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Immune checkpoint proteins PD-1 and TIM-3 are both highly expressed in liver tissues and correlate with their gene polymorphisms in patients with HBV-related hepatocellular carcinoma

Zhu Li, PhD, MD[a], Na Li, PhD, MD[a], Fang Li, ScM[a], Zhihua Zhou, BM[a], Jiao Sang, BM[a], Yanping Chen, MD[a,b], Qunying Han, MD[a], Yi Lv, MD, PhD[c,d], Zhengwen Liu, MD, PhD[a,d,*]

Abstract

Immune checkpoint proteins programmed death-1 (PD-1) and T-cell immunoglobulin domain and mucin domain containing molecule-3 (TIM-3) expression and their gene polymorphisms have separately been shown to be associated with hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC). This study simultaneously examined PD-1 and TIM-3 expression in liver tissues and *PD1* and *TIM3* polymorphisms and analyzed their correlations in 171 patients with HBV-related HCC and 34 patients with HBV-related cirrhosis.

PD-1 and TIM-3 expression in liver tissues were examined by immunohistochemistry and the genotypes of *PD1* rs10204525 and *TIM3* rs10053538 polymorphisms were determined using genomic DNA extracted from peripheral blood as template.

Both PD-1 and TIM-3 expressions in liver infiltrating lymphocytes of HCC tumor tissues were significantly higher than those in tumor adjacent tissues or cirrhotic tissues. The elevated PD-1 and TIM-3 expressions were significantly associated with higher tumor grades. The levels between PD-1 and TIM-3 expression in tumor tissues and tumor adjacent tissues had a significant positive intercorrelation. The expressions of PD-1 and TIM-3 in tumor tissues, tumor adjacent tissues, and cirrhotic tissues were significantly associated with *PD1* and *TIM3* polymorphisms, with genotype AA of *PD1* rs10204525 and genotypes GT+TT of *TIM3* rs10053538 being associated with significantly increased PD-1 and TIM-3 expression, respectively.

These findings support the potential to improve the efficiency of immune checkpoint-targeted therapy and reduce resistance to the therapy by blocking both PD-1 and TIM-3 and suggest the potential to apply the genotype determination of *PD1* rs10204525 and *TIM3* rs10053538 as biomarkers of immune checkpoint-directed therapies.

Abbreviations: HBV = hepatitis B virus, HCC = hepatocellular carcinoma, IHC = immunohistochemistry, LC = liver cirrhosis, PBS = phosphate buffered saline, PD-1 = programmed death-1, TIM-3 = T-cell immunoglobulin domain and mucin domain containing molecule-3.

Keywords: hepatitis B virus, hepatocellular carcinoma, immune checkpoint, PD-1, polymorphism, TIM-3

1. Introduction

Hepatitis B virus (HBV) infection is a major public health problem. About 2 billion people worldwide have been infected with HBV and among them nearly 250 million people are chronically infected with the virus.[1] Chronic HBV infection manifests heterogeneous clinical outcomes, ranging from asymptomatic chronic HBV carrier status, chronic hepatitis, and cirrhosis to hepatocellular carcinoma (HCC).[2,3] Increasing evidence shows that host immune responses to HBV play an important role in determining the clinical outcomes.[4–9] Insufficient or dysregulated immune responses, T cell exhaustion and excessive activation of immune suppressive cells such as regulatory T cells (Tregs) in particular, have been shown to primarily contribute to the immunopathogenesis of persistent HBV infection and HBV-associated HCC.[10–14]

A major characteristic of the exhaustion of T cells and the excessive activation of Tregs involved in chronic HBV infection, and the development of HBV-associated HCC is the upregulated and sustained expression of multiple negative coinhibitory molecules, especially immune checkpoint proteins programmed death-1 (PD-1) and T-cell immunoglobulin domain and mucin

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[a] Department of Infectious Diseases, First Affiliated Hospital of Xi'an Jiaotong University, [b] Department of Infectious Diseases, Yanan University Affiliated Hospital, Yanan, [c] Department of Hepatobiliary Surgery, First Affiliated Hospital of Xi'an Jiaotong University, [d] Institute of Advanced Surgical Technology and Engineering, Xi'an Jiaotong University, Xi' an, Shaanxi, China.

