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Prognostic impact of the tumor-infiltrating regulatory T-cell (Foxp3$^+$)/activated cytotoxic T lymphocyte (granzyme B$^+$) ratio on resected left-sided pancreatic cancer

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Abstract. Among the subsets of tumor-infiltrating lymphocytes (TILs), activated cytotoxic T lymphocytes (granzyme B$^+$) have an antitumor effect, while regulatory T lymphocytes [forkhead box P3 (Foxp3)$^+$] suppress the antitumor immune response. The aim of the present study was to investigate the possible associations between TIL subsets and survival outcomes in patients with left-sided pancreatic ductal adenocarcinoma (PDAC). From January 2000 to December 2008, 30 patients who underwent curative distal pancreatectomy without neoadjuvant chemoradiotherapy due to left-sided PDAC were enrolled in the present study. TIL subsets were enumerated by immunohistochemical staining for cluster of differentiation (CD)3, CD4, CD8, Foxp3 and granzyme B in the intra-tumoral areas of tissue blocks. Patients were divided into two groups according to the median value of the absolute counts and relative ratios of TIL subsets. In the univariate analysis, age, gender, tumor size, nodal stage, tumor differentiation and lymphovascular/perineural invasion were not significantly associated with survival outcome. However, low levels of preoperative cancer antigen (CA) 19-9 were associated with a longer overall survival (OS), although the association was not significant (37 vs. 18 months; P=0.061). A high level of granzyme B$^+$ was associated with enhanced disease-free survival (DFS) (25 vs. 10 months; P=0.023), and a low Foxp3$^+$/granzyme B$^+$ ratio was associated with a favorable prognosis in terms of DFS (25 vs. 8 months; P=0.008) and OS (47 vs. 17 months; P=0.003). In the multivariate analysis, the ratio of Foxp3$^+$/granzyme B$^+$ was an independent prognostic factor for determining DFS [Exp(B), 3.060; 95% confidence interval (CI), 1.259-47.436; P=0.014] and OS [Exp(B), 3.580; 95% CI, 1.460-8.780; P=0.005]. Among the clinicopathological factors, low levels of CA 19-9 were significantly associated with a low Foxp3$^+$/granzyme B$^+$ ratio (P=0.016). The results of the present study suggested that a low Foxp3$^+$/granzyme B$^+$ ratio may be useful for predicting a good prognosis in surgically resected left-sided PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a fatal cancer with an overall 5-year survival rate of <5%. Surgical treatment has the most favorable outcome, with a 5-year survival rate of ~20%; however, only 15-20% of patients are candidates for surgical resection (1,2). Standard treatment modalities, including chemotherapy and chemoradiotherapy, have been shown to be ineffective for improving survival in patients with pancreatic cancer (1,2). Therefore, novel treatments are urgently required for this devastating disease.

Lymph node metastasis, a high tumor grade, a large tumor size, lymphovascular invasion, perineural invasion, a high level of preoperative cancer antigen (CA) 19-9, persistently elevated postoperative levels of CA 19-9 and positive margins of resection are typically considered the main prognostic factors for PDAC (2-5). In addition to clinicopathological features, the tumor-specific host immune response has been reported to have a crucial role in disease-related survival outcomes for numerous types of cancers (6-15). Among the parameters representing tumor-specific immune function, tumor infiltrating lymphocytes (TILs) are often observed in resected cancer tissue and are thought to participate in the host immune response against cancer (16). Interactions between the tumor microenvironment and the immune system significantly affect cancer development and progression (17). TILs are considered prognostic factors because they represent local host antitumor immunity (16,17).

TILs consist of functionally distinct subsets, including the antitumor effectors, CD8$^+$ T lymphocytes and CD4$^+$ helper T lymphocytes, which are associated with a favorable

prognosis (7). Conversely, regulatory T lymphocytes (Tregs) suppress the antitumor immune response and have been shown to adversely affect patient survival (6,9,13,14,18). The forkhead/winged helix transcription factor, forkhead box P3 (Foxp3), which is genetically defective in an autoimmune and inflammatory syndrome in humans and mice, is specifically expressed in naturally arising $CD4^+$ Tregs (19). Tregs weaken host antitumor immunity by suppressing T-cell proliferation, antigen presentation and cytokine production (20).

