The Korean Journal of Pathology 2010; 44: 9-15
DOI: 10.4132/KoreanJPathol.2010.44.1.9

The Prognostic Significance of the Tumor-Infiltrating FoxP3-Positive Regulatory T Cells in Gastric Carcinoma

Sang Jae Noh · Shin Young Park
Kyung Ryoul Kim[1]
Chan Young Kim[2]
Keun Sang Kwon[3]
Ho Sung Park · Ho Lee[4]
Myoung Ja Chung · Woo Sung Moon
Kyu Yun Jang

Department of Pathology, Research Institute of Clinical Medicine and Institute for Medical Sciences, Chonbuk National University Medical School, Jeonju; [1]Department of Forensic Medicine, National Institute of Scientific Investigations, Seoul; Departments of [2]Surgery, [3]Preventive Medicine, and [4]Forensic Medicine, Research Institute of Clinical Medicine and Institute for Medical Sciences, Chonbuk National University Medical School, Jeonju, Korea

Received : April 7, 2009
Accepted : August 31, 2009

Corresponding Author
Kyu Yun Jang, M.D.
Department of Pathology, Chonbuk National University Medical School, San 2-20 Geumam-dong, Deokjin-gu, Jeonju 561-180, Korea
Tel: 063-270-3071
Fax: 063-270-3135
E-mail: kyjang@chonbuk.ac.kr

*This study was supported by a grant of the Ministry of Science & Technology/Korea Science & engineering Foundation through the Diabetes Research Center of Chonbuk National University (R13-2008-005-0000-0).

Background : Regulatory T cells (Tregs) are known to be key regulators of immune responses in patients with autoimmune disease and infection and also for attenuating antitumor immunity by the host. It has been reported that high numbers of tumor-infiltrating Tregs might be associated with poor clinical outcomes for several malignant tumors. Therefore, this study aimed to examine the impact of tumor-infiltrating Tregs on the prognosis of gastric carcinoma patients. **Methods :** The immunohistochemical staining for anti-fork head Box P3 (FoxP3) antibody was performed by using a 3 mm core from the tumor specimens of each of the 173 gastric cancer patients for constructing a tissue microarray. FoxP3-positive Tregs were quantified by calculating the numbers of positive cells per 5 high-power fields on light microscopy. Thereafter, the 173 patients were subdivided into the low Tregs group ($\leq$ 3/5 high power fields [HPF], n = 41) and the high Tregs group (> 3/5 HPF, n = 132). **Results :** The high Tregs group was significantly associated with a higher stage, more invasion depth and lymph node metastasis (p = 0.009, p = 0.036, p = 0.006, respectively). The high Tregs group showed significantly poorer overall survival and event-free survival (p = 0.004, p = 0.017, respectively) on the univariate analysis. The Tregs group and the tumor, node and metastasis stage were also independent prognostic factors that were significantly associated with overall survival (p = 0.025, p < 0.001, respectively) by multivariate analysis. **Conclusions :** Our results indicated that a high number of tumor-infiltrating FoxP3-positive Tregs could be an indicator of poor long term survival for gastric carcinoma patients.

Key Words : T-lymphocytes, regulatory; FoxP3 protein, human; Stomach neoplasms

Regulatory T cells (Tregs) are important T cells for preserving peripheral tolerance and they play a central role in several disease such as autoimmune disease, graft versus host disease and infectious diseases. The best defined Tregs CD4+ cells that constitutively express CD25.[1] For identifying CD4+CD25+ T cell, several markers such as CD45RB, CD38 and CD62L have been introduced in murine models.[2,3] Fork head Box P3 (FoxP3) has recently been identified as a specific marker of CD4+CD25+ T cell. FoxP3 is consistently expressed in naturally occurring CD4+CD25+ Tregs populations and it is the master gene for development and differentiation of Tregs in the thymus and the periphery.[4]

The tumor microenvironment is composed of tumor cells, immune cells and stromal cells, and this is an important factor in tumor growth, progression and regression. Tumor cells grow and propagate as a result of escaping attack of immune cells. Tregs show a high prevalence in malignant tumor patients and they

attenuate antitumor immunity.[5] Especially, FoxP3-positive Tregs have been focused on because of their roles in the development and progression of tumors. Recent studies using FoxP3 as a marker of Tregs have also shown that tumor-infiltrating Tregs are associated with a worse prognosis for human malignant tumors such as breast,[6] lung,[7] ovary,[8] and liver tumors.[9]

Gastric carcinoma is one of the most common types of cancer. Although the incidence and mortality of gastric carcinoma have fallen, it remains the second most common cause of cancer deaths worldwide.[10] In Asian countries, including Korea, Japan, and China, gastric carcinoma causes major public health problems. However, the role of Tregs as a prognostic factor for gastric carcinoma is not understood. Therefore, we investigated the tumor-infiltrating FoxP3-positive Tregs in gastric carcinoma and their correlations with the clinicopathologic prognostic factors and gastric cancer patients' long-term survival.

