

Association between Tim-3 and Gal-9 expression and gastric cancer prognosis

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Abstract. The T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3)/galectin 9 (Gal-9) pathway, which serves a pivotal role in immune regulation, is similar to the programmed death (PD)-1/PD-ligand 1 pathway. Recent evidence has suggested that Tim-3 is differentially regulated in a variety of tumors and is a potential therapeutic target. The aim of the present study was to evaluate Tim-3 and Gal-9 expression and cluster of differentiation (CD)3⁺, CD8⁺ and forkhead box (FOX)p3⁺ T cell tumor-infiltration in gastric cancer, as well as their impact on prognosis. Tissue samples from 587 patients with gastric cancer were used to create a tissue microarray (TMA). The immune markers Tim-3, Gal-9, CD3, CD8 and FOXp3 were immunostained in the TMA, and correlations with clinicopathological findings and prognosis were analyzed. Several Gene Expression Omnibus gastric cancer databases and the K-M plotter website were used to analyze the association between the expression of Tim-3, Gal-9 and CD8A RNA and patient survival. The results demonstrated that Tim-3 was mainly expressed in immune cells, with minimal expression in gastric cancer cells. Its ligand, Gal-9, was significantly overexpressed in tumor cells. Tim-3 and Gal-9 expression and Foxp3⁺ T cell density were negatively associated with the patient overall survival (OS) rate. The density of CD8⁺ T cells was positively associated with the patient OS rate. Tim-3 expression and CD8⁺ T cell density were revealed to be independent prognostic factors for patients with gastric cancer.

Introduction

Gastric cancer (GC) is the fifth most common type of malignancy and the third leading cause of cancer-associated mortality worldwide (1). Half of all cases worldwide occur in East Asia (1). The prognosis of GC is generally poor, particularly in patients with advanced stages of the disease (1). At present, the routine treatment for advanced GC involves surgical resection and perioperative chemotherapy (2). Immunotherapeutic drugs, particularly immune checkpoint inhibitors, including anti-programmed death (PD)1 (nivolumab and pembrolizumab) and anti-cytotoxin T-lymphocyte-associated protein 4 (CTLA4) (ipilimumab) drugs, were recently approved by the Food and Drug Administration to treat a number of solid tumor types. Although there have been successful cases of immune checkpoint inhibitor use in these tumor types, certain patients have been revealed to be resistant to therapies targeting CTLA-4 and PD-1 (3). The T-cell immunoglobulin and mucin domain-containing protein 3 (Tim-3)/galectin 9 (Gal-9) pathway, which is yet to be entirely characterized, may be a potential site of immune checkpoint blockade in immunotherapy, which could be used to overcome drug resistance (3).

Tim-3 was first identified as a molecule expressed in interferon (IFN)- γ -producing cluster of differentiation (CD)4⁺ T helper 1 (Th1) and CD8⁺ T cytotoxic 1 (Tc1) cells (4). It has been reported that Tim-3 dysregulation in CD4⁺ and CD8⁺ T cells is associated with tumorigenesis (5). When Tim-3 interacts with its ligand, Gal-9, the Th1 response is blocked, resulting in the death of IFN-induced Th1 cells. In a previous study, it was demonstrated that Tim-3 reduces the antigen-specific T-cell response and downregulates antitumor immunity *in vivo* by inhibiting the Th1 response (6).

Galectins are characterized by their β -galactoside-binding affinity, and have an evolutionarily conserved carbohydrate recognition domain (CRD) (7). Galectins are classified according to their CRD and are subdivided into three groups: Prototype galectins (galectin-1, -2, -7, -10, -13 and -14), chimera-type galectins (galectin-3) (each has a single CRD) and tandem-repeat-type galectins (galectin-4, -8, -9 and -12) (each has two CRDs joined by a flexible peptide linker). Gal-9 is a tandem-repeat-type galectin that is known to serve key roles in eosinophil chemoattraction and activation (8).

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Forkhead box (FOX)p3, a member of the forkhead family, is specifically expressed in Treg cells, including CD4⁺ CD25^{high} Treg cells and CD8⁺ CD25^{high} Treg cells (9). Treg cells are important factors in the development of immune suppression and tolerance (10). A previous study confirmed that high FOXP3 expression in Tregs allows tumor cells to escape immune surveillance, thereby promoting the proliferation and development of tumor cells (11).

To assess the association between the Tim-3/Gal-9 pathway and GC prognosis, Gal-9 and Tim-3 expression was measured in 587 patients with gastric cancer using Gene Expression Omnibus (GEO) databases and the K-M plotter website. The association between CD3⁺, CD8⁺ and FOXP3⁺ T cell infiltration and GC prognosis was also analyzed in the present study.

Materials and methods

Patients and tissue microarray (TMA) construction. The primary objective of the present study was to evaluate the association between immune characteristics, clinicopathological features and GC prognosis. This was a retrospective analysis of 587 patients with primary GC who underwent gastrectomy at the Department of Gastrointestinal Surgery, Ren Ji Hospital, (School of Medicine, Shanghai Jiao Tong University, Shanghai, China) between January 2006 and December 2011. The final follow-up date was December 31, 2015 for all cases examined. The mean age of the patients was 61.6 years (range, 22–89 years), including 401 males and 186 females. A total of 251 cancer-associated mortalities occurred. All patients received standard treatments, including D2 radical resection [excluding 6 cases with Tumor-Node-Metastasis (TNM) (12) stage IV] and first-line adjuvant chemotherapy (for patients with advanced GC) according to the National Comprehensive Cancer Network guidelines (<https://www.nccn.org/>). Lauren type was divided into intestinal, diffuse and mixed as previously described (13). The location of the lesion was classified into top, middle and bottom through linking the corresponding trisection points of the lesser gastric curvature and the greater gastric curvature. No patients had received neoadjuvant chemotherapy or human epidermal growth factor receptor 2 targeted therapy. A number of patients with advanced GC did not complete the standard chemotherapy regimen for personal reasons or an inability to tolerate side effects. There was no difference in the number of patients not completing the standard chemotherapy regimen between the Tim-3 high and low expression groups. The following exclusion criteria were used: i) Recurrent GC following radical surgery; ii) receipt of previous neoadjuvant chemotherapy or radiotherapy; iii) the presence of other malignant tumors; and iv) evidence of autoimmune or immunodeficiency diseases.

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from the Pathology Department of Ren Ji Hospital. TNM staging was performed based on the American Joint Committee on Cancer (7th Edition) staging system (12). For each case, the diagnosis was confirmed by two senior pathologists through a review of H&E-stained slides. Representative FFPE blocks were selected for construction of the TMA using a tissue arrayer of 5- μ m thickness.

