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Original Article

Correlation between cellular immune function and prognosis of gastric cancer

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Abstract: Objective: To investigate the functions of Treg cells and immune cells of the cancer tissues, para-cancerous tissues and normal tissues. Method: Tumor tissues, para-carcinoma tissue and normal tissues from 265 gastric patients were collected, and the expression of Treg cells, CD3, CD4, CD8, NK cell and DC cells (dendritic cells) were evaluated. Through statistical methods of t test, chi-square test, Cox univariate survival analysis and Multivariate survival analysis, we studied the correlation between varieties of immune cells with clinical prognosis. Result: (1) The average number $Foxp3^+$ Treg cell and CD3, CD4, CD8, NK cell, DCs in tumor tissues and para-carcinoma tissues was higher than that of normal tissues ($P<0.05$), but there was no difference in the number of immune cells between tumor tissues and para-carcinoma tissues ($P>0.05$). (2) The regional lymph node metastasis was significantly different between high and low infiltration groups ($P<0.05$). (3) The recurrence, metastasis of tumor and survival condition between high and low infiltration groups were not statistically significant ($P>0.05$). (4) Cox univariate survival analysis and multivariate survival analysis indicated the content and infiltrating of $Foxp3^+$ Treg cells is an independent factor for the prognosis of patients with gastric cancer. Conclusion: The number of immune cells in tumor tissue and para-carcinoma tissues were significantly more than those in normal tissues. $Foxp3^+$ Treg cell infiltration in tumor tissue may indicate the survival prognosis of gastric cancer patients and will benefit for cancer immunotherapy against tumors.

Keywords: Immune microenvironment, T-Lymphocytes, regulatory lymphocytes, tumor-infiltrating, gastric cancer, radical operation

Introduction

Gastric cancer is one of the most common cancers that derived from epithelial malignant tumors, namely gastric adenocarcinoma, which accounts for 95% of the malignant gastric tumors [1]. Gastric cancer is more commonly seen in middle-aged men. In recent years, with the development of modern life style, as well as the bad living habits and environmental pollution, there is increasing number of young stomach cancer patients [2]. Long-term consumption of the roast and salt food, which contains carcinogens such as nitrite, mycotoxins, polycyclic aromatic hydrocarbons, would result in high incidence of distal gastric cancer; smokers have 50% higher risk of stomach cancer than the non-smokers [3]. Gastric cancer has high incidence in East Asia with an obvious regional difference. Especially in China, the huge population base, the higher morbidity and mortality has made gastric cancer a serious threat to people's life and health.

Gastric cancer occurs more frequently in patients over 50 years old with a male/female ratio of 2:1 [4]. Most patients have nausea, vomiting or ulcer-like upper gastrointestinal symptoms at their early stage, however, a small number of patients shows no symptoms; as the illness progresses, epigastric pain, loss of appetite and fatigue become more obvious [5]. Pain and weight loss are the most common clinical symptoms at late stage. Different lesion locations may have different symptoms. Patients with carcinoma of gastric cardia may suffer retrosternal pain and difficult in swallowing; and patients with lesions on near pylorus may have pyloric obstruction. Once the lesions invade blood vessels, patients will show symp-

toms like hematemesis, black stool and gastrointestinal bleeding. Continuous pain in abdomen often suggests tumor extending beyond the lining of the stomach, with symptoms like supraclavicular lymph node enlargement, ascites, jaundice, abdominal mass, rectal before concave ammonites and lump, etc [6]. Advanced gastric cancer patients often show symptoms like anemia, emaciation, malnutrition, and even cachexia, etc. Proliferation and metastasis of gastric cancer has following approaches: direct infiltration, hematogenous metastasis, peritoneal planting metastasis and lymphatic metastasis.

