Differences in immune contextures among different molecular subtypes of gastric cancer and their prognostic impact

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Abstract

Background Gastric cancers have been recently classified in accordance with their molecular characteristics, thus demonstrating the complex nature of cancers and an association with the immune contexture within the tumor microenvironment. This study aimed to investigate the correlation between the molecular subtype and immune contexture of gastric cancers.

Methods The immune contexture, including the type, density, and location of tumor-infiltrating lymphocytes (TILs), of gastric cancer patients was examined and immune subtypes were classified based on it. In particular, PD-L1 expression on tumor cells and TILs and Foxp3⁺ TILs was assessed in accordance with molecular subtypes.

Results High levels of visual TIL estimates and Foxp3⁺ TILs were markedly associated with increased overall survival ($P=0.001$, $P<0.001$, separately). Immune subtypes were associated with tumor size, gross type, depth of invasion, lymph node metastatic status, lymphovascular invasion, perineural invasion, and microsatellite instability status. EBV-positive (C1) and MSI (C2) gastric cancers, considered subtypes with better prognosis, were significantly associated with high TIL levels ($P<0.05$). In contrast, epithelial–mesenchymal transition (EMT, C3) gastric cancers with poor overall survival displayed low levels of Foxp3⁺ TILs. Type II tumors (low level of TILs/low PD-L1 expression) displayed a significant correlation with poor overall survival ($P=0.004$) and accounted for the highest proportion in the aberrant p53-expressing (C4) gastric cancers.

Conclusion The molecular subtype of gastric cancers is correlated with the immune subtype, including immune contexture and PD-L1 expression, within the tumor microenvironment.

Keywords Gastric cancer · Tumor microenvironment · Tumor-infiltrating lymphocytes · Survival

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Introduction

Cancer is a morphologically and genetically complex disease with major pathognomonic hallmarks [1]. Recently, The Cancer Genome Atlas (TCGA) research network has profiled and analyzed gastric cancers and revealed DNA, RNA, protein, and epigenetic aberrations and classified gastric cancers on the basis of their molecular characteristics [2]. This molecular classification of gastric cancer is associated with distinct clinical and histological characteristics and delineates the key features of genetic alterations for putative targeted therapy [3, 4].

In addition to the understanding of intrinsic molecular alterations in cancer, the tumor microenvironment has received increasing attention in cancer biology. During tumor development and progression, tumor cells interact with various host cells such as infiltrating immune cells, including antigen-presenting cells, neutrophils, T cells, and

B cells, and the tumor microenvironment is influenced by the host immune system [5].

To decrease immunogenicity, tumor cells activate the immune checkpoint pathway, which is critical for maintaining self-tolerance. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) are immune checkpoint receptors that inhibit the T cell response and have been extensively studied in the clinical context of immunotherapy [6]. Immune checkpoint inhibitors targeting the CTLA4 and PD1 pathways have shown remarkable clinical effects [7–9]. Most recently, anti-PD-1 checkpoint immunotherapy with pembrolizumab was approved for patients with recurrent or metastatic PD-L1⁺ gastric cancer. However, favorable results were obtained for a small subset [10–12].

Therefore, to administer effective immune-based cancer treatments to gastric cancer patients, comprehensive assessment of the immune contexture is essential. In this study, the immune contexture, including the type, density, and location of tumor-infiltrating lymphocytes (TILs), was examined in gastric cancer patients. In particular, the correlation between the immunologic and molecular subtypes of gastric cancers was evaluated on the basis of PD-L1 expression and TIL levels. Ultimately, this study aimed to determine the difference in the tumor microenvironment in accordance with the molecular subtype of gastric cancer and to identify their potential therapeutic relevance.

Materials and methods

Tumor samples and histopathologic evaluation

In total, 578 gastric adenocarcinoma tissue samples from patients who underwent gastrectomy with lymph node dissection as first-line treatment at the Pusan National University Hospital (PNUH, Busan, Korea) between 2009 and 2010 were reviewed, of which 406 were used to construct the tissue microarray (TMA). Finally, 297 cases, including those of early gastric cancer with submucosal invasion and advanced gastric cancer, were included. Since we aimed to investigate the pathologic characteristics and immune contextures of the tumor center and its invasive margin, patients with early gastric cancer without submucosal invasion were excluded. All included patients completed follow-up evaluation until February 2016.