The present study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of

Medicine. Written informed consent was obtained from all enrolled patients prior to their inclusion in the study.

TMA staining, fluorescence-activated cell sorting (FACS) and evaluation. Immunohistochemistry (IHC) was performed on 5- μ m thick TMA sections with antibodies specific to Tim-3 (dilution, 1:200; cat. no. 45208; Cell Signaling Technology, Inc., Danvers, MA, USA), Gal-9 (dilution, 1:250; cat. no. ab69630; Abcam, Cambridge, UK), CD3 (dilution, 1:200; cat. no. GB11014; Wuhan Goodbio Technology Co., Ltd., Wuhan, China), CD8 (dilution, 1:100; cat. no. GB11068; Wuhan Goodbio Technology Co., Ltd.) and FOXP3 (dilution, 1:200; cat. no. 98377; Cell Signaling Technology, Inc.). In brief, tissue sections were deparaffinized, rehydrated in a graded ethanol series, and incubated with citrate antigen retrieval solution for 15 min at 95°C and 3% hydrogen peroxide for 30 min at 37°C. Additionally, 1X PBS was used as washing reagent. Tissue sections were blocked with 10% BSA (Sangon Biotech Co., Ltd., Shanghai, China) at room temperature for 1 h. Tissues were subsequently incubated with primary antibodies at 4°C overnight, followed by incubation with a horseradish peroxidase-conjugated rabbit secondary antibody (dilution, 1:20,000; cat. no. 31460; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 1 h. Positive staining was visualized using 3,3'-diaminobenzidine substrate liquid (Gene Tech Biotechnology Co., Ltd., Shanghai, China) and counterstained with hematoxylin at room temperature for 10 sec. All sections were observed and images were captured using an optical microscope (magnification, x200 and x400; Zeiss GmbH, Jena, Germany).

In the subsequent analysis, immune cells in vessels, lymph nodes, lymphatics, necrotic tissue or necrosis-adjacent areas were excluded. Tim-3 was mainly expressed in immune cells with low expression in tumor cells, whereas Gal-9 was more highly expressed in tumor cells. Tumor cell staining was used to evaluate Gal-9 expression. Based on the staining intensity and area, patients were divided into high and low expression groups. Staining intensity was defined as follows: Weak staining, 1; medium staining, 2; and strong staining, 3. The median staining intensity value obtained was multiplied by the percentage of the tissue area that was stained. Nikon DR-Si2 cell counting and imaging software (version 4.30.01; Nikon DR-Si2; Nikon Corporation, Tokyo, Japan) was used for this analysis. A total of 4 randomly selected fields of view (magnification, x200; 0.34 mm²) were used to analyze each case, and the average number of Tim-3⁺, CD3⁺, CD8⁺ and FOXP3⁺ cells was counted. According to the median number of stained immune cells (Tim-3) or T cells (CD3 and CD8), patients were divided into low and high infiltration groups as previously described (14). To evaluate FOXP3⁺ T cells, positive staining was defined as >5 stained cells/high-power field (HPF) and negative staining was defined as \leq 5 cells/HPF. The results were verified by two senior pathologists who were blinded to the clinicopathological data of the patients.

Detection of Tim-3 expression in CD3⁺ T cells by FACS staining. Surgical resection of tumor tissues from one male patient with GC (age, 62 years; TNM stage III) was performed in the present study. A total of 1 g dissociated tissue was

treated with collagenase IV (cat. no. C5138; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 90 min at 37°C on a rotating shaker. The homogenates were filtered through membranes of 48 μ m (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to remove cell mass and fiber agglomerates. Tumor tissue-derived single cells were obtained by centrifugation (400 \times g at room temperature for 10 min) and suspended in 1X PBS. Subsequently, the cells were incubated with a mouse anti-human Tim-3 antibody (PE-CF594; cat. no. 565561; dilution, 1:200; BD Biosciences, Franklin Lakes, NJ, USA) and a fluorescein isothiocyanate-conjugated CD3 antibody (cat. no. ab 210316; dilution, 1:200, Abcam) for 30 min at 4°C in a dark environment. Following washing in 1X PBS, Tim-3 expression was detected in CD3⁺ T cells using a flow cytometer (BD Biosciences). CD3 was considered to be a molecular marker of T cells in the present study. FlowJo version 10 software was used for analysis (FlowJo LLC, Ashland, OR, USA).

Cell culture and reagents. The human gastric cancer AGS, BGC-823, HGC-27, MGC-803, MKN-45 and SGC-7901 cell lines were all maintained in the Shanghai Cancer Institute, Ren Ji Hospital. All cells were cultured in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.) or F-12 Medium (Gibco; Thermo Fisher Scientific, Inc.) according to ATCC protocols and supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics (100 μ g/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified incubator at 5% CO₂. The cell medium was replaced every 2-3 days, and 1X PBS was used for cell washing prior to the medium being replaced. Cells were collected in the logarithmic growth phase for protein and RNA extraction.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The thermocycling conditions were as follows: 60°C for 34 sec and 95°C for 15 sec for 40 cycles. Total RNA was extracted from AGS, BGC-823, HGC-27, MGC-803, MKN-45 and SGC-7901 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) and reverse transcribed using a PrimeScript RT-PCR kit (Takara Bio., Inc., Otsu, Japan), according to the manufacturer's protocols. RT-qPCR was performed with SYBR Premix Ex Taq (Takara Bio., Inc.) using a 7500 Real-time PCR system (Thermo Fisher Scientific, Inc.). The primer sequences used in the present study were as follows: Tim-3 forward, 5'-CTGCTGCTACTACTTACAAGGTC-3' [melting temperature (T_m)=60.1°C] and reverse, 5'-GCA GGGCAGATAGGCATTCT-3' (T_m=61.8°C); Gal-9 forward, 5'-TCTGGGACTATTCAAGGAGGTC-3' (T_m=60.3°C) and reverse, 5'-CCACTGGAGCTGAGAACGG-3' (T_m=62.0°C); and β -actin forward, 5'-CATGTACGTTGCTATCCAGGC-3' (T_m=60.8°C) and reverse, 5'-CTCCTTAATGTCACGCACGAT-3' (T_m=60.2°C). The 2^{- $\Delta\Delta$ C_q} method (15) was used to quantify relative Tim-3 and Gal-9 expression, which was normalized to β -actin.