A subset of TILs has been identified in PDAC (21); however, the relationship between the TILs and patient prognosis is largely unexplored. To evaluate the prognostic value of TILs in PDAC, the present study constrained the investigation to left-sided PDAC, as some cases of cancer of the pancreatic head cannot be differentiated from distal bile duct, ampulla of Vater or duodenal cancers. The present study aimed to evaluate the association between TILs in surgically resected left-sided PDAC and patient outcomes.

Materials and methods

Patients. To avoid potential contamination with other periampullary cancers, such as distal bile duct, ampulla of Vater and duodenal cancers, only left-sided pancreatic cancers were considered. A total of 30 patients who underwent a curative distal pancreatectomy due to left-sided PDAC at Severance Hospital, Yonsei University College of Medicine (Seoul, Korea) between January 2000 and December 2008 were enrolled in the present study. In addition, paraffin-embedded tissue blocks from the patients were included in the TIL analysis. The present study retrospectively analyzed patient demographics, histopathological findings and survival outcomes. Follow-up was completed on May 30, 2012. Patients who received neoadjuvant chemotherapy or chemoradiotherapy, or had another primary tumor, were excluded from the study. Overall survival (OS) time was defined as the interval between surgery and death, or between surgery and the last observation of surviving patients. The disease-free survival (DFS) time was defined as the interval between surgery and recurrence. Data were censored at the last follow-up for living patients. This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University college of Medicine.

Immunohistochemical (IHC) staining and quantification of TIL subsets. IHC staining for TILs was performed as described previously (11). Briefly, paraffin-embedded PDAC tissue sections at a thickness of 4-μm were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed in citrate buffer in a microwave oven. Endogenous peroxidase activity was blocked by incubating the tissues with 3% hydrogen peroxide in methanol for 5 min. The sections were then incubated for 60 min at room temperature with primary monoclonal antibodies against cluster of differentiation (CD)3 (cat. no. RM-9107-S; 1:100; Lab Vision Corporation, Fremont, CA, USA), CD4 (cat. no. NCL-L-CD4-1F6; 1:100; Novocastra™ Primary Antibodies; Leica Microsystems, Ltd., Milton Keynes, UK), CD8 (cat. no. IS62330; 1:100; Dako, Glostrup, Denmark), Foxp3 (cat. no. ab20034; 1:100; Abcam, Cambridge, UK), and granzyme B (cat. no. MS-1157-S;1:100; Lab Vision Corporation), which were used to identify total numbers of T lymphocytes, helper T lymphocytes, cytotoxic T lymphocytes (CTLs), Tregs and activated CTLs, respectively. After washing the sections twice with 0.05 mol/l Tris-buffered saline containing 0.2% Tween-20, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. K5007; ready to use; Dako EnVision® Detection system; Dako), followed by development with diaminobenzidine and counterstaining with hematoxylin. Normal human tonsil tissue obtained from a healthy volunteer was used as the positive control. The negative control for immunostaining was prepared by incubating tissue sections without primary antibody, according to a previous study (11).

IHC staining was quantified by two experienced pathologists who were blinded to the patient data. Three intense foci of staining in the tumor sections were selected and four high-power fields (magnification, x400) from each slide were selected for calculation of the IHC staining results. Fields with necrosis or hemorrhage in the tumor portion were avoided. The median value of positively stained cells in each part was recorded. Using the absolute counts and relative ratios of lymphocytes stained by each antibody (CD3, CD4, CD8, Foxp3 and granzyme B; Fig. 1), the patients were divided into low and high groups.

Statistical analysis. All statistical analyses were performed with SPSS 20.0 software (IBM SPSS, Armonk, NY, USA). Categorical data were compared using χ^2 or Fisher's exact tests. Absolute counts of TIL subsets and the relative ratios between two different TIL subsets were dichotomized in the survival analysis using cut-off values derived by the median, as described previously (6,10,11,22). OS and DFS times were calculated using the Kaplan-Meier method and significance was evaluated using the log-rank test. Cox proportional hazard models were used for univariate and multivariate survival analysis. $P<0.05$ was considered to indicate a statistical significance.

