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ORIGINAL ARTICLE

Intratumoral $CD8^+$ T/$FOXP3^+$ cell ratio is a predictive marker for survival in patients with colorectal cancer

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Abstract The human immune system consists of a balance between immune surveillance against non-self antigens and tolerance of self-antigens. $CD8^+$ T cells and $CD4^+$ regulatory T cells (Tregs) are the main players for immune surveillance and tolerance, respectively. We examined immunohistochemically the immunological balance at the tumor site using 94 surgically resected colorectal cancer tissues. Forkhead box P3 $(FOXP3)^+$ cells were considered to be Tregs in the present study. The number of intratumoral $FOXP3^+$ cells ($itFOXP3^+$ cells) was positively correlated with lymph node metastases ($P = 0.030$). $itCD8^+$ T/$itFOXP3^+$ cell ratio negatively correlated with pathological stages ($P = 0.048$). Next, relationship between the number of $itCD8^+$ T cells or $itFOXP3^+$ cells and survival prognosis in 94 patients who underwent a curative resection was analyzed. Only $itCD8^+$ T/$itFOXP3^+$ cell ratio positively correlated with disease-free survival (0.023) and overall survival ($P = 0.010$). Multivariate analysis indicated that $itCD8^+$ T/$itFOXP3^+$ cell ratio is an independent prognostic factor ($P = 0.035$) of overall survival. The number of $itFOXP3^+$ cells positively correlated with transforming growth factor-beta TGF-β production at the tumor site ($P = 0.020$). In conclusion, $itCD8^+$ T/$itFOXP3^+$ cell ratio is a predictive marker for both disease-free survival time and overall survival time in patients with colorectal cancer. Importantly, $itCD8^+$ T/$itFOXP3^+$ cell ratio may be an independent prognostic factor. And, tumor-producing TGF-β may contribute to the increased number of $itFOXP3^+$ cells.

Keywords Colorectal cancer · Intratumoral $CD8^+$ T cells · Intratumoral $FOXP3^+$ regulatory T cells · $CD8^+$ T cell/$FOXP3^+$ cell ratio · Prognostic factor

Abbreviations

Tregs	$CD4^+$ regulatory T cells
FOXP3	Forkhead box P3
$itCD8^+$ T cells	Intratumoral CD8-positive T cells
$itFOXP3^+$ cells	Intratumoral FOXP3-positive cells
$itCD8^+$ T/ $itFOXP3^+$ cell ratio	Ratio of number of intratumoral CD8-positive T cells to number of intratumoral FOXP3-positive cells
TILs	Tumor-infiltrating lymphocytes
PBMCs	Peripheral blood mononuclear cells
TGF-β	Transforming growth factor-beta

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Introduction

For many years, the immune system has been generally considered to be able to control and eliminate tumors that develop spontaneously, so-called immune surveillance [9]. Indeed, tumor cells can express antigens which become targets for a T cell-mediated immune response [4]. To date, there have been many investigations supporting this concept. For example, correlation has been made between the

degree of tumor invasion by tumor-infiltrating lymphocytes (TILs) and better survival in patients with various types of tumors, including colorectal cancer [18, 27, 38]. In a report analyzing subpopulations of TILs, a correlation between an increase in $CD8^+$ T cells and better survival has been shown in colorectal cancer [24]. Recently, it has been reported that an increase of memory T cells, especially memory $CD8^+$ T cells, correlates with increased survival in large cohorts of human colorectal cancer patients [12].

On the other hand, cancer cells evade antitumor T cell response by multiple immunosuppressive mechanisms [8]. In the 1980s, several investigators had already proposed an involvement of suppressor T cells in the immune responses against tumors [3]. Recent evidence has revealed the existence of a unique $CD4^+$ T cell population, designated regulatory T cells (Tregs), as an important suppressor T cell population [22, 29, 32]. Tregs were originally identified as $CD4^+$ T cells that constitutively expressed the interleukin (IL)-2 receptor α-chain (CD25) [28]. More recent studies have shown that the transcription factor forkhead box P3 (FOXP3) is not only a key intracellular marker but also a crucial developmental and functional factor for $CD4^+CD25^+$ Tregs [17]. Thus, it is now generally considered that $CD25^+FOXP3^+CD4^+$ T cells are Tregs. Tregs are considered to act as players in immune tolerance against self-antigens. Most tumor-associated antigens (TAAs) are self-antigens. This means that TAAs themselves may induce an increased number of Tregs in cancer patients and that the increased Tregs may negatively control the antitumor immune response. Indeed, increased numbers of Tregs in the peripheral blood or in tumor tissues have been shown in several types of tumors, including colorectal cancer [7, 20, 21, 35], and a possible suppression of antitumor immunity by Tregs has been indicated [30, 37]. It should be noted that the increased numbers of Tregs infiltrating tumors corresponded to poor prognosis in patients with ovarian cancer [7].