[*] Correspondence: Zhengwen Liu, Department of Infectious Diseases, First Affiliated Hospital of Xi'an Jiaotong University, No. 277 Yanta West Road, Xi'an, 710061 Shaanxi Province, the People's Republic of China (e-mail: liuzhengwen@medmail.com.cn).

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domain containing molecule-3 (TIM-3).[15–22] Importantly, the expressions of PD-1 and TIM-3 in liver tissues of chronic HBV infection and HBV-related HCC are related to the severity of liver inflammation and T cell exhaustion and the prognosis of HCC patients.[17,19,21,23–26] The polymorphisms of *PD1* and *TIM3* are associated with the development of HBV-associated liver diseases and the prognosis of patients with HBV-related HCC.[27–30]

However, whether there are associations between PD-1 expression and TIM-3 expression in liver tissues and whether there are links between PD-1 and TIM-3 expressions, and their gene polymorphisms in patients with HBV-associated liver diseases remain unknown. Therefore, in the present study, we simultaneously determined the expression of PD-1 and TIM-3 in liver tissues of HBV-related HCC and liver cirrhosis (LC) and *PD1* and *TIM3* polymorphisms and analyzed the associations between PD-1 and TIM-3 expressions and between PD-1 and TIM-3 expressions and *PD1* and *TIM3* polymorphisms in Chinese patient populations.

2. Materials and methods

2.1. Patients

From January 1, 2011 to December 31, 2013, 171 patients who were diagnosed with HBV-related HCC and received surgical treatment and 34 patients who were diagnosed with HBV-associated LC were recruited from the First Affiliated Hospital of Xi'an Jiaotong University. The diagnosis of HBV-related HCC was based on HBsAg, HBeAg, or anti-HBe, and anti-HBc seropositivity for more than 6 months, and pathological examination of surgical liver tissues in combination with angiography, ultrasound, computed tomography, and magnetic resonance imaging. The diagnosis of HBV-related LC was based on pathological cirrhosis of liver biopsy in combination with HBsAg, HBeAg, or anti-HBe, and anti-HBc seropositivity for more than 6 months, and abnormal liver function, portal hypertension, esophageal varices, splenomegaly, ascites, and imaging features of cirrhosis in ultrasound/computed tomography/magnetic resonance imaging.[31] All patients were non-kin Han Chinese adults. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. All patients gave written informed consent.

The patients with HBV-related HCC received no other treatments, including radiofrequency therapy, chemotherapy, and immunotherapy before surgery and the HCCs were confirmed by postoperative liver tissue pathology. The tumor tissue and tumor adjacent tissue (from the cancer tissue 2 cm) were taken by surgical resection in each patient. The liver tissue in patients with LC was taken by liver biopsy. Patients' demographic and clinical data were collected, and the data pertinent to tumor grade, size, and metastasis in HCC patients were also collected.

2.2. Genotyping of polymorphisms

Whole blood of 2 mL was collected intravenously in ethylenediaminetetraacetic acid anticoagulated tube from each patient after over-night fasting and cryopreserved at −20 °C until use for the extraction of human genomic DNA. Human genomic DNA was extracted from the whole blood and the genotyping of *PD1* +8669 G/A (rs10204525) and *TIM3* −1516 G/T (rs10053538) polymorphisms was carried out by using methods as described previously.[27,28]