Results

Patient demographics. A total of 54 patients underwent curative distal pancreatectomy with or without splenectomy for left-sided PDAC. Among them, 7 patients who underwent neoadjuvant chemoradiotherapy were excluded. Paraffin-embedded tissue blocks were not available for 9 patients and the qualities of the paraffin-embedded tissue blocks were not good for 8 patients. Therefore, 30 patients were enrolled in this study. The mean age of the enrolled patients was 62.4±8.9 years and 21 patients (70%) were male. The mean operation time was 327±190 min. Combined organ resection was performed in 8 patients (26.7%), and an intraoperative transfusion was required in 9 patients (30%). The mean tumor size was 3.9±1.5 cm. The pathological T stage was T2 in 3 patients (10%), T3 in 25 patients (83.3%) and T4 in 2 patients (6.7%); the pathological N1 stage was observed in 13 patients (43.3%). Lymphovascular invasion and perineural invasion were observed in 8 patients (26.7%) and 11 patients (36.7%), respectively. In terms of tumor differentiation, there were 7 well-differentiated, 20 moderately-differentiated and 2 poorly-differentiated tumors, as well as 1 case of an

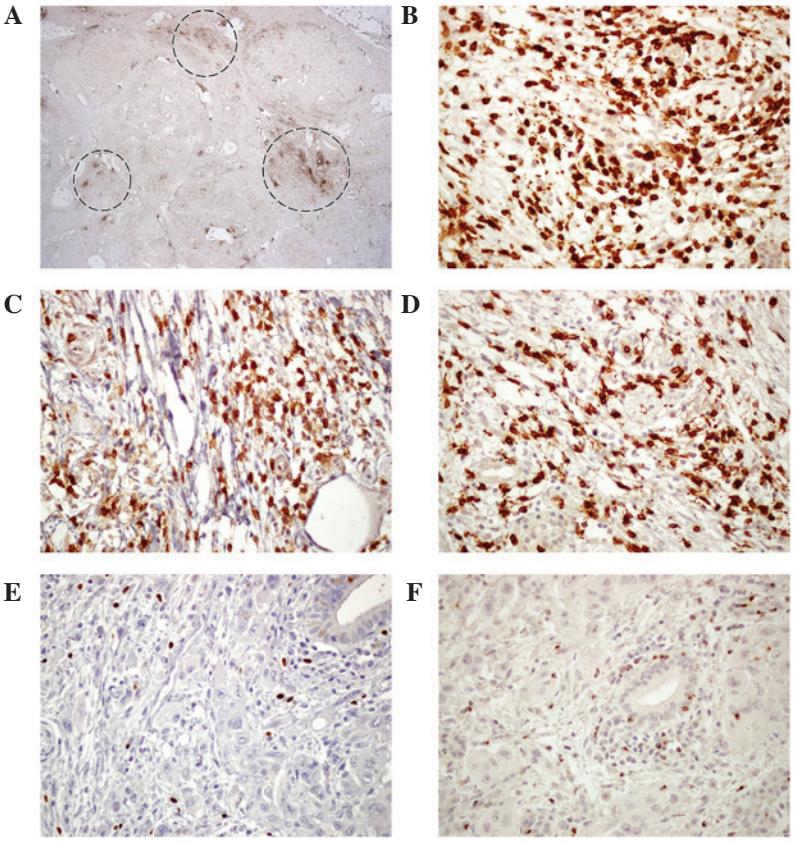

Figure 1. Immunohistochemical staining of tumor-infiltrating T lymphocytes. (A) After reviewing the tissue sections, three intense foci of staining were selected (magnification, x12). Immunohistochemical detection of (B) $CD3^+$ T lymphocytes, (C) $CD4^+$ helper T lymphocytes, (D) $CD8^+$ cytotoxic T lymphocytes, (E) forkhead box $P3^+$ regulatory T lymphocytes and (F) granzyme B^+ activated cytotoxic T lymphocytes in consecutive sections (magnification, x400). CD, cluster of differentiation.

undifferentiated tumor. Resection margin status was R0 in 27 patients, R1 in 1 patient and R2 in 2 patients. The median duration of follow-up was 23 months (range, 5-94 months).

