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Original Article

Expression of Tim-3 in gastric cancer tissue and its relationship with prognosis

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Abstract: As a negative regulatory molecule, T-cell immunoglobulin–and mucin domain-3 (Tim-3) plays a crucial role in the tumor immunological tolerance. In the present study, we aimed to determine the Tim-3 expression in gastric cancer tissue and its relationship with clinicopathological parameters and prognosis. The Tim-3 expression was assessed in 52 gastric cancer specimens and 15 gastritis tissues by flow cytometry, and gastritis tissues served as the control. As a result, we found that the Tim-3 expressions on $CD4^+$T cells and $CD8^+$T cells in gastric cancer tissue was significantly higher than those in gastritis tissue (P=0.022, P=0.047, respectively). The median expression level of Tim-3 on $CD4^+$T cells were significantly correlated with clinicopathological parameters, such as tumor size, lymph node metastasis, the depth of tumor invasion and TNM staging (P=0.042, P=0.026, P=0.001, P=0.003, respectively), while it was not correlated with sex, age and histological subtype (all P>0.05). In $CD8^+$T cells, the Tim-3 expression was relevant to tumor invasion and TNM staging (P=0.035, P=0.017, respectively), while it was irrelevant to other clinicopathological parameters (all P>0.05). Additionally, Kaplan-Meier survival curves showed that the median overall survival time of patients with lower Tim-3 expression was greater than that of patients with higher Tim-3 expression in $CD4^+$T cells and $CD8^+$T cells (χ^2=18.036, P<0.001 and χ^2=18.036, P<0.001, respectively). Moreover, the multivariate analysis revealed that the Tim-3 expression and TNM stage were independent prognostic factors for gastric cancer patients (P=0.029, P=0.043 and P=0.003, respectively). These results suggest that Tim-3 played an important role in the development and progression of gastric cancer, and it could be used as an independent prognostic factor for gastric cancer patients.

Keywords: T-cell immunoglobulin-and mucin domain-3 (Tim-3), flow cytometry, gastric cancer, prognosis

Introduction

Gastric cancer is one of the most common types of cancer worldwide in terms of incidence and mortality [1]. China is the country with the highest morbidity of gastric cancer, seriously threatening the public health. Although standardized operational technology and diversified therapies have been constantly improved, the survival rate and life quality of gastric cancer patients remain poor. Therefore, it is very urgent to seek new indicators for gastric cancer prognosis and effective therapies.

T-cell immunoglobulin-and mucin domain-3 (Tim-3) was primarily defined as the specific expression in Th1 cells. At present, the Tim-3 expression can be detected in $CD8^+$T cells, Treg, dendritic cells (DC), Th17, natural killer (NK) cells, melanoma, monocytes, mast cells, lung cancer cells and other lymphocyte subpopulations [2-8]. In recent years, Tim-3 was regarded as a negative regulatory molecule, which plays a crucial role in anti-tumor immunity. However, its anti-tumor mechanism remains unknown. In the present study, we investigated the Tim-3 expression of 52 gastric cancer and 15 gastritis specimens by flow cytometry. We aimed to determine the Tim-3 expression in gastric cancer tissue and its relationship with clinicopathological parameters and prognosis.

Materials and methods

Selection of patients

A total of 52 gastric cancer specimens were collected in Gastrointestinal Surgery Department

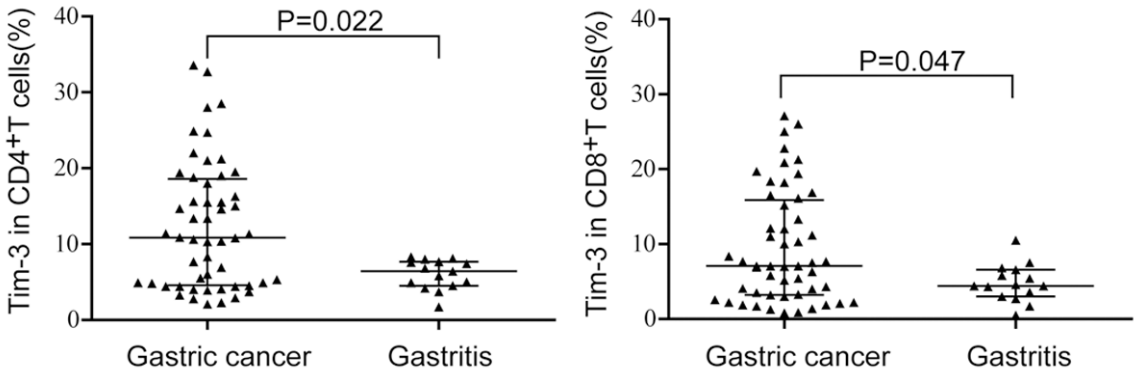

Figure 1. Percentage of Tim-3 expression on $CD4^+$T cells and $CD8^+$T cells in gastric cancer and gastritis patients.