The human immune system consists of an elegant balance between immune surveillance and immune tolerance of self-antigens. Tregs play an important role in the prevention of autoimmune disorders by controlling the activity of autoreactive T cells [22]. On the other hand, Tregs may play critical roles in immune tolerance against cancers [25, 30, 37]. Recently, several studies have shown the importance of a balance between immune surveillance and immune evasion in the tumor microenvironment [11, 13, 31]. First, the tumor-infiltrating $CD8^+$ T cell/Tregs ratio was associated with survival prognosis in ovarian cancer [31]. Second, an inverse relationship between intratumoral $CD8^+$ T cells and intratumoral Tregs in hepatocellular carcinoma (HCC) tissues was shown. In addition, an increased quantity of circulating Tregs was associated with reduced survival time of these patients. However, no significant correlation between numbers of TILs and survival prognosis was found [11]. Third, the possibility of an intratumoral balance of Tregs and activated $CD8^+$ T cells, as an independent predictor of survival in HCC was shown [13].

Transforming growth factor-β (TGF-β) is a potent regulatory cytokine with diverse effects on hemopoietic cells. The role of TGF-β in the induction and maintenance of Tregs has attracted much attention. Importantly, it has been shown that TGF-β1 can convert $CD4^+CD25^-$ T cells into Tregs in vitro [5]. In addition, a reduced number of peripheral Tregs has been shown in TGF-$\beta1^{-/-}$ mice [23]. Peng et al. [26] have also shown in mouse that TGF-β may regulate the in vivo expansion of Tregs. Taken together, TGF-β may play an important role in in vivo induction and/or maintenance of Tregs.

In the present study, the absolute numbers of tumor-infiltrating $CD8^+$ T cells and $FOXP3^+$ cells were measured using surgically resected specimens. We indicate for the first time that the tumor-infiltrating $CD8^+$ T/$FOXP3^+$ cell ratio may be a predictive marker for disease-free survival in patients with colorectal cancer. In addition, we suggest that tumor-produced TGF-β contributes to the increased number of intratumoral Tregs.

Patients and methods

Patients and samples

Ninety-five patients with primary colorectal cancer underwent resection at the Department of Surgery and Oncology, Kyushu University (Fukuoka, Japan). All patients who were enrolled in the present study provided informed consent before surgical treatment. Primary colorectal cancer surgical specimens with adjacent normal colonic mucosa were fixed in 10% formalin and embedded in paraffin. Sections were cut serially into 4 μm sections. All tumors were staged according to the TMN classification system of the International Union Against Cancer (UICC).

Immunohistochemistry

Single or double color DAB immunoperoxidase staining was performed as described previously [31]. Anti-human CD8 mouse monoclonal antibody (CM154 BIOCARE, Concord, USA), FOXP3 rabbit monoclonal antibody (ab20034, Abcam, Cambridge, UK), TGF-β rabbit polyclonal antibody (sc-146, Santa Cruz, CA, USA), and CD3 mouse monoclonal antibody (N1617, Dako, Tokyo, Japan) were used. Slides were incubated with each primary antibody at 4°C overnight, washed in phosphate buffered saline (PBS) three times for 5 min each, and incubated with secondary antibodies (goat anti-mouse and rabbit IgG;

Nichirei Corp, Ltd, Tokyo, Japan) at room temperature for 30 min. Immunoreactivity was visualized by the development of brown pigment via a standard 3,3′-diaminobenzidine protocol, and of red pigment via a New Fuchsin solution kit (Nichirei Corp., Ltd, Tokyo). Sections were then counterstained lightly with hematoxylin. CD8 and FOXP3 stained cells are described as $CD8^+$ T cells and $FOXP3^+$ cells, respectively.

Evaluation of immunostaining

Evaluation of immunostaining was performed as previously described but with minor modifications [31]. Each entire tumor section was evaluated for TILs by microscopic examination, (×400; BX51; Olympus, Tokyo, Japan) and ten independent areas that had the most abundant numbers of TILs were selected and photographed with a digital camera (Binary Planner 4490; Jenoptil, Jena, Germany). Absolute numbers of labeled tumor-infiltrating cells, excluding tumor cells, were counted manually. The count was performed two times for each photograph by the same investigator without knowledge of the corresponding clinical data. Total numbers of the ten selected areas were represented as intratumoral $CD8^+$ T cells ($itCD8^+$ T cells) or $itFOXP3^+$ cells. A ratio of $itCD8^+$ T cells to $itFOXP3^+$ cells was represented as the $itCD8^+$ T/$itFOXP3^+$ cell ratio.