Survival outcomes. Table I shows survival outcomes based on a univariate analysis according to the clinicopathological parameters and operative findings. No factors were significantly predictive of DFS or OS. However, low levels of preoperative CA 19-9 were associated with a longer OS, with a marginal statistical significance (37 vs. 18 months; P=0.061).

Table II shows survival outcomes based on the univariate analysis according to the TIL subsets. High levels of granzyme B^+ TILs were significantly related to a longer DFS time (25 vs. 10 months; P=0.023), and a low $Foxp3^+$/granzyme B^+ ratio was significantly associated with a favorable DFS time (25 vs. 8 months; P=0.008) and OS (47 vs. 17 months; P=0.003). High levels of $CD4^+$ were marginally related to good DFS (25 vs. 8 months; P=0.063). In the multivariate survival analysis, the ratio of $Foxp3^+$/granzyme B^+ was an independent prognostic factor for determining DFS [Exp(B), 3.060; 95% confidence interval (CI), 1.259-7.436; P=0.014) and OS [Exp(B), 3.580; 95% CI, 1.460-8.780; P=0.005) (Table III and Fig. 2).

Association between TIL subsets and clinicopathological factors. Among the clinicopathological factors, age, gender, tumor size, pathological nodal stage, combined organ resection, lymphovascular invasion, perineural invasion and transfusion were not associated with TIL subsets ($CD4^+$, granzyme B^+ and $Foxp3^+$/granzyme B^+). However, low levels of CA 19-9 were significantly associated with a low $Foxp3^+$/granzyme B^+ ratio (P=0.016; Table IV).

Discussion

The present study demonstrated the clinical impact of TILs in left-sided PDAC. Although lymph node metastasis, a high tumor grade, a large tumor size, lymphovascular invasion, perineural invasion, high levels of preoperative CA19-9, persistently elevated postoperative CA19-9 levels and positive margins of resection are typically considered prognostic factors for pancreatic cancer (2-5), none were significantly correlated with DFS and OS in the present study. Instead, the ratio of $Foxp3^+$/granzyme B^+ was an independent prognostic factor in a multivariate analysis and low levels of CA19-9 were associated with a low $Foxp3^+$/granzyme B^+ ratio, despite the small sample size.

Previous studies have reported that the host immune response to tumors has a critical role in disease-associated survival outcomes (6-15), suggesting that host immune factors may offer a useful tool for predicting prognosis (23). Although the status of peripheral blood lymphocytes has been reported as a prognostic factor (24), TILs in resected cancer specimens are thought to be a more reliable measure of the host immune

Table I. Univariate survival analysis according to clinicopathological and operative findings.

	Disease-free survival		Overall survival	
Characteristic	Months (median)	P-value	Months (median)	P-value
Age, years		0.273		0.261
<59 (n=13)	11		18	
≥59 (n=17)	12		29	
Gender		0.483		0.419
Male (n=21)	12		22	
Female (n=9)	10		23	
CA 19-9, U/ml[a]		0.152		0.061
≤109 (n=15)	13		37	
>109 (n=14)	7		18	
Tumor size, cm		0.404		0.467
<3.5 (n=15)	12		35	
≥3.5 (n=15)	8		18	
N stage		0.219		0.137
N0 (n=17)	12		35	
N1 (n-13)	8		18	
Combined organ resection		0.100		0.480
No (n=22)	12		25	
Yes (n=8)	7		20	
Differentiation		0.178		0.219
Well (n=7)	25		37	
Moderate (n=20)	10		20	
Poor (n=2)	3		9	
Undifferentiated (n=1)	7		21	
Lymphovascular invasion[b]		0.799		0.685
No (n=20)	11		22	
Yes (n=8)	7		25	
Perineural invasion[b]		0.682		0.438
No (n=17)	12		20	
Yes (n=11)	10		25	
Transfusion		0.084		0.091
No (n=21)	12		35	
Yes (n=9)	7		18	

[a]Data unavailable for 1 patient. [b]Data unavailable for 2 patients. CA 19-9, cancer antigen 19-9.