of the Third Affiliated Hospital of Soochow University (Jiangsu Province, China) from Oct. 2010 to Nov. 2011, and all of them were post-operatively pathologically diagnosed. Among these collected specimens, 41 were males and 11 were females. The median age of patients was 64.5 years old, ranging from 33 to 82 years old. All patients did not receive chemotherapy and other therapies before operation. In addition, 15 gastritis specimens, including 5 males and 10 females with a median age of 45 years old, were obtained from the medical outpatients at the Third Affiliated Hospital of Soochow University during September, 2011. Written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.

Reagents and instruments

Anti-human Tim-3 was purchased from R&D Systems (USA); anti-human CD4-FITC, anti-human CD8-FITC, isotype control IgG2a-PE and IgG1-FITC were obtained from Beckman Coulter (USA); and collagenase IV, type I DNA enzyme and hyaluronidase were supplied by Sigma Company (USA). Flow cytometric analysis was performed using EPICS XL flow cytometer of Beckman Coulter (USA).

Flow cytometry analysis

Fresh tissue was cut into pieces of 1 mm^3 and then added into 50 mL RPMI-1640 medium. Tissue was digested with 0.1% collagenase IV, 0.002% type I DNA enzyme and 0.01% hyaluronidase at 37°C for 45 min. Subsequently, the tissue pieces were transferred to the steel mesh, and single cell suspensions were obtained through mechanical grinding. Tim-3-PE/CD4-FITC mAb, Tim-3-PE/CD8-FITC mAb and isotype control IgG2a-PE and IgG1-FITC were respectively added to the tubes containing cells. Other experimental procedures were carried out based on the instructions, and stained cells were then subjected to flow cytometry analysis.

Statistical analysis

All data were analyzed by the SPSS software package 13.0 (SPSS, Chicago, IL). Due to non-normal distribution, the Mann-Whitney U-test was used for comparison between groups. Survival time was defined as the time from the date of diagnosis to the date of death or the date of the last follow-up. Survival curves were analyzed using Kaplan-Meier curves, and survival differences were examined using the log-rank test. The COX model was performed to evaluate the prognosis factors for gastric cancer by the univariate and multivariate analysis. *P*-values less than 0.05 were considered as statistically significant.

Results

Expression of Tim-3 in the tissue of gastric cancer and gastritis

Our research shows that Tim-3 was expressed on the surface of the freshly isolated $CD4^+$T cells and $CD8^+$T cells. **Figure 1** exhibits that the Tim-3 expression on $CD4^+$T cells in gastric cancer tissue (median of 10.85% with a range from 2.10 to 33.6%) was significantly higher than that in gastritis (median of 6.40% with a range

Table 1. The Correlation between the Tim-3 expression and clinic pathological features of patients

Characteristics	N	$Tim\text{-}3^+CD4^+T$ cells			$Tim\text{-}3^+CD8^+T$ cells		
		Median (range)	Z	P	Median (range)	Z	P
Sex			1.188	0.235		0.571	0.568
Female	11	16.30 (2.90-22.00)			7.10 (2.10-27.10)		
Male	41	10.40 (2.10-33.60)			7.10 (0.80-25.00)		
Age			0.188	0.851		1.289	0.198
≤60	20	10.15 (2.10-33.60)			5.60 (1.30-20.90)		
>60	32	10.85 (2.30-28.50)			7.70 (0.80-27.10)		
Tumor Size			2.036	0.042		1.916	0.055
<5 cm	29	6.90 (2.10-33.60)			5.80 (0.80-27.10)		
≥5 cm	23	14.7 (2.30-28.50)			11.00 (1.90-25.00)		
Histological Types			0.165	0.869		0.348	0.728
Differentiated	26	10.45 (3.30-28.50)			7.05 (0.80-27.10)		
Poorly Differentiated	26	14.00 (2.10-33.60)			7.40 (0.90-20.90)		
Invasion			3.191	0.001		2.013	0.035
No	13	4.80 (2.80-13.40)			4.10 (0.90-26.00)		
Yes	39	14.70 (2.10-33.60)			8.40 (1.80-27.10)		
Lymph node metastasis			2.232	0.026		1.945	0.052
No	22	7.15 (2.10-28.00)			5.60 (0.90-27.10)		
Yes	30	15.25 (2.30-33.60)			11.10 (0.80-25.00)		
TNM Staging			2.992	0.003		2.390	0.017
I+II	23	5.75 (2.10-28.00)			5.30 (0.90-27.10)		
III+IV	29	15.50 (2.30-33.60)			11.60 (0.80-25.00)		

from 1.70 to 8.30%) (P=0.022). Similarly, the expression of Tim-3 on $CD8^+$T cells in gastric cancer tissue (median of 7.10% with a range from 0.80 to 27.10%) was also significantly increased compared with gastritis (median of 4.40% with a range from 0.50 to 10.50%) (P=0.047).