To examine whether TGF-β contributes to increased $itFOXP^+$ cell numbers, we analyzed the relationship between TGF-β expression at the tumor site and the numbers of $itFOXP^+$ cells in each specimen. As described above, ten independent areas that were the most abundant in TILs were selected. When TGF-β-expressing areas occupied over 30% of the total of the 10 independent areas, specimens were considered to be high TGF-β-expressing specimens [15].

Cell culture and fluorescence-activated cell sorting (FACS) analysis

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque (Life Technologies, Gaithersburg, MD, USA) density gradient centrifugation from five healthy volunteers. $CD4^+$ T cells were purified from PBMCs with a CD4-positive isolation kit (Dynabeads, Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions. The positive-selection process yielded over 98% $CD4^+$ T cells. $CD4^+$ T cells (5.0×10^5 cells/well) were cultured with GMP-grade RPMI 1640 (Hy-medium; Nipro, Tokyo, Japan) containing 5% human albumin, 100 IU/mL human recombinant IL-2 (Nipro) and immobilized monoclonal antibody to CD3 (10 μg/mL, OKT-3; Jansen-Kyowa, Tokyo, Japan) for 5 days in the presence or absence of recombinant human TGF-β (100-B;R& D systems, Weisbaden, Germany). After 5 days, the cells were harvested and intracellular staining of FOXP3 was conducted using a PE-conjugated anti-human FOXP3 Staining Set (clone PCH101; e-Bioscience, SanDiego, CA, USA) according to the manufacturer's instructions. Two-color flow cytometry was performed using a FACSCalibur™ (Becton–Dickinson CA, USA). $FOXP3^+$ cells, after gaiting of $CD4^+$ lymphocytes, were analyzed. The percentage of $FOXP3^+$ cells among the total $CD4^+$ T cells was determined.

Statistical analysis

All statistical analyses were performed with JMP Statistical Software (SAS Institute, Inc). The correlations between subsets of TILs ($itCD8^+$ T cells, $itFOXP3^+$ cells, and the $itCD8^+$ T/$itFOXP3^+$ cell ratio) and the clinicopathological features listed were analyzed by Spearman's test. The statistical significance of difference between the two groups was determined using the Mann–Whitney nonparametric *U* test. Kruskal–Wallis *H* nonparametric test was applied to multiple comparisons. All items were treated as dichotomous variables. Survival was measured from the date of operation to the time of death/relapse or the time the patient was last seen. The log-rank test was used to perform univariate analyses and Kaplan–Meier curves were used to estimate survival rates. Survival rates were compared by the log-rank test via the SAS/STAT PHREG procedure. Prognostic factors for survival were evaluated in multivariate analyses by Cox proportional hazards regression. One patient with distant metastasis was excluded from this analysis. In the remaining 94 patients, $itCD8^+$ T cells and $itFOXP3^+$ cells were dichotomized by a cutoff point of 151 and 14, respectively, on the basis of the mean value. Since no $itFOXP3^+$ cells were observed in a few specimens, it was not suitable to divide patients into two groups based on the mean value of the $itCD8^+$ T/$itFOXP3^+$ ratio. Therefore, patients were divided into equal-sized groups. As a result, the $itCD8^+$ T/$itFOXP3^+$ cell ratios were dichotomized by a cutoff point of 12, i.e., $\geq$12; 47 cases versus $\leq$12; 47 cases. A P value of <0.05 was considered to be significant.

Results

Immunohistochemical characteristics

In this study, we used surgically resected tumor specimens. By hematoxylin and eosin staining, TILs distribution was found to be relatively homogenous within a tumor, excluding necrotic and fibrotic areas, although TILs were usually more abundant in stromal areas compared with epithelial areas, in agreement with previous reports [19, 21]. To

