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Original Article

Contrasting Prognostic Effects of Tumor-Infiltrating Lymphocyte Density in Cardia and Non-cardia Gastric Adenocarcinomas

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ABSTRACT

Purpose: This study sought to investigate the prognostic significance of tumor-infiltrating lymphocytes (TILs) in relation to tumor location within the stomach.
Materials and Methods: The densities and prognostic significance of TIL subsets were evaluated in 542 gastric cancer patients who underwent gastrectomy. Immunohistochemical staining for CD3, CD4, CD8, forkhead/winged helix transcription factor (Foxp3), and granzyme B was performed.
Results: Cardia cancer was associated with significantly lower densities of CD8 T-cells and higher densities of Foxp3 and granzyme B T-cells than non-cardia tumors. Multivariate analysis showed that advanced age (hazard ratio [HR], 1.023; 95% confidence interval [CI], 1.006–1.040), advanced T classification (HR, 2.029; 95% CI, 1.106–3.721), lymph node metastasis (HR, 3.319; 95% CI, 1.947–5.658), low CD3 expression (HR, 0.997; 95% CI, 0.994–0.999), and a high Foxp3/CD4 ratio (HR, 1.007; 95% CI, 1.001–1.012) were independent predictors of poor overall survival in cardia cancer patients. In non-cardia cancer patients, total gastrectomy (HR, 2.147; 95% CI, 1.507–3.059), advanced T classification (HR, 2.158; 95% CI, 1.425–3.266), lymph node metastasis (HR, 1.854; 95% CI, 1.250–2.750), and a low Foxp3/CD4 ratio (HR, 0.978; 95% CI, 0.959–0.997) were poor prognostic factors for survival.
Conclusions: The densities and prognostic effects of TILs differed in relation to the location of tumors within the stomach. The contrasting prognostic effects of Foxp3/CD4 ratio in cardia and non-cardia gastric cancer patients suggests that clinicians ought to consider tumor location when determining treatment strategies.

Keywords: Gastric cancer; Tumor-infiltrating lymphocytes; Tumor location; Cardia; Non-cardia

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

INTRODUCTION

Gastric cancer is a common cancer and a leading cause of cancer-related deaths worldwide [1]. Although staging remains the best tool to determine the prognosis and adjuvant treatment strategy for gastric cancer patients, outcomes can differ between individuals with the same disease stage. Studies have shown that prognosis can be influenced by both intrinsic tumor cell characteristics and tumor-associated factors, such as the tumor microenvironment and host immune status [2-4]. Tumor-infiltrating lymphocytes (TILs) are known to be prognostic factors in solid organ cancers, including gastric cancer [5,6], while regulatory T-cells are thought to induce tolerance to altered self-antigens, resulting in an immune response that is deleterious to the host [7].

While some studies have shown regulatory T-cells to be associated with poor prognoses in patients with gastric cancer [6,8-10], others have reported that regulatory T-cell expression is associated with a favorable prognosis [11,12]. It is possible that disregarding the tumor location during risk assessment could account for the disparate findings between these studies [8,9]. Indeed, recent studies have documented distinct molecular and pathophysiologic mechanisms of carcinogenesis in cardia and non-cardia cancers [13,14]. Cardia cancers have been associated with gastroesophageal reflux [15,16] and obesity [15,17]; in contrast, non-cardia cancers have been associated with decreased acidity [18,19]. Such findings suggest that a single factor such as acidity can exert opposing effects on carcinogenesis depending on the anatomical location of the tumor. Furthermore, different staging systems are used to assess cardia and non-cardia cancers (esophageal cancer and stomach cancer, respectively) [20,21]. Therefore, we sought to determine whether immune responses to gastric cancer differ according to tumor location (cardia and non-cardia regions).

This study aimed to characterize and evaluate the prognostic effects of $CD3^+$, $CD4^+$, $CD8^+$, forkhead/winged helix transcription factor $(Foxp3)^+$, and granzyme B $(GZB)^+$ T-cells in patients with gastric cancer according to the location of the tumor within the stomach.