response to cancer (16,23,25). TILs consist of functionally distinct subsets. Tumor-infiltrating $CD8^+$ CTLs and $CD4^+$ helper T lymphocytes operate as antitumor effectors and are associated with a favorable prognosis (7). Activated $CD8^+$ T-cells attack tumor cells presenting tumor-associated antigens via the peptide/major histocompatibility complex class I on the tumor cell surface (26,27). Activated $CD8^+$ CTLs express granzyme B on their surface (22,28). $CD4^+$ T-cells have a central role in initiating and maintaining the host immune response against cancer through numerous mechanisms. $CD4^+$ T-cells provide crucial help to the priming of $CD8^+$ T-cells via activation of antigen-presenting cells. Furthermore, $CD4^+$ T-cells secrete cytokines required for maintaining $CD8^+$ T-cell function and proliferation, and can also inhibit tumor growth directly or indirectly. In addition, $CD4^+$ T-cells promote B-cell activation (29,30). Tregs, which make up a small fraction (5-6%) of the overall $CD4^+$ T-cell population, reduce the antitumor immune response by suppressing effector T-cells and the production of several immunosuppressive cytokines, including interleukin-10 and transforming growth factor-β (31,32). Tregs have been shown to adversely affect patient survival (6,9,13,14,18). The role of Tregs in PDAC is well-understood, and circulating Tregs and PDAC tissue-specific Treg cells are significantly higher in patients with pancreatic cancer compared with healthy controls (24,33-37). Furthermore, the presence of Tregs in tumor tissue correlates with the stage and progression of pancreatic cancer (24,33-37).

Table II. Univariate survival analysis according to TIL subset counts.

TIL subset	Disease-free survival		Overall survival	
	Months (median)	P-value	Months (median)	P-value
Absolute count				
$CD3^+$		0.290		0.643
Low (<256, n=15)	11		21	
High (≥256, n=15)	13		25	
$CD4^+$		0.063		0.164
Low (<160, n=15)	8		18	
High (≥160, n=15)	25		37	
$CD8^+$		0.116		0.208
Low (<115, n=15)	8		18	
High (≥115, n=15)	13		35	
Granzyme B^+		0.023[a]		0.084
Low (<24, n=15)	10		18	
High (≥24, n=15)	25		37	
$Foxp3^+$		0.538		0.603
Low (<28, n=15)	12		22	
High (≥28, n=15)	10		23	
Relative ratio				
$Foxp3^+/CD3^+$		0.408		0.421
Low (<0.111, n=15)	13		29	
High (≥0.111, n=15)	10		20	
$Foxp3^+/CD4^+$		0.104		0.094
Low (<0.169, n=15)	14		37	
High (≥0.169, n=15)	10		18	
$Foxp3^+/CD8^+$		0.173		0.124
Low (<0.026, n=15)	13		37	
High (≥0.026, n=15)	10		18	
$Foxp3^+$/granzyme B^+		0.008[a]		0.003[a]
Low (<0.110, n=15)	25		47	
High (≥0.110, n=15)	8		17	

[a]P<0.05. CD, cluster of differentiation; Foxp3, forkhead box P3; TIL, tumor-infiltrating lymphocyte.

Table III. Multivariate survival analysis according to TIL subset counts.

TIL	Disease-free survival			Overall survival		
	Exp(B)	95% CI	P-value	Exp(B)	95% CI	P-value
$CD4^+$ (low vs. high)	1.738	0.725-4.167	0.215	1.617	0.684-3.825	0.273
Granzyme B^+ (low vs. high)	1.405	0.485-4.068	0.531	0.979	0.331-2.895	0.970
$Foxp3^+$/granzyme B^+ (high vs. low)	3.060	1.259-7.436	0.014[a]	3.580	1.460-8.780	0.005[a]

[a]P<0.05. CD4, cluster of differentiation 4; 95% CI, 95% confidence interval; Foxp3, forkhead box P3; TIL, tumor-infiltrating lymphocyte.

Fukunaga *et al* (25) reported that the presence of $CD4^+$ T-cells together with $CD8^+$ T-cells was negatively correlated with tumor depth and tumor-node-metastasis stage in pancreatic cancer. Furthermore, in multivariate analyses, they demonstrated that a $CD4^+/CD8^+$ status was an independent favorable prognostic factor (25). Ino *et al* (35) reported

Table IV. Association between tumor-infiltrating lymphocyte subsets and clinicopathological factors.