MATERIALS AND METHODS

Patients and tissue samples

The patients who underwent radical gastrectomy for gastric carcinoma at Chonbuk National University Hospital between January 1997 and December 2005 were included in the present study. To minimize the confounding effects, a matching process was conducted. All of the patients who were in stage IV (n = 50) were included in this analysis. Thereafter, the patients who were in stage I, II or III were matched accoding to the following variables; gender, age (± 2 years), and the calendar year of the surgical operation (± 2 years). Among the 200 selected cases of gastric cancer, the paraffin-embedded tissue blocks of 27 cases were unavailable. Therefore, 173 cases of gastric cancer were finally included in the present study. All of these cases were reviewed and reclassified according to the World Health Organization classification criteria.[11] The pathologic staging was reviewed based on the tumor, node and metastasis (TNM) staging system of the American Joint Committee on Cancer. This study received the approval of the local ethics committee from Chonbuk National University Hospital's institutional review board. Informed consent was provided according to the Declaration of Helsinki. The patients were grouped according to their age, gender, the preoperative serum carcinoembryonic antigen level, the serum carbohydrate antigen (CA) 19-9 level, the serum CA72-4 level, the stage (I and II vs III and IV), the depth of tumor invasion, the presence of lymph node metastasis, the presence of distant metastasis, the histologic types and differentiation, and Lauren's classification

Immunohistochemical staining for the tissue microarray

The hematoxylin and eosin-stained sections from each paraffin-embedded, formalin-fixed block were utilized to define the diagnostic areas which were principally composed of tumor cells and stromal cells, and one 3.0 mm core was obtained from each case. These cores were inserted in a grid pattern into a recipient block by using a tissue arrayer. The 4 μm-sections were then cut from each tissue microarray and immunohistochemical staining for FoxP3 was performed by using the DAKO Envision system (DAKO, Carpinteria, CA, USA), which uses dextran polymers conjugated with horseradish peroxidase, to avoid any endogenous biotin contamination. Briefly, after deparaffinization, the tissue sections underwent a microwave antigen retrieval procedure in sodium citrate buffer (pH 9.0) for 10 minutes. After blocking the endogenous peroxidase, the sections were incubated with Protein Block Serum-Free (DAKO) at room temperature for 10 minutes to block the nonspecific staining, and then the sections were incubated for 12 hours at 4℃ with monoclonal antibodies against FoxP3 (1 : 50, clone 236A/E7, eBioscience, SanDiego, CA, USA). The peroxidase activity was detected with the enzyme substrate 3-amino-9-ethyl carbazole. For positive controls, normal tonsillar tissue sections were treated in the same manner. For negative controls, the sections were treated in the same manner except they were incubated with Tris-buffered saline without the primary antibody.

Quantitative assessment of the numbers of FoxP3-positive Tregs

For the quantification of the tumor infiltrating FoxP3-positive Tregs, the total numbers of FoxP3-positive cells in the intratumoral stroma of 5 high-power fields (HPF) were counted by two pathologists with consensus in all cases. Considering the limited area of the tumor component in some cases, and especially in the cases of early gastric carcinoma, the 5 HPFs were thoroughly examined.

The counting of FoxP3-positive Tregs was conducted without the pathologists having any clinical information about the cases. Slide examinations were performed using a Nikon ECLIPSE 80i light microscope (Nikon, Tokyo, Japan) with 40 × objective lens (Plan Fluor 40 × / 0.75, Nikon). The field area of one HPF was 0.307 mm^2. Therefore, the total area that was analyzed per

case was 1.535 mm^2.

