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## ***Helicobacter pylori* Infection Affects the Tumor Immune Microenvironment of Esophageal Cancer Patients**

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**Abstract.** *Background/Aim:* We herein examined T cell immunity in esophageal cancer patients with and without *Helicobacter pylori* infection to establish a foundation for immunotherapeutic strategies targeting esophageal cancer in the presence of *H. pylori* infection. *Materials and Methods:* Twenty-six patients with esophageal squamous cell carcinoma between 2015 and 2017 were enrolled in the present study. Serum antibodies against *H. pylori* were measured. Fresh tumor tissues were obtained by endoscopic biopsy or from surgical resection. A cell suspension of these tissues was subjected to a flow cytometric analysis. *Results:* Among the 26 patients analyzed, 10 (38.5%) were seropositive for *H. pylori*. The flow cytometric analysis of tumor-infiltrating lymphocytes revealed that the percentage of CD103<sup>+</sup>CD4<sup>+</sup> T cells in esophageal tumors was significantly lower in *H. pylori*-positive patients than in *H. pylori*-negative patients ( $p=0.0105$ ). Conversely, the percentage of CD45RA-CD25hi effector Treg cells in esophageal tumors was significantly higher in *H. pylori*-positive patients than in *H. pylori*-negative patients ( $p=0.0022$ ), indicating an immunosuppressive tumor microenvironment in the former. Following neoadjuvant chemotherapy, the number of CD45RA-CD25hi effector Treg cells decreased ( $p=0.0248$ ). *Conclusion:* The tumor immune microenvironment of esophageal cancer patients with *H. pylori* infection exhibited an immunosuppressive phenotype. The targeting of Treg cells has potential in immunotherapy for this patient population.

*Helicobacter pylori* infection in the stomach, as well as pre-existing *H. pylori* infection, increases the risk of gastric cancer (1-4). Immunological reactions are critical for infection and cancer, including *H. pylori* and gastric cancer. Regarding T cell immunity, previous studies indicated that regulatory T (Treg) cells were abundant in the gastric tumor tissues of patients with *H. pylori* infection (5-8). In a recent study that performed multiplex immunohistochemistry on 170 patients with gastric cancer, gastric tumors from *H. pylori*-positive patients showed higher densities of PD-L1<sup>+</sup> cells and non-exhausted CD8<sup>+</sup> T cells, indicating a “hot” tumor immune microenvironment (9).

The efficacy of immunotherapy for *H. pylori*-positive gastric cancer patients is controversial. A retrospective analysis of 215 patients with gastric cancer treated with immune checkpoint inhibitors indicated that progression-free survival (PFS) was significantly shorter in *H. pylori*-positive patients than in *H. pylori*-negative patients (10). In contrast, a retrospective study on 636 patients with Epstein-Barr virus-negative microsatellite-stable gastric cancer treated with anti-PD-1/PD-L1 therapy showed that PFS was significantly longer in *H. pylori*-positive patients than in *H. pylori*-negative patients (9). This study also examined 319 patients with esophageal squamous cell carcinoma and found that PFS was significantly shorter in *H. pylori*-positive patients than in *H. pylori*-negative patients (9). In contrast to gastric cancer, limited information is currently available on the relationship between *H. pylori* infection and immunological aspects in patients with esophageal cancer.

Gastroesophageal reflux disease is a common gastrointestinal disorder in which gastric contents flow back into the esophagus. In a previous study that examined the histological progression of esophagitis in animal tissue, reflux esophagitis started with lymphocytic infiltration of the submucosa, which then progressed to the epithelial surface (11). Esophageal biopsies of patients with reflux

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*Key Words:* *Helicobacter pylori*, T cells, esophageal cancer.

esophagitis after the discontinuation of proton pump inhibitors showed significant increases in intraepithelial lymphocytes, which were predominantly T cells (12). These findings suggest that the refluxate does not directly cause cell death, but instead stimulates inflammatory reactions involving T cell immunity in the pathogenesis of reflux esophagitis (13). In contrast to these findings on reflux esophagitis, the involvement of T cell immunity as well as *H. pylori* infection in the pathogenesis of esophageal cancer has yet to be elucidated.