The clinicopathological data for each patient were obtained by reviewing the patient's electronic medical records and pathologic reports; these included patient age and sex, tumor location, size and gross type, Lauren and WHO histologic type, depth of invasion (pT stage), lymph node metastatic status (pN stage), lymphovascular and

perineural invasion, microsatellite instability (MSI) status, mucin phenotype, and HER2 status.

TMA construction

For TMA analysis, sections of formalin-fixed paraffin-embedded tissues were prepared and stained with hematoxylin and eosin (H&E). All available H&E-stained slides were reviewed for appropriate tumor areas with the highest TIL population at the tumor center and invasive margin. From each tumor, two representative cores of 2.0-mm diameter were obtained using the TMA instrument and inserted in a grid pattern into a recipient block.

Immunohistochemical analysis and Epstein–Barr virus in situ hybridization (EBV ISH)

Four-micrometer-thick sections from each TMA were probed with anti-CD3 (1:400, Dako, Carpinteria, CA, USA), anti-CD8 (1:200, 4B11, Leica, Microsystems, Wetzlar, Germany), anti-Foxp3 (1:100, 236A/E7, Abcam, Cambridge, UK), anti-PD-L1 (1:200, E1L3N, Cell signaling Technology, Danvers, MA, USA), anti-MLH1 (1:150, ES05, Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK), anti-E-cadherin (1:100, 36B5, Novocastra), and anti-p53 (1:400, DO-7, Novocastra) antibodies, using automatic immunohistochemical staining devices (Benchmark XT; Ventana Medical System, Tucson, AZ, USA and Bond-Max; Leica Microsystems, Wetzlar, Germany).

First, the study cohort was classified into five molecular subtypes (C-subtypes) in accordance with MLH-1, E-cadherin, and p53 mRNA and protein expression levels [4]. Aberrant MLH-1 expression was defined as complete loss of nuclear staining. Tumor cells with diffuse, completely obliterated, or upregulated p53 nuclear staining was considered to aberrantly express p53. Cells displaying complete loss of membranous or cytoplasmic staining were considered to aberrantly express E-cadherin.

To evaluate the immune contexture of each molecular subtype (C-subtypes), including visually estimated TILs, CD3⁺, CD8⁺, and Foxp3⁺ T cells, the absolute numbers of labeled TILs at the tumor center (TC) and invasive margin (IM) were determined. Visually estimated TILs were defined as the number of eyeball-measured lymphoid cells in the H&E staining. CD3 and CD8 were detected in the cytoplasm of the TILs, Foxp3, nucleus. Two representative areas with the highest cell density at high magnification (400×) were counted, and the higher value was selected. Regarding TILs, infiltration levels were categorized as low and high based on the median number of immunoreactive immune cells and visually estimated immune cells in the TC and IM (Supplementary Fig. 1). For statistical analysis, total TIL levels were determined from the sum of the level in the TC and

IM. Low TIL levels in both the TC and IM indicated low total TIL levels.

PD-L1 expression was evaluated separately for tumor cells and TILs at the TC and IM. Membrane staining was performed to detect PD-L1, using human placental tissue as a positive control. Staining intensity was graded using a 4-point scale as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), or 3 (strong staining) (Supplementary Fig. 2 and 3). The percentage of positive cells was assessed at 5% increments. PD-L1-positive staining included more than 5% of positive cells showing moderate and strong intensity [13]. PD-L1 expression levels on tumor cells and TILs were categorized as low and high upon positive staining of both the TC and IM. Tumor cells or TILs with negative PD-L1 staining in both the TC and IM were considered to display PD-L1 downregulation. The others (PD-L1 negative in the TC and PD-L1 positive in the IM; PD-L1 positive in TC and PD-L1 negative in the IM; PD-L1 positive in the TC and PD-L1 positive in the IM) were considered to display PD-L1 upregulation. Furthermore, tumors were categorized as high and low in accordance with their total PD-L1 status in tumor cells and TILs. PD-L1 downregulation in both tumor cells and TILs indicated a low PD-L1 status; the others were considered to have high PD-L1 status.