Statistical analysis

The cut-off number of FoxP3-positive Tregs was assessed by the area under the curve (AUC) of the receiver operating characteristic (ROC) curve for overall survival, and this was determined by the highest positive likelihood ratio (sensitivity/[1-specificity]). The cut-off number for the ideal test is presented as the AUC 1.000 with sensitivity of 1 and specificity of 1. If the area under the ROC curve of a test is greater than 0.5, then the cut-off number has a positive value and it can be calculated. Then, using this cut-off of number of Tregs, the patients were separated into the low and high Tregs subgroups for further analysis. The comparisons between the numbers of tumor-infiltrating FoxP3-positive Tregs and the clinical factors were tested by t-tests and chi-square tests. The end points of interest were overall survival (OS) and event-free survival (EFS). The end point of follow-up was the date of the last contact or the date of death through November 2007. The patients who were alive at last contact were treated as censored for OS analysis. The EFS was calculated from the time of diagnosis to the date of recurrence, death or the last contact. The patients who were alive at the last contact and who had not recurred were treated as censored data for the EFS analysis. Univariate and multivariate Cox proportional hazards regression analyses were performed to estimate the impact of the numbers of tumor infiltrating FoxP3-positive Treg and the clinicopathologic factors on OS and EFS. The Kaplan-Meier survival curves were constructed to further illustrate the impact on OS and EFS, when indicated. p-values less than

Table 1. Tumor-infiltrating FoxP3-positive regulatory T cell numbers and relations with patient characteristics

Characteristics		No. of patients	Tregs group		p-value
			Low	High	
Age (yr)	< 60	52	14	38	0.513
	≥ 60	121	27	94	
Gender	Male	134	36	98	0.069
	Female	39	5	34	
TNM stage	Stage I & II	79	26	53	0.009
	Stage III & IV	94	15	79	
T stage	T1	37	15	22	0.036
	T2	37	9	28	
	T3	75	14	61	
	T4	24	3	21	
N stage	N0	60	23	37	0.006
	N1	57	9	48	
	N2	25	6	19	
	N3	31	3	28	
M stage	M0	169	41	128	0.259
	M1	4	0	4	
CEA	Normal	111	23	88	0.868
	Elevated	31	6	25	
CA19-9	Normal	125	26	99	0.849
	Elevated	16	3	13	
CA72-4	Normal	125	26	99	0.524
	Elevated	16	3	13	
Lauren's classification	Diffuse type	73	19	54	0.827
	Intestinal type	77	17	60	
	Mixed type	23	5	18	
Growth pattern	Infiltrative type	90	16	74	0.184
	Expanding type	17	6	11	
	Mixed type	28	4	24	

FoxP3, fork head Box P3; Tregs, regulatory T cells; TNM, tumor, node and metastasis; CEA, carcinoembryonic antigen; CA, carbohydrate antigen.

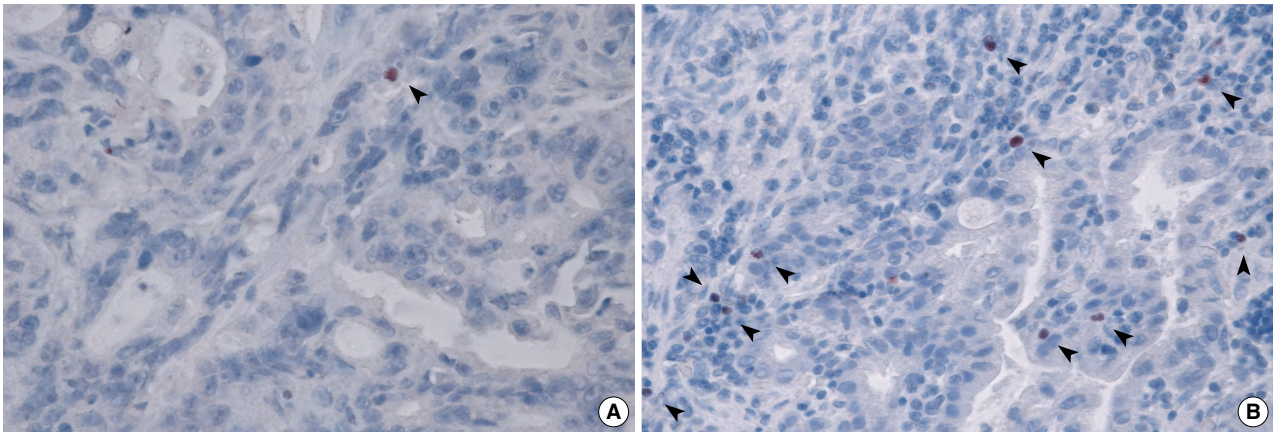

Fig. 1. Immunohistochemical staining for fork head Box P3 (FoxP3). FoxP3-positive regulatory T cells (Tregs) are indicated by arrowheads. (A) A case of FoxP3-positive Tregs less than three per 5 high-power fields. (B) A case of FoxP3-positive Tregs more than three per 5 high-power fields.