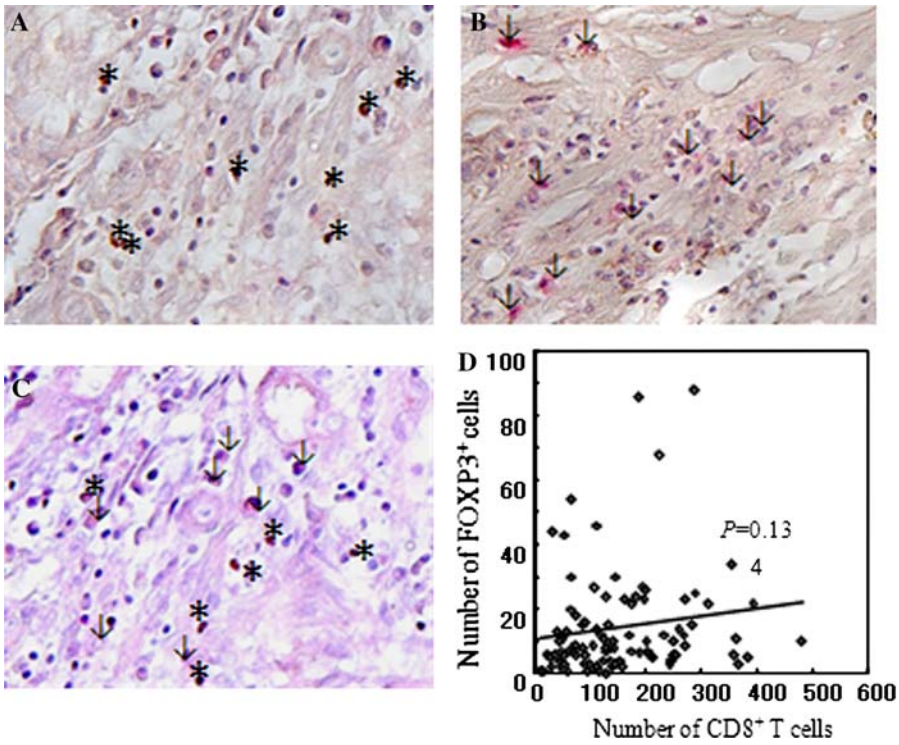

Fig. 1 Immunohistochemical staining shows that FOXP3$^+$ lymphocytes are distinguishable from CD8$^+$ lymphocytes in colorectal cancer. **a** FOXP3 positive cells are stained *brown*, as shown by *asterisks* (×400). **b** CD8 positive cells are stained *red*, as shown by *arrows* (×400). **c** Double staining of FOXP3 (*asterisks*, in cell nucleus) and CD8 (*arrows*, on cell membrane) (×400). **d** The correlation between CD8$^+$ T cells and FOXP3$^+$ cells is shown (Mann–Whitney *U* test). *P* values less than 0.05 were considered significant (color figure online)

estimate the number of TILs, ten independent areas that were the most abundant in TILs were selected and total number of labeled cells was expressed as itCD8$^+$ T cells or as itFOXP3$^+$ cells. ItFOXP3$^+$ cells exhibited distinct nuclear staining (Fig. 1a). When TILs were doubly stained with anti-FOXP3 and anti-CD3 antibodies, most itFOXP3$^+$ cells were positive for CD3 (date not shown), indicating that itFOXP3$^+$ cells are T cells. itCD8$^+$ T cells exhibited homogeneous cytoplasmic staining, while nuclei were not stained (Fig. 1b). When TILs were doubly stained with anti-FOXP3 and anti-CD8 antibodies, itFOXP3$^+$ cells and itCD8$^+$ T cells could be counted separately as different cells (Fig. 1c). When the correlation between the number of itCD8$^+$ T and itFOXP3$^+$ cells was analyzed, no significant correlation was detected (Fig. 1d).

Correlation of TIL subtypes with clinicopathological features

The relationships of itCD8$^+$ T and itFOXP3$^+$ cells with traditional pathological measures for the 94 colorectal cancer specimens were analyzed statistically (Table 1). The number of itCD8$^+$ T cells showed no correlation with any pathological parameters. On the other hand, the number of itFOXP3$^+$ cells was positively correlated with lymph node metastases ($P = 0.030$). The ratio of the number of itCD8$^+$ T cells to the number of itFOXP3$^+$ cells (itCD8$^+$ T/itFOXP3$^+$ cell ratio) was negatively correlated with pathological stages ($P = 0.048$).

Correlation of TIL subtypes with survival prognosis

To determine whether itCD8$^+$ T and itFOXP3$^+$ cell numbers are significant prognostic factors for survival, 95 patients who underwent a curative resection were analyzed (Fig. 2). One patient was excluded from this analysis, because he had distant metastases and thus underwent a non-curative resection. The 94 patients were divided into two groups by cutoff points of 151 and 14 for the number of itCD8$^+$ T cells and itFOXP3$^+$ cells, respectively, as described in the "Patients and methods" section. The number of itCD8$^+$ T cells did not significantly correlate with disease-free survival (Fig. 2a) and overall survival (Fig. 2b). The number of itFOXP3$^+$ cells also showed no significant correlation with disease-free survival (Fig. 2c) and overall survival (Fig. 2d). Interestingly, when the itCD8$^+$ T/itFOXP3$^+$ cell ratio was dichotomized by a cutoff point of 12, itCD8$^+$ T/itFOXP3$^+$ cell ratio showed a significant positive correlation with disease-free survival ($P = 0.023$; Fig. 2e) and overall survival ($P = 0.010$; Fig. 2f).