Therefore, we herein investigated T cell immunity in esophageal cancer patients with or without *H. pylori* infection to provide the rationale of immunotherapy for esophageal cancer with *H. pylori* infection.

## Materials and Methods

**Patient selection and data collection.** Patients with esophageal squamous cell carcinoma at Osaka University Hospital between 2015 and 2017 were enrolled in the present study. Data were collected from medical charts. Serum antibodies to *H. pylori* were measured using a *H. pylori* IgG ELISA Kit (E-plate, Eiken, Japan). Fresh tumor tissues were obtained by endoscopic biopsy or from surgical resection.

**Sample preparation.** Tumor tissues were minced and digested to a single cell suspension using a Tumor Dissociation Kit for humans (Miltenyi Biotec, Bergisch Gladbach, Germany) and gentleMACS Dissociator (Miltenyi Biotec) according to the manufacturer's instructions. The cell suspension was subjected to a flow cytometric analysis.

**Flow cytometry analysis.** A flow cytometric analysis was performed on BD LSRII Fortessa X-20 (Becton Dickinson, Franklin Lakes, NJ, USA) with FACSDiva software (Becton Dickinson). Surface marker staining was conducted after the FcR block using the Human TruStain FcX Fc Receptor blocking solution (BioLegend, San Diego, CA, USA). Cells were incubated with the Zombie NIR Fixable Viability Kit (BioLegend). The antibodies used for surface marker staining are shown in Table I. The gating strategy of the flow cytometric analysis is shown in Figure 1.

**Statistical analysis.** A two-tailed Student's *t*-test was used to examine the significance of differences between samples. Overall survival (OS) was analyzed by using the Kaplan-Meier method with differences between groups being calculated using the Log-rank test. OS was measured from the date of surgery to death. The cut-off date for data collection was March 1, 2024. OS for living patients was censored at the date of last known contact. A *p*-value <0.05 was considered to be significant. These evaluations were performed using JMP software (SAS Institute, Inc.).

**Ethical considerations.** The study protocol was approved by the Institutional Ethics Committee of Osaka University Hospital (IRB number 13266). Written informed consent was obtained from participants before their inclusion in the study. The present study was conducted according to the principles of the Declaration of Helsinki.

Table I. Antibody list for the flow cytometry analysis.

Antigen	Clone	Manufacturer
CD45	H130	BD Biosciences
CD3	UCHT1	BD Biosciences
CD4	RPA-T4	BD Biosciences
CD8	RPA-T8	BD Biosciences
CD45RA	cH100	BD Biosciences
CD25	cBC96	BD Biosciences
PD-1	EH12	BD Biosciences
CD103	Ber-ACT8	BD Biosciences

## Results

**Baseline patient characteristics.** Baseline patient characteristics are shown in Table II. Among the 26 patients with esophageal squamous cell carcinoma enrolled in this study, 10 (38.5%) were seropositive for *H. pylori*. There were no significant differences in age, sex, or clinical stages between the *H. pylori*-seropositive and -seronegative groups. OS after surgery did not significantly differ between the two groups (Figure 2).

**Effects of *H. pylori* infection on the tumor immune microenvironment of esophageal cancer patients.** To investigate the effects of *H. pylori* infection on the tumor immune microenvironment of esophageal cancer patients, a flow cytometric analysis of esophageal tumor tissues from *H. pylori*-positive and -negative patients was performed. Esophageal tumor tissues were obtained by biopsy before neoadjuvant chemotherapy. The flow cytometric analysis showed that the percentage of PD-1<sup>+</sup>CD8<sup>+</sup> T cells in esophageal tumors was slightly lower in *H. pylori*-positive patients than in *H. pylori*-negative patients (*p*=0.1784) (Figure 3A). The percentage of CD103<sup>+</sup>CD4<sup>+</sup> T cells in esophageal tumors was lower in *H. pylori*-positive patients than in *H. pylori*-negative patients (*p*=0.0105) (Figure 3B). On the other hand, the percentage of CD45RA-CD25hi effector Treg cells in esophageal tumors was higher in *H. pylori*-positive patients than in *H. pylori*-negative patients (*p*=0.0022) (Figure 3C). These results on T cell profiles indicated the immune-inactive tumor microenvironment of esophageal cancer with *H. pylori* infection.