MATERIALS AND METHODS

Study design and patients

We performed a retrospective analysis of 542 gastric cancer patients who underwent gastrectomy at Severance Hospital between September 1996 and June 2011. Cardia cancer was defined as a lesion(s) with an epicenter located within 5 cm of the esophagogastric junction [20,21]. The incidence of cardia cancer is low in Korea; therefore, we included a higher number of cardia cancer patients in the study to obtain a comparable dataset (cardia: non-cardia = 2:3). The median follow-up duration was 95 months (interquartile range: 78 months), and the final follow-up date was February 28, 2018. Patients with a history of other primary cancers and those who died of surgery-related causes were excluded; patients whose paraffin blocks were unsuitable for immunohistochemical staining and those who had missing or unclear information on the longitudinal location of their tumors were also excluded. Staging was performed according to the American Joint Committee on Cancer 8th Edition [21]. Patients with stage II/III cancer were prescribed 5-fluorouracil-based adjuvant chemotherapy, while those with stage IV cancer received palliative chemotherapy with best supportive care. Patients underwent follow-up evaluations every 3 months for 1 year, every 6 months for 2 years, and annually thereafter for the duration of the scheduled follow-up period. This study

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was approved by the Yonsei Institutional Review Board (4-2017-0753), and the requirement for informed consent was waived because of the retrospective design of the study.

TILs

Immunohistochemical (IHC) staining and quantification of TILs were performed as previously described [6]. Briefly, IHC staining was performed on paraffin-embedded cancer tissue sections that had been serially sectioned at a thickness of 4 mm after hematoxylin and eosin staining. The sections were deparaffinized, rehydrated, and treated for antigen retrieval, after which they were incubated for 60 min at room temperature with primary monoclonal antibodies against the following antigens (**Fig. 1**): CD3 (total T lymphocytes, 1:100, Labvision Corporation, Fremont, CA, USA), CD4 (helper T lymphocytes, 1:100, Novocastra, Newcastle upon Tyne, UK), CD8 (cytotoxic T lymphocytes, 1:100, Novocastra), Foxp3 (regulatory T-cells, 1:100, Abcam, Cambridge, UK), and GZB (activated cytotoxic T lymphocytes, 1:100, Labvision Corporation). Sections were then incubated with horseradish peroxidase-conjugated secondary antibodies, developed with diaminobenzidine, and counterstained with hematoxylin.

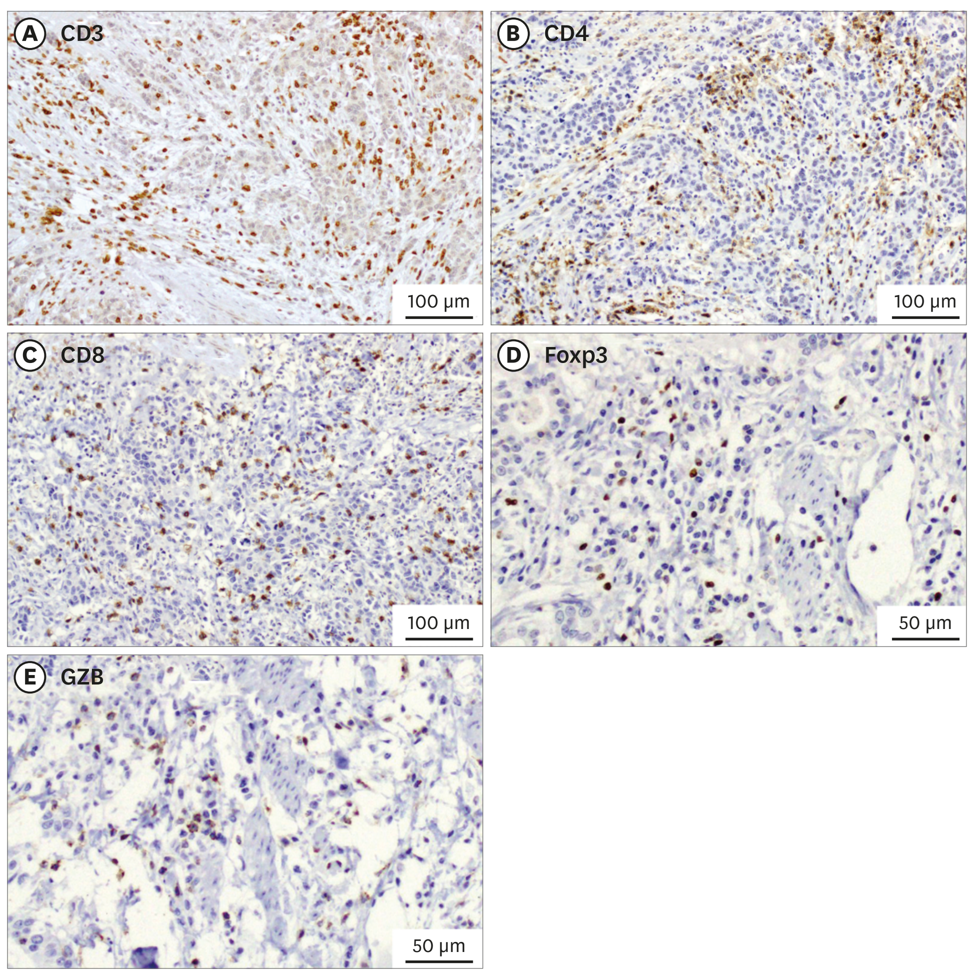

Fig. 1. Representative images of CD3, CD4, CD8, Foxp3, and granzyme B staining in gastric adenocarcinoma specimens. Immunohistochemical staining for (A) CD3 (T lymphocytes), (B) CD4 (helper T lymphocytes), (C) CD8 (cytotoxic T lymphocytes), (D) Foxp3 (regulatory T lymphocytes), and (E) GZB (activated cytotoxic T lymphocytes) in gastric adenocarcinoma specimens.
Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B.

