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The Prognostic Significance of the Tumor-infiltrating Programmed Cell Death-1$^+$ to CD8$^+$ Lymphocyte Ratio in Patients with Colorectal Cancer

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Abstract. *Background/Aim: Tumor-infiltrating lymphocytes (TILs) have been reported to reflect the antitumor immunity of the host and correlate with the therapeutic outcomes and survival. Nowadays TILs are attracting attention as new biomarkers of diseases such as colorectal cancer. TILs are classified into several subsets, among which CD8$^+$ T cells directly attack cancer cells and play a central role in antitumor immunity. A high density of CD8$^+$ TILs has been reported to correlate with a better clinical outcome. Programmed cell death-1 (PD-1) is recognized to be a surface marker for dysfunction of T lymphocytes. However, the prognostic significance of PD-1$^+$ TILs remains unclear. The aim of this study was to evaluate the prognostic significance of the number of PD-1$^+$ TILs and the tumor-infiltrating PD-1$^+$ to CD8$^+$ lymphocyte ratio (PD-1/CD8 ratio) in patients with colorectal cancer (CRC). Patients and Methods: A total of 90 patients with stage II/III CRC who underwent curative surgery were enrolled in this study. Immunohistochemistry was used to assess the densities of PD-1$^+$ TILs and CD8$^+$ TILs. The PD-1/CD8 ratio was defined as the number of PD-1$^+$ TILs divided by the number of CD8$^+$ TILs. The optimum cut-off value for the number of PD-1$^+$ TILs and the PD-1/CD8 ratio was determined via a receiver operating characteristic analysis. We then assessed the prognostic significance of the number of PD-1$^+$ TILs and the PD-1/CD8 ratio. Results: The relapse-free and overall survival rates were significantly worse in the high-PD-1/CD8 ratio group than in the low-PD-1/CD8 ratio group (relapse-free survival: p=0.0257, overall survival: p=0.0363), although the number of PD-1$^+$ TILs showed no prognostic significance. Conclusion: The PD-1/CD8 ratio may, therefore, be a useful prognostic marker for stage II/III CRC. What is important for predicting the prognosis may be the PD-1/CD8 ratio rather than the absolute number of PD-1$^+$ TILs.*

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The tumor microenvironment as well as the characteristics of cancer cells influence the biological behavior of the tumor. Recently, antcancer immunity was recognized to correlate with cancer progression. Tumor-infiltrating lymphocytes (TILs) have been reported to reflect the anticancer immunity and to correlate with the therapeutic outcome and survival in patients with various types of cancer, including colorectal cancer (CRC), breast cancer, and lung cancer (1-3). Therefore, TILs are attracting attention as new biomarkers.

TILs are classified into several subsets, each with its own function. CD8$^+$ T cells directly attack cancer cells and play a central role in antitumor immunity (4, 5). Therefore, a high density of CD8$^+$ TILs has been reported to correlate with a better clinical outcome (6-8). However, the numbers of reports regarding immune escape are increasing in recent years. Continuous exposure to tumor antigens results in the exhaustion of lymphocytes, inducing dysfunction of the lymphocytes and a worse clinical outcome (9, 10). Programmed cell death-1 (PD-1), which is a cell surface receptor molecule on T cells, is a marker for dysfunction of T lymphocytes (11, 12). Programmed cell death-ligand 1 (PD-L1), which is the ligand for PD-1, is expressed on cancer cells, dendritic cells and macrophages (13). By binding PD-1, PD-L1 can deliver an inhibitory signal to PD-1$^+$ T lymphocytes, leading to immune suppression by inducing anergy, apoptosis and dysfunction of T lymphocytes (14-16). However, the prognostic significance of PD-1$^+$ TILs remains unclear.

The aim of this study was to assess the prognostic significance of the number of PD-1$^+$ TILs and the tumor-

infiltrating PD-1$^+$ to CD8$^+$ lymphocytes ratio (PD-1/CD8 ratio) in patients with CRC.