**Effects of neoadjuvant chemotherapy on the tumor immune microenvironment of esophageal cancer patients.** To examine the effects of neoadjuvant chemotherapy on the tumor immune microenvironment of esophageal cancer patients, we compared T cell profiles in esophageal tumor tissues before and after neoadjuvant chemotherapy. Esophageal tumor tissues were obtained from each patient by biopsy before neoadjuvant chemotherapy and by surgery after neoadjuvant chemotherapy.

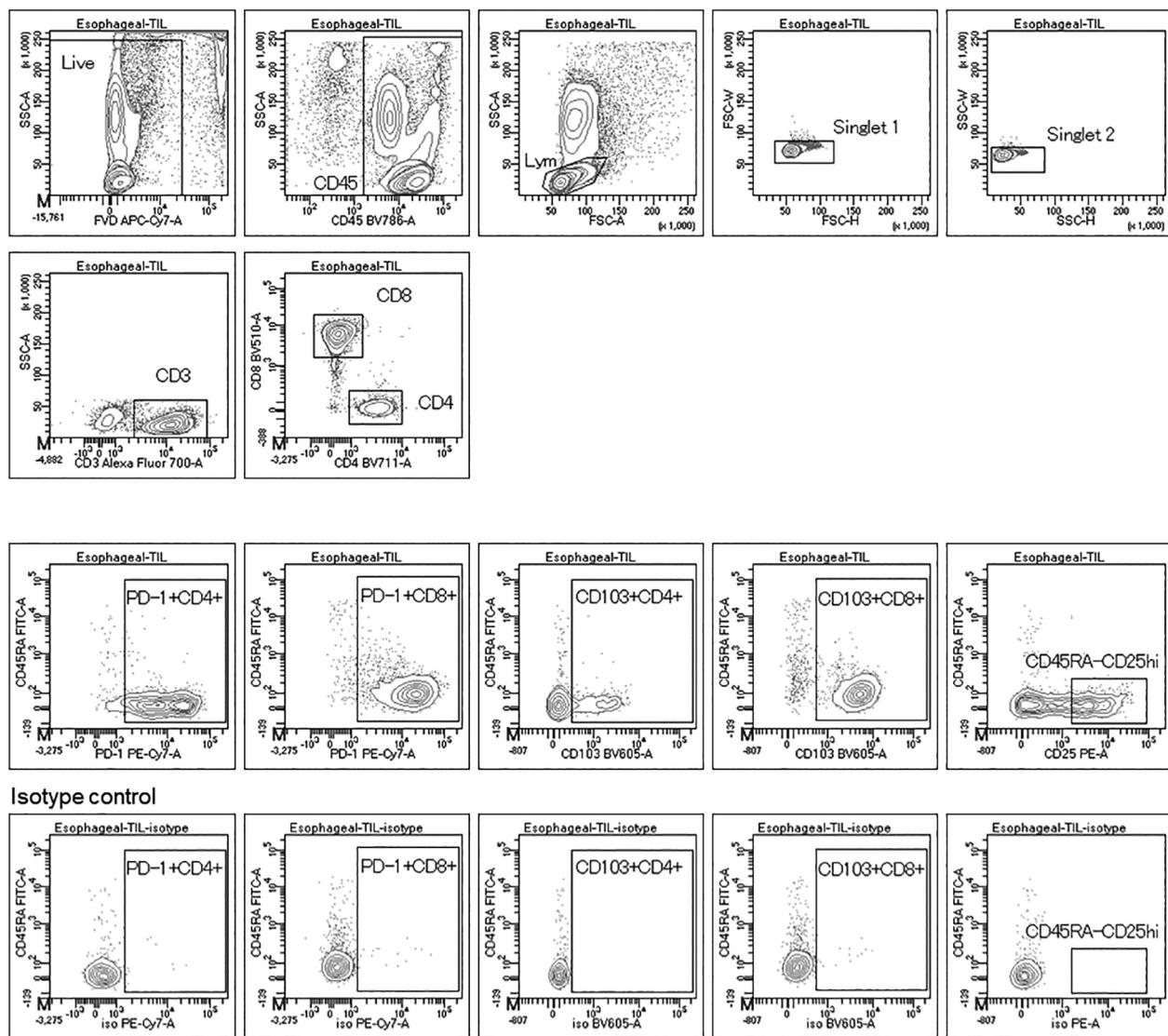


Figure 1. Gating strategy for a flow cytometric analysis of T cell subsets. The gating strategy for the flow cytometric analysis of surface marker expression is shown using BD LSRFortessa X-20 with FACSDiva software.

Nine patients received neoadjuvant chemotherapy with docetaxel, cisplatin, and 5-fluorouracil. The flow cytometric analysis of these tissues showed that the percentage of PD-1<sup>+</sup>CD4<sup>+</sup> T cells slightly increased after neoadjuvant chemotherapy ( $p=0.0752$ ) (Figure 4A). In contrast, the percentage of CD45RA-CD25hi effector Treg cells decreased after neoadjuvant chemotherapy ( $p=0.0248$ ) (Figure 4B). These changes in the T cell profile suggest that neoadjuvant chemotherapy for esophageal cancer modified the tumor immune microenvironment to be favorable for anti-PD-1 immunotherapy by decreasing effector Treg cells and increasing PD-1<sup>+</sup> T cells.

## Discussion

In the present study, we found a low number of PD-1<sup>+</sup> and CD103<sup>+</sup> T cells and a high number of effector Treg cells in the tumor tissues of esophageal cancer patients with *H. pylori* infection, indicating resistance to anti-PD-1 therapy. On the other hand, neoadjuvant chemotherapy increased PD-1<sup>+</sup> T cells and decreased effector Treg cells in the tumor tissues of patients with esophageal cancer, suggesting favorable changes in the tumor immune microenvironment for anti-PD-1 therapy.