Western blotting. Total protein was extracted using a total protein extraction buffer (Beyotime Institute of Biotechnology, Haimen, China) and the protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (30 μ g per lane) were separated

using 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Following blocking with 1% BSA at room temperature for 1 h, the membrane was probed with Tim-3 (dilution, 1:1,000; cat. no. 45208; Cell Signaling Technology, Inc.), Gal-9 (dilution, 1:1,000; cat. no. ab69630; Abcam) or β -actin (dilution, 1:1,000; cat. no. 20536-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) primary antibodies overnight at 4°C, and a rabbit immunoglobulin G (H+L) secondary antibody (dilution, 1:10,000; cat. no. 31460; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Proteins were visualized using the Molecular Imager ChemiDoc XRS⁺ System (Bio-Rad Laboratories, CA, USA).

Analysis using K-M plotter. To analyze the association between immune marker expression and patient prognosis, a number of GEO databases (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) regarding Tim-3, Gal-9 and CD8A expression levels in GC and the K-M plotter website (<http://kmplot.com/analysis/>) (16) were consulted. The following GEO datasets were used: gse14210 (17), gse15459 (18), gse22377 (19), gse29272 (20), gse51105 (21) and gse62254 (22).

Statistical analysis. Associations between Tim-3 expression and clinicopathological factors were analyzed using the χ^2 test or Fisher's exact test. Survival analysis was performed using the Kaplan-Meier method and the log-rank test. Univariate and multivariate analyses were conducted using the Cox proportional hazards model to identify prognostic factors. All statistical tests were two-sided and P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 13.0 statistical package software (SPSS, Inc., Chicago, IL, USA) or GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

Clinicopathological features of patients with GC. To study the association between Tim-3 expression and patient clinicopathological features, patient age, sex, tumor location, tumor diameter, Lauren type, perineuronal invasion, blood-vessel invasion and TNM stage were analyzed. Follow-up information was available for all 587 patients. The follow-up time ranged between 1 and 117 months following surgery, with a median follow-up time of 48 months.

Association between patient prognosis and immune characteristics. For immune characteristic analysis, immunohistochemical staining was performed on the TMA for immune checkpoint Tim-3 and Gal-9, as well as T-cell markers, CD3 (tumor infiltrating lymphocytes, TILs), CD8 (cytotoxic T lymphocytes, CTLs) and FOXP3 (T regulatory cells, Tregs). Images of Tim-3 and Gal-9 expression are shown in Fig. 1A and B. The majority of the GC tissues exhibited cytoplasmic and extracellular Gal-9 staining. However, few gastric cancer cells exhibited Tim-3 staining, which is strongly expressed in infiltrating immune cells. Considerable infiltration of the GC parenchyma by CD3⁺ and CD8⁺ T cells was evident in the patients with a favorable prognosis. The number of FOXP3⁺ T cells that infiltrated the tumor parenchyma was less than that of the other types of

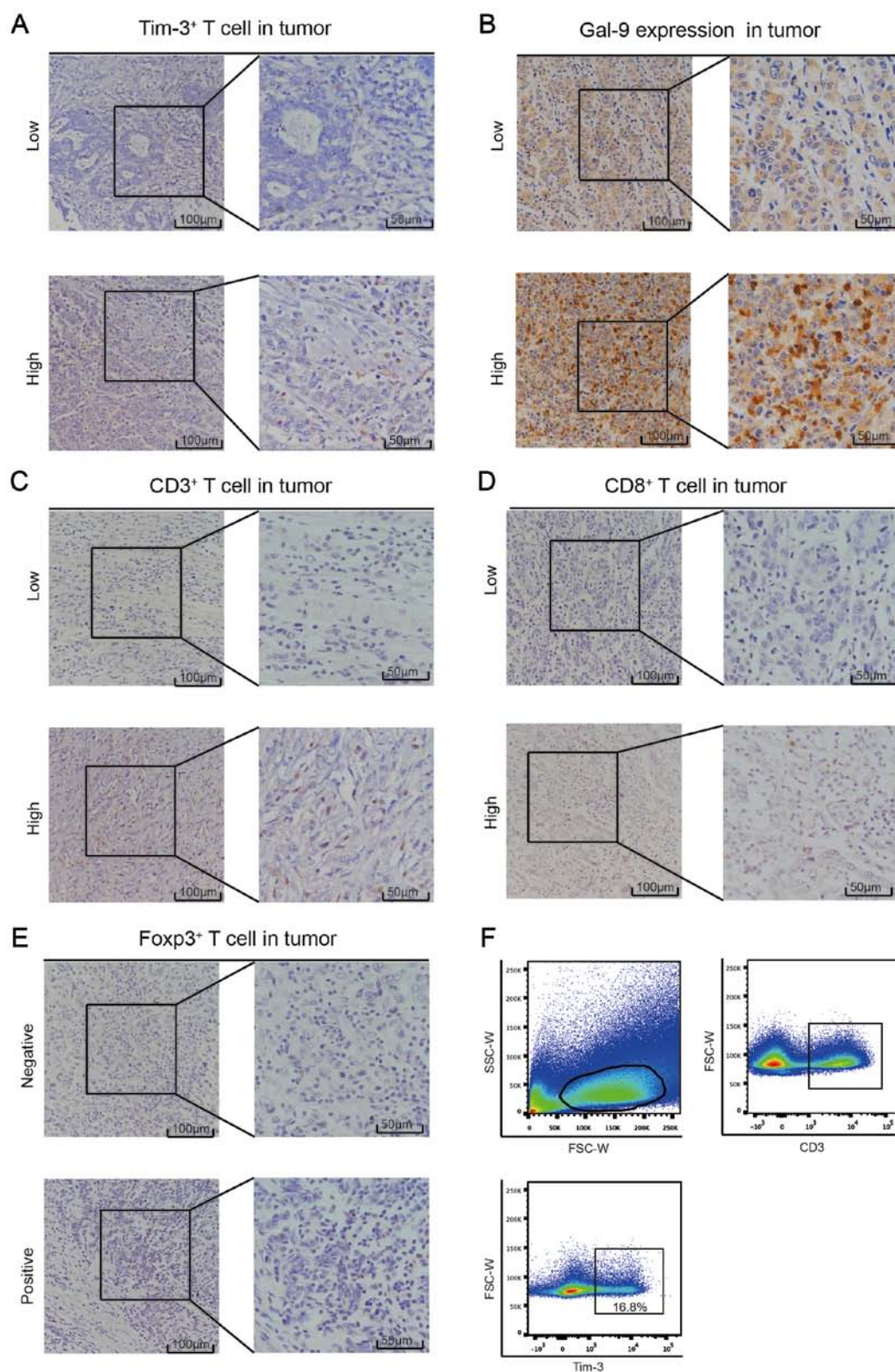


Figure 1. Tim-3, Gal-9, CD3, CD8 and FOXP3 expression in GC tissues. (A) Immunohistochemical detection of Tim-3 protein in patient TMAs. Tim-3 staining was observed in infiltrating immune cells in the tumor parenchyma. Tumor cells are minimally stained. (B) Immunohistochemical detection of Gal-9 protein using TMA. Gal-9 staining was observed in tumor cells. Representative examples of (C) CD3⁺ high and low density, (D) CD8⁺ high and low density and (E) FOXP3⁺ positive and negative immunohistochemical staining in GC parenchyma. Scale bar, 100 μm (left panel) and 50 μm (right panel). (F) Tim-3 expression in CD3⁺ T cells as detected using fluorescence-activated cell sorting. Tim-3, T-cell immunoglobulin and mucin domain-containing protein 3; Gal-9, galectin 9; CD, cluster of differentiation; FOXP3, forkhead box p3; GC, gastric cancer; TMAs, tissue microarrays.