For survival analysis, the study cohort was segregated into four immune subtypes (I-subtypes) based on the TIL levels and PD-L1 expression in tumor cells, similar to Teng's classification [14]: type I, high level of TILs and high PD-L1 status (TILs H/PD-L1 H); type II, low level of TILs and low PD-L1 status (TILs L/PD-L1 L); type III, low level of TILs and high PD-L1 status (TILs L/PD-L1 H); type IV, high level of TILs and low PD-L1 status (TILs H/PD-L1 L). Herein, levels of visual TIL estimates were considered a representative value of TIL levels.

The presence of EBV in tumor cells was confirmed via chromogenic ISH with EBV-encoded small RNA (EBER-ISH), using an automatic staining device (BOND-MAX), with a Novocastra EBER probe for EBV (Leica Biosystems). Strong nuclear staining of the tumor cells rendered them EBV positive.

Statistical analysis

All statistical analyses were performed using SPSS 24.0 (IBM Corporation, New York, NY, USA). Immune contexture and PD-L1 expression were analyzed for differences among subtypes, using the χ^2 test, Fisher's exact test and Kruskal–Wallis test. The correlation between different immune cell densities was analyzed using the Spearman rank correlation test. Differences in clinicopathologic characteristics based on PD-L1 status were analyzed using the Student's *t* test, χ^2 test, or Fisher's exact test. Survival curves were derived from Kaplan–Meier estimates, and the curves

were compared using a log-rank test. For all tests, statistical significance was set at $P < 0.05$.

Results

Clinical impact of the immune contexture and PD-L1 expression on gastric cancer

The association between the clinicopathological characteristics and immune contexture of TILs, including cell type (CD3⁺, CD8⁺, and Foxp3⁺ TILs), and density is summarized in Table 1. The high level of visual TIL estimates was significantly associated with various histopathologic features including younger age ($P = 0.007$) and location in the body ($P = 0.005$), non-excavated gross type ($P = 0.014$), lower pT ($P < 0.000$) and pN stages ($P = 0.002$), and the absence of lymphovascular and perineural invasion ($P = 0.003$ and $P < 0.000$, separately). Moreover, Foxp3 upregulation was significantly associated with large tumor size ($P = 0.015$), and low level of Foxp3⁺ TILs was statistically associated with high stage of pT ($P < 0.001$) and pN ($P = 0.015$) and the presence of lymphovascular invasion ($P = 0.050$). Regarding the prognostic impact of immune cells on gastric cancer, high levels of visual TIL estimates were significantly associated with a better prognosis ($P = 0.001$, Fig. 1a). Furthermore, Foxp3 upregulation was significantly associated with better treatment outcomes ($P < 0.001$, Fig. 1d). However, a high level of CD3⁺ and CD8⁺ TILs were associated with Lauren classification ($P = 0.005$ and $P = 0.011$, respectively), perineural invasion ($P = 0.017$ and $P = 0.007$, respectively) and MSI-H ($P = 0.021$ and $P = 0.023$, respectively), but not with pT and pN stages and prognosis ($P = 0.185$ and $P = 0.179$, respectively, Fig. 1b and c).

The association between PD-L1 expression and the clinicopathological features is shown in Table 2. Among the histopathologic parameters, tumor size ($P = 0.024$), pT stage ($P = 0.043$), lymphovascular invasion ($P = 0.029$), and MSI ($P = 0.033$) were significantly associated with PD-L1 expression on tumor cells. However, PD-L1 expression of TILs was not associated with pT and pN stages and lymphovascular invasion. Moreover, PD-L1 upregulation on tumor cells and TILs was slightly, but not significantly associated with improved overall survival ($P = 0.133$ and $P = 0.644$, respectively, Fig. 2).