*H. pylori* infects the gastric mucosa, but not the esophageal mucosa. Therefore, the effects of *H. pylori* infection on the

Table II. Baseline characteristics of patients with esophageal squamous cell carcinoma enrolled in the present study.

	<i>H. pylori</i> -positive (n=10)	<i>H. pylori</i> -negative (n=16)	p-Value
Age, years			0.4989*
Median	67	67	
Range	63-82	52-78	
Sex, n (%)			0.3402**
Male	7 (70.0)	14 (87.5)	
Female	3 (30.0)	2 (12.5)	
Tumor location, n (%)			
Ce	1 (10.0)	0 (0)	
Ut	3 (30.0)	2 (12.5)	
Mt	3 (30.0)	11 (68.7)	
Lt	3 (30.0)	3 (18.8)	
cT, n (%)			
T1	0 (0)	0 (0)	
T2	1 (10.0)	1 (6.2)	
T3	4 (40.0)	9 (56.3)	
T4	5 (50.0)	6 (37.5)	
cN, n (%)			
N0	0 (0)	1 (6.2)	
N1	5 (50.0)	9 (56.3)	
N2	2 (20.0)	5 (31.3)	
N3	3 (30.0)	1 (6.2)	
cM, n (%)			
M0	6 (60.0)	9 (56.3)	
M1	4 (40.0)	7 (43.7)	
cStage (UICC-7 <sup>th</sup> edition), n (%)			0.6967**
I	0 (0)	0 (0)	
II	1 (10.0)	2 (12.5)	
III	6 (60.0)	12 (75.0)	
IV	3 (30.0)	2 (12.5)	

Student's *t*-test. \*\*Fisher's exact test.

tumor immune microenvironment of esophageal cancer were considered to be indirect. In a previous study, the efficacy of immunotherapy including anti-CTLA-4/PD-L1 or cancer vaccines to MC38 colon adenocarcinoma or B16-OVA melanoma-bearing mice was significantly lower in *H. pylori*-infected mice than in *H. pylori* non-infected mice (14). A decreased number and activation status of tumor-specific CD8<sup>+</sup> T cells were observed in the tumors of *H. pylori*-infected mice treated with cancer immunotherapies. Additionally, in a retrospective clinical study, *H. pylori* seropositivity was associated with shorter survival in non-small cell lung cancer (NSCLC) patients receiving anti-PD-1 therapy (14).