Patients and Methods

Patients. A total of 90 patients with stage II/III CRC were enrolled in this study. All patients underwent potentially curative surgery for CRC at the Department of Surgical Oncology of Osaka City University between 2007 and 2008. Patients who received preoperative therapy were excluded from this study. The indications for adjuvant chemotherapy were high-risk stage II or stage III disease. The presence of T4, lymphatic involvement, venous involvement, or high-grade histology was defined as high-risk stage II disease. Some patients did not undergo adjuvant chemotherapy because of their performance status or their wishes. As adjuvant chemotherapy, patients received a 5-fluorouracil based regimen. The resected specimens were pathologically classified according to the seventh edition of the UICC TNM classification of malignant tumors (17). All patients were followed-up regularly with physical and blood examinations, including measurements of the levels of tumor markers, such as carcinoembryonic antigen (CEA) and mandatory screening using colonoscopy and computed tomography until December 2016 or death. This research conformed to the provisions of the Declaration of Helsinki. All patients were informed of the investigational nature of this study and provided their written informed consent. This retrospective study was approved by the ethics committee of Osaka City University (approval No. 926).

Immunohistochemistry staining for PD-1 and CD8. Surgically-resected specimens were retrieved to perform the immunohistochemistry. Sections 4 μm in thickness were deparaffined and rehydrated and then subjected to endogenous peroxidase blocking in 1% H_2O_2 solution in methanol for 15 min. Antigen retrieval was performed by autoclaving the sections at 105˚C for 10 min, each in Dako Target Retrieval Solution (Dako, Glostrup, Denmark). Serum blocking was performed with 10% normal rabbit serum (Nichirei, Tokyo, Japan) for 10 min. After H_2O_2 and serum blocking, the slides were incubated with primary monoclonal mouse anti-human antibody against PD-1 (NAT105, 1:50 dilution; Abcam, Cambridge, UK) at 4˚C overnight, or CD8 (C8/144B, 1:100 dilution; Dako, Glostrup, Denmark) at room temperature for 30 min. The secondary antibody was biotin-labeled rabbit anti-mouse IgG (1:500; Nichirei, Tokyo, Japan). Detection was performed with a DAB kit (Histofine simple stain kit; Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin.

Immunohistochemical evaluation. An immunohistochemical evaluation was carried out by two independent pathologists who were blinded to clinical information. The number of immunoreactive lymphocytes at the invasive margin was counted with a light microscope in a randomly selected field at a magnification of 400-fold (Figure 1). The mean of values obtained in five different areas was used for the data analysis. The PD-1/CD8 ratio was defined as the number of PD-1$^+$ TILs divided by the number of CD8$^+$ TILs.

Statistical analyses. A receiver operating characteristic (ROC) analysis was performed for each relapse-free and overall survival event to determine the optimum cut-off value for the number of PD-1$^+$ TILs and the PD-1/CD8 ratio. Relapse-free survival events were defined as cancer recurrence or death from any cause, and overall survival events were defined as death from any cause. The duration of the survival was calculated using the Kaplan-Meier method. Differences in the survival curves were assessed using the log-rank test. A multivariate analysis was performed according to the Cox proportional hazard model. All of the statistical analyses were conducted using the SPSS version 19.0 statistical software (IBM, Armonk, NY, USA). *p*-Values of <0.05 were considered to indicate statistical significance.

Table I. *Patients characteristics*.

Gender	
Male	49
Female	41
Age (years)	
Median (range)	65 (26-81)
Location of primary tumor	
Right side	20
Left side	70
Tumor depth	
T1-3	57
T4	33
Tumor diameter (cm)	
≥5	59
5>	31
Histological type	
Well, Moderately	79
Poorly, Mucinous	11
Lymphatic involvement	
Negative	18
Positive	72
Venous involvement	
Negative	71
Positive	19
Lymph node metastases	
Negative	47
Positive	43
CEA (ng/ml)	
≥5	57
5>	33
Adjuvant chemotherapy	
Absent	27
Present	63
Number of PD-1$^+$ lymphocyte (/field)	
Median (range)	1.5 (0-22.8)
Number of CD8$^+$ lymphocyte (/field)	
Median (range)	7.5 (0.4-37.8)

CEA: Carcinoembryonic antigen; PD-1: programmed cell death-1.

Results

Patient characteristics. The patient characteristics are listed in Table I. The distribution of TNM stage was stage II in 47 patients and stage III in 43 patients. The median number of PD-1$^+$ lymphocyte was 1.5, with a range of 0-22.8. The median number of CD8$^+$ TILs was 7.5, with a range of 0.4-37.8.