The study cohort was segregated into four immune subtypes (I-subtypes) on the basis of TIL level and PD-L1 expression in tumor cells as mentioned in “Materials and methods”: type I (TILs H/PD-L1 H), type II (TILs L/PD-L1 L), type III (TILs L/PD-L1 H), and type IV (TILs H/PD-L1 L), and the association between I-subtypes and clinicopathological characteristics was analyzed. These subtypes were associated with tumor size ($P = 0.013$), gross type

Table 1 Relationship between the immune contexture and clinicopathologic characteristics of gastric cancer

	Level of visual TIL estimates			Level of CD3 ⁺ TILs			Level of CD8 ⁺ TILs			Level of Foxp3 ⁺ TILs		
	Low	High	P value	Low	High	P value	Low	High	P value	Low	High	P value
<i>Number (%)</i>	151 (50.8)	146 (49.2)		150 (50.5)	147 (49.5)		149 (50.2)	148 (49.8)		154 (51.9)	143 (48.1)	
<i>Age</i>												
Mean ± SD	64.07 ± 10.524	60.64 ± 11.455	0.007	61.75 ± 10.898	63.03 ± 11.317	0.324	61.48 ± 11.044	63.30 ± 11.132	0.158	61.55 ± 10.987	63.29 ± 11.203	0.177
<i>Sex</i>												
Male	106 (70.2)	98 (67.1)	0.568	95 (63.3)	109 (74.1)	0.044	100 (67.1)	104 (70.3)	0.558	104 (67.5)	100 (69.9)	0.656
Female	45 (29.8)	48 (32.9)		55 (36.7)	38 (25.9)		49 (32.9)	44 (29.7)		50 (32.5)	43 (30.1)	
<i>Size (mm)</i>												
Mean ± SD	58.13 ± 30.979	52.07 ± 31.352	0.095	54.81 ± 31.138	55.50 ± 31.482	0.851	57.22 ± 33.280	53.07 ± 29.044	0.253	59.40 ± 34.488	50.58 ± 26.731	0.015
<i>Location</i>												
Cardia, EGJ	6 (4.0)	9 (6.2)	0.005	9 (6.0)	6 (4.1)	0.230	9 (6.0)	6 (4.1)	0.711	8 (5.2)	7 (4.9)	0.095
Body	54 (35.8)	80 (54.8)		60 (40.0)	74 (50.3)		65 (43.6)	69 (46.6)		62 (40.3)	72 (50.3)	
Antrum, pylorus	87 (57.6)	53 (36.3)		78 (52.0)	62 (42.2)		72 (48.3)	68 (45.9)		82 (53.2)	58 (40.6)	
N/A	4 (2.6)	4 (2.7)		3 (2.0)	5 (3.4)		3 (2.0)	5 (3.4)		2 (1.3)	6 (4.2)	
<i>Gross type</i>												
Non-excavated	123 (81.5)	101 (69.2)	0.014	108 (72.0)	116 (78.9)	0.167	107 (71.8)	117 (79.1)	0.147	123 (79.9)	101 (70.6)	0.065
Excavated	28 (18.5)	45 (30.8)		42 (28.)	31 (21.1)		42 (28.2)	31 (20.9)		31 (20.1)	42 (29.4)	
<i>Grade & WHO classification</i>												
W/D, M/D	80 (53.0)	69 (47.3)	0.085	79 (52.7)	70 (47.6)	0.197	80 (53.7)	69 (46.6)	0.209	72 (46.8)	77 (53.8)	
P/D	49 (32.5)	55 (37.7)		45 (30.0)	59 (40.1)		45 (30.2)	59 (39.9)		57 (37.0)	47 (32.9)	
Signet ring cell	15 (9.9)	21 (14.4)		20 (13.3)	16 (10.9)		18 (12.1)	18 (12.2)		18 (11.7)	18 (12.6)	
Mucinous	7 (4.6)	1 (0.7)		6 (4.0)	2 (1.4)		6 (4.0)	2 (1.4)		7 (4.5)	1 (0.7)	
<i>Lauren type</i>												
Intestinal	71 (47.0)	59 (40.4)	0.511	75 (50.0)	55 (37.4)	0.005	75 (50.3)	55 (37.2)	0.011	63 (40.9)	67 (46.9)	0.571
Diffuse	57 (31.7)	61 (41.8)		60 (40.0)	58 (39.5)		58 (38.9)	60 (40.5)		65 (42.2)	53 (37.1)	
Mixed	23 (15.2)	26 (17.8)		15 (10.0)	34 (23.1)		16 (10.7)	33 (22.3)		26 (16.9)	23 (16.1)	
<i>pT stage</i>												
T1	19 (12.6)	53 (36.3)	0.000	44 (29.3)	28 (19.0)	0.139	44 (29.5)	28 (18.9)	0.151	23 (14.9)	49 (34.3)	0.000
T2	26 (17.2)	30 (20.5)		26 (17.3)	30 (20.4)		24 (16.1)	32 (21.6)		25 (16.2)	31 (21.7)	
T3	34 (22.5)	26 (17.8)		25 (16.7)	35 (23.8)		27 (18.1)	33 (22.3)		33 (21.4)	27 (18.9)	
T4	72 (47.7)	37 (25.3)		55 (36.7)	54 (36.7)		54 (36.2)	55 (37.2)		73 (47.4)	36 (25.2)	
<i>pN stage</i>												
N0	49 (32.5)	71 (48.6)	0.002	64 (42.7)	56 (38.1)	0.741	61 (40.9)	59 (39.9)	0.544	52 (33.8)	68 (47.6)	0.015
N1	23 (15.2)	29 (19.9)		24 (16.0)	28 (19.0)		24 (16.1)	28 (18.9)		25 (16.2)	27 (18.9)	
N2	35 (23.2)	15 (10.3)		23 (15.3)	27 (18.4)		22 (14.8)	28 (18.9)		27 (17.5)	23 (16.1)	