In recent years, studies have shown that local immune microenvironment and prognosis of malignant tumors have a close relationship [7]. As reported in literature: Treg cells can inhibit immune cells from killing the tumor cells by inhibiting the $CD4^+$ $CD25^+$ activation and inhibiting $CD8^+$ cell proliferation, resulting in poor prognosis [8]. Study also showed that the expression of Treg cells can be used as an independent prognostic factor for some cancers, and it plays an important role in the secretion of cytokines in spleen tumor [9]. This study observed Treg cell infiltration in gastric cancer and detected the expression of various cytokines in order to explore the relationship between Treg cells and prognosis of gastric cancer.

Material and methods

Patients

Two hundreds and sixty-five patients with gastric cancer treated in Xinjiang Medical University Affiliated Tumor Hospital between January 2013 and January 2016 were included in this study. The patients were consisted of 174 male and 91 female, with an average age of 65 years old (range from 48 to 82 years old). Exclusion criteria include: (1) patients with malfunctions in heart, lung, liver, kidney and other organs; (2) patients with other endocrine, metabolic, and autoimmune diseases; (3) patients with severe postoperative complications. Inclusion criteria: (1) pathologically confirmed gastric cancer patients; (2) no previous history of radiotherapy, chemotherapy or biological immunotherapy; (3) patents with KARNOFSKY score (KPS) over 60 points; (4) patients without history of autoimmune disease [10, 11]. The surgeries were performed by the same surgical team to guarantee the consistency of surgical quality. The study was approved by Ethical Committee of Xinjiang Medical University Affiliated Tumor Hospital, and the written informed consent was obtained from each participant.

Methods

Tissues from tumor lesions, tissues adjacent to tumor lesions (1 cm), and normal gastric wall tissues were obtained during the surgery to perform immunohistochemical staining for the observation of TNM stage, differentiation degree, and infiltration as well as metastasis.

Regents

Primary antibodies include: mouse anti human S100 monoclonal antibody (labeled by DC cell marker); mouse anti human CD3 monoclonal antibody (labeled by CD3 cell); mouse anti human cd45rot monoclonal antibody (labeled by memory T-lymphocyte); mouse anti human cd8t monoclonal antibody (labeled by cytotoxic T lymphocyte); and mouse anti human CD57 monoclonal antibody (labeled by NK cell) (titers were 1:100, all monoclonal antibodies were purchased from Beijing Zhongshan Biotechnology Co. company); foxp3-fitc (PCH 101), fox P3 staining buffer (purchased from bioscience company) (Foxp3 is a specific human Treg cell antibody); Secondary antibodies were derived from Goat anti Mouse and anti-rabbit; DAB solution (purchased from Beijing Zhongshan Biotechnology Co., Ltd.).

Immunohistochemical staining: Elivision two-step process: the tissues were fixed by 10% neutral formalin, embedded by paraffin, and then sliced with thickness of 4 μm; de-waxed then soaked in 3% H_2O_2; add primary antibody and incubated in 4°C refrigerator overnight before adding secondary antibody; dye with DAB first, and then washed with l% HCl alcohol before secondary dye with hematoxylin.

Observe $Foxp3^+$ Treg cells and CD3, CD4, CD8, NK cells, DCs under Optical microscope.

The concentration of $Foxp3^+$ Treg cells was detected by flow cytometry. The residue blood was washed by PBS and single cell suspension was prepared. 106 cells got Treg cell surface antibody staining, Add fixed rupture fluid and

Table 1. Clinical data of patients

Characteristics		Percentage n (%)
Gender	Male	174 (65.66%)
	Female	91 (34.34%)
Age (years)	≥65	98 (36.98%)
	<65	167 (63.02%)
BMI (kg/m^2)	<18.5	220 (83.03%)
	18.5~25	43 (16.22%)
	>25	2 (0.75%)

incubated at 4°C in the dark environment. Add Foxp3-FITC after washing with PBS, resuspended and then performed flow cytometry. The mean fluorescence intensity (M FI) represents the Foxp3$^+$ Treg concentration.