2.3. Immunohistochemistry (IHC)

Paraffin-embedded liver tissue sections were prepared from paraformaldehyde-fixed tissues. Antigen retrieval was performed by heating the sections in ethylenediaminetetraacetic acid antigen repair solution in a microwave for 10 minutes. Endogenous peroxidase activity was inactivated by 3% H_2O_2 deionized water, and the sections were blocked with bovine serum albumin at 37 °C for 10 to 15 minutes after washing with phosphate buffered saline (PBS). The sections were incubated with primary antibodies mouse monoclonal anti-PD-1 antibody (Abcam, Cambridge, MA) at a dilution of 1:50 or rabbit polyclonal anti-TIM-3 antibody (BioVision, Milpitas, CA) at a dilution of 1:150 at 37 °C for 30 minutes. Then, the sections were incubated with secondary horse radish peroxidase goat antimouse IgG antibody (Abgent, San Diego, CA) or horse radish peroxidase goat anti-rabbit IgG antibody (Abgent) at room temperature for 15 minutes after washing with PBS. The stain was developed with 3,3-diaminobenzidine at room temperature. After washing with distilled water, the sections were counter stained with hematoxylin and then mounted with neutral resin. Negative controls were carried out by replacing the primary antibodies with PBS for each IHC reaction.

Immunohistochemical staining was qualitatively and semi-quantitatively examined for each section under a light microscopy at 200× magnification in 5 non-overlapping random fields according to the specific staining of the cells. The expression of PD-1 or TIM-3 was observed as positive staining when the cell membranes displayed yellow, brown, or dark-brown granules. The expression levels of both proteins were determined by taking into account both the percentage of positive cells and the staining intensity score.[32] The percentage of positive cells (A) was estimated by counting 100 cells in each field and then rated as: 1 = 0% to 4%, 2 = 5% to 19%, 3 = 20% to 39%, 4 = 40% to 59%, 5 = 60% to 79%, and 6 = 80% to 100%. The staining intensity score (B) was graded as: 0 = no staining, 1 = light yellow staining, 2 = brown staining, and 3 = dark-brown staining. Finally, the value of A × B in each section was regarded as the IHC score of the protein expression.[23,32]

2.4. Statistical analysis

Statistical analysis was carried out by SPSS16.0 software. Demographic and laboratory data including TIM-3 and PD-1 expression levels in HBV-related HCC and LC patients were compared using univariate logistic regression analysis with *P* values being corrected for age and sex. The differences of TIM-3 and PD-1 expression between tumor tissue, tumor adjacent tissue, and cirrhosis tissue were tested using χ^2 test. The association between the levels of TIM-3 and PD-1 expression was examined by Pearson correlation. The correlation between the clinicopathological features and TIM-3 and PD-1 expression was tested by one-way analysis of variance. The association of TIM-3 and PD-1 expression levels with the genotypes of *TIM3* and *PD1* polymorphisms was also tested using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Demographic and clinical characteristics of the study subjects

The 171 HBV-related HCC patients included 144 males and 27 females. The 34 HBV-related LC patients included 19 males and

Table 1

Demographic and clinical data in the patients with HBV-related HCC and LC.

Variables	HCC (n = 171)	LC (n = 34)	*P*
Gender (male/female)	144/27	19/15	<0.001
Age, years	49.43 ± 11.26	38.09 ± 10.00	<0.001
HBV DNA, log IU/mL	4.23 ± 1.51	5.46 ± 1.80	0.004
HBeAg (+/−)	38/133	16/18	0.009
ALT, IU/L	38.00 (8.00–704.20)	39.00 (9.00–308.00)	0.650
AST, IU/L	45.00 (15.00–721.90)	27.00 (14.00–261.00)	0.188
TBIL, μmol/L	16.40 (3.40–274.80)	12.20 (4.00–29.30)	0.051
ALB, g/L	37.90 (24.55–52.75)	43.50 (36.00–50.10)	<0.001

ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, LC = liver cirrhosis, TBIL = total bilirubin.

