageous to virus survival through making a difference in Th1/Th2 cytokine balance, although the underlying mechanisms remain unclear.

The gene *TBX*21 encodes for transcription factor T-bet, which appears to act as a master switch for Th1 development responsible for the induction of Th1 cells and the repression of Th2 cells from naive T lymphocytes [15]. In addition, T-bet also shares an effect on Th1/Th2 cytokine dysregulation and it could increase IFN-γ production in Th1 cells, whereas represses the expression of Th2 cytokines such as IL-4, IL-5 and IL-13 [15, 31–34]. This could play an important role in disease progression in chronically infected patients.

Although the relationship between the genetic variations in *TBX*21 and persistent HCV infection has not been clearly demonstrated yet, several SNPs in *TBX*21 have been known to affect T-bet expression levels and be associated with disease susceptibility [17–20]. In our study, we showed that genetic variation in the *TBX*21 gene promoter, specifically the rs4794067 polymorphism, was significantly associated with sustainability to chronic HCV infection. A minor allele with C instead of T in this position was significantly more common among persistent HCV-infected subjects than healthy controls. This result was in accordance with genetic variation of *TBX*21 in hepatitis B virus carriers in Chinese Han people, as demonstrated by Chen et al. [22]. In addition, Sullivan et al. showed that lacking T-bet might increase susceptibility to *Mycobacterium tuberculosis* infection with increased IL-10 and decreased IFN-γ production in mice [35]. More recently, Yao et al. demonstrated that HCV could replicate in T cells and inhibit T cells proliferation through the Jak/T-bet/STAT pathway [36, 37]. A previous report also has demonstrated that *TBX*21 T-rs4794067C is a common

functional polymorphism that suppresses *TBX*21 promoter activity in activated $CD4^+$ T cells and that such suppression is mediated through binding affinity to the Yin Yang 1 (YY1) transcription factor. The *TBX*21 promoter carrying rs4794067C allele possesses significantly stronger affinity to the YY1 which affects T-bet expression and Th1 cytokine production [38]. In summary, the rs4794067 polymorphism may participate in T-bet expression and interact with infection factors, such as bacteria or virus. The results of cooperation seem to lead to inefficient T cells response with lower proliferation and imbalance in production of Th1/Th2 cytokines, which might be involved in the development of chronic HCV and help to provide a comfortable microenvironment for pathogen existence and new product generation.

In our present study, a minor allele with C instead of T in rs4794067 was significantly more common in anti-F protein seropositive subjects compared with anti-F protein seronegative subjects. The frequency of TAC haplotype at positions rs17250932, rs2074190 and rs4794067 was also significantly higher. Thus, carriage of the C allele in the rs4794067 gene locus may increase the risk of HCV F protein generation. Other studies also considered that the expression of the F protein would relatively decrease virus pathogenicity and play a protective role in the host. The hypothesis was building on lower HCV viral load in anti-F protein seropositive individuals [13]. However, HCV RNA load showed no differences in the distribution of anti-F seronegative subjects and anti-F seropositive subjects in our present study. The difference was possibly due to the variety of subjects or the small sample size in a previous experiment. Additionally, our previous results showed that F protein may participate in a protective T-cell-mediated immune response to protect liver cell injury from acute inflammation, with increased Th2 cytokine (IL-4) production and decreased Th1 cytokine (IFN-γ) production [13, 14]. This was according to Fyall et al., who reported that rs4794067C polymorphism was associated with decreased IFN-γ production [21]. The allele frequencies of rs17250932T/C and rs2074190A/G polymorphism were low in the population of our study and no significant associations were observed with the susceptibility of HCV infection or F protein generation.

The previous report declared that the differences in disease exposure should also be taken into account in disease-related characteristics [39]. So we assessed the genotype frequency of rs17250932, rs2074190 and rs4794067 in several subgroups. It has been publicly accepted that both pathogen and host characteristics can influence the development and progress of the disease. In our study, the frequency of rs4794067 C allele was significantly different between anti-F protein seropositive subjects and anti-F protein seronegative subjects in subgroups of HCV 1b genotype and patients with mild fibrosis, whereas the rs2074190 G allele showed increased frequency in the HCV non-1b genotype subgroup between the two groups. This pathogen-host interaction may provide a novel way to understand the complex mechanism of the formation of chronic infection with different viral genotypes. We also conducted an analysis stratified by age and sex. It showed that the carriage of C allele in the rs4794067 gene locus appeared to be associated with an increasing risk of persistent HCV infection in a younger subgroup and female subgroup. This was contrary to popular belief with young subjects showing a higher rate of spontaneous clearance in HCV infection [40]. The possible interpretations were that an appropriate immune response in younger infections was to protect liver cell injury from acute inflammation and a reduced immune response by the action of estradiol in females [41]. In addition, the results also revealed that rs4794067 C allele could increase the risk of anti-F protein antibody generation in an older subgroup. In a word, these results suggested a complex relationship between age, viral genotype, and genomic factors.

In summary, we studied the association of the SNPs of *TBX*21 gene rs17250932, rs2074190 and rs4794067 with susceptibility to HCV infection and the F protein generation in a high-risk Chinese population for the first time. To reduce the inescapable selection bias, we applied many strategies to control and minimize the potential confounding factors, such as matching the age and residential area, taking the stage of liver fibrosis and viral genotype into account, and testing anti-F protein antibody in patients with chronic HCV infection. However, the number of subjects in our study was relatively limited. So this conclusion needs to be verified by similar studies in other populations. With accumulating researches revealing that the genetic variations in *TBX*21 may be involved in the etiology of susceptibility and/or sustainability to HCV infection, our study conducted a novel pathogenic antigen (HCV F protein) to provide a new perspective to understand the mechanism of HCV evasion strategy for the first time. However, further studies are necessary to demonstrate how these *TBX*21 gene polymorphisms are implicated in the pathogenesis of chronic HCV infection.

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