Flow Cytometry: The cell membrane and cytoplasmic staining appears brown (DC and NK cells were larger, round or irregular in shape as positive cells, while the memory T cell/cytotoxic T cells/CD3 cell was smaller. Selected lymphocyte-intensive cells as positive cells in low magnification randomly, counts the total number of positive cells in 400× magnification to represents the number of immune cells in the gastric tissue microenvironment. Calculate the number of positive cells by two pathologists under double-blind according to the computer system of image acquisition and image analysis software (NIH ImageJ 1.46 bundled with 64-bit Java).

Statistical analysis

Statistical analysis was performed with SPSS 21.0. Measurement data were presented by mean ± standard deviation (x ± s), and examined by T test. Enumeration data were presented by percentage and tested by chi-squaretest. COX univariate regression analysis [13] was performed to analyze the relationship between immune parameters and the survival. Cox multivariate regression analysis was performed to analyze the correlation between activity of immunefunction and the prognosis. P<0.05 was regarded as statistically significant.

Results

Among the 265 patients, there were 174 male and 91 female, the average age of the patients was (65.2 ± 17.2) years old. Prehospital BMI of patients was (18.85 ± 2.83) kg/m^2, and the BMI one year after surgery was (21.77 ± 3.38) kg/m^2. Ninety-two cases were lost during follow-up with a ratio of 34.72%, in which 87 patients died within a year after surgery with a mortality rate of 32.83%. The main causes of death were postoperative tumor recurrence and metastasis. One hundred and twelve patients were readmitted for surgical factors, including 6 cases of postoperative infection, 2 cases of gastric ulcer complicated with infection, and 104 cases of recurrence after surgery. For details, please see **Tables 1** and **2**.

Immunohistochemical staining was performed to observe the positive expression of Foxp3$^+$ Treg cells, CD3, CD4, CD8, NK cells, and DCs in tumor tissues, para-carcinoma tissues and normal gastric tissues. The average numbers of above cells in tumor tissues and para-carcinoma tissues were higher than that of normal gastric tissues (all P<0.05), see **Table 3**.

Regarding on tumor metastasis, patients with high infiltration of positive Foxp3$^+$ Treg cells, CD3, CD4, CD8, NK cells, and DCs were significantly more than those with low infiltration (P<0.05), however, there was no difference between high and low infiltration group regarding on tumor differentiation, infiltrative depth, TNM stage (P>0.05). For details, see **Table 4**.

In the immune microenvironment of gastric cancer, the comparison between different degree of Foxp3$^+$ Treg cells infiltration in tumor metastasis, infiltration depth, TNM stage and survival condition was statistically significant (P<0.05), the high infiltration of Foxp3$^+$ Treg cells suggests the presence of metastasis and deep infiltration, poor TNM staging, may lead to poor survival status. For details, see **Table 5**.

Cox univariate analysis showed that factors including lymph node metastasis, tumor diameter, TNM staging, and infiltration of Foxp3$^+$ Treg cells were closely related with the prognosis of gastric cancer patients; however, age, gender, BMI and histological differentiation degree, CD3/CD4/CD8 cell content and NK cell and DC cell content had no correlation with the prognosis. For details, see **Table 6**.

According to the Cox univariate analysis, lymph node metastasis, tumor diameter, TNM staging and Treg infiltration had a significant relationship with prognosis of the disease. Therefore, the above four factors were taken into Cox mul-

Table 2. Clinicopathologic data

Clinicopathologic data		Percentage n (%)
Histopathologic atypia	High	201 (75.85%)
	Moderate	60 (22.64%)
	Low	4 (1.51%)
Tumor differentiation	Well	6 (2.26%)
	Moderate	62 (23.40%)
	Poorly	197 (74.34%)
Infiltration range	T1~2	63 (23.77%)
	T3~4	202 (76.23%)
TNM stage	TNM 0~II	86 (32.45%)
	TNM III~IV	179 (67.55%)
Transfer condition	Lymph mode metastasis	168 (63.40%)
	Hematogenous metastasis	22 (8.30%)
	Implantation metastasis	15 (5.66%)
	Negative	60 (22.64%)
Recurrence	Positive	104 (39.24%)
	Negative	161 (60.76%)
Survival condition	Survive	178 (67.17%)
	Death	87 (32.83%)
Secondary treatment	Postoperative infection	6 (2.26%)
	Postoperative gastric ulcer Complicated with infection	2 (0.75%)
	Tumor recurrence	104 (39.24%)
Diameter of tumor tissue	≤4 cm	185 (69.81%)
	>4 cm	80 (30.19%)
Missing follow-up	Death	87 (32.83%)
	Others	5 (1.89%)