15 females. HCC patients had higher proportion of males than cirrhosis patients ($P < 0.001$); the average age in the HCC patients (49.43 ± 11.26 years) was older than that in the LC patients (38.09 ± 10.00 years, $P < 0.001$). The HBV DNA level in HCC patients (4.23 ± 1.51 log IU/mL) was lower than that in cirrhosis patients (5.46 ± 1.80 log IU/mL, $P = 0.004$). The HBeAg positivity in patients with cirrhosis (16/18) was more frequent than in HCC patients (38/133) ($P = 0.009$). The albumin (ALB) level in HCC patients (37.90 [24.55–52.75] g/L) was lower than that in cirrhosis patients (43.50 [36.00–50.10] g/L, $P < 0.001$). The alanine aminotransferase, aspartate aminotransferase, and total bilirubin levels between HCC and cirrhosis patients had no significant difference (Table 1).

3.2. PD-1 and TIM-3 expression in liver tissues

PD-1 staining was mainly observed in the lymphocyte cell membrane and cytoplasm of portal area in the liver tissues, and the positive cells were regionally distributed or clustered as brown or dark-brown staining (Fig. 1). The staining intensity of PD-1 expression in HCC tumor tissue (Fig. 1A) was significantly higher than that in tumor adjacent tissues (Fig. 1B) or cirrhosis tissues (Fig. 1C). The IHC score of PD-1 in the tumor tissues (5.06 ± 1.11) was significantly higher than that in the tumor adjacent tissues (3.07 ± 1.25, $P < 0.001$) or the cirrhosis tissues (1.35 ± 1.18, $P < 0.001$, Fig. 1D).

TIM-3 staining was primarily observed in the cell membrane and cytoplasm of lymphocytes in the portal area manifested as brown or dark-brown granular or massed multiregional distribution (Fig. 2). The staining intensity of TIM-3 expression in HCC tumor tissue (Fig. 2A) was also significantly higher than that in tumor adjacent tissues (Fig. 2B) or cirrhosis tissues (Fig. 2C). The IHC score of TIM-3 in the tumor tissues (5.68 ± 1.34) was also significantly higher than that in the tumor adjacent tissues (3.63 ± 1.13, $P < 0.001$) and LC tissues (2.65 ± 1.07, $P < 0.001$, Fig. 2D).

The analysis of between PD-1 and TIM-3 expression and the clinical characteristics of HBV-related HCC patients showed that the PD-1 and TIM-3 increased expression levels paralleled the tumor grades of HCC. The HBV-related HCC patients with grades III + IV had significantly higher IHC score (5.32 ± 0.95) of PD-1 expression than those with grades I + II (4.88 ± 1.18, $P = 0.010$, Table 2). Similarly, the HBV-related HCC patients with grades III + IV had significantly higher IHC score (5.97 ± 0.63) of TIM-3 expression than those with grades I + II (5.48 ± 1.35, $P = 0.005$, Table 2). The IHC score of PD-1 expression in patients with Child-Pugh score B (5.35 ± 0.94) was significantly higher than those with Child-Pugh score A (4.87 ± 1.18, $P = 0.005$, Table 2). The IHC score of TIM-3 expression in patients with Child-Pugh score B (5.82 ± 1.08) was higher than that in those with Child-Pugh score A (5.59 ± 1.17) but the difference was not significant ($P = 0.193$, Table 2).

3.3. Association between PD-1 and TIM-3 expressions

There was a significant positive correlation between the levels of PD-1 and TIM-3 expression in tumor tissues ($r = 0.551$, $P < 0.001$) and tumor adjacent tissues ($r = 0.626$, $P < 0.001$). The expressions between PD-1 and TIM-3 in cirrhotic liver tissues had no significant correlation ($r = 0.150$, $P = 0.397$). There was also a significant positive correlation between PD-1 and TIM-3