T cells (Fig. 1C-E). FACS was performed in order to detect Tim-3 expression in CD3⁺ T cells. The results showed that

16.8% of CD3⁺ T cells exhibited notable expression of Tim-3 protein. Therefore, it was demonstrated that Tim-3 is notably

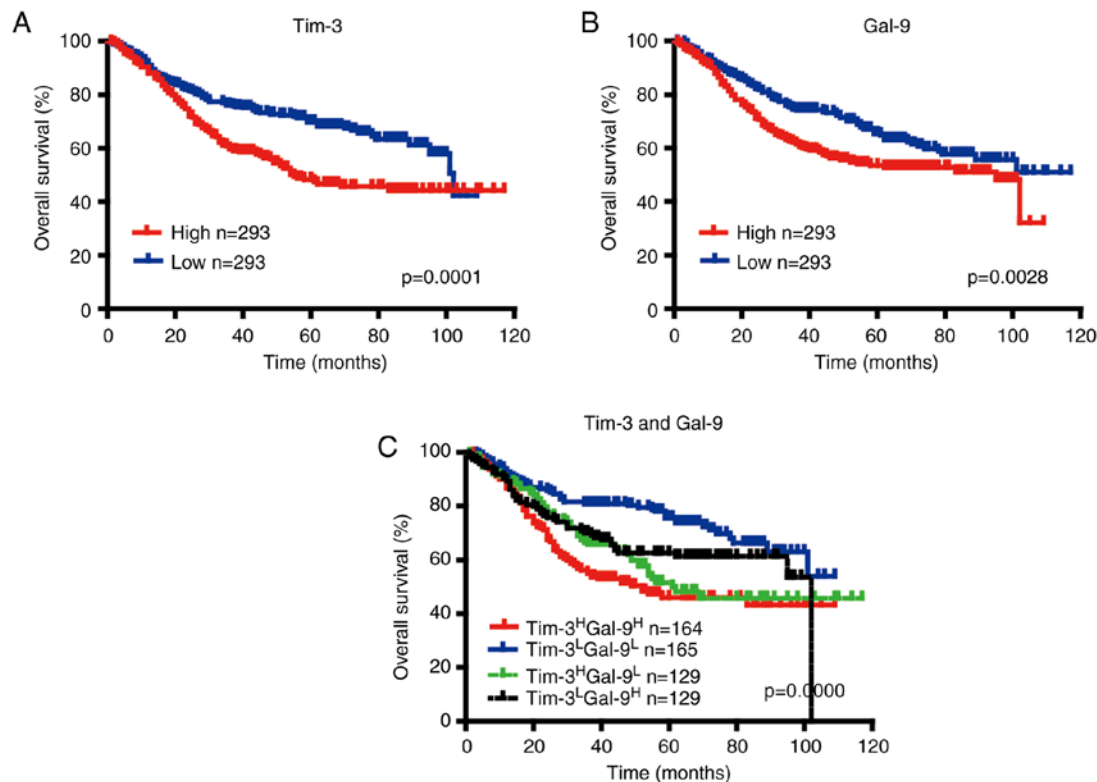


Figure 2. High Tim-3 and Gal-9 expression in gastric cancer is associated with a poor OS rate. The association between OS and (A) Tim-3 or (B) Gal-9 protein expression. (C) Combined analysis of the association between Tim-3 and Gal-9 expression, and patient OS rate. Tim-3, T-cell immunoglobulin and mucin domain-containing protein 3; Gal-9, galectin 9; OS, overall survival.

expressed in the tumor-infiltrating lymphocytes of patients with GC (Fig. 1F).

The present study analyzed the association between Tim-3 expression in immune cells in patients with GC and patient prognosis. It was revealed that the expression of Tim-3 in immune cells was significantly associated with patient survival. Increased Tim-3⁺ immune cell infiltration into the tumor was associated with a lower OS rate ($P=0.001$; Fig. 2A). Gal-9 expression in GC was also compared with patient prognosis. Patients exhibiting high Gal-9 expression had a relatively poor prognosis and a poorer OS rate ($P=0.0028$; Fig. 2B). As Gal-9 is a ligand of Tim-3, combined analysis involving the expression of the two factors was performed. Patients with low Tim-3 and Gal-9 expression had a significantly greater OS rate than other patients ($P<0.001$; Fig. 2C).

TIL, CTL and Treg infiltration into the tumor parenchyma was analyzed by measuring the number of CD3⁺, CD8⁺ and FOXP3⁺ T cells. CD3⁺ T-cell density in tumor tissues was not associated with OS rate ($P=0.0776$; Fig. 3A), while CD8⁺ T cell density in tumor tissue was positively associated with OS rate ($P=0.0395$; Fig. 3B). However, a high density of FOXP3⁺ T cells in tumor tissue was associated with a poorer OS rate ($P=0.0164$; Fig. 3C).

Association between patient clinicopathological parameters and immune characteristics. It was demonstrated that age and tumor diameter were significantly different in the Tim-3 high and low expression groups. Tumor location and blood vessel invasion were significantly different in the Gal-9 high and low expression groups. The Tim-3 or Gal-9

expression status was positively associated with N stage and TNM stage (Tables I and II). However, in terms of the association between TIL, CTL and Tregs density, and patient clinicopathological parameters, only tumor diameter was significantly different between the CTL high- and low-density groups (Tables III-V).

Association between biomarker expression levels. CD3⁺ T cell density in tumor tissue was correlated with CD8⁺ T cell density ($r=0.6281$, $P<0.0001$, Fig. 3D). However, there was no correlation between Tim-3⁺ immune cell density and CD3⁺ or CD8⁺ T cell density ($r=0.2405$, $P<0.0001$ and $r=0.1550$, $P=0.0002$, respectively; Fig. 3E and F).