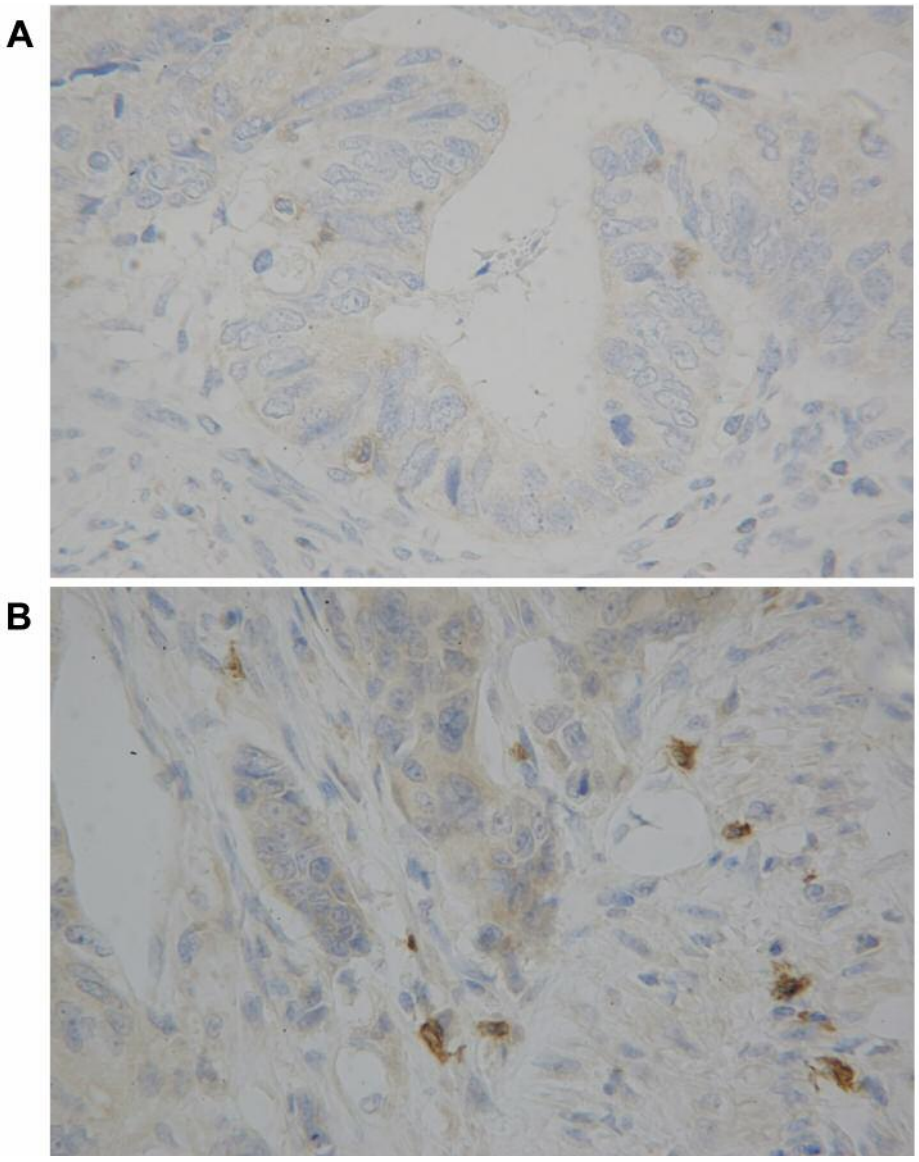


Figure 1. *Immunohistochemical staining for primary antibodies against programmed cell death-1 (A) and CD8 (B). Original magnification, ×400.*

Cut-off value of the number of PD-1$^+$ TILs and the PD-1/CD8 ratio. Of the 90 patients with stage II/III CRC, 19 (21.1%) developed disease recurrence, and 12 (13.3%) died within the follow-up period. A ROC analysis showed that the optimal cut-off value of the number of PD-1$^+$ TILs was 0.9 for the relapse-free survival (sensitivity of 75.0%, specificity of 37.8%) and 1.9 for the overall survival (sensitivity of 58.3%, specificity of 59.0%) (Figure 2). Furthermore, the ROC analysis showed that the optimum cut-off value of the PD-1/CD8 ratio was 0.22 for the relapse-free survival (sensitivity of 76.2%, specificity of 52.2%) and 0.548 for the overall survival (sensitivity of 58.3%, specificity of 69.2%) (Figure 3).

Survival analyses according to the number of PD-1$^+$ TILs. The number of PD-1$^+$ TILs showed no prognostic significance (Figure 4).