Quantification of TILs

An experienced pathologist (SJS) who was blinded to the patient data reviewed the hematoxylin and eosin-stained slides for each case. The tumoral center, as well as the centers of four tumor quadrants drawn within the borders of each lesion, were analyzed. A representative high-power field (×400) was chosen for each area and captured as an image. Regions of necrosis or hemorrhage were avoided, as were areas of stroma with few tumor glands (less than 10% of the total area). The densities of cells with positive reactions to each antibody were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The mean numbers of positively stained cells in each area were recorded. Subsequently, the absolute numbers of positive cells per high-power field (×400) were calculated for each antigen (CD3, CD4, CD8, Foxp3, and GZB) by adding the mean numbers of positive cells in each of the five areas of interest (tumoral center and four tumor quadrants) and dividing by five.

Statistical analyses

Age, sex, body mass index, tumor size, circular location, longitudinal location, histologic type, lymphovascular invasion (LVI), perineural invasion, T and N classification, and stage were evaluated as clinical variables. Categorical data were compared using the χ^2 or Fisher's exact tests. Absolute numbers of cells positive for each stain and the relative ratio between two different stains were compared using the Student's t-test or analysis of variance (ANOVA). Median counts were used to divide patients into low- and high-density groups. The Kaplan–Meier method was used to construct survival curves, and their differences were assessed using the log-rank test. Cox proportional hazard models were applied for univariate analysis. The proportionality assumption for the Cox model was evaluated by Schoenfeld residuals and Grambsch-Therneau tests. Independent predictors of survival were assessed using multivariate Cox forward stepwise regression. Age, sex, and factors with P-values <0.10 in the univariate analysis were subjected to multivariate analysis. A 2-tailed P-value of 0.05 or less was considered statistically significant. All statistical analyses were performed using SAS software, version 9.4 (SAS Institute, Cary, NC, USA).

RESULTS

Patient demographics and TILs

Table 1 shows the clinicopathological parameters associated with the subsets of TILs stained with monoclonal antibodies against CD3, CD4, CD8, Foxp3, and GZB (**Fig. 1**). The density of $CD3^+$ cells was higher in undifferentiated histologic-type tumors (P=0.038), whereas $CD4^+$ cell density was higher in early stage lesions (P=0.027). The density of $CD8^+$ cells was significantly higher in men and in tumors with advanced T classification (P=0.038 and P=0.044, respectively). $Foxp3^+$ cell density was significantly higher in patients who were negative for perineural invasion (P=0.001), whereas GZB^+ levels were significantly lower in patients with small tumor sizes (P=0.024) and early T classification (P<0.001).

Tumor location and TILs

The associations between the anatomic locations of the tumors and TIL subsets are shown in **Fig. 2**. While levels of $CD3^+$ and $CD4^+$ cells were not associated with longitudinal locations, $CD8^+$ cell densities were significantly lower in cardia cancers (**Fig. 2A**). The densities of $Foxp3^+$ and GZB^+ cells were also significantly higher in cardia cancers than in tumors located elsewhere. No association was found among circular locations and the distribution of TILs (**Fig. 2B**).

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Table 1. Clinical parameters and TILs