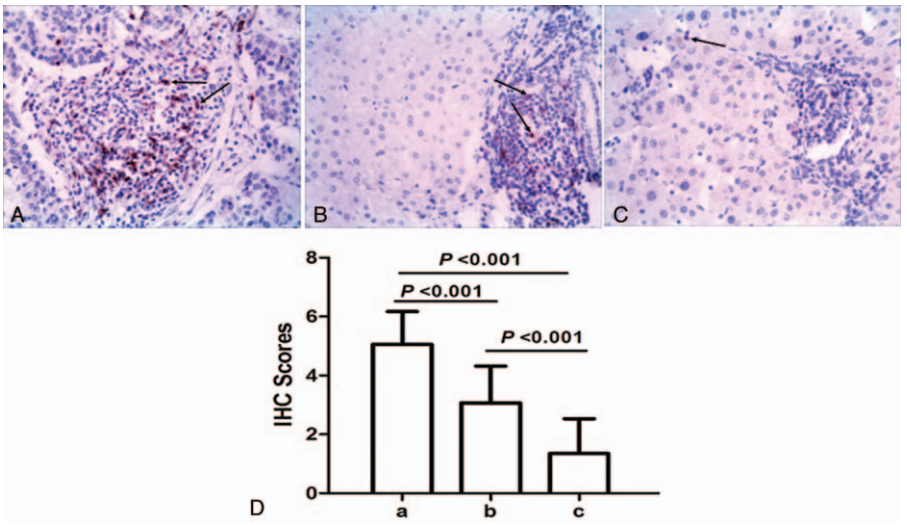

Figure 1. PD-1 expression in liver tissues (×200). (A) Tumor tissues of HCC; (B) tumor adjacent tissues of HCC; (C) liver tissues of cirrhosis; (D) IHC score of PD-1 expression in (a) tumor tissues of HCC, (b) tumor adjacent tissues of HCC, and (c) liver tissues of cirrhosis. Black arrows indicate examples of positive staining cells. HCC = hepatocellular carcinoma, IHC = immunohistochemistry, PD-1 = programmed death-1.

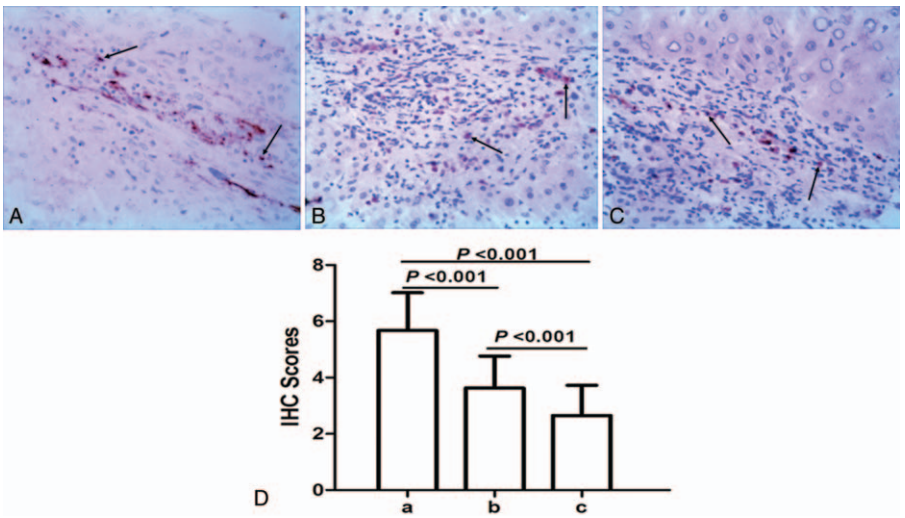

Figure 2. TIM-3 expression in liver tissues (×200). (A) Tumor tissues of HCC; (B) tumor adjacent tissues of HCC; (C) liver tissues of cirrhosis; (D) IHC score of TIM-3 expression in (a) tumor tissues of HCC, (b) tumor adjacent tissues of HCC, and (c) liver tissues of cirrhosis. Black arrows indicate examples of positive staining cells. HCC = hepatocellular carcinoma, IHC = immunohistochemistry, TIM-3 = T-cell immunoglobulin domain and mucin domain containing molecule-3.

expression when all the liver tissues (tumor tissues, tumor adjacent tissues, and cirrhotic liver tissues) were included in the analysis ($r = 0.699$, $P < 0.001$).