Survival analyses according to the PD-1/CD8 ratio. The relapse-free survival rate was significantly worse in the high-PD-1/CD8 ratio group than in the low-PD1/CD8 ratio group (p=0.0257) (Figure 5A). Furthermore, the overall survival

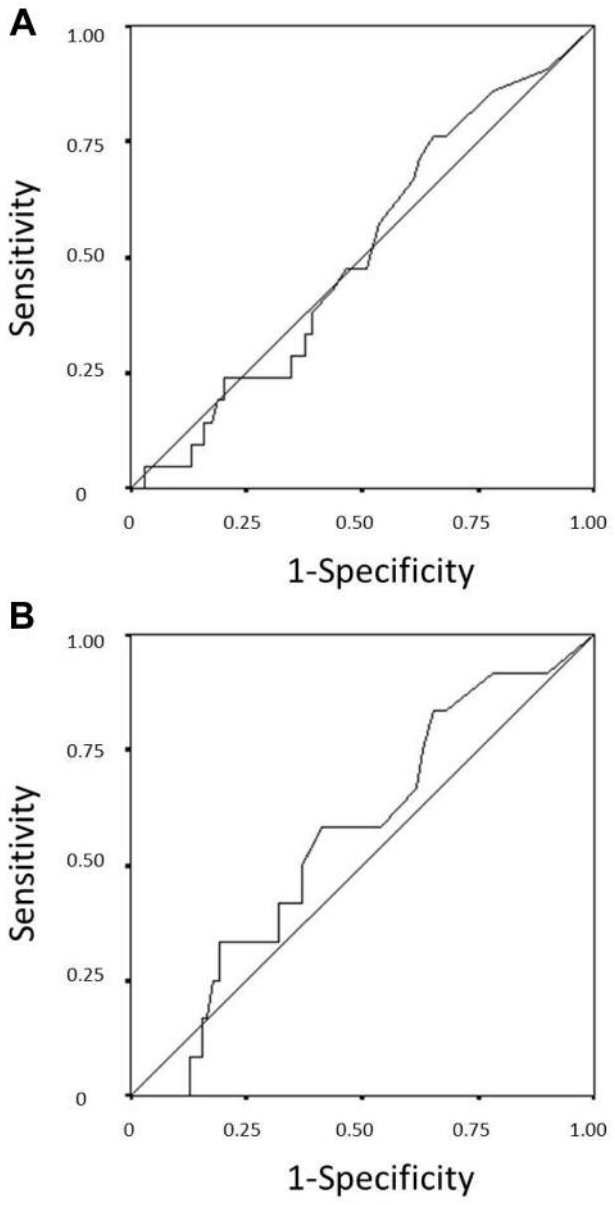


Figure 2. *ROC curve of the number of PD-1⁺ TILs for the survival status. (A) Relapse-free survival status. Area under the curve=0.508, 95% confidence interval=0.374-0.643, p=0.909. (B) Overall survival status. Area under the curve=0.562, 95% confidence interval=0.401-0.724, p=0.487.*