Previous studies demonstrated that *H. pylori* dampened the functions of dendritic cells (DCs) and promoted the generation of Treg cells (15-17). *H. pylori*-stimulated DCs retain a semi-mature phenotype and secrete low levels of proinflammatory factors. Semi-mature DCs stimulated by *H. pylori* secrete increased levels of IL-10 and TGF- $\beta$ , a process that is required

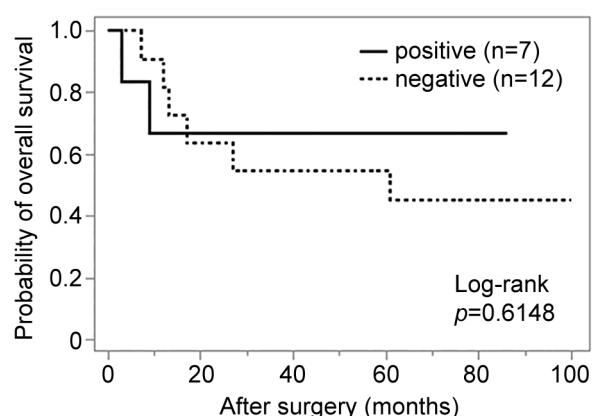


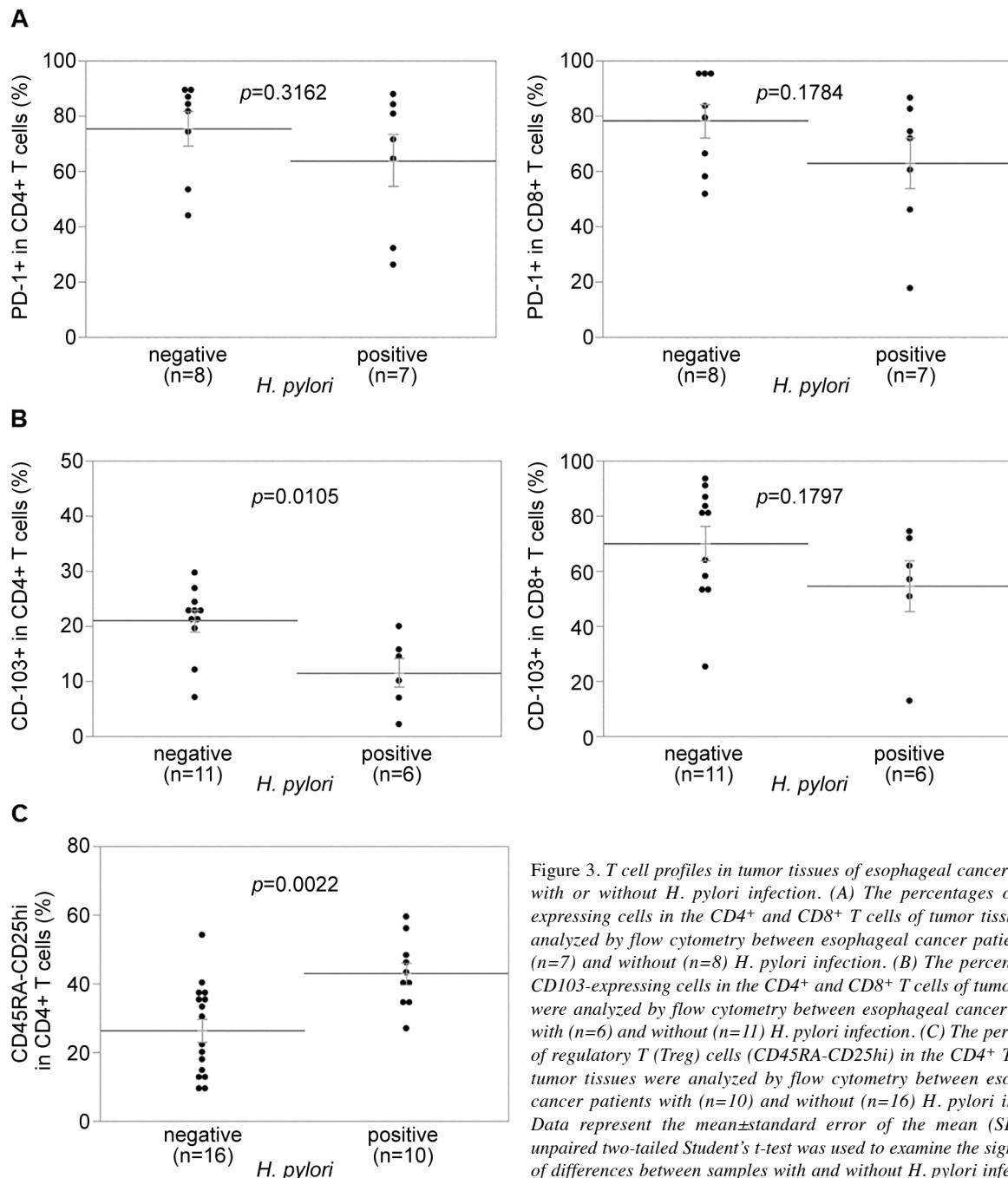
Figure 2. Kaplan-Meier curves for the overall survival of patients with esophageal squamous cell carcinoma were compared between *H. pylori*-positive (n=7) and -negative (n=12) patients.