To estimate the value of TIL numbers as a prognostic factor, the correlation of traditional clinicopathological factors, such as lymph node metastases, with disease-free survival (Table 2) and overall survival (Table 3) was analyzed. Venous invasion, lymph node metastases, pathological stages, and itCD8$^+$ T/itFOXP3$^+$ cell ratio correlated with disease-free survival in this cohort (Table 2, upper panel). We next examined whether the itCD8$^+$ T/itFOXP3$^+$ cell ratio is able to be an independent prognostic indicator for

Table 1 Correlation analysis between subtype of TILs and clinicopathological parameters

Parameters	Number of cases	TILs (*P* value: one-sided)		
		itCD8$^+$ T cells	it FOXP3$^+$ cells	itCD8$^+$T/itFOXP3$^+$ ratio
Age				
~65 years	45	0.247	0.967	0.467
66 years~	49			
Gender				
Female	41	0.560	0.057	0.159
Male	53			
Tumor location				
Colon	58	0.110	0.861	0.198
Rectum	36			
Depth of invasion				
m, sm, mp	13	0.497	0.546	0.143
ss,se	81			
Lymphnode metastases				
n(−)	47	0.940	0.030(+)*	0.084
n(+)	47			
Lymphatic invasion				
ly(−)	40	0.244	0.982	0.489
ly(+)	54			
Venous invasion				
v(−)	66	0.406	0.305	0.899
v(+)	28			
Pathological stages				
I + II	43	0.316	0.116	0.048(−)*
III + IV	51			

M musosa, *sm* submucosa, *mp* muscularis propria, *ss* subserosa, *se* serosa, (+) positive correlation, (−) negative correlation

* Significant

disease-free survival. Multivariate analysis was performed between these four factors (Table 2, lower panel). Only venous invasion significantly correlated with disease-free survival ($P = 0.045$). On the other hand, venous invasion, pathological stages, and itCD8$^+$ T/itFOXP3$^+$ cell ratio correlated with overall survival (Table 3, upper panel). When multivariate analysis was performed between these four factors, venous invasion ($P = 0.050$) and itCD8$^+$ T/itFOXP3$^+$ cell ratio ($P = 0.035$) significantly correlated with overall survival (Table 3, lower panel).

Correlation between TGF-β distribution within tumor tissues and the number of itFOXP3$^+$ cells

We tried to evaluate quantitatively TGF-β expression in 31 out of the 94 specimens. TGF-β expression was estimated by a ratio (%) of the TGF-β-stained area to the total area of ten independent tumor areas that had the most abundant number of TILs. When this ratio was over 30% [15], the specimens were considered to be high TGF-β-expressing specimens (Fig. 3a), and other specimens were considered to be low TGF-β-expressing specimens (Fig. 3b). Specimens were doubly stained with anti-TGF-β antibody and anti-FOXP3 antibody (Fig. 3c). When the numbers of itFOXP3$^+$ cells were over 14, the specimens were considered to be high itFOXP3$^+$ cells number specimens as described in "Patients and methods". A ratio of TGF-β-expressing area positively correlated with the number of itFOXP3$^+$ cells ($P = 0.020$; Table 4).

Discussion

We show for the first time that a balance of itCD8$^+$ T and itFOXP3$^+$ cells, the itCD8$^+$ T/itFOXP3$^+$ cell ratio, is an independent indicator for overall survival in patients with colorectal cancer who have undergone curative surgery. In addition, itCD8$^+$ T/itFOXP3$^+$ cell ratio shows a positive correlation with disease-free survival time and overall survival time. We also suggest that tumor-producing TGF-β at least partly contributes to the increased number of itFOXP3+ cells.

A correlation of increased memory CD8$^+$ T cells with increased survival in large cohorts of colorectal cancer patients has been shown [12], and importantly, our data may recapitulate these findings (Fig. 2a, b).