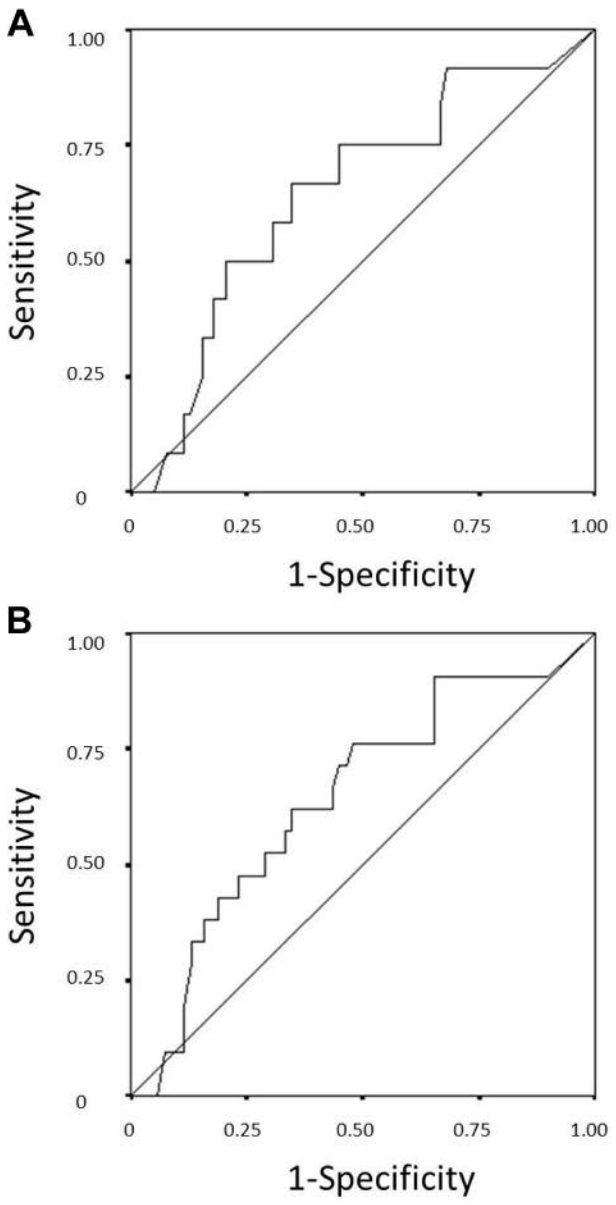
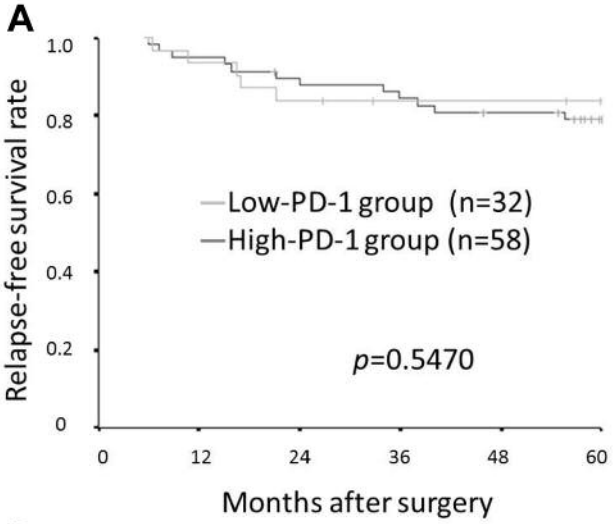


Figure 3. *ROC curve of the PD-1/CD8 ratio for the survival status. (A) Relapse-free survival status. Area under the curve=0.643, 95% confidence interval=0.509-0.778, p=0.048. (B) Overall survival status. Area under the curve=0.646, 95% confidence interval=0.481-0.840, p=0.105.*

rate was also significantly worse in the high-PD1/CD8 ratio group than in the low-PD1/CD8 ratio group (p=0.0363) (Figure 5B).

Prognostic factors influencing the survival. The correlations between the progression-free survival and the clinicopathological factors are shown in Table II. According to the results of a univariate analysis, the progression-free survival showed significant relationships with the PD-1/CD8 ratio (p=0.034), lymph node metastasis (p=0.043), and CEA (p=0.046). A multivariate analysis indicated that the PD-1/CD8 ratio (HR: 3.214; 95% CI: 1.123-9.196; p=0.030) and CEA (HR: 2.682; 95% CI: 1.090-6.601; p=0.032) were independent prognostic factors for the progression-free survival.



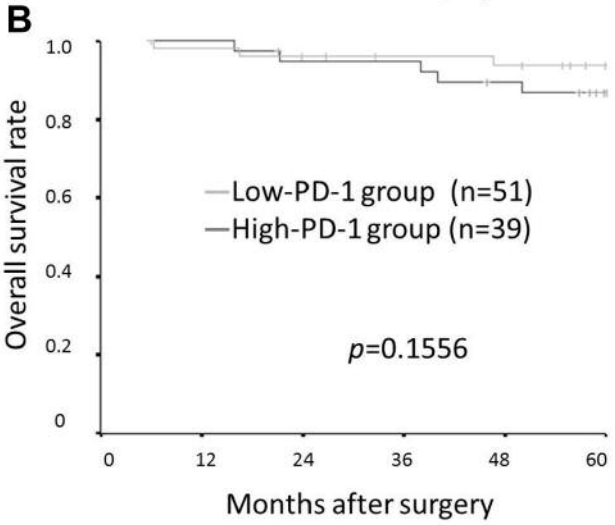
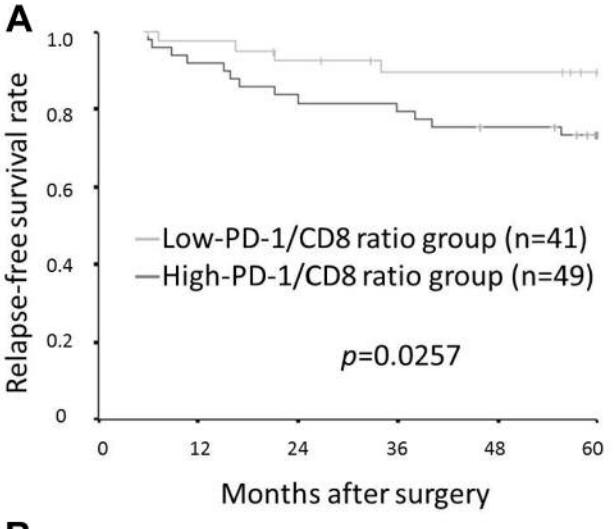