Characteristics	Subsets of TILs										
	Number	CD3	P-value	CD4	P-value	CD8	P-value	Foxp3	P-value	GZB	P-value
Age (yr)			0.284		0.645		0.779		0.265		0.912
<60	272 (50.2%)	172±64.8		96.8±50.1		80.2±41.4		18.7±15		20.3±17.7	
≥60	270 (49.8%)	166.2±59.5		94.9±48.8		81.1±36.7		20.2±15.7		20.5±17	
Sex			0.119		0.844		0.038		0.898		0.484
Male	347 (64.0%)	166±60.3		96.2±51.1		83.3±39.7		19.5±15.3		20.8±17	
Female	195 (36.0%)	174.7±65.2		95.3±46.5		76±37.7		19.4±15.5		19.6±18	
Body mass index (kg/m²)			0.869		0.603		0.284		0.264		0.587
Low	271 (50.0%)	168.7±61.9		96.9±51.4		78.8±39.2		20.2±17.8		20±15.2	
High	271 (50.0%)	169.5±62.6		94.7±47.4		82.4±39		18.7±12.4		20.8±19.1	
Tumor size (>4 cm)			0.769		0.251		0.166		0.718		0.024
No	268 (49.4%)	168.3±62.9		98.3±50.5		78.3±35.8		19.2±16.9		18.7±15.5	
Yes	274 (50.6%)	169.9±61.6		93.4±48.4		82.9±42		19.7±13.7		22.1±18.8	
Histologic type*			0.038		0.240		0.115		0.177		0.598
Differentiated	217 (40.0%)	162.3±61.1		92.8±48.8		77.5±33.8		20.6±16.9		19.9±16.2	
Undifferentiated	325 (60.0%)	173.6±62.6		97.9±49.8		82.7±42.2		18.7±14.1		20.7±18.1	
LVI†			0.544		0.699		0.887		0.095		0.477
No	162 (29.9%)	171.3±58.1		99.2±45.9		81.5±39.3		17.3±9.7		17.5±15.7	
Yes	205 (37.8%)	174.9±54.6		101.2±52		82.1±35.4		15.7±8.8		18.8±16.6	
Perineural invasion†			0.906		0.709		0.844		0.001		0.515
No	203 (37.5%)	175.2±54.5		101.6±48.6		82.3±35.7		17.7±9.8		17±13.2	
Yes	144 (26.6%)	174.5±53.9		103.6±50.5		81.5±37.7		14.6±7.9		18.3±18.3	
T classification			0.087		0.931		0.044		0.166		<0.001
T1, T2	211 (38.9%)	163.4±60.2		96.1±45.7		76.4±35.3		18.4±14.2		17.4±13.3	
T3, T4	331 (61.1%)	172.8±63.3		95.7±51.7		83.4±41.2		20.2±16		22.5±19.4	
N classification			0.436		0.061		0.343		0.064		0.913
N−	256 (47.2%)	166.9±62		100±50		82.3±41.7		20.8±16.9		20.3±17.9	
N+	286 (52.8%)	171.1±62.4		92.1±48.7		79.1±36.7		18.3±13.7		20.5±16.7	
Stage			0.827		0.027		0.198		0.050		0.370
I, II	317 (58.5%)	168.6±60.7		99.8±49.2		82.4±41.2		20.5±15.9		19.9±17.3	
III, IV	225 (41.5%)	169.8±64.5		90.3±49.4		78.1±35.9		17.9±14.5		21.3±17.3	

TIL, tumor-infiltrating lymphocyte; LVI, lymphovascular invasion; Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B.
*Histologic type divided to 2 type: 1) Differentiated: papillary, well-differentiated adenocarcinoma and moderately differentiated adenocarcinoma; 2) Undifferentiated: poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous cancer, and other undifferentiated carcinomas. †Patients with missing information (175, lymphovascular invasion; 195, perineural invasion) were excluded from the analysis.