0.05 were considered to indicate statistical significance. The SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

The association of tumor infiltrating FoxP3-positive Tregs with the clinicopathologic characteristics of the gastric carcinoma patients

The expression of FoxP3-positive Tregs in the gastric cancer tissues, as revealed by immunohistochemical staining, is shown in Fig. 1. The average number of tumor-infiltrating FoxP3-positive Tregs per 5 HPFs was 15.91 (range, 0 to 181).

In this study, the cut-off number of Tregs was determined by using the AUC of the ROC curve that yielded the maximal sensitivity plus specificity for overall survival. The AUC was 0.525, and the cut-off level was determined to be 3 Tregs per 5 HPFs. According to the cut-off number of Tregs, the 173 gastric cancer patients were classified into two groups; the low Tregs group (≤ 3/5 HPF, n = 41), and the high Tregs group (> 3/5 HPF, n = 132).

For the 173 gastric cancer patients, there were significant correlations between the high Tregs group and a higher TNM stage (p = 0.009), a greater invasion depth (p = 0.036), and the presence of lymph node metastasis (p = 0.006). There was no significant correlation between the Tregs groups and the tumor differentiation, Lauren's classification, the tumor growth pattern, the presence of distant metastasis and the pre-operative serum tumor markers. The clinicopathologic features of the gastric carcinoma patients and their relations between the Tregs group are shown in Table 1.

Table 2. Clinicopathologic factors and their effect on event-free survival and overall survival by univariate Cox proportional hazards regression analysis

Characteristics		No. of patients	OS			EFS		
			HR	95% CI	p-value	HR	95% CI	p-value
CEA	Normal	111	1.000			1.000		
	Elevated	31	1.924	1.050-3.528	0.034	2.056	1.177-3.592	0.011
CA19-9	Normal	125	1.000			1.000		
	Elevated	16	2.636	1.276-5.447	0.009	2.693	1.390-5.217	0.003
TNM stage	I & II	79	1.000			1.000		
	III & IV	94	7.607	3.735-15.495	< 0.001	7.954	4.056-15.596	< 0.001
T stage	T1	37	1.000		< 0.001	1.000		< 0.001
	T2	37	2.765	0.865-8.833	0.086	4.419	1.635-12.609	0.012
	T3	75	4.595	1.627-12.975	0.004	5.277	1.877-14.836	0.002
	T4	24	11.109	3.704-33.314	< 0.001	12.236	4.109-36.434	< 0.001
N stage	N0	60	1.000		< 0.001	1.000		< 0.001
	N1	57	4.558	1.710-12.152	0.002	5.189	1.963-13.714	0.001
	N2	25	11.567	4.151-32.227	< 0.001	15.314	5.614-41.773	< 0.001
	N3	31	18.540	7.044-48.795	< 0.001	21.749	8.325-56.824	< 0.001
Tregs group	Low	41	1.000			1.000		
	High	132	2.664	1.267-5.600	0.004	2.066	1.086-3.931	0.017

OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; CEA, carcinoembryonic antigen; CA, carbohydrate antigen; TNM, tumor, node and metastasis; Tregs, regulatory T cells.

Table 3. Multivariate Cox proportional hazards regression analysis for overall survival and event-free survival

Characteristics	No. of patients	OS			EFS		
		HR	95% CI	p-value	HR	95% CI	p-value
Tregs group	Low	1.000			1.000		
	High	3.830	1.181-12.415	0.025	1.841	0.784-4.325	0.161
TNM stage	I & II	1.000			1.000		
	III & IV	6.137	2.736-13.766	< 0.001	6.796	3.183-14.512	< 0.001

Variables considered in the analysis were the preoperative serum level of CEA and CA19-9, TNM stage and Tregs group.
OS, overall survival; EFS, event-free survival; HR, hazard ratio; CI, confidence interval; Tregs, regulatory T cells; TNM, tumor, node and metastasis.