Figure 4. *Kaplan-Meier survival curves for the relapse-free (A) and overall survival (B) according to the number of PD-1$^+$ TILs. The number of PD-1$^+$ TILs showed no prognostic significance.*



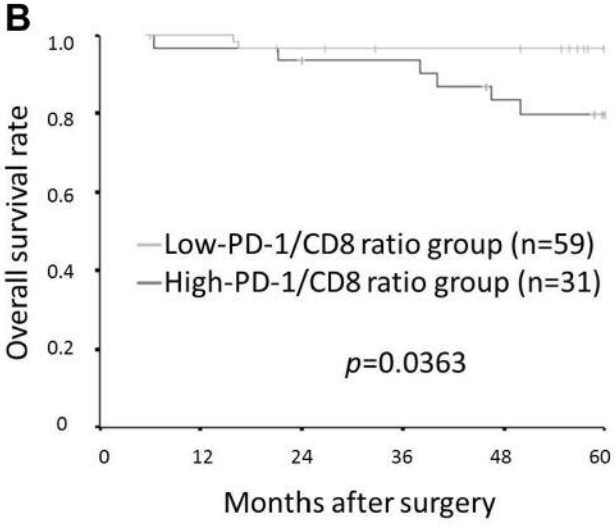


Figure 5. *Kaplan-Meier survival curves according to the PD-1/CD8 ratio. (A) The relapse-free survival rate was significantly worse in the high-PD-1/CD8 ratio group than in the low-PD-1/CD8 ratio group (p=0.0257). (B) The overall survival rate was significantly worse in the high-PD-1/CD8 ratio group than in the low-PD-1/CD8 ratio group (p=0.0363).*

The correlations between the overall survival and the clinicopathological factors are shown in Table III. According to the results of a univariate analysis, the overall survival showed significant relationships with the PD-1/CD8 ratio (p=0.049), venous involvement (p=0.004), and CEA (p=0.044). A multivariate analysis indicated that the PD-1/CD8 ratio (HR: 4.809; 95% CI: 1.313-17.606; p=0.018), venous involvement (hazard ratio: 5.371; 95% confidence interval: 1.582-18.236; p=0.007) and CEA (HR: 3.712; 95% CI: 1.060-12.994; p=0.040) were independent prognostic factors for the overall survival.

Discussion

In this study, a high-PD-1/CD8 ratio was found to be associated with poor relapse-free and overall survival rates, although the number of PD-1$^+$ TILs showed no prognostic significance. To our knowledge, this is the first report to investigate the prognostic significance of the PD-1/CD8 ratio in patients with CRC.

CD8$^+$ T cells are cytotoxic T lymphocytes that directly attack cancer cells and play a central role in anti-cancer immunity (5). A previous study uncovered substantial

Table II. *The correlations between the progression-free survival and the clinicopathological factors.*

	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-Value	HR	95%CI	*p*-Value
Tumor depth (T4 *vs.* T1-3)	1.194	0.493-2.893	0.694			
Tumor diameter (>5 cm *vs.* ≤5 cm)	1.179	0.492-2.828	0.711			
Histological type (Poorly, Mucinous *vs.* Well, Moderately)	2.552	0.926-7.029	0.070			
Location of the tumor (Right side *vs.* Left side)	1.259	0.442-3.752	0.679			
Lymphatic involvement (Positive *vs.* Negative)	5.733	0.774-43.067	0.087			
Venous involvement (Positive *vs.* Negative)	1.853	0.712-4.827	0.206			
Lymph node metastasis (Positive *vs.* Negative)	2.560	1.031-6.357	0.043	1.659	0.641-4.289	0.297
CEA (>5 ng/ml *vs.* ≤5 ng/ml)	2.418	1.015-5.760	0.046	2.682	1.090-6.601	0.032
Adjuvant chemotherapy (Negative *vs.* Positive)	1.657	0.602-4.556	0.328			
The PD-1/CD8 ratio (>0.220 *vs.* ≤0.220)	2.982	1.089-8.165	0.034	3.214	1.123-9.196	0.030

HR: Hazard ratio; CI: confidence interval; CEA: carcinoembryonic antigen; PD-1: programmed cell death-1.