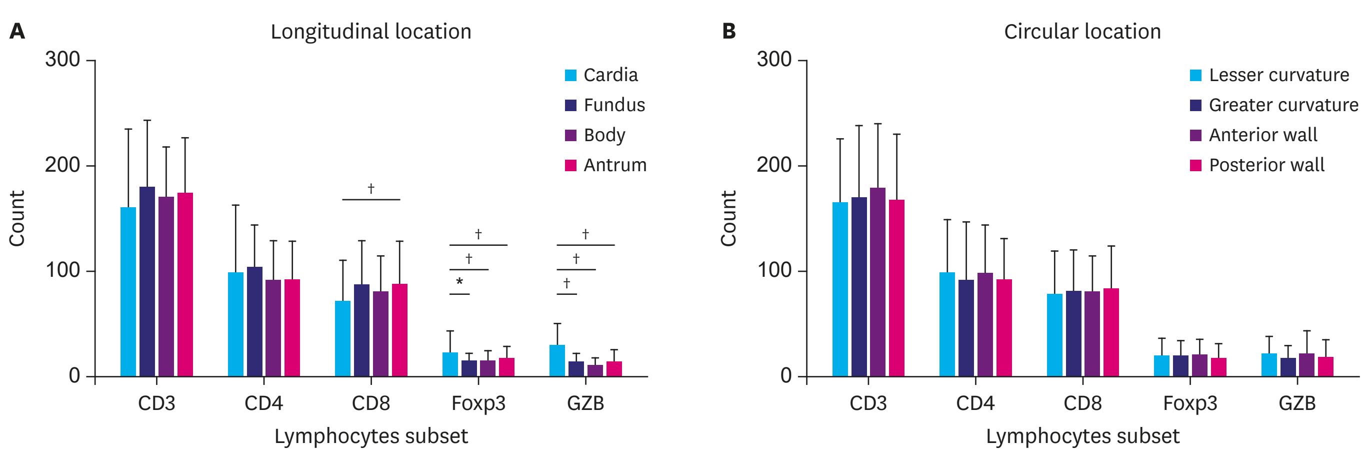

Fig. 2. Tumor location and TIL expression. (A) Immune responses according to longitudinal location: cardia tumors were associated with significantly different distributions of CD8-, Foxp3-, and GZB-positive TILs. (B) Immune responses according to circular location: no significant associations were noted.
TIL, tumor-infiltrating lymphocyte; Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B.
*P<0.05 with Bonferroni correction; †P<0.01 with Bonferroni correction.

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Comparison of cardia and non-cardia tumors

Cardia cancers were larger in size, showed more LVI, were more commonly located in the lesser curvature of the stomach, and had more advanced T classification and stage than non-cardia cancers (**Table 2**). In terms of immune responses, cardia cancers were strikingly different from their non-cardia counterparts. Cardia cancers showed fewer total lymphocytes ($CD3^+$; P=0.030) and fewer cytotoxic T lymphocytes ($CD8^+$; P<0.001) than non-cardia cancers, with no difference in helper T lymphocyte levels ($CD4^+$; P=0.347). We recorded fewer cytotoxic T lymphocytes and an increased number of activated cytotoxic T lymphocytes (GZB^+; P<0.001) in cardia cancers, findings that are suggestive of immune exhaustion. We also noted a greater absolute number of regulatory T lymphocytes ($Foxp3^+$; P<0.001) and a

Table 2. Comparison of the characteristics of patients with cardia and non-cardia tumors

Characteristics	Cardia (n=207)	Non-cardia (n=335)	P-value
Age (yr)			0.492
<60	100 (48.3%)	172 (51.3%)	
≥60	107 (51.7%)	163 (48.7%)	
Sex			0.119
Male	141 (68.1%)	206 (61.5%)	
Female	66 (31.9%)	129 (38.5%)	
Body mass index (kg/m^2)	23.2±3.2	23.1±3.3	0.886
Tumor size (>4 cm)			0.029
No	90 (43.5%)	178 (53.1%)	
Yes	117 (56.5%)	157 (46.9%)	
Histologic type*			0.068
Differentiated	93 (44.9%)	124 (37.0%)	
Undifferentiated	114 (55.1%)	211 (63.0%)	
LVI†			0.026
No	38 (35.2%)	124 (47.9%)	
Yes	70 (64.8%)	135 (52.1%)	
Perineural invasion†			0.151
No	48 (52.2%)	155 (60.8%)	
Yes	44 (47.8%)	100 (39.2%)	
Circular location‡			0.031
Lesser curvature	107 (54.6%)	154 (46.5%)	
Greater curvature	14 (7.1%)	52 (15.7%)	
Anterior wall	29 (14.8%)	49 (14.8%)	
Posterior wall	46 (23.5%)	76 (23%)	
T classification			<0.001
T1, T2	60 (29%)	151 (45.1%)	
T3, T4	147 (71%)	184 (54.9%)	
N classification			0.130
N0	86 (41.5%)	170 (50.7%)	
N1	27 (13%)	45 (13.4%)	
N2	39 (18.8%)	45 (13.4%)	
N3	55 (26.6%)	75 (22.4%)	
Stage			<0.001
I, II	103 (49.8%)	214 (63.9%)	
III, IV	104 (50.2%)	121 (36.1%)	
CD3	161.1±74.3	174±53.0	0.030
CD4	98.7±63.9	94.1±37.9	0.347
CD8	71.9±38.1	86.0±38.8	<0.001
Foxp3	23.2±21	17.2±9.8	<0.001
GZB	29.9±21.5	13.9±9.4	<0.001
Foxp3/CD4 (%)	31.6±30.7	19.6±11.9	<0.001

LVI, lymphovascular invasion; Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B.
*Histologic type divided to 2 type: 1) Differentiated: papillary, well-differentiated adenocarcinoma and moderately differentiated adenocarcinoma; 2) Undifferentiated: poorly differentiated adenocarcinoma, signet-ring cell carcinoma, mucinous cancer, and other undifferentiated carcinomas. †Patients with missing information (175, lymphovascular invasion; 195, perineural invasion) were excluded from the analysis. ‡Circular location unknown (n=15).

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stable number of $CD4^+$ T lymphocytes in cardia cancers, resulting in a higher $Foxp3^+/CD4^+$ ratio (P<0.001) in cardia cancers than in non-cardia cancers.