Table 3. Immune cell infiltration in different site (μ ± S)

Immune cell types	Tumor tissues	Para-carcinoma tissues	Normal gastric tissues
DCs	41.35 ± 6.25*	37.38 ± 5.03#	27.65 ± 2.02
NK cells	88.38 ± 5.68*	84.46 ± 4.88#	63.89 ± 3.58
CD4T-lymphocyte	95.08 ± 5.38*	93.35 ± 5.64#	75.89 ± 6.12
CD8T-lymphocyte	87.28 ± 5.52*	87.92 ± 6.21#	63.31 ± 4.05
CD3 T cells	97.21 ± 3.85*	93.82 ± 4.09#	38.36 ± 4.21
$Foxp3^+$ Treg cells	11.36 ± 0.97*	8.95 ± 4.12#	4.35 ± 0.82

*tumor tissues group, shows obvious different from normal gastric tissues group; #para-carcinoma tissues, shows obvious different from normal gastric tissues group.

tivariate analysis, and the results showed that the four factors could be used as independent factors for the prognosis in tumor tissues. For details, see **Table 7**.

Discussion

Tumor development and immune function are closely related with each other, especially in immune microenvironment of a tumor. Although the immune microenvironment of a tumor cannot directly reflect the immune function of the body, it is useful for analyzing the progression of tumor and prognosis [14].

The mechanism of the aggregation of various types of immune cells

Infiltration of $Foxp3^+$ Treg cells, CD3, CD4, CD8, NK cells, and DCs in tumor tissues and para-carcinoma tissues was higher than that in normal gastric tissues ($P<0.05$), however, the comparison between infiltration status in tumor tissues and para-carcinoma tissues was not statistically significant ($P>0.05$). It's commonly thought to be related to the following 3 mechanisms: (1) Stimulation of tumor associated antigens (TAAs) induced activation and proliferation of various kinds of immune cells; (2) The chemo-

Table 4. Relationship between the immune cell infiltration and clinicopathological status in each group

Clinicopathological features		T cells (CD3/CD4/CD8)			NK cells			DCs		
		High	Low	P	High	Low	P	High	Low	P
Tumor metastasis	Yes	155	50	0.000	151	54	0.000	132	73	0.022
	No	32	28		29	31		25	35	
Tumor differentiation	Well/moderate	31	37	0.363	36	32	0.382	35	33	0.452
	Poor	141	56		128	69		109	88	
Infiltration depth	T1~2	41	22	0.142	37	26	0.445	33	30	0.893
	T3~4	133	69		137	65		142	60	
TNM stage	0~II	48	38	0.163	50	36	0.121	53	33	0.092
	III~IV	134	45		133	46		136	43	
Survival condition	Death	64	23	0.332	63	24	0.188	60	27	0.373
	Survival	125	53		115	63		106	72	

Table 5. Relationship between infiltration level of Foxp3$^+$ Treg cell and clinicalpatholocical features

Clinicopathological features		Low infiltration	High infiltration	χ^2	P
Tumor metastasis	Positive	32	173	-7.73	0.025
	Negative	31	29		
Tumor differentiation	Well/moderate	30	38	1.08	0.385
	Poor	107	90		
Infiltration depth	T1~2	44	19	10.89	0.000
	T3~4	81	121		
TNMstage	0~II	45	41	25.52	0.000
	III~IV	136	43		
Survival condition	Survival	119	59	5.52	0.018
	Death	55	32		

kines secreted by tumor cells induced the aggregation of immune cells in tumor lesion; (3) Secretion and induction of some cytokines [15]. Maybe the Treg cells gathering in tumor tissues is the basis of its immunosuppressive effect, and the suppression function further promotes the accumulation of Treg cells [16].