K-M plotter analysis. Tim-3, Gal-9 and CD8A mRNA expression was compared with patient survival using the following GEO databases on the K-M plotter website: gse14210, gse15459, gse22377, gse29272, gse51105 and gse62254. High Tim-3 expression in patients with GC was associated with relatively short survival times (Fig. 4A and B). High Gal-9 expression in patients with GC was also associated with relatively short survival times (Fig. 4C). High CD8A expression was associated with relatively long survival times (Fig. 4D).

Expression of Tim-3 and Gal-9 in GC cell lines. To detect Tim-3 and Gal-9 expression in tumor cell lines, western blotting and RT-qPCR were performed using AGS, BGC-823, HGC-27, MGC-803, MKN-45 and SGC-7901 cells. GC cells exhibited low expression levels of Tim-3 but relatively high Gal-9 expression (Fig. 4E-G).

Table I. Association between tumor Tim-3 expression and clinicopathological parameters in patients with gastric cancer.

Clinicopathological feature	Tim-3 expression		P-value
	Low (n=294)	High (n=293)	
Age, years			
≤65 (n=371)	198	173	0.0370 ^a
>65 (n=216)	96	120	
Sex			
Male (n=401)	199	202	0.7439
Female (n=186)	95	91	
Tumor location			
Top (n=118)	63	55	0.7212
Middle (n=202)	99	103	
Bottom (n=267)	132	135	
Diameter, cm			
≤5 (n=375)	202	173	0.0148 ^a
>5 (n=212)	92	120	
Pathological Lauren type			
Intestinal (n=194)	106	88	0.1715
Diffuse (n=326)	152	174	
Mixed (n=67)	36	31	
Perineuronal invasion			
Negative (n=518)	263	255	0.3617
Positive (n=69)	31	38	
Blood vessel invasion			
Negative (n=493)	253	240	0.1711
Positive (n=94)	41	53	
pT stage			
T1 (n=94)	56	38	0.0795
T2 (n=80)	44	36	
T3 (n=151)	66	85	
T4 (n=262)	128	134	
pN stage			
N0 (n=234)	133	101	0.0478 ^a
N1 (n=105)	50	55	
N2 (n=114)	54	60	
N3 (n=134)	57	77	
TNM stage			
I (n=137)	81	56	0.0221 ^a
II (n=165)	85	80	
III (n=279)	127	152	
IV (n=6)	1	5	

^aP<0.05. Tim-3, T-cell immunoglobulin and mucin domain-containing protein 3; TNM, Tumor-Node-Metastasis.

Table II. Association between tumor Gal-9 expression and clinicopathological parameters in patients with gastric cancer.

Clinicopathological feature	Gal-9 expression		P-value
	Low (n=294)	High (n=293)	
Age, years			
≤65 (n=371)	194	177	0.1613
>65 (n=216)	100	116	
Sex			
Male (n=401)	202	199	0.8327
Female (n=186)	92	94	
Tumor location			
Top (n=118)	72	46	0.0291 ^a
Middle (n=202)	95	107	
Bottom (n=267)	127	140	
Diameter, cm			
≤5 (n=375)	199	176	0.0547
>5 (n=212)	95	117	
Pathological Lauren type			
Intestinal (n=194)	106	88	0.0841
Diffuse (n=326)	150	176	
Mixed (n=67)	38	29	
Perineuronal invasion			
Negative (n=518)	263	255	0.3617
Positive (n=69)	31	38	
Blood vessel invasion			
Negative (n=493)	256	237	0.0410 ^a
Positive (n=94)	38	56	
pT stage			
T1 (n=94)	49	45	0.3397
T2 (n=80)	41	39	
T3 (n=151)	66	85	
T4 (n=262)	138	124	
pN stage			
N0 (n=234)	134	100	0.0060 ^b
N1 (n=105)	55	50	
N2 (n=114)	53	61	
N3 (n=134)	52	82	
TNM stage			
I (n=137)	75	62	0.0292 ^a
II (n=165)	94	71	
III (n=279)	123	156	
IV (n=6)	2	4	

^aP<0.05, ^bP<0.01. Gal-9, galectin 9; TNM, Tumor-Node-Metastasis.

CD8⁺ T cell density and Tim-3⁺ immune cell infiltration are independent prognostic factors in GC. Table VI shows the univariate and multivariate analysis of clinicopathological

factors. The univariate analysis revealed that the following factors were significantly associated with patient postoperative survival: CD8⁺ T cell density (P=0.025), FOXP3⁺ T cell density (P=0.017), Tim-3⁺ immune cell density (P<0.0001),

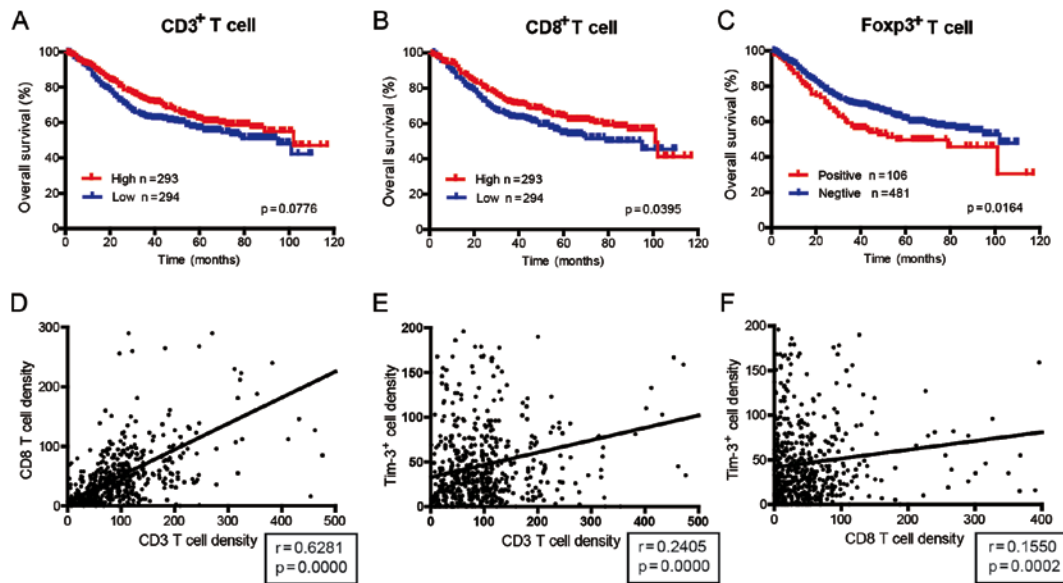


Figure 3. Association between patient survival and CD3⁺, CD8⁺ and Foxp3⁺ T cell infiltration in gastric cancer parenchyma. (A) No statistically significant association was observed between CD3⁺ T-cell infiltration, while (B) CD8⁺ T-cell infiltration in GC was associated with a high OS. (C) Foxp3⁺ T cell infiltration in GC is associated with a low OS. Association between (D) CD3 and CD8, (E) CD3 and Tim-3 and (F) CD8 and Tim-3 expression. CD, cluster of differentiation; FOXp3, forkhead box p3; GC, gastric cancer; OS, overall survival; Tim-3, -cell immunoglobulin and mucin domain-containing protein 3.