Prognostic implications of the immune response according to tumor location

High $CD3^+$ and $CD4^+$ cell densities were associated with a good prognosis only in cardia cancers (**Fig. 3A-D**), while a high $Foxp3^+$ cell density was associated with a good prognosis only in non-cardia cancers (**Fig. 3E and F**). Remarkably, a high $Foxp3^+/CD4^+$ ratio was associated with poor prognosis in cardia cancer patients and with good prognosis in non-cardia cancer patients (**Fig. 3G and H**). $CD8^+$ and GZB^+ expression was not associated with a difference in survival according to tumor location (data not shown).

Univariate Cox regression analysis of overall survival for the entire study cohort revealed that total gastrectomy, larger tumor size, advanced T classification, lymph node metastasis, and low $CD4^+$ and $CD8^+$ cell densities were poor prognostic factors (**Table 3**, whole cohort). Multivariate analysis showed that total gastrectomy (hazard ratio [HR], 1.75; 95% confidence interval [CI], 1.291–2.378), advanced T classification (HR, 1.99; 95% CI, 1.42–2.79), lymph node metastasis (HR, 2.31; 95% CI, 1.69–3.14), and low $CD8^+$ cell density (HR, 0.99; 95% CI, 0.992–0.999) were independent predictors of overall survival.

In the subgroup analysis of cardia cancer (**Table 3**, cardia only), advanced age (HR, 1.023; 95% CI, 1.01–1.04), higher T classification (HR, 2.029; 95% CI, 1.11–3.72), lymph node metastasis (HR, 3.319; 95% CI, 1.95–5.66), low $CD3^+$ cell density (HR, 0.997; 95% CI, 0.994–0.999), and a high $Foxp3^+/CD4^+$ ratio (HR, 1.007; 95% CI, 1.00–11.012) were independent predictors of an unfavorable prognosis. In non-cardia cancer (**Table 3**, non-cardia only), total gastrectomy (HR, 2.147; 95% CI, 1.51–3.06), higher T classification (HR, 2.158; 95% CI, 1.43–3.27), lymph node metastasis (HR, 1.85; 95% CI, 1.25–2.75), and a low $Foxp3^+/CD4^+$ ratio (HR, 0.978; 95% CI, 0.96–0.99) were independent predictors of an unfavorable prognosis.

Table 3. Univariate and multivariate analyses of factors associated with overall survival

Factors	Whole cohort (n=542)			Cardia (n=207)			Non-cardia (n=335)		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Univariate									
Age	0.998	0.988–1.008	0.687	1.004	0.988–1.019	0.652	0.994	0.98–1.007	0.350
Sex	1.181	0.906–1.541	0.218	0.837	0.544–1.288	0.419	1.545	1.092–2.188	0.014
Total gastrectomy	2.148	1.589–2.904	<0.001	N/A			2.479	1.747–3.519	<0.001
Tumor size (>4 vs. <4 cm)	2.040	1.559–2.669	<0.001	2.110	1.392–3.197	<0.001	1.974	1.385–2.815	<0.001
T classification (T3/T4 vs. T1/T2)	2.810	2.057–3.838	<0.001	3.002	1.759–5.125	<0.001	2.676	1.812–3.951	<0.001
N positive vs. negative	3.082	2.312–4.108	<0.001	3.800	2.366–6.103	<0.001	2.603	1.799–3.766	<0.001
CD3	0.998	0.996–1.000	0.109	0.997	0.995–1.000	0.034	1.001	0.997–1.004	0.701
CD4	0.997	0.994–1.000	0.043	0.996	0.992–0.999	0.009	1.001	0.996–1.005	0.763
CD8	0.996	0.992–0.999	0.020	0.996	0.991–1.001	0.160	0.997	0.992–1.001	0.159
Foxp3	0.994	0.985–1.003	0.196	0.998	0.989–1.007	0.700	0.972	0.953–0.992	0.006
GZB	0.998	0.990–1.006	0.628	0.993	0.983–1.002	0.139	0.983	0.961–1.006	0.159
Foxp3/CD4	1.004	0.998–1.009	0.166	1.006	1.000–1.011	0.034	0.974	0.956–0.993	0.007
Multivariate									
Age				1.023	1.006–1.040	0.008			
Total gastrectomy	1.752	1.291–2.378	<0.001				2.147	1.507–3.059	<0.001
T classification (T3/T4 vs. T1/T2)	1.991	1.419–2.793	<0.001	2.029	1.106–3.721	0.022	2.158	1.425–3.266	<0.001
N positive vs. negative	2.307	1.694–3.142	<0.001	3.319	1.947–5.658	<0.001	1.854	1.250–2.750	0.002
CD3				0.997	0.994–0.999	0.016			
CD8	0.995	0.992–0.999	0.010						
Foxp3/CD4				1.007	1.001–1.012	0.022	0.978	0.959–0.997	0.022

Forward stepwise elimination with a threshold of P=0.10 was used to select variables for inclusion.
HR, hazard ratio; CI, confidence interval; N/A, not applicable; Foxp3, forkhead/winged helix transcription factor; GZB, granzyme B.