The relationship between the Tim-3 expression and clinicopathological features in gastric cancer patients

The Tim-3 expression was correlated with clinicopathologic characteristics, such as sex, age, tumor size, histological type, lymph node metastasis, the depth of tumor invasion and TNM staging (**Table 1**). We found that the median expression level of Tim-3 on $CD4^+$T cells was significantly correlated with clinicopathological parameters, including tumor size, lymph node metastasis, the depth of tumor invasion and TNM staging (P=0.042, P=0.026, P=0.001, P=0.003, respectively), while it was not correlated with sex, age and histological subtype (all P>0.05). In $CD8^+$T cells, the Tim-3 expression was relevant to tumor invasion and TNM staging (P=0.035, P=0.017, respectively), while it was irrelevant to other clinicopathological parameters (all P>0.05).

The relationship between the Tim-3 expression and prognosis

Further analysis revealed that there was a significant correlation between the survival time of gastric patients and the Tim-3 expression level. In $CD4^+$T cells and $CD8^+$T cells, the median overall survival time of patients with lower Tim-3 expression was greater compared with patients with higher Tim-3 expression (χ^2=18.036, P<0.001 and χ^2=18.036, P<0.001, respectively) (**Figure 2**). **Table 2** shows that the tumor size, TNM staging and Tim-3 expression were significantly related to overall survival time (P=0.026, P<0.001 and P<0.001, respectively). With the adjustment of such factors, sex, age, tumor size and TNM staging, the multivariate analysis revealed that the death risk of patients with higher Tim-3 expression was significantly greater than those with lower Tim-3 expression (P=0.029 and P=0.043 for $CD4^+$T cells and $CD8^+$T cells, respectively). All these

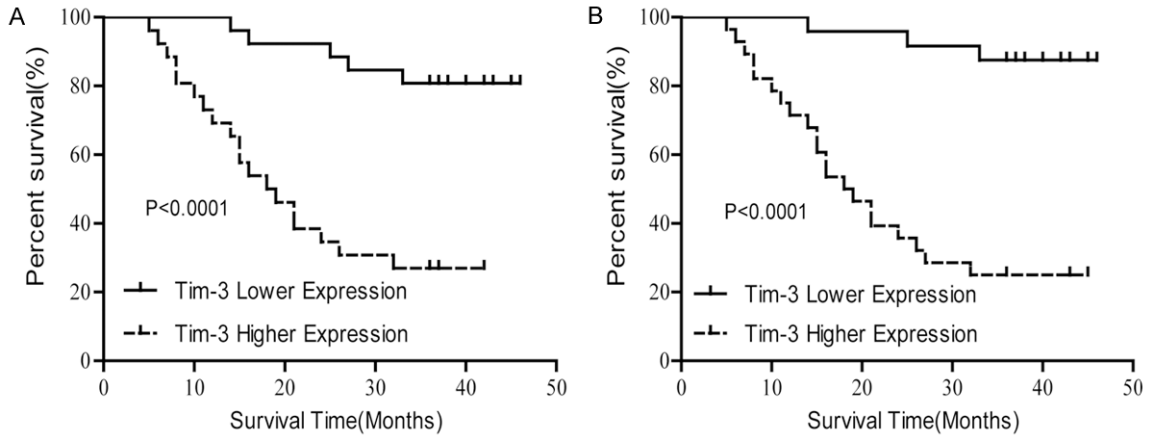

Figure 2. Patients with higher Tim-3 levels had a significantly lower survival rate compared with those with lower Tim-3 levels. A. The Tim-3 expression on $CD4^+$T cells. B. The Tim-3 expression on $CD8^+$T cells. *P*-Values are shown.