Correlations between various types of immune cells and clinical pathological features

The immune cell infiltration in tumor tissues was positively correlated to regional lymph node metastasis, and higher infiltration indicated the possibility of lymph node metastasis (P<0.05); however, the immune cell infiltration was not obviously correlated to the degree of tumor differentiation, infiltrative depth, TNM stage as well as survival (P<0.05). The possible mechanism might be that the abnormal expression of the protein in immune microenvironment may contribute to the metastasis of tumor cells; these proteins can be recognized and destroyed by immune cells. The patients with high immune cell infiltrationmay have a more healthy immune systems. Immune cells are easier to invade and damage tumor cells in lymph nodes [17]. But recent years study shows that the protein also contributes to the accumulation of Treg cells [18].

Correlations between Treg cells and clinical pathological features

Treg cells have two functions: immune anergy and immune suppression [19]. Treg cells can inhibit the tumor specific T cell responsesin the tumor microenvironment, thus impeding the anti-tumor reaction. The study showed that infiltration of Foxp3$^+$ Treg cells in tumor tissue was increased significantly compared with those in normal gastric tissue (P<0.05), confirming the significance of Foxp3$^+$ Treg cells in promoting the proliferation of tumorcells. Effective antitumor immunity can inhibit potential invasive ability of tumor cells [20]. In contrast, Treg cells with immunosupression ability may allow invasion, proliferation and degeneration of tumor cells [21], that was confirmed by the significant correlation of high infiltration of Foxp3$^+$ Treg

Table 6. Cox univariate analysis of factors affecting the prognosis of patients with gastric cancer

Clinical data		N (%)	Overall survival (95% CI)	P
Gender	Male	174 (65.66%)	22.8~41.5	0.798
	Female	91 (34.34%)	22.5~41.1	
Age	≥65	98 (36.98%)	25.8~51.5	0.058
	<65	167 (63.02%)	21.6~48.6	
BMI	<18.5/>25	222 (83.78%)	18.8~47.6	0.099
	18.5~25	43 (16.22%)	21.3~52.6	
Tumor differentiation	Well/moderate	68 (25.66%)	23.8~56.4	0.142
	Poor	197 (74.34%)	21.9~53.8	
Lymph mode metastasis	Positive	205 (77.36%)	18.2~33.5	0.012
	Negative	60 (22.64%)	32.2~78.3	
Tumor diameter	≤4 cm	185 (69.81%)	24.3~55.6	0.007
	>4 cm	80 (30.19%)	15.9~34.2	
TNM stage	TNM 0~II	86 (32.45%)	43.5~62.8	0.001
	TNM III~IV	179 (67.55%)	17.3~35.2	
CD3	High	147 (55.47%)	25.5~61.8	0.135
	Low	118 (44.53%)	27.3~63.3	
CD4	High	162 (61.13%)	24.7~58.2	0.107
	Low	103 (38.87%)	22.9~56.6	
CD8	High	176 (66.42%)	30.5~61.3	0.066
	Low	89 (33.58%)	27.9~56.2	
NK	High	152 (57.36%)	28.3~56.4	0.078
	Low	113 (42.64%)	25.1~52.3	
DC	High	164 (61.89%)	27.7~54.8	0.089
	Low	101 (38.11%)	24.3~51.9	
$Foxp3^+$ Treg	High	189 (71.32%)	28.2~71.5	0.000
	Low	76 (28.68%)	14.8~56.3	