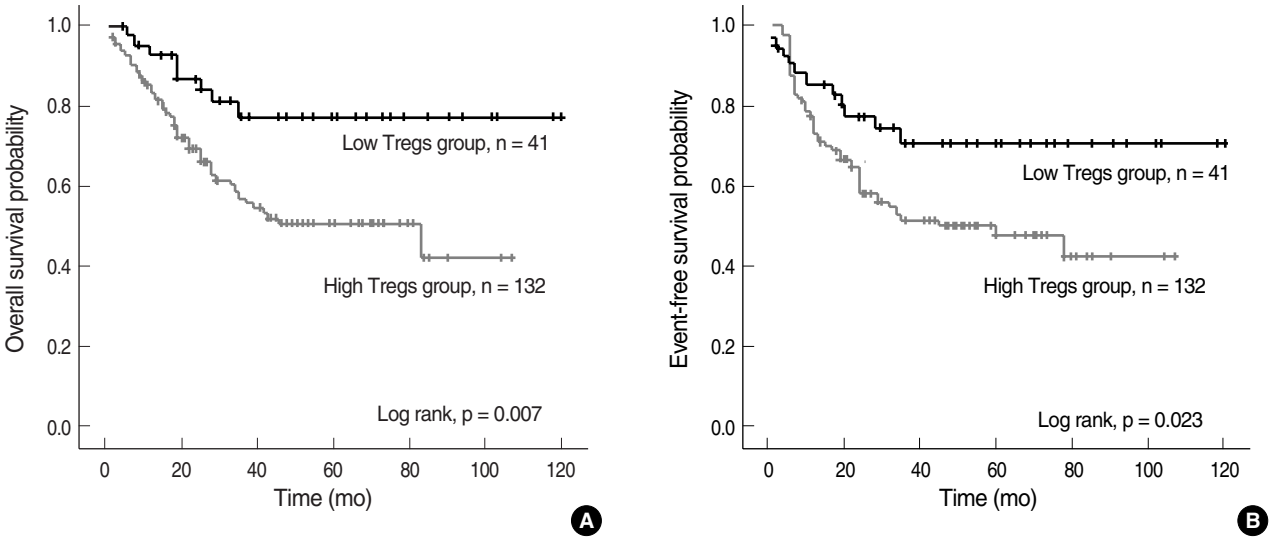

Fig. 2. Kaplan-Meier survival analysis for overall survival and event-free survival. (A) High regulatory T cells (Tregs) group showed poor overall survival in comparison to low Tregs group. (B) High Tregs group showed poor event-free survival in comparison to low Tregs group.

The prognostic significance of infiltrating FoxP3-positive Tregs in the gastric carcinoma patients

The mean follow-up duration of the 173 gastric cancer patients was 36.79 months (range, 1 to 120 months). The patients in the high Tregs group also had significantly poorer OS ($p = 0.004$) and EFS ($p = 0.017$) than the patients in the low Tregs group, according to the univariate analysis (Table 2). By multivariate analysis, the Tregs group ($p = 0.025$) and the TNM stage ($p < 0.001$) were independent predictors of OS (Table 3). However, on the multivariate analysis for EFS, the TNM stage was the only significant independent prognostic marker. The OS and EFS according to the Tregs groups are plotted in Fig. 2.

DISCUSSION

This study demonstrated that having more than 3 tumor-infiltrating FoxP3-positive Tregs per 5 HPFs (1.535 mm^2) was significantly associated with advanced cancer characteristics, such as advanced tumor invasion, lymph node metastasis and a high TNM stage. Moreover, the patients in the high Tregs group showed a significant shorter OS and EFS as compared with that of the low Tregs group patients. These results indicate that Tregs may play an important role in tumor progression and they may be a clinically significant prognostic indicator of gastric carcinoma. The cut-off number of 3 Tregs per 5 HPFs that we found is comparable with that (6 Tregs per 10 HPFs) reported by Perrone *et al.*[12] In agreement with our findings, Perrone *et al.*[12] have shown that a high number of intratumoral FoxP3-positive Tregs were associated with an adverse prognosis in 110 gastric cancer patients. Although FoxP3 was not used as a marker of Tregs, there are some reports that Tregs are associated with progression of gastric carcinoma.[13,14] Increased numbers of CD4+CD25+ Tregs in the peripheral blood of gastric cancer patients were associated with a high tumor stage and poor survival,[13] and the populations of CD4+CD25+ T cells in the tumor-infiltrating lymphocytes of gastric cancer patients with advanced disease were significantly higher than that of the tumor-infiltrating lymphocytes in the patients with early stage disease.[14] Furthermore, increasing evidences has indicated that tumor-infiltrating Tregs play a major role in tumor progression and they have an influence on patients' survival; the importance of Tregs in tumor immunology has been emphasized in various human malignant tumors in addition to gastric carcinoma. The number of Tregs is a predictor of recurrence in patients with stage I non-small cell lung cancer.[7] FoxP3-positive Tregs have been reported to be a novel marker for identifying late-relapse patients with breast cancer.[6] Sasada *et al.*[15] have reported that patients with recurrent gastric carcinoma show a significantly higher relative prevalence of the CD25+ T cell subset in the peripheral blood than that of patients with primary gastric carcinoma. On the other hand, some conflicting results also have been reported. According to the report of Mizukami *et al.*,[16] the number of tumor-infiltrating Tregs was not associated with the survival of gastric carcinoma patients. In terms of the localization patterns of Tregs,