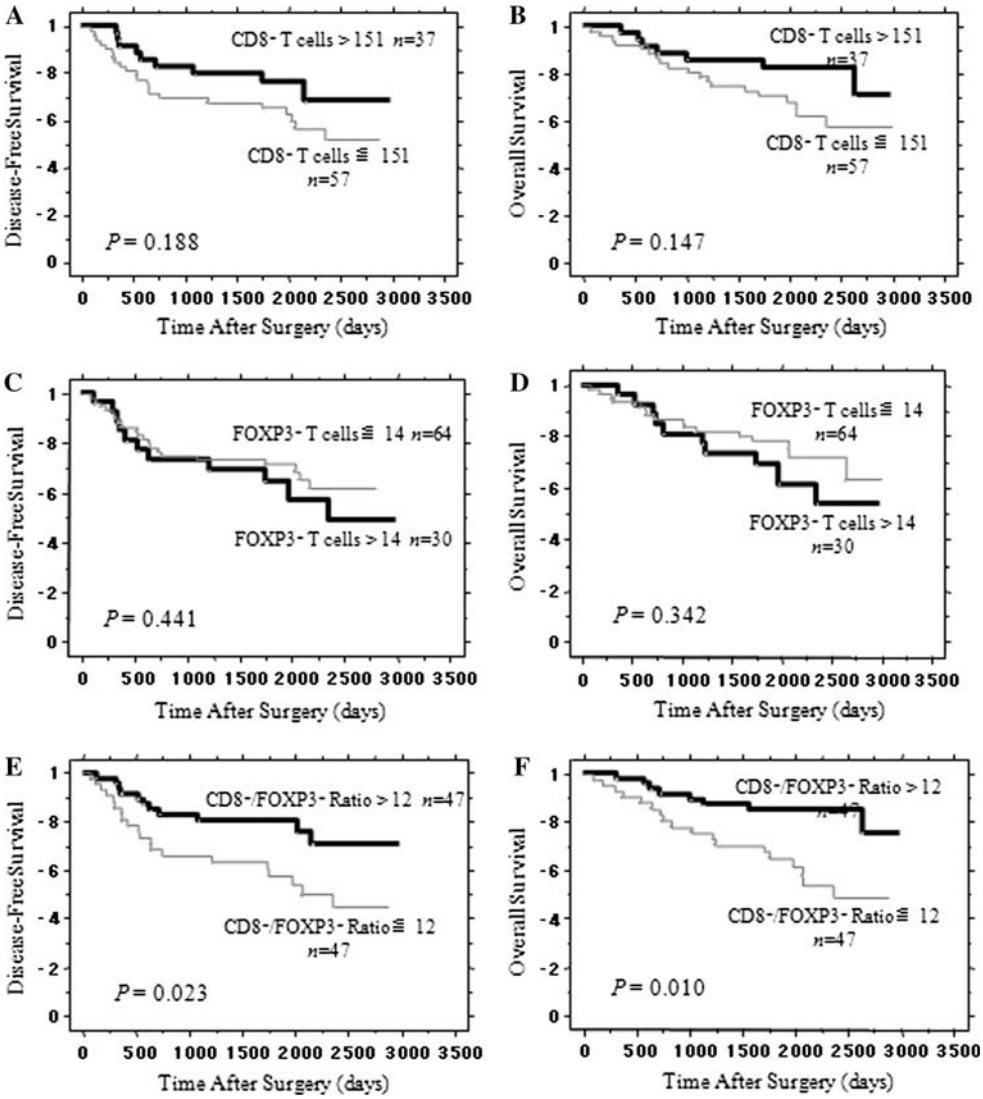

Fig. 2 The itCD8$^+$ T/itFOXP3$^+$ cell ratio predicts poor disease-free survival and overall survival in 94 colorectal cancer patients. itCD8$^+$ T cells (**a** and **b**) and itFOXP3$^+$ cells (**c** and **d**) correlated with neither disease-free survival nor with overall survival. Disease-free survival and overall survival of the patients showing high an itCD8$^+$ T/itFOXP3$^+$ cell ratio were significantly better compared with those of the patients showing a low itCD8$^+$ T/itFOXP3$^+$ cell ratio (**e** and **f**). *P* values less than 0.05 were considered significant

Many investigators have observed that Tregs are significantly higher in a number in tumor tissues, especially in stromal areas, compared with normal tissues [7, 13, 20, 21], and that tumor-infiltrating Tregs (itTregs) may play an important role in the suppression of the antitumor immune response [7, 11, 19, 30, 37]. If this is so, numbers of itTregs should correlate inversely with survival prognosis. In fact, there are several studies indicating an inverse correlation of itTregs with survival in certain kinds of tumors, including colorectal cancer [2, 7, 13, 16, 36]. On the other hand, there are also some investigations indicating no significant correlation between itTregs and survival [11, 14, 21]. In most of these studies, FOXP3$^+$ T cells are classified as itTregs [2, 7, 11, 14, 16, 19, 21, 31, 36]. In the present study, most itFOXP3$^+$ cells were also positive for CD3, indicating that itFOXP3$^+$ cells are T cells (data not shown). Thus, we also considered itFOXP3$^+$ cells to be itTregs.

Our present data did not show a significant correlation between itFOXP3$^+$ cells and survival (Fig. 2c, d). Lymph node metastases, which is one of the important prognostic indicators in colorectal cancer [6], did correlate with disease-free survival time ($P = 0.018$; Table 2, upper panel) and overall survival time ($P = 0.069$; Table 3, upper panel). Thus, although cohort used in the present study is small, it seems that this cohort may be relatively suitable for analysis of prognostic factors. However, it is still controversial as to whether the number of itFOXP3$^+$ cells is a prognostic indicator in colorectal cancer. Nevertheless, it is noteworthy that the number of itFOXP3$^+$ cells showed a significant positive correlation with the status of lymph node metastases ($P = 0.030$; Table 1). This finding indicates that the number of itFOXP3$^+$ cells may be a predictive factor for lymph node metastases.