3.4. Association between PD-1 and TIM-3 expression and PD1 and TIM3 polymorphisms

The IHC scores of PD-1 expression in the tumor tissues and tumor adjacent tissues of HCC patients with *PD1* +8669 genotype AA (5.45 ± 0.94 and 3.53 ± 1.09, respectively) were significantly higher than those in HCC patients with genotypes AG+GG (4.43 ± 1.08 and 2.34 ± 1.15, respectively; both $P <$ 0.001, Fig. 3). The IHC score of PD-1 expression in the liver tissues of cirrhotic patients with *PD1* +8669 genotype AA (1.70 ± 1.17) was also significantly higher than that in cirrhotic patients with *PD1*+8669 genotypes AG+GG (0.86 ± 1.03, $P = 0.038$, Fig. 3). For TIM-3 expression, the IHC scores in the tumor tissues and tumor adjacent tissues of HCC patients with *TIM3* −1516 genotypes GT+TT (6.11 ± 0.82 and 4.22 ± 1.05, respectively) were significantly higher than those in HCC patients with genotype GG (5.57 ± 1.18 and 3.47 ± 1.09, respectively; $P =$ 0.011 and $P < 0.001$, respectively, Fig. 4). The IHC score of TIM-3 expression in the liver tissues of cirrhotic patients with *TIM3*-1516 genotypes GT+TT (3.60 ± 0.89) was also significantly higher than that in cirrhotic patients with genotype GG (2.48 ± 1.02, $P = 0.029$, Fig. 4).

Table 2

Expressions of PD-1 and TIM-3 in liver tissues (IHC score) according to demographics and clinicopathological characteristics of tumors in the HBV-related HCC patients.

Variables	n	PD-1	*P*	TIM-3	*P*
Gender			0.172		0.405
Male	144	5.01 ± 1.13		5.65 ± 1.21	
Female	27	5.33 ± 0.96		5.85 ± 0.53	
Age, years			0.159		0.966
≤50	96	4.95 ± 1.12		5.69 ± 1.24	
>50	75	5.20 ± 1.09		5.68 ± 0.99	
Tumor grade			0.010		0.005
I + II	100	4.88 ± 1.18		5.48 ± 1.35	
III + IV	71	5.32 ± 0.95		5.97 ± 0.63	
Child-Pugh score			0.005		0.193
A	103	4.87 ± 1.18		5.59 ± 1.17	
B	68	5.35 ± 0.94		5.82 ± 1.08	
AFP, ng/mL			0.096		0.443
≤200	77	4.96 ± 1.11		5.61 ± 1.12	
>200	94	5.15 ± 1.12		5.74 ± 1.14	
Tumor size, cm			0.750		0.075
≤5	98	5.04 ± 1.19		5.55 ± 1.24	
>5	73	5.10 ± 1.00		5.86 ± 0.96	

AFP = alpha-fetoprotein, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, IHC = immunohistochemistry, PD-1 = programmed death-1, TIM-3 = T-cell immunoglobulin domain and mucin domain containing molecule-3.