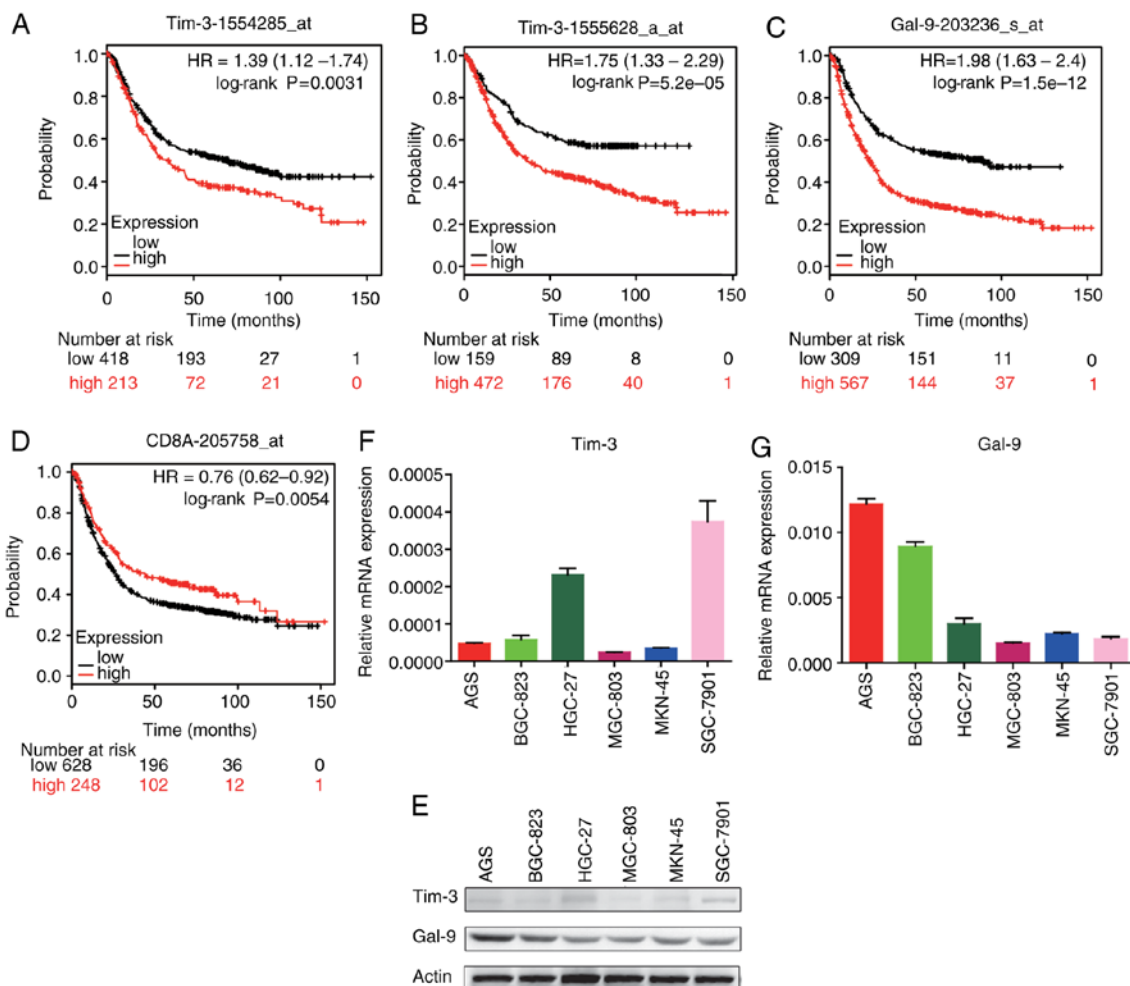


Figure 4. K-M plotter website analysis and expression of Tim-3/Gal-9 in gastric cancer cell lines. Association between patient survival and Tim-3 mRNA expression using the K-M plotter website. The analysis contains two Affy IDs, (A) 1554285_at and (B) 1555628_a_at. (C) Association between patient survival and Gal-9 mRNA expression level. (D) Association between patient survival and CD8A mRNA expression level. (E) Expression of Tim-3 and Gal-9 protein and (F and G) mRNA in gastric cancer cell lines. Tim-3, -cell immunoglobulin and mucin domain-containing protein 3; Gal-9, galectin 9; CD, cluster of differentiation; HR, hazard ratio.

Table III. Association between tumor CD3 expression and clinicopathological parameters in patients with gastric cancer.

Clinicopathological feature	CD3 expression		P-value
	Low (n=294)	High (n=293)	
Age, years			
≤65 (n=371)	183	188	0.6298
>65 (n=216)	111	105	
Sex			
Male (n=401)	203	198	0.7017
Female (n=186)	91	95	
Tumor location			
Top (n=118)	61	57	0.8201
Middle (n=202)	103	99	
Bottom (n=267)	130	137	
Diameter, cm			
≤5 (n=375)	181	194	0.2412
>5 (n=212)	113	99	
Pathological Lauren type			
Intestinal (n=194)	106	88	0.3006
Diffuse (n=326)	156	170	
Mixed (n=67)	32	35	
Perineuronal invasion			
Negative (n=518)	256	262	0.3778
Positive (n=69)	38	31	
Blood-vessel invasion			
Negative (n=493)	245	248	0.6656
Positive (n=94)	49	45	
pT stage			
T1 (n=94)	41	53	0.1804
T2 (n=80)	37	43	
T3 (n=151)	72	79	
T4 (n=262)	144	118	
pN stage			
N0 (n=234)	116	118	0.3688
N1 (n=105)	48	57	
N2 (n=114)	65	49	
N3 (n=134)	65	69	
TNM stage			
I (n=137)	63	74	0.6838
II (n=165)	82	83	
III (n=279)	146	133	
IV (n=6)	3	3	

CD, cluster of differentiation; TNM, Tumor-Node-Metastasis.