patients with diffuse localization of Tregs had a poorer survival than did the patients with a peritumoral localization of Tregs in the same study.

In the tumor microenvironment, Tregs are recruited by tumor-derived factors such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and interleukin (IL)-10.[17] A subset of dendritic cells that was exposed to tumor cells acquired the capacity to secrete TGF-β and to promote the proliferation of naturally occurring Tregs *in vivo*.[18] It has been suggested that Tregs' suppressive functions occurs by four basic pathways: inhibitory cytokines, cytolysis, metabolic disruption, and modulation of dendritic cell maturation or fuction.[19] Tregs suppress the proliferation and differentiation of the naïve T cells and they also suppress the activity of differentiated CD4+ and CD8+ T cells, natural killer cells and dendritic cells.[20] In addition, Tregs suppress B cells by inhibiting antibody production and proliferation.[21] For the results of the present study, tumor-infiltrating Tregs were associated with a high stage and advanced invasiveness of gastric carcinoma. Therefore, our study also supports the suggestion that Tregs might be involved in the suppression of anti-tumor immunity.

In this study, tumor-infiltrating FoxP3-positive Tregs were associated with lymph node metastasis. In addition, all of the four cases with distant metastasis were included in the high Tregs group and no cases of the low Tregs group showed distant metastasis. In the tumor microenvironment, the tumor cells and the induced Tregs release excessive TGF-β. The released TGF-β induces the expression of VEGF[22] and the cancer cells also produce VEGF.[17] In addition, the overexpression of VEGF by the tumor increases the presence of regulatory T cells.[23] VEGF participates in not only angiogenesis, but also lymphangiogenesis,[24] and a previous study has shown positive relationships between the FoxP3 expression, TGF-β, VEGF and angiogenesis.[25] It also has been reported that the number of Tregs are increased in the draining lymph nodes of gastric cancer patients.[26] Moreover, in human metastatic melanoma lymph nodes, FoxP3 expressing CD4+CD25+ T cells are overexpressed and they inhibit the CD4+CD25- T cells as well as the CD8+ T cells.[27] These findings suggested that the recruited Tregs not only inhibit the immune response to tumor antigens, but they may also induce angio- and lymphangiogenesis at the primary and metastatic sites, and then they promote tumor growth and spread into distant sites such as lymph nodes or other organs.

The therapeutic strategies for cancer by inducing the depletion of Tregs have been evaluated in several animal models and clinical trials. Administration of anti-CD25 monoclonal antibody facilitated anti-tumor immunity and it induced tumor regression *in vivo*.[28] Denileukin diftitox, which is a recombinant IL-2 diphteria toxin conjugate, selectively eliminates CD25-expressing Tregs from the peripheral blood of cancer patients.[29] The administration of low dose cyclophosphamide induces decreased numbers of Tregs and it inhibits the suppressive capability of Tregs.[30] Considering these findings that high numbers of Tregs are associated with advanced cancer characteristics and a poor prognosis, the use of new therapeutic strategies for modulating Tregs may be helpful for improving the prognosis of gastric carcinoma patients.

In conclusion, we showed that tumor-infiltrating FoxP3-positive Tregs were associated with the tumor stage, the depth of tumor invasion, lymph node metastasis and patient survival, and these findings support that Tregs may play a key role in the tumor progression of patients with gastric carcinoma. Furthermore, these findings may be helpful for establishing novel immunotherapy for gastric carcinoma.