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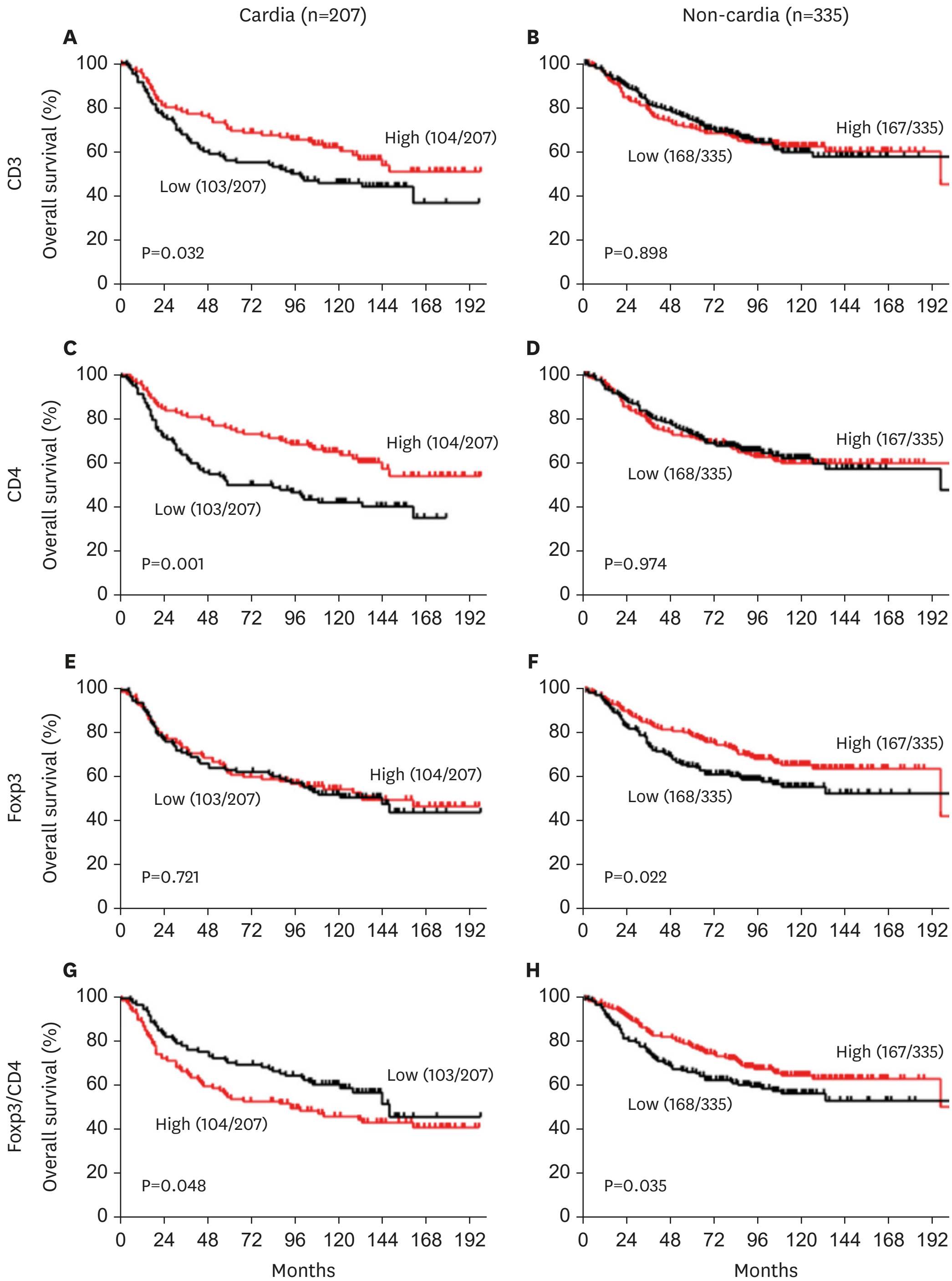

Fig. 3. Survival according to immune responses in cardia and non-cardia tumors. Kaplan–Meier analysis of overall survival in cardia and non-cardia patients as a function of (A, B) CD3 (T lymphocytes), (C, D) CD4 (helper T lymphocytes), and (E, F) Foxp3 (regulatory T lymphocytes) cell densities and (G, H) Foxp3/CD4 ratio. High (red) versus low (black) expression groups were divided according to median values.
TIL, tumor-infiltrating lymphocyte; Foxp3, forkhead/winged helix transcription factor.