	CD4$^+$			Granzyme B$^+$			Foxp3$^+$/granzyme B$^+$		
Characteristic	Low	High	P-value	Low	High	P-value	Low	High	P-value
Age, years			0.713			0.269			0.713
<59 (n=13)	6 (40.0)	7 (46.7)		8 (53.3)	5 (33.3)		7 (46.7)	6 (40.0)	
≥59 (n=17)	9 (60.0)	8 (53.3)		7 (46.7)	10 (66.7)		8 (53.3)	9 (60.0)	
Gender			0.427			0.427			1.000
Male (n=21)	9 (60.0)	12 (80.0)		9 (60.0)	12 (80)		10 (66.7)	11 (73.3)	
Female (n=9)	6 (40.0)	3 (20.0)		6 (40.0)	3 (20)		5 (33.3%)	4 (26.7)	
Tumor size, cm			0.715			0.715			0.715
<3.5 (n=15)	8 (53.3)	7 (46.7)		7 (46.7)	8 (53.3)		8 (53.3)	7 (46.7)	
≥3.5 (n=15)	7 (46.7)	8 (53.3)		8 (53.3)	7 (46.7)		7 (46.7)	8 (53.3)	
Nodal stage			0.713			0.713			0.269
N0 (n=17)	8 (53.3)	9 (60.0)		8 (53.3)	9 (60.0)		10 (66.7)	7 (46.7)	
N1 (n=13)	7 (46.7)	6 (40.0)		7 (46.7)	6 (40.0)		5 (33.3)	8 (53.3)	
Combined resection			1.000			1.000			0.682
No (n=22)	11 (73.3)	11 (73.3)		11 (73.3)	11 (73.3)		12 (80.0)	10 (66.7)	
Yes (n=8)	4 (26.7)	4 (26.7)		4 (26.7)	4 (26.7)		3 (20.0)	5 (33.3)	
Lymphovascular invasion[b]			0.678			1.000			1.000
No (n=20)	11 (78.6)	9 (64.3)		11 (73.3)	9 (69.2)		9 (69.2)	11 (73.3)	
Yes (n=8)	3 (21.4)	5 (35.7)		4 (26.7)	4 (46.2)		4 (30.8)	4 (26.7)	
Perineural invasion[b]			0.699			0.488			0.934
No (n=17)	8 (57.1)	9 (64.3)		10 (66.7)	7 (53.3)		8 (61.5)	9 (60.0)	
Yes (n=11)	6 (42.9)	5 (35.7)		5 (33.3)	6 (46.2)		5 (38.5)	6 (40.0)	
Transfusion			1.000			1.000			1.000
No (n=21)	11 (73.3)	10 (66.7)		11 (73.3)	10 (66.7)		11 (73.3)	10 (66.7)	
Yes (n=9)	4 (26.7)	5 (33.3)		4 (26.7)	5 (33.3)		4 (26.7)	5 (33.3)	
CA 19-9, U/ml[c]			0.573			0.356			0.016[a]
≤109 (n=15)	8 (57.1)	7 (46.7)		6 (42.9)	9 (60.0)		11 (73.3)	4 (28.6)	
>109 (n=14)	6 (42.9)	8 (53.3)		8 (57.1)	6 (40.0)		4 (26.7)	10 (71.4)	

Data are presented as n (%). [a]P<0.05. [b]Data unavailable for 2 patients. [c]Data unavailable for 1 patient. CA 19-9, cancer antigen 19-9; CD4, cluster of differentiation 4; Foxp3, forkhead box P3.