REFERENCES

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995; 155: 1151-64.
2. Lepault F, Gagnerault MC. Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in nonobese diabetic mice. J Immunol 2000; 164: 240-7.
3. Read S, Mauze S, Asseman C, Bean A, Coffman R, Powrie F. CD38+ CD45RB (low) CD4+ T cells: a population of T cells with immune regulatory activities in vitro. Eur J Immunol 1998; 28: 3435-47.
4. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-61.
5. Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. Clin Cancer Res 2003; 9: 606-12.
6. Bates GJ, Fox SB, Han C, *et al.* Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006; 24: 5373-80.
7. Petersen RP, Campa MJ, Sperlazza J, *et al.* Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. Cancer 2006; 107: 2866-72.
8. Wolf D, Wolf AM, Rumpold H, *et al.* The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is asso-

ciated with poor prognosis in ovarian cancer. Clin Cancer Res 2005; 11: 8326-31.
9. Sasaki A, Tanaka F, Mimori K, *et al.* Prognostic value of tumor-infiltrating FOXP3+ regulatory T cells in patients with hepatocellular carcinoma. Eur J Surg Oncol 2008; 34: 173-9.
10. Crew KD, Neugut AI. Epidemiology of gastric cancer. World J Gastroenterol 2006; 12: 354-62.
11. Hamilton SR, Aaltonen LA. World Health Organization Classification of Tumours: pathology and genetics of tumours of the digestive system. Lyon: IARC Press, 2000.
12. Perrone G, Ruffini PA, Catalano V, *et al.* Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer. Eur J Cancer 2008; 44: 1875-82.
13. Kono K, Kawaida H, Takahashi A, *et al.* CD4(+)CD25high regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers. Cancer Immunol Immunother 2006; 55: 1064-71.
14. Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H, 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: 4404-8.
15. Sasada T, Kimura M, Yoshida Y, Kanai M, Takabayashi A. CD4+CD25+ regulatory T cells in patients with gastrointestinal malignancies: possible involvement of regulatory T cells in disease progression. Cancer 2003; 98: 1089-99.
16. Mizukami Y, Kono K, Kawaguchi Y, *et al.* Localisation pattern of Foxp3+ regulatory T cells is associated with clinical behaviour in gastric cancer. Br J Cancer 2008; 98: 148-53.
17. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005; 5: 263-74.
18. Ghiringhelli F, Puig PE, Roux S, *et al.* Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation. J Exp Med 2005; 202: 919-29.
19. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008; 133: 775-87.
20. Onishi Y, Fehervari Z, Yamaguchi T, Sakaguchi S. Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc Natl Acad Sci U S A 2008; 105: 10113-8.
21. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. Activated CD-4+CD25+ T cells selectively kill B lymphocytes. Blood 2006; 107: 3925-32.
22. Donovan D, Harmey JH, Toomey D, Osborne DH, Redmond HP, Bouchier-Hayes DJ. TGF beta-1 regulation of VEGF production by breast cancer cells. Ann Surg Oncol 1997; 4: 621-7.
23. Li B, Lalani AS, Harding TC, *et al.* Vascular endothelial growth factor blockade reduces intratumoral regulatory T cells and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. Clin Cancer Res 2006; 12: 6808-16.
24. Zhang B, Zhao WH, Zhou WY, Yu WS, Yu JM, Li S. Expression of vascular endothelial growth factors-C and -D correlate with evidence of lymphangiogenesis and angiogenesis in pancreatic adenocarcinoma. Cancer Detect Prev 2007; 31: 436-42.
25. Gupta S, Joshi K, Wig JD, Arora SK. Intratumoral FOXP3 expression in infiltrating breast carcinoma: its association with clinicopathologic parameters and angiogenesis. Acta Oncol 2007; 46: 792-7.
26. Kawaida H, Kono K, Takahashi A, *et al.* Distribution of CD4+CD25 high regulatory T-cells in tumor-draining lymph nodes in patients with gastric cancer. J Surg Res 2005; 124: 151-7.
27. Viguier M, Lemaître F, Verola O, *et al.* Foxp3 expressing CD4+CD25 (high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. J Immunol 2004; 173: 1444-53.
28. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res 1999; 59: 3128-33.
29. Dannull J, Su Z, Rizzieri D, *et al.* Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. J Clin Invest 2005; 115: 3623-33.
30. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005; 105: 2862-8.