Table 2. Univariate and multivariate analyses of overall survival in patients

Clinicopathological parameters	Comparison reference	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Tim-3 expression in $CD4^+$T cells	Low/High	0.153 (0.057-0.413)	<0.001	0.252 (0.073-0.871)	0.029
Tim-3 expression in $CD8^+$T cells	Low/High	0.094 (0.028-0.320)	<0.001	0.219 (0.050-0.950)	0.043
Gender	Female/Male	0.957 (0.357-2.564)	0.930	0.514 (0.149-1.767)	0.291
Age	<60/≥60	0.771 (0.330-1.803)	0.548	1.680 (0.588-4.802)	0.333
Tumor size	<5 cm/≥5 cm	0.389 (0.170-0.894)	0.026	1.393 (0.514-3.777)	0.515
TNM stage	I, II/III, IV	0.071 (0.017-0.306)	<0.001	0.092 (0.019-0.439)	0.003

data indicated that the Tim-3 expression was an independent prognostic factor for patients with gastric cancer. Moreover, the TNM staging was also positively correlated with the death risk of gastric patients (P=0.003).

Discussion

Tim-3 refers to the membrane molecules expressed by the sub-category of some T cells. Previous studies showed that it can adjust the immune response of Th1 cells and mediate the cell immune tolerance [9, 10]. Tim-3 also plays an essential role in autoimmune diseases, anaphylactic diseases, immune tolerance and even anti-tumor immunity [11-13]. When Tim-3 is interacted with its ligand, galectin-9, the Th1 responses is a blockade by negatively regulating IFN gamma secretion [10, 14]. Gao et al. [15] reported that Tim-3 plays a significant role in tumor progression by maintaining the tumor immunosuppressive environment via regulatory T cells. Many recent studies indicated that Tim-3 is expressed in many types of tumor tissues, including lung cancer [3], gastric cancer [8], prostate cancer [16], ovarian cancer [17], cervical cancer [7], glioma [18], acute myeloid leukemia [19] and so on. In the present study, we investigated the Tim-3 expression on $CD4^+$T cells and $CD8^+$T cells of gastric cancer and gastritis tissues by flow cytometry. However, the Tim-3 expression in gastric caner tissue was distinctly higher than that in chronic gastritis tissue, suggesting that Tim-3 was involved in the pathogenesis of gastric cancer via its regulation on various immune cells.

The interaction between Tim-3 and its ligand, galectin-9, plays a negative regulatory role in the immune response mediated by Th1 cells [20]. It has been reported that the Tim-3 expression in the vascular endothelial cells can activate the interleukin-6-STAT3 pathway, leading to suppressed activation of $CD4^+$T cells [21]. In this study, we found that the Tim-3 expression on $CD4^+$T cells was significantly correlated with tumor size, lymph node metastasis, the depth of tumor invasion and TNM staging. Our results

indicated that the Tim-3 expression could be used as a marker for the development and progression of gastric cancer by regulating $CD4^+$T-cell subsets.

It is well known that $CD8^+$T cells play an important role in the tumor microenvironment. Fourcade et al. [22] discovered that Tim-3 was expressed on NY-ESO-1-specific $CD8^+$T cells in patients with advanced melanoma. They found that the blockade of both Tim-3 and PD-1 pathways can reverse tumor-induced T cell exhaustion in patients with advanced melanoma. Our results revealed that the Tim-3 expression on $CD8^+$T cells was relevant to tumor invasion and TNM staging, which might be due to Tim-3-induced T cell exhaustion, resulting in the tumor occurrence.

Recent reports suggested that the higher expression of Tim-3 was negatively correlated with the survival time of cancer patients [3, 7, 8]. Our work clearly exhibited a correlation between the Tim-3 expression and survival rate of gastric cancer patients. The multivariate analysis revealed that the death risk of patients with higher Tim-3 expression was significantly greater than that of patients with lower Tim-3 expression. Our findings were in agreement with previous reports showing that the Tim-3 expression could be used as an independent prognostic factor for gastric cancer patients.

Kaori et al. [23] reported that the co-expression of Tim-3 and PD-1 can be found in the tumor infiltrating lymphocytes of the solid tumor in mice. The multi-targeted therapy of Tim-3 and PD-1 has a high vitality in controlling the tumor growth. Our work revealed that the Tim-3 expression in gastric cancer tissue was negatively correlated with clinicopathological parameters. Moreover, Our previous study indicated that as a negative regulatory molecule, PD-L1 is distinctly expressed in the gastric cancer tissue [24]. However, the underlying mechanism remains unclear. Therefore, further research should be performed on the multi-targeted therapy of Tim-3 and PD-1 for the treatment of gastric cancer.

In summary, our study showed that the Tim-3 expression was up-regulated on $CD4^+$T cells and $CD8^+$T cells in fresh tissue in gastric cancer. In addition, the Tim-3 expression on $CD4^+$T cells and $CD8^+$T cells was closely correlated with clinicopathological parameters. Our results suggested that Tim-3 was an independent prognostic factor for gastric cancer patients. However, further studies are required to understand the underlying mechanism on how Tim-3 affects the development and progression of gastric cancer.

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Disclosure of conflict of interest

None.

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