A direct link between itFOXP3$^+$ cells and prognostic survival remains unclear in the present study. However, recent evidence indicates that a balance of itTregs and itCD8$^+$ T cells is a more sensitive predictor for recurrence and survival than itTregs or itCD8$^+$ T cells alone [13, 31].

Table 2 Univariate and multivariate analysis of parameters associated with disease-free survival time

	Hazard ratio	95% Cl	*P* value
Parameters			
Age	0.129	0.399–1.626	0.528
Gender	1.161	0.424–1.747	0.678
Tumor location	1.608	0.307–1.259	0.186
Depth of invasion	5.682	0.024–1.294	0.088
Lymph node metastases	2.494	0.189–0.852	0.018(−)*
Lymphatic invasion	1.667	0.315–1.380	0.269
Venous invasion	2.725	0.185–0.762	0.007(−)*
Pathological stages	2.933	0.152–0.764	0.009(−)*
itCD8$^+$ T cells	1.678	0.274–1.298	0.193
itFOXP3$^+$ cells	1.335	0.639–2.788	0.443
itCD8$^+$T/itFOXP3$^+$ ratio	2.229	0.208–0.908	0.027(+)*
Variables			
Lymph node metastases	1.314	0.174–3.316	0.716
Venous invasion	2.110	0.228–0.984	0.045(−)*
Pathological stages	1.805	0.112–2.748	0.469
itCD8$^+$T/itFOXP3$^+$ ratio	1.869	0.251–1.137	0.104

Each cutoff point of parameters is the same as the parameters of Table 1

(+), positive correlation; (−), negative correlation

* Significant

Table 3 Univariate and multivariate analysis of parameters associated with overall survival time

	Hazard Ratio	95% Cl	*P* value
Parameters			
Age	1.032	0.474–2.247	0.936
Gender	1.252	0.369–1.729	0.569
Tumor location	1.046	0.474–2.304	0.912
Depth of invasion	4.310	0.031–1.718	0.153
Lymph node metastases	2.123	0.210–1.059	0.069
Lymphatic invasion	1.134	0.399–1.948	0.756
Venous invasion	2.857	0.162–0.759	0.008(−)*
Pathological stages	2.618	0.160–0.909	0.030(−)*
itCD8$^+$ T cells	1.883	0.274–1.298	0.193
itFOXP3$^+$ cells	1.465	0.663–3.234	0.345
itCD8$^+$T/itFOXP3$^+$ ratio	2.841	0.153–0.811	0.014(+)*
Variables			
Venous invasion	2.222	0.203–0.998	0.050(−)*
Pathological stages	2.045	0.201–1.191	0.115
itCD8$^+$T/itFOXP3$^+$ ratio	2.475	0.173–0.940	0.035(+)*

Each cutoff point of parameters is the same as the parameters of Table 1

(+) positive correlation, (−) negative correlation

* Significant

Our data may be compatible with this new theory. Namely, itFOXP3$^+$ cells alone were not an indicator for survival, whereas the itCD8$^+$ T/itFOXP3$^+$ cell ratio correlated well with disease-free survival (Fig. 2e) and overall survival (Fig. 2f).

The next question is why a balance of itFOXP3$^+$ and itCD8$^+$ T cells is a more sensitive marker of recurrence or survival compared with itFOXP3$^+$ cells or itCD8$^+$ T cells alone. One possible answer is that antitumor immunity is determined by the immunological balance in the microenvironment of the tumor site. The molecular mechanisms governing the intratumor accumulation of CD8$^+$ T cells may be different from those regulating Tregs. Namely, CD8$^+$ T cells and FOXP3$^+$ cells are likely to accumulate independently at the tumor site. In the present study, the number of itFOXP3$^+$ cells correlated with lymph node metastases ($P = 0.030$), whereas the number of itCD8$^+$ T cells did not ($P = 0.940$) (Table 1). This finding may indirectly indicate an independent accumulation of CD8$^+$ T cells and of FOXP3$^+$ cells at the tumor site. Thus, we suggest that the CD8$^+$ T/FOXP3$^+$ cell ratio at the tumor site reflects more strongly the patient's total antitumor immunity than simply the numbers of CD8$^+$ T or FOXP3$^+$ cells.