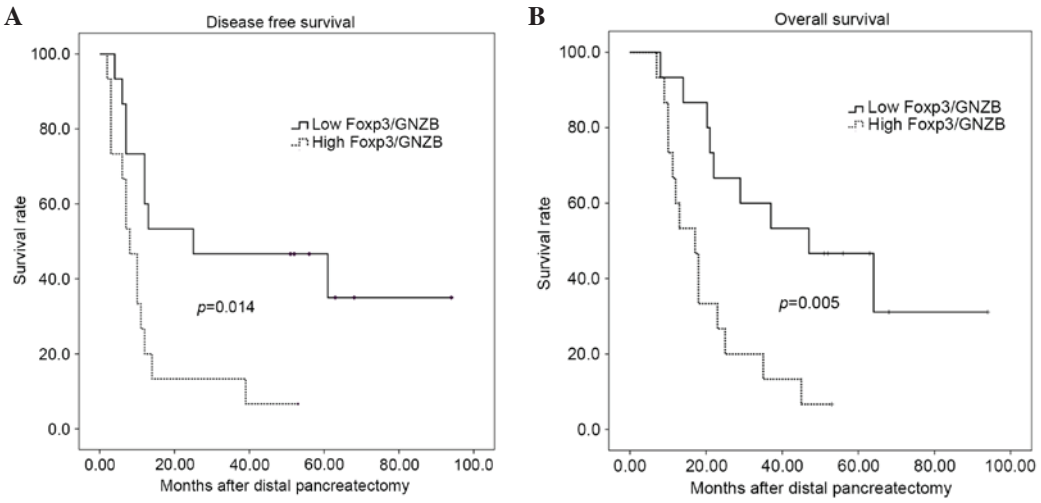

Figure 2. Kaplan-Meier analysis of (A) disease-free survival and (B) overall survival according to the Foxp3$^+$/granzyme B$^+$ ratio (low vs. high). The low Foxp3$^+$/granzyme B$^+$ ratio group showed favorable survival outcomes in terms of disease-free (P=0.014) and overall (P=0.005) survival. Foxp3, forkhead box P3; GNZB, granzyme B.

that higher levels of tumor-infiltrating $CD4^+$ and $CD8^+$ T-cells were significantly associated with a longer survival in patients with PDAC. In the present study, patients with higher $CD4^+$ T-cell counts had longer DFS and OS times, but the results did not reach statistical significance. High $CD8^+$ T-cell counts were also associated with longer DFS and OS times, but the trend also failed to reach statistical significance.

Granzyme B is exclusively expressed on the surface of activated $CD8^+$ CTLs (38). Activated CTLs (granzyme B^+) have been identified as a favorable prognostic factor in various cancers (22,28,39-41); however, their role in PDAC is unknown. In the present study, patients with high granzyme B^+ CTL counts showed significantly improved DFS (25 vs. 10 months; P=0.023) and longer OS in the univariate survival analysis (37 vs. 18 months; P=0.084).

The balance between effector T-cells ($CD4^+$, $CD8^+$ and granzyme B^+ T-cells) and Tregs may more effectively reflect prognosis than absolute counts alone. A ratio of high effector cell-to-low Treg density has been reported as a promising independent predictor for prognosis in various tumors (9,11,14,22,23,42). Accordingly, the present study analyzed the ratios of $Foxp3^+/CD4^+$, $Foxp3^+/CD8^+$ and $Foxp3^+$/granzyme B^+ as prognostic factors on the basis that they were more representative of the biological characteristics of TILs. The univariate survival analysis according to the relative ratios of TIL subsets indicated that patients with a low ratio of $Foxp3^+$/granzyme B^+ had a significantly improved DFS (25 vs. 8 months; P=0.008) and OS (47 vs. 17 months; P=0.003). In the multivariate survival analysis, a low $Foxp3^+$/granzyme B^+ ratio remained a significant independent prognostic marker with a higher hazard ratio for DFS [Exp(B), 3.060; 95% CI, 1.259-7.436; P=0.014] and OS [Exp (B), 3.580; 95% CI, 1.460-8.780; P=0.005).

The present study was limited by the small number of patients, particularly since we did not observe any statistically significant differences, even for conventional prognostic factors. However, despite the small size, the present study demonstrated that a low $Foxp3^+$/granzyme B^+ ratio predicted a significantly improved prognosis. Further studies with larger sample sizes are required to clarify the prognostic meaning of TILs in association with other clinicopathological parameters. This study also showed that low levels of CA19-9 were significantly associated with a low $Foxp3^+$/granzyme B^+ ratio.

In conclusion, the present study demonstrated that a low $Foxp3^+$/granzyme B^+ ratio was an independent favorable prognostic marker following surgical resection of left-sided PDAC. This immunological parameter may be useful for stratifying patients and planning adjuvant treatment.

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