In multivariate analysis of relapse-free survival, immune responses were not associated with prognosis. Only T classification and lymph node metastasis were identified as independent risk factors for both cardia cancers (HR, 4.57; 95% CI, 2.66–7.86 and HR=3.306; 95%

CI=2.09–5.23, respectively) and non-cardia cancers (HR, 3.951; 95% CI, 2.23–6.99 and HR, 3.099; 95% CI, 1.88–5.12, respectively).

DISCUSSION

We previously demonstrated the prognostic significance of Foxp3$^+$/CD4$^+$ ratio in a cohort of 180 cardia cancer patients [6]. In this study, we accumulated data from additional cardia (n=207) and non-cardia (n=335) cancer patients to examine the role of immune cells in gastric cancer. Our findings demonstrate that the nature and prognostic significance of the immune response differs between cardia and non-cardia cancers. Cardia tumors exhibited lower CD3$^+$ and CD8$^+$ cell densities but higher Foxp3$^+$ and GZB$^+$ cell densities than non-cardia tumors. Furthermore, we found that a high Foxp3$^+$/CD4$^+$ ratio was associated with a poor prognosis in cardia cancer and a favorable prognosis in non-cardia cancer, corroborating our previous findings.

Immune responses that are specific to certain tumor locations within a single organ have also been observed in colorectal cancer, in which tumors in the left colon exhibit clinicopathologic [22] and molecular [23] characteristics that are distinct from those exhibited by tumors in the right colon. A study of the linear distribution of immune cells in normal colons revealed that the numbers of CD3$^+$ and CD8$^+$ cells progressively decreased from the ascending colon to the rectum [24]. Another recent study comparing cancers in the right colon, left colon, and rectum showed that high Foxp3$^+$ cell density was a favorable prognostic factor only in patients with rectal tumors [25]. Such findings may also translate to gastric cancer and could explain the favorable prognostic effect of Foxp3$^+$ cells in non-cardia cancer.

In general, high infiltration of regulatory (Foxp3$^+$) T lymphocytes is known to be associated with a poor prognosis, which is consistent with our data from patients with cardia cancer. Paradoxically, colorectal [26,27] and oral cavity [28] cancers, which are expected to have a high bacterial burden, demonstrate a favorable prognosis when levels of regulatory T lymphocytes are high, indicating that the suppression of inflammation by regulatory cells confers an antitumor effect [29,30]. In our study, patients with non-cardia cancers with high regulatory T lymphocyte infiltration showed a favorable prognosis. Achlorhydria caused by atrophic gastritis and *Helicobacter pylori* infection results in bacterial overgrowth and dysbiosis in the stomach [31]. As such, the infiltration of Foxp3$^+$ TILs might improve a patient's prognosis by suppressing inflammation and subsequent carcinogenesis [32-34]. As stated above, reduced acidity is an underlying cause of non-cardia cancer while high acidity supports the development of cardia cancer [18,19]. These opposing carcinogenic mechanisms may be reflected in the contrasting prognostic effects of regulatory T-cells in gastric cancers.

Generally, cytotoxic T-cells (CD8$^+$) are regarded as important prognostic factors in gastric cancer [9,12]. In this study, we found that a high CD8$^+$ count was independently associated with a favorable prognosis when the data were analyzed as a whole, as shown in **Table 3**. These findings corroborate those of other studies [9,12]. However, a high CD8$^+$ count was not significantly associated with prognosis when data for cardia and non-cardia cancers were analyzed separately. These results indirectly support the importance of considering tumor location in the analyses and interpretation of data for gastric cancer.

This study has some limitations. First, owing to the retrospective nature of the study, there may have been bias during patient selection. Second, we did not evaluate the status of

H. pylori infection, which could be a confounding factor since it is associated with acidity and inflammation. Third, the current findings may not be generalizable to other solid organ cancer types. Despite these limitations, we found that gastric tumors exhibit significantly divergent characteristics depending on their gastric locations after adjusting for stage and other clinicopathological factors [5]. Moreover, to the best of our knowledge, our study is the first to investigate differences in immune cell characteristics in gastric cancer according to the topographic locations of tumors within the stomach.

In summary, we found that the densities and prognostic effects of TILs differ according to the tumor location in the stomach. Most notably, we observed opposing prognostic effects associated with a high Foxp3/CD4 ratio between patients with cardia and non-cardia gastric cancer. Overall, our study indicates that the longitudinal location of a tumor within the stomach should be considered when designing individualized immunotherapy protocols for patients with gastric cancer.

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