It is very rare to detect FOXP3$^+$ cells in normal colorectal tissues (data not shown), therefore, why does the number of FOXP3$^+$ cells increase within tumor tissues? A unique mechanism of specific recruitment of Tregs in ovarian cancer tissues has been proposed [7], whereby chemoattractants for Tregs are produced by tumors and/or by tumor-infiltrating macrophages. Similar chemoattractants may be produced within the colorectal cancer tissues; however, this suggestion requires further investigation. Another possibility is the conversion of FOXP3$^-$ T cells to FOXP3$^+$ T cells or the expansion of the FOXP3$^+$ T population within the tumor microenvironment [1, 34]. It has been shown that TGF-β, which is present at high levels in the tumor microenvironment, can mediate this conversion or expansion [5, 10, 33]. Indeed, we confirmed the production of TGF-β in the colorectal cancer tissues examined. Importantly, a larger number of FOXP3$^+$ cells were found in high TGF-β-expressing specimens compared with low TGF-β expressing specimens (Table 4). In addition, co-culture of peripheral CD4$^+$ T cells with recombinant TFG-β1 increased the number of FOXP3$^+$ cells (data not shown). Although these findings support a possible conversion of FOXP3$^-$ T cells to FOXP3$^+$ T cells or the expansion of the FOXP3$^+$ T cell population in the colorectal cancer microenvironment, it is difficult to precisely evaluate this possibility from our surgically resected specimens.

In conclusion, we want to stress that the CD8$^+$ T/FOXP3$^+$ cell ratio may be an independent prognostic factor for colorectal cancer patients who have undergone curative resection. This finding will improve our understanding of

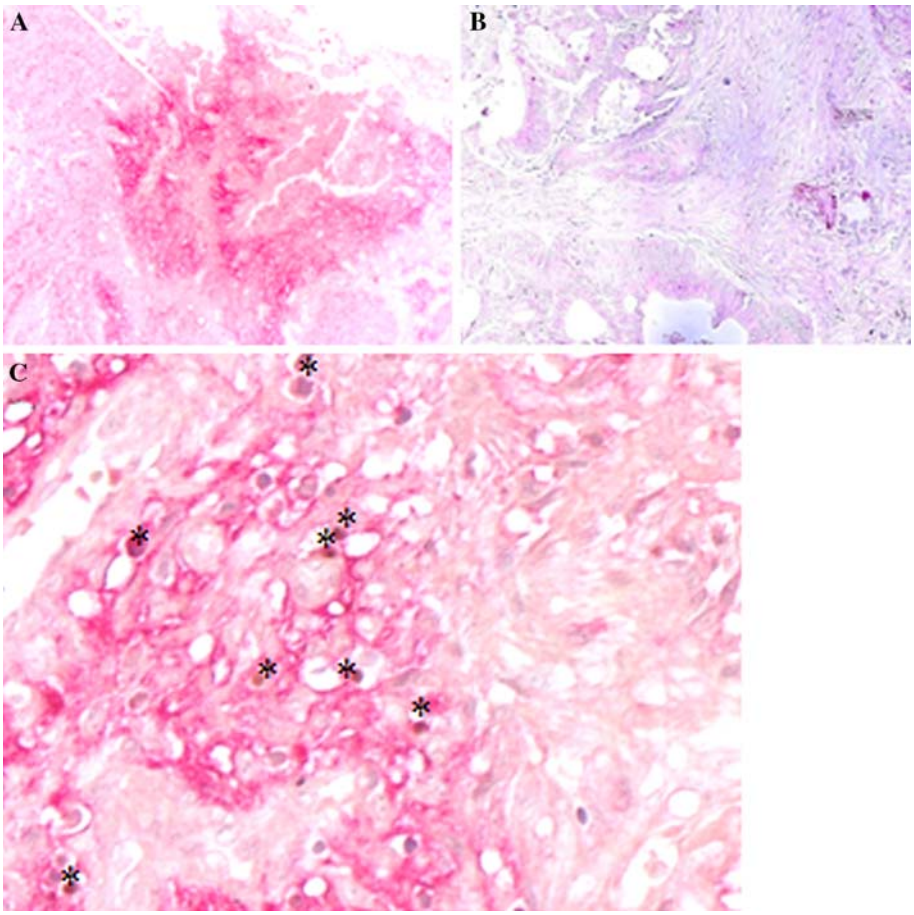

Fig. 3 Representative pictures of immunohistochemical double staining. **a** A high TGF-β-expressing specimen (*red*) (×400). **b** A low TGF-β-expressing specimen are shown (×400). **c** There are a high number of FOXP3$^+$ cells (*brown*) indicated by asterisks, in the high TGF-β expressing area (×400) (color figure online)

Table 4 Significant relation between number of itFOXP3$^+$ cells and TGF-β expression

TGF-β expression	Number of itFOXP3$^+$ cells	
	>14	<14
High	15 specimens	7 specimens
Low	1 specimens	8 specimens

When TGF-β-expressing areas occupied over 30% of the total of the 10 independent areas, specimens were considered to be high TGF-β-expressing specimens

Significant at $P = 0.020$ for positive correlation

clinical significance of immune cells existing in the tumor microenvironment.

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