Table 7. Cox Multivariate survival analysis of gastric cancer patients

Affective factors		HR	Survival (95% CI)	P
Center of tumor tissue	Lymph mode metastasis (P/N)	3.42	0.15~0.43	0.012
	Tumor diameter (≤4/>4 cm)	2.51	2.85~8.83	0.000
	TNM stage (T 0~II/T III~IV)	0.48	3.02~9.97	0.000
	$Foxp3^+$ Treg infiltration (high/low)	2.89	2.27~7.98	0.001
Peritumoral 1 cm	Lymph mode metastasis (P/N)	0.35	0.83~1.35	0.089
	Tumor diameter (≤4/>4 cm)	0.012	0.78~1.42	0.093
	TNM stage (T 0~II/T III~IV)	0.003	1.12~1.43	0.133
	$Foxp3^+$ Treg infiltration (high/low)	0.025	1.08~1.56	0.215
Normal gastric tissues	Lymph mode metastasis (P/N)	0.000	1.03~1.11	0.562
	Tumor diameter (≤4/>4 cm)	0.000	0.96~1.07	0.628
	TNM stage (T 0~II/T III~IV)	0.000	0.98~1.05	0.891
	$Foxp3^+$ Treg infiltration (high/low)	0.006	1.12~1.25	0.245

cells with metastasis, deep infiltration, poor TNM stage and poor survival.

COX regressionanalysis on the factors affecting the prognosis of gastric cancer

The prognosis of gastric cancer is influenced by many factors; Cox univariate analysis showed that lymph node metastasis, tumor diameter, TNM stage and $Foxp3^+$ Treg invasion were the factors related to the prognosis of the disease. It is reported that $Foxp3^+$ Treg cells in tumor tissue can be used to predict the prognosis of tumor such as colorectal cancer and ovarian cancer [22]. Shen *et al.* also found that $Foxp3^+$ Treg infiltration in hepatocellular carcinoma was significantly correlated with the prognosis of cancer. The possible mechanisms might be: 1. direct or indirect inhibiting T cell proliferation and activation through a variety of ways: 2. inhibiting the killing effect of $CD8^+$ T cells and NK cells on tumor cells; 3. competitively inhibiting IL-2 to influence the immune response of T cells to tumor cells [23].

In conclusion, lymph node metastasis, tumor diameter, TNM staging and other independent factors can be used as reference for clinical treatment of diseases. The prognosis of patients with gastric cancer can bejudged by detecting $Foxp3^+$ Treg cells. In

addition, target inhibiting tumor inflammatory factors and chemokines to reduce the infiltration of $Foxp3^+$ Treg cells may be effect to achieve tumor growth inhibition.

Disclosure of conflict of interest

None.

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References

[1] ICH Harmonised Tripartite Guideline. Statistical principles for clinical trials. International Conference on Harmonisation E9 Expert Working Group. Stat Med 1999; 18: 1905-1942.

[2] Jan M, Chao MP, Cha AC, Alizadeh AA, Gentles AJ, Weissman IL and Majeti R. Prospective separation of normal and leukemic stem cells based on differential expression of TIM3, a human acute myeloid leukemia stem cell marker. Proc Natl Acad Sci U S A 2011; 108: 5009-5014.

[3] Huang X, Bai X, Cao Y, Wu J, Huang M, Tang D, Tao S, Zhu T, Liu Y, Yang Y, Zhou X, Zhao Y, Wu M, Wei J, Wang D, Xu G, Wang S, Ma D and Zhou J. Lymphoma endothelium preferentially expresses Tim-3 and facilitates the progression of lymphoma by mediating immune evasion. J Exp Med 2010; 207: 505-520.

[4] Sato S, Ouellet M, St-Pierre C and Tremblay MJ. Glycans, galectins, and HIV-1 infection. Ann N Y Acad Sci 2012; 1253: 133-148.

[5] Wu FH, Yuan Y, Li D, Lei Z, Song CW, Liu YY, Li B, Huang B, Feng ZH and Zhang GM. Endothelial cell-expressed Tim-3 facilitates metastasis of melanoma cells by activating the NF-kappaB pathway. Oncol Rep 2010; 24: 693-699.