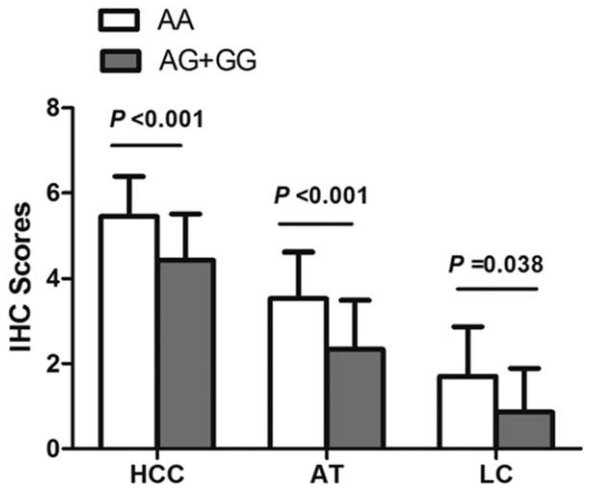

Figure 3. PD-1 expression in tumor tissues of HBV-related HCC (n = 171), tumor adjacent tissues of HCC (AT, n = 171), and cirrhotic liver tissues (LC, n = 34) according to the genotypes of *PD1* polymorphism. AA, AG, and GG = genotype AA, genotype AG, and genotype GG of *PD1* +8669 G/A (rs10204525) polymorphism, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, IHC = immunohistochemistry, AT = adjacent tissues of HCC, LC = liver cirrhosis, PD-1 = programmed death-1.

4. Discussion

Various studies have demonstrated that the elevated expressions of PD-1 and TIM-3 on lymphocytes, especially exhausted T cells and Tregs and within the liver, are closely associated with the dysfunction of immune response in chronic HBV infection and HBV-related HCC.[15–24] Consistent with these studies, we in this study showed that both PD-1 and TIM-3 were highly expressed on liver infiltrating lymphocytes and the expression levels displayed a significant increase in tumor tissue compared to tumor adjacent tissue or cirrhotic liver tissue. The levels of PD-1 and TIM-3 expression were associated with the tumor grades and PD-1 expression was also associated with the Child-Pugh score in HBV-related cirrhotics, supporting the role of PD-1 and TIM-3 in the development of HBV-related liver diseases and the immune escape of HBV-related HCC.

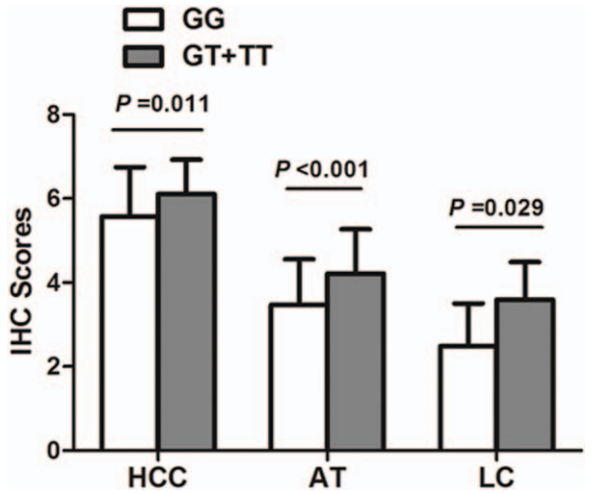

Figure 4. TIM-3 expression in tumor tissues of HBV-related HCC (n = 171), tumor adjacent tissues of HCC (AT, n = 171), and cirrhotic liver tissues (LC, n = 34) according to the genotypes of *TIM3* polymorphism. GG, GT, and TT = genotype GG, genotype GT, and genotype TT of *TIM3* −1516 G/T (rs10053538) polymorphism, HBV = hepatitis B virus, HCC = hepatocellular carcinoma, IHC = immunohistochemistry, AT = adjacent tissues of HCC, LC = liver cirrhosis, TIM-3 = T-cell immunoglobulin domain and mucin domain containing molecule-3.