Table III. *Correlations between the overall survival and the clinicopathological factors.*

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Tumor depth (T4 *vs.* T1-3)	1.021	0.298-3.495	0.974			
Tumor diameter (>5 cm *vs.* ≤5 cm)	1.510	0.481-4.743	0.480			
Histological type (Poorly, Mucinous *vs.* Well, Moderately)	2.791	0.739-10.547	0.130			
Location of the tumor (Right side *vs.* Left side)	1.663	0.355-7.783	0.518			
Lymphatic involvement (Positive *vs.* Negative)	2.719	0.347-21.287	0.341			
Venous involvement (Positive *vs.* Negative)	5.903	1.777-19.610	0.004	5.371	1.582-18.236	0.007
Lymph node metastasis (Positive *vs.* Negative)	3.596	0.971-13.319	0.055			
CEA (>5 ng/ml *vs.* ≤5 ng/ml)	3.454	1.034-11.530	0.044	3.712	1.060-12.994	0.040
Adjuvant chemotherapy (Negative *vs.* Positive)	2.103	0.454-9.741	0.342			
PD-1/CD8 ratio (>0.548 *vs.* ≤0.548)	3.436	1.005-11.752	0.049	4.809	1.313-17.606	0.018

HR: Hazard ratio; CI: confidence interval; CEA: carcinoembryonic antigen; PD-1: programmed cell death-1.

evidence that the density of $CD8^+$ TILs was associated with the long-term survival in patients with various types of cancer (5, 18, 19). Furthermore, the density of $CD8^+$ TILs was also reported to be associated with the therapeutic effectiveness of chemotherapy and radiotherapy (7, 19, 20).

PD-1 inhibits the immunological function of T cells by binding with its ligand, PD-L1 (14-16). Therefore, PD-1 is recognized as an immunosuppressive marker for T cells (11, 12). Previous studies have suggested that the number of $PD\text{-}1^+$ TILs is associated with the survival in patients with breast cancer and gastric cancer (21, 22). However, others have suggested that the number of $PD\text{-}1^+$ TILs was not associated with the survival in patients with nasopharyngeal carcinoma (23). In the present study, there were no relationships between the number of $PD\text{-}1^+$ TILs and survival. In studies regarding anti-cancer immunity, it may be necessary to consider the balance between the functional effector T cells and immune escape (24). In clinical practice, the efficacy of anti-PD-1 antibody has been reported to correlate with the density of $CD8^+$ TILs in patients with non-small cell lung cancer (25).

Several limitations associated with the present study warrant mentioning. First, we evaluated a relatively small number of patients, and the study design was retrospective. Second, double immunofluorescence staining was not performed in this study. Therefore, the expression rate of PD-1 in $CD8^+$ TILs was not evaluated in this study. However, we did evaluate the number of $PD\text{-}1^+$ TILs among $CD4^+$ TILs. According to the previous reports, which lymphocyte subsets express PD-1 is important (10). Third, we did not evaluate the expression of PD-L1 in cancer cells, dendritic cells, or macrophages in this study. When considering the interaction between PD-1 and PD-L1, not only the expression of PD-1 but also the expression of PD-

L1 may need to be taken into consideration. Fourth, as has been argued in previous reports (26, 27), the results may change with each measurement due to heterogeneity with regard to the location of TILs. A new method able to evaluate the immune status of the whole tumor accurately should be developed.

Currently, the clinical application of immunocheckpoint inhibitors is being explored, with some progress reported. Success in the development of new drugs for preventing immunosuppression will lead to the improvement of the survival outcomes in patients with various types of malignancies.

Conclusion

In conclusion, the PD-1/CD8 ratio may be a useful prognostic marker for stage II/III CRC. What is important for predicting the prognosis may be the PD-1/CD8 ratio rather than the absolute number of PD-1$^+$ TILs.

Conflicts of Interest

The Authors declare that they have no competing interests.

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