for the differentiation of immunosuppressive Treg cells. Through lymphocyte recirculation mechanisms, these Treg cells in the gastric mucosa migrate to other lymphoid tissues in different organs to exert their immunoregulatory effects. Based on these mechanisms, *H. pylori* infection in the stomach appears to exert immunosuppressive effects on the esophageal mucosa and has been shown to exert similar effects in other diseases. Epidemiological studies and animal experiments revealed an inverse correlation between *H. pylori* infection and the onset of inflammatory bowel disease (18-20), suggesting that colonization by *H. pylori* exerts protective effects against autoimmune diseases.

**Study limitations.** The number of participants enrolled in the present study was small. There was also a limited cohort of participants in the previous study that investigated the tumor immune microenvironment of esophageal squamous cell carcinoma with neoadjuvant therapy (21). Therefore, prospective large cohort studies are needed to confirm the results obtained. The association between *H. pylori* infection and various factors, such as smoking, alcohol consumption, and genotypes warrants investigation (22). Furthermore, we did not examine the outcomes of immune checkpoint inhibitors in esophageal cancer patients with *H. pylori* infection. Our analysis of the tumor immune microenvironment in esophageal cancer patients with *H. pylori* infection suggests the unsuitability of anti-PD-1 immunotherapy. The relationship between these immune profiles and the outcomes of anti-PD-1 immunotherapy will support our discussion.

## Conclusion

The tumor immune microenvironment of esophageal cancer patients with *H. pylori* infection showed an immunosuppressive



**Figure 3.** *T cell profiles in tumor tissues of esophageal cancer patients with or without *H. pylori* infection. (A) The percentages of PD-1-expressing cells in the CD4<sup>+</sup> and CD8<sup>+</sup> T cells of tumor tissues were analyzed by flow cytometry between esophageal cancer patients with (n=7) and without (n=8) *H. pylori* infection. (B) The percentages of CD103-expressing cells in the CD4<sup>+</sup> and CD8<sup>+</sup> T cells of tumor tissues were analyzed by flow cytometry between esophageal cancer patients with (n=6) and without (n=11) *H. pylori* infection. (C) The percentages of regulatory T (Treg) cells (CD45RA-CD25hi) in the CD4<sup>+</sup> T cells of tumor tissues were analyzed by flow cytometry between esophageal cancer patients with (n=10) and without (n=16) *H. pylori* infection. Data represent the mean $\pm$ standard error of the mean (SEM). An unpaired two-tailed Student's t-test was used to examine the significance of differences between samples with and without *H. pylori* infection.*

phenotype. Treg cells in these patients have potential as targets for immunotherapy.

### Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

### Authors' Contributions

HM: Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing. KI: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization. TT: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Review & Editing. RK: Methodology, Validation,

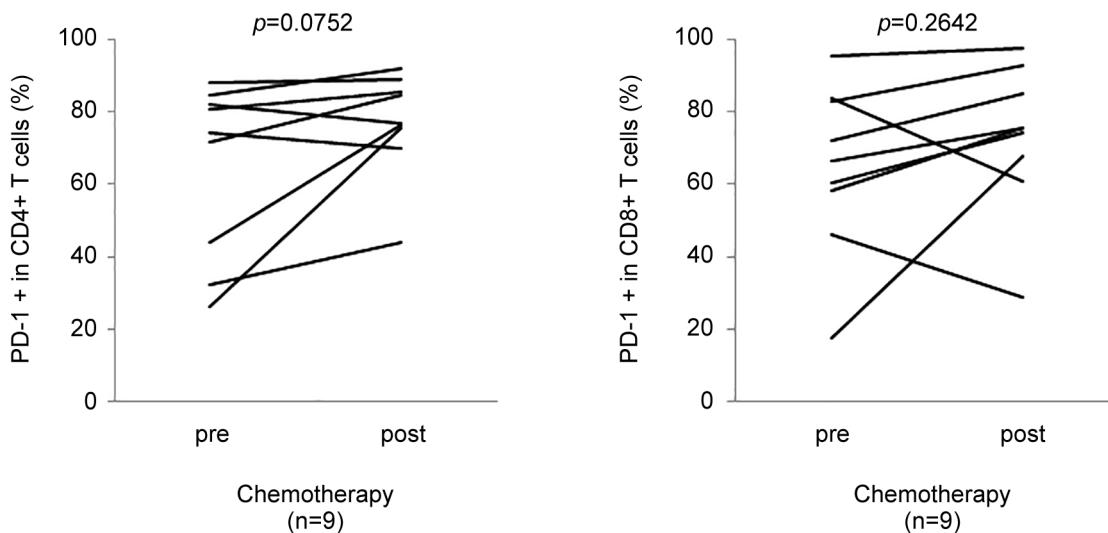
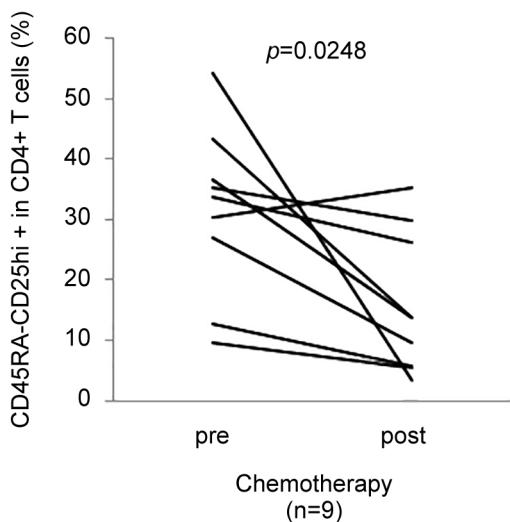
**A****B**

Figure 4. Changes in T cell profiles in tumor tissues of esophageal cancer patients before and after chemotherapy. (A) The percentages of PD-1-expressing cells in the CD4<sup>+</sup> and CD8<sup>+</sup> T cells of tumor tissues were analyzed by flow cytometry before and after chemotherapy (n=9). (B) The percentages of regulatory T (Treg) cells (CD45RA-CD25hi) in the CD4<sup>+</sup> T cells of tumor tissues were analyzed by flow cytometry before and after chemotherapy (n=9). A paired two-tailed Student's *t*-test was used to examine the significance of differences between samples before and after chemotherapy.

Formal analysis, Investigation, Data Curation, Writing – Review & Editing. SU: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – Review & Editing. TS: Writing - Review & Editing. TM: Writing – Review & Editing. HE: Supervision, Writing - Review & Editing. YD: Supervision, Writing – Review & Editing. HW: Supervision, Writing – Review & Editing.

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