Table IV. Association between tumor CD8 expression and clinicopathological parameters in patients with gastric cancer.

Clinicopathological feature	CD8 expression		P-value
	Low (n=294)	High (n=293)	
Age, years			
≤65 (n=371)	185	186	0.8889
>65 (n=216)	109	107	
Sex			
Male (n=401)	206	195	0.3601
Female (n=186)	88	98	
Tumor location			
Top (n=118)	58	60	0.8629
Middle (n=202)	99	103	
Bottom (n=267)	137	130	
Diameter, cm			
≤5 (n=375)	174	201	0.0176 ^a
>5 (n=212)	120	92	
Pathological Lauren type			
Intestinal (n=194)	106	88	0.1245
Diffuse (n=326)	151	175	
Mixed (n=67)	37	30	
Perineuronal invasion			
Negative (n=518)	257	261	0.5315
Positive (n=69)	37	32	
Blood vessel invasion			
Negative (n=493)	247	246	0.9856
Positive (n=94)	47	47	
pT stage			
T1 (n=94)	50	44	0.6614
T2 (n=80)	36	44	
T3 (n=151)	73	78	
T4 (n=262)	135	127	
pN stage			
N0 (n=234)	109	125	0.0736
N1 (n=105)	48	57	
N2 (n=114)	69	45	
N3 (n=134)	68	66	
TNM stage			
I (n=137)	70	67	0.3494
II (n=165)	73	92	
III (n=279)	148	131	
IV (n=6)	3	3	

^aP<0.05. CD, cluster of differentiation; TNM, Tumor-Node-Metastasis.

Gal-9 expression (P=0.002), age (P=0.009), tumor diameter (P<0.0001), Lauren type (P=0.001), perineuronal invasion (P<0.0001), blood vessel invasion (P<0.0001) and TNM stage (P<0.0001). Multivariate regression analysis

indicated that CD8⁺ T cell density (P=0.031), Tim-3⁺ immune cell density (P=0.012), tumor diameter (P=0.001), blood vessel invasion (P=0.003) and TNM stage (P<0.001) were independent prognostic factors in GC.

Table V. Association between tumor FOXP3 expression and clinicopathological parameters in patients with gastric cancer.

Clinicopathological feature	FOXP3 expression		P-value
	Negative (n=481)	Positive (n=106)	
Age, years			
≤65 (n=371)	309	62	0.2664
>65 (n=216)	172	44	
Sex			
Male (n=401)	332	69	0.4313
Female (n=186)	149	37	
Tumor location			
Top (n=118)	95	23	0.7776
Middle (n=202)	164	38	
Bottom (n=267)	222	45	
Diameter, cm			
≤5 (n=375)	304	71	0.4634
>5 (n=212)	177	35	
Pathological Lauren type			
Intestinal (n=194)	165	29	0.0785
Diffuse (n=326)	257	69	
Mixed (n=67)	59	8	
Perineuronal invasion			
Negative (n=518)	427	91	0.3974
Positive (n=69)	54	15	
Blood-vessel invasion			
Negative (n=493)	408	85	0.2389
Positive (n=94)	73	21	
pT stage			
T1 (n=94)	84	10	0.1746
T2 (n=80)	67	13	
T3 (n=151)	122	29	
T4 (n=262)	208	54	
pN stage			
N0 (n=234)	197	37	0.7098
N1 (n=105)	85	20	
N2 (n=114)	92	22	
N3 (n=134)	107	27	
TNM stage			
I (n=137)	120	17	0.1364
II (n=165)	133	32	
III (n=279)	222	57	
IV (n=6)	6	0	

FOXP3, forkhead box p3; TNM, Tumor-Node-Metastasis.

Discussion

At present, the treatment options available for GC are limited, particularly for patients with advanced stages of disease (23).

In China, early diagnosis remains problematic and therefore the majority of patients have advanced GC at the time of diagnosis (23). This is likely because gastroendoscopy is not performed as regularly in China as in other developed countries (23). The current conventional treatments used for GC include surgery and perioperative chemotherapy (2). Immunotherapies, particularly immune checkpoint inhibitors, have great potential as effective treatments for GC in the future and have been used successfully to treat other solid tumors.

Tim-3 is selectively expressed in IFN- γ -producing CD4⁺ Th1 and CD8⁺ Tc1 cells (4). Previous studies have demonstrated that Tim-3 is required to induce immune tolerance, as Tim-3-deficient mice and mice treated with a Tim-3 Ig protein had defects in the induction of antigen-specific tolerance (24,25). Tim-3, as an immune checkpoint receptor, limits the duration and magnitude of the Th1 and Tc1 T-cell response. In the present study, Tim-3-positive immune cells were demonstrated to infiltrate GC tissue. GC cells rarely express Tim-3 protein but Tim-3 expression in tumor cells has been previously reported (26); however, the majority of previous studies have indicated that Tim-3⁺ cells are immune cells, including T cells, macrophages and dendritic cells (27-29). In the present study, a significant association between Tim-3⁺-cell tumor infiltration, and tumor diameter and TNM stage was observed. Furthermore, the survival of patients exhibiting high Tim-3⁺-cell tumor infiltration was significantly lower than that in those exhibiting low infiltration. Univariate and multivariate analyses also revealed that Tim-3⁺ infiltrating immune cells in tumors were associated with a poor prognosis. The mechanism behind this association is not yet fully understood. However, it is possible that the Tim-3/Gal-9 pathway down-regulates T-cell responses by mediating apoptosis. A recent study revealed a negative regulatory effect by Tim-3 expression on CD4⁺ and CD8⁺ T cell viability (30). Furthermore, the Tim-3/Gal-9 pathway contributes toward the suppressive tumor microenvironment via Treg activation upon TCR activation (31). Expression of the Tim-3 surface protein by CD8⁺ T cells provides immune tolerance upon encounter of cancer cells highly expressing Gal-9.

Gal-9, a galectin protein, regulates the survival, proliferation and cytokine synthesis of effector helper and cytotoxic T cells (32). Gal-9 has recently become a major molecule of interest due to the identification of its negative influence on the adaptive immune response. In the present study, Gal-9 was demonstrated to be highly expressed in GC and subcellularly localized to the extracellular area and cytoplasm of tumor cells. A significant association between Gal-9 expression, blood vessel invasion and TNM stage in GC was observed. Additionally, high expression of Gal-9 was associated with poor patient survival. Gal-9 and other galectins have been indicated to be prognostic markers in other types of cancer (33-35). Tim-3/Gal-9 binding interactions inhibit Th17 polarization, driving the proliferation of FOXP3⁺ Tregs (26). Tim-3/Gal-9 binding can also induce apoptosis or necrosis in pro-inflammatory T cell subsets (37). While Gal-9 and other galectins induce pro-apoptotic features in pro-inflammatory T cell subsets, they function through Bcl-2 blocking Gal-9-induced apoptosis (34). *In vitro* studies using lung cancer-specific T-cell lines revealed that apoptosis is induced in Tim-3⁺ CD8⁺ T-cell clones following interaction with

Table VI. Univariate and multivariate analysis of prognostic parameters for the overall survival of patients with gastric cancer.