[6] DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim YL, Lee HH, Karisola P, Pichavant M, Kaplan GG, Umetsu DT, Freeman GJ and Casasnovas JM. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. J Immunol 2010; 184: 1918-1930.

[7] Chiba S, Baghdadi M, Akiba H, Yoshiyama H, Kinoshita I, Dosaka-Akita H, Fujioka Y, Ohba Y, Gorman JV, Colgan JD, Hirashima M, Uede T, Takaoka A, Yagita H and Jinushi M. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol 2012; 13: 832-842.

[8] Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, Kirkwood JM, Kuchroo V and Zarour HM. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J Exp Med 2010; 207: 2175-2186.

[9] Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK and Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J Exp Med 2010; 207: 2187-2194.

[10] Wherry EJ. T cell exhaustion. Nat Immunol 2011; 12: 492-499.

[11] Yang ZZ, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, Witzig TE and Ansell SM. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. J Clin Invest 2012; 122: 1271-1282.

[12] Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, Murphy WJ, Azuma M, Anderson AC, Kuchroo VK and Blazar BR. Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. Blood 2011; 117: 4501-4510.

[13] Gao X, Zhu Y, Li G, Huang H, Zhang G, Wang F, Sun J, Yang Q, Zhang X and Lu B. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. PLoS One 2012; 7: e30676.

[14] Zhao JZ, Zhang RP, Yu JP, Li H, Wang G, Wang XJ, Xue Q, Li FX, Ren XB. Relationship between Immunocytes in the Tumor Microenvironment and Prognosis of Gastric Carcinoma. Chinese Journal of Clinical Oncology 2010; 37: 1290-1292.

[15] Zhang Y, Ma CJ, Wang JM, Ji XJ, Wu XY, Moorman JP and Yao ZQ. Tim-3 regulates pro- and anti-inflammatory cytokine expression in human CD14+ monocytes. J Leukoc Biol 2012; 91: 189-196.

[16] Brown KE, Freeman GJ, Wherry EJ and Sharpe AH. Role of PD-1 in regulating acute infections. Curr Opin Immunol 2010; 22: 397-401.

[17] Gleason MK, Lenvik TR, McCullar V, Felices M, O'Brien MS, Cooley SA, Verneris MR, Cichocki F, Holman CJ, Panoskaltsis-Mortari A, Niki T, Hirashima M, Blazar BR and Miller JS. Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. Blood 2012; 119: 3064-3072.

[18] Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, Schoeffler EC, Fujita T, Nixon DF and Lanier LL. Tim-3 marks human

natural killer cell maturation and suppresses cell-mediated cytotoxicity. Blood 2012; 119: 3734-3743.

[19] da Silva IP, Jimenez-Baranda S, Gallois A, Kuchroo V, Osman I and Bhardwaj N. Tim-3 expression and function in natural killer cells from advanced melanoma patients. Journal of Clinical Oncology 2012: 8571.

[20] Zhuang X, Zhang X, Xia X, Zhang C, Liang X, Gao L and Ma C. Ectopic expression of TIM-3 in lung cancers: a potential independent prognostic factor for patients with NSCLC. Am J Clin Pathol 2012; 137: 978-985.

[21] Li C, Yu X, Zhu Y, Wu X, Ma X, Liu H, Ye L, Ma C, Xia R, Sun A, Ruan C, Chen S, Depei W. TIM-3 Is Highly Expressed On Blast Cells In Patients With Acute Myeloid Leukemia. Blood 2013; 21: 4931-4931.

[22] Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H and Fujii H. Increased populations of regulatory T cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. Clin Cancer Res 2003; 9: 4404-4408.

[23] Shen LS, Wang J, Shen DF, Yuan XL, Dong P, Li MX, Xue J, Zhang FM, Ge HL and Xu D. CD4(+) CD25(+) CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. Clin Immunol 2009; 131: 109-118.