Targeting the immune checkpoints, especially PD-1, has been clinically or experimentally applied in many cancers,[33] but resistance to the therapies has increasingly been observed. Studies in animal models showed that combined targeting of the PD-1 and TIM-3 pathways is more effective in improving CD8 T cell response, controlling chronic virus infection, and suppressing tumor growth.[34–36] Our present study showed that the expressions of PD-1 and TIM-3 in liver infiltrating lymphocytes of HCC were both significantly upregulated and had a significant positive intercorrelation. In solid tumor-bearing mice, all TIM-3 positive tumor-infiltrating lymphocytes were found to coexpress PD-1, and PD-1 and TIM-3 dual positive tumor-infiltrating lymphocytes were shown to account for the predominant fraction of T cells infiltrating tumors.[35] Interestingly, a recent study indicates that the adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of TIM-3.[37,38] Therefore, although further studies are necessary to clarify the interrelationship between PD-1 and TIM-3 expression as well as other immunoinhibitory molecules such as interleukin 10 in various tumors, it is suggested that the application of combinatorial blocking of PD-1 and TIM-3 may not only reinforce the effectiveness of immunotherapy but also prevent or reduce the occurrence of adaptive resistance to the blockade of a single checkpoint.

This study demonstrated that the increased expressions of PD-1 and TIM-3 in liver infiltrating lymphocytes in tumor tissues, tumor adjacent tissues, and cirrhotic liver tissues were associated with the *PD1* and *TIM3* polymorphisms, with the *PD1* +8669 genotype AA and *TIM3* −1516 genotypes GT+TT being associated with increased PD-1 and TIM-3 expression, respectively, and thus displaying a predisposing role for HCC. These results are consistent with previous findings, showing that the *PD1* +8669 genotype AA and *TIM3* −1516 genotypes GT+TT are associated with either increased PD-1 mRNA expression in peripheral blood nuclear cells and disease progression in chronic HBV infection[27,39] or higher tumor grade and more frequent lymph node metastasis in HBV-related HCC.[28] Furthermore, these genotypes have been shown to be related to the poor survival of HBV-related HCC patients.[30] Therefore, our findings in the present study provide further evidence linking the host genetic background and immune dysfunction, supporting the importance of host immunogenetic background in the development, progression, and prognosis of HBV-associated liver diseases.

Biomarkers are necessary to identify patients most appropriate to use immunotherapy through blocking immune checkpoints. Currently, programmed death ligand 1 (PD-L1) expression is an extensively examined potential predictive biomarker for PD-1-directed therapy, but it has a low prediction accuracy[40,41] and may only be used for PD-1-targeted therapy. Therefore, additional biomarkers are necessary to accurately predict patients most appropriate to benefit from not only PD-1-targeted therapy but also other immune checkpoints-directed therapies or combinatorial therapies such as PD-1 and TIM-3 dually targeted therapy. The demonstration that there is a close relationship between *PD1* +8669G/A (rs10204525) and *TIM3* −1516G/T (rs10053538) polymorphisms and PD-1 and TIM-3 expressions, respectively, in this study, together with the previous findings

showing that these 2 polymorphisms are associated with the prognosis of HBV-related HCC patients, implies that these polymorphisms may be candidate immunogenetic biomarkers for future personalized checkpoints-directed therapies.

It should be noted that this study has some limitations, including the small sample size in the patient population especially in the cirrhotic patient population and the unavailability of the inclusion of liver tissues from normal healthy individuals. Therefore, additional studies with large patient population and healthy control population liver tissues are warranted to confirm our findings in this study.

In conclusion, this study showed that both PD-1 and TIM-3 are highly expressed on liver infiltrating lymphocytes of HBV-related HCC tumor tissues and tumor adjacent tissues, especially tumor tissues. The levels of PD-1 and TIM-3 expression are significantly associated with their gene polymorphisms *PD1* rs10204525 (+8669A/G) and *TIM3* rs10053538 (−1516G/T), respectively. These findings support the potential to improve the potency of immune checkpoint targeted therapy and to reduce resistance to the therapy by blocking both PD-1 and TIM-3 and suggest the potential to apply the genotype determination of *PD1* rs10204525 and *TIM3* rs10053538 polymorphisms as biomarkers of personalizing, monitoring, and predicting checkpoints-targeted therapies. However, more studies are needed to replicate and confirm these findings and to test the potential to use these immunogenetic biomarkers to predict the responses to immunotherapies.

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