Prognostic parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Age	1.397	1.087-1.797	0.009 ^b	1.084	0.826-1.424	0.559
Sex	1.154	0.889-1.499	0.281			
Tumor location						
Top	1	Reference				
Middle	0.824	0.594-1.144	0.248			
Bottom	0.858	0.608-1.211	0.383			
Diameter	0.358	0.279-0.459	<0.001 ^b	0.623	0.474-0.818	0.001 ^b
Lauren type (intestinal/mixed vs. diffuse)	1.613	1.218-2.137	0.001 ^b	1.120	0.837-1.499	0.445
Perineuronal invasion	0.441	0.318-0.612	<0.001 ^b	0.785	0.557-1.105	0.165
Blood vessel invasion	0.370	0.277-0.493	<0.001 ^b	0.620	0.455-0.846	0.003 ^b
TNM stage	0.213	0.160-0.283	<0.001 ^b	0.295	0.217-0.402	<0.001 ^b
CD3	0.781	0.609-1.001	0.051			
CD8	0.753	0.587-0.966	0.025 ^a	0.755	0.585-0.975	0.031 ^a
FOXP3	1.439	1.067-1.941	0.017 ^a	1.176	0.887-1.559	0.260
Tim-3	1.710	1.327-2.203	<0.001 ^b	1.395	1.078-1.807	0.012 ^a
Gal-9	1.489	1.160-1.912	0.002 ^b	1.205	0.933-1.556	0.153

^aP<0.05, ^bP<0.01. HR, hazard ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis; CD, cluster of differentiation; FOXP3, forkhead box p3; Tim-3, T-cell immunoglobulin and mucin domain-containing protein 3; Gal-9, galectin 9.

Gal-9, which can be inhibited by the addition of anti-Gal-9 or anti-Tim-3 antibodies. The release of soluble Gal-9 can therefore negatively regulate T-cell function or induce apoptosis via Tim-3 (35). Another study demonstrated that Gal-9 induces Tim-3⁺ Th1, Th17 and Tc1 T cell apoptosis in hyperimmune conditions (36). However, the function of Gal-9 in breast cancer, cervical carcinoma and malignant melanoma has also been reported, which is in contrast to the results of the present study (38-40). This may be due to individual variation in the immune state, including differing Tim-3 protein expression and cytokine levels. Univariate analysis demonstrated that Gal-9 overexpression in tumor cells is associated with a poor prognosis in GC. However, multivariate analysis did not reveal any statistical significance, suggesting that Gal-9 functions as a tumorigenesis promoter depending on the immune state of the patient. High expression of Tim-3 and Gal-9 was associated with a poor survival in patients with GC.

Regarding CD3⁺ and CD8⁺ T cell infiltration in tumors and survival in patients with GC, previous studies have reported an association between high CD3⁺ and CD8⁺ T cell density and a favorable prognosis (41,42). The same results were observed in the present study; the density of CD8⁺ T cells in GC tumors was associated with tumor size. Univariate and multivariate analyses also revealed that high CD8⁺ T cell infiltration was associated with a good prognosis. CTLs are also associated with a good prognosis. In adaptive immunity, CTLs directly kill tumor cells in the tumor microenvironment. Adaptive immunity, which is mediated by T cells, has been suggested to serve a major role in antitumor immunity (42).

Forkhead box protein 3 (FOXP3), a member of the forkhead transcription factor family, is considered to be a distinctive molecular marker of regulatory T cells (43). It has been suggested that Tregs can suppress the majority of immune cells, including CD4⁺ and CD8⁺ T cells, B cells, natural killer cells and NK T cells. Tregs also inhibit the proliferation of effector T cells, reduce cytokine secretion, promote B-cell anergy, impede antibody production, inhibit the expression of co-stimulatory and antigen-presenting molecules, and reduce the ability to stimulate T cell responses (44). A high frequency of Tregs is generally considered to be a marker of poor prognosis in various types of cancer. Tregs may mediate the suppression of antitumor immunity, which promotes tumor growth. The present study revealed a high level of Tregs infiltrating the tumor tissue, which was associated with a poor survival in patients with GC. A previous study demonstrated that enhanced FOXP3⁺ T cells expression was associated with a low OS rate and a poor prognosis (45). A high density of Tregs in tumors and peripheral blood is therefore generally considered to be a marker of a poor prognosis in cancer.

The present study has a number of limitations. The IHC of the TMA may not completely represent the immune marker expression. Previous studies have used large areas of tissue for immunostaining (46). Tim-3 is mainly expressed in T cells but is also expressed in other immune cells. Future studies should aim to define the detailed function of Tim-3 and the immune cells in which it is expressed in GC.

While two previous studies have investigated the association between Tim-3 expression on T cells and NK cells, and patient clinicopathological features (47,48). However, the

present study compared its ligand Gal-9 expression and T-cell infiltration in tumor tissues with patient clinicopathological features and survival. The large patient sample size made the conclusions of the present study more robust. The association between Tim-3 expression and Gal-9 expression was also discussed in the present study. Tim-3 is primarily expressed by infiltrating T cells, while Gal-9 is expressed by GC cells. A large number of TILs and CTLs were revealed to infiltrate GC, and CTL infiltration was associated with OS. Univariate and multivariate analyses revealed that Tim-3 expression and CD8⁺ T cell density in tumors are associated with GC prognosis and can act as independent prognostic factors. However, the mechanism by which Tim-3 functions in GC and its interaction with Gal-9 remain unclear and require investigation in the future. Finally, the Tim-3/Gal-9 pathway may be a valuable immunotherapy target for the treatment of GC and other types of solid tumor.

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Availability of data and materials

The datasets generated and analyzed during the study are available in Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/pubmed>) and Kaplan Meier-Plotter website (<http://kmplot.com/analysis/index.php?p=service&cancer=gastric>). Other datasets used in the study are available from the corresponding author upon reasonable request.

Authors' contributions

HC and GZ conceived and designed the study. YW, EZ and ZZ performed the experiments. YW wrote the study. HC, GZ, EZ and ZZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). Written informed consent was obtained from all enrolled patients prior to their inclusion in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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