Table 1 (continued)

	Level of visual TIL estimates			Level of CD3 ⁺ TILs			Level of CD8 ⁺ TILs			Level of Foxp3 ⁺ TILs		
	Low	High	P value	Low	High	P value	Low	High	P value	Low	High	P value
N3	44 (29.1)	31 (21.2)	0.003	39 (26.0)	36 (24.5)	0.274	42 (28.2)	33 (22.3)	0.859	50 (32.5)	25 (17.5)	0.050
<i>Lymphovascular invasion</i>												
Positive	84 (55.6)	56 (38.4)		66 (44.0)	74 (50.3)		71 (47.7)	69 (46.6)		81 (52.6)	59 (41.3)	
Negative	67 (44.4)	90 (61.6)		84 (56.0)	73 (49.7)		78 (52.3)	79 (53.4)		73 (47.4)	84 (58.7)	
<i>Perineural invasion</i>												
Positive	112 (74.2)	74 (50.7)	0.000	84 (56.0)	102 (69.4)	0.017	82 (55.0)	104 (70.3)	0.007	104 (67.5)	82 (57.3)	0.070
Negative	39 (25.8)	72 (49.3)		66 (44.0)	45 (30.6)		67 (45.0)	44 (29.7)		50 (32.5)	61 (42.7)	
<i>MSI</i>												
MSI-H	15 (9.9)	17 (11.6)	0.635	10 (6.7)	22 (15.0)	0.021	10 (6.7)	22 (14.9)	0.023	13 (8.4)	19 (13.3)	0.178
MSI-I/L	136 (90.1)	129 (88.4)		140 (93.3)	125 (85.0)		139 (93.3)	126 (85.1)		141 (91.6)	124 (86.7)	

TILs indicates tumor-infiltrating lymphocytes; Foxp3 forkhead box protein 3, SD standard deviation, EGI esophagogastric junction, N/A not available, W/D we differentiated tubular adenocarcinoma, M/D moderately differentiated tubular adenocarcinoma, P/D poorly differentiated tubular adenocarcinoma, MSI microsatellite instability, EBV Epstein–Barr virus, EMT epithelial–mesenchymal transition

($P=0.000$), pT stage ($P=0.000$), pN stage ($P<0.000$), lymphovascular invasion ($P=0.000$), perineural invasion ($P<0.000$), and MSI status ($P<0.000$) (Table 3). Accordingly, type II tumors were significantly correlated with poor overall survival rate; type I, better overall survival rate; type III and IV, intermediate overall survival rate ($P=0.004$, Fig. 3a).

Differences in immune contexture and PD-L1 expression among molecular subtypes and the association between immune and molecular subtypes within the tumor microenvironment

According to a simple taxonomic sequence in our previous study [3], the cohort was divided into the following five molecular subtypes (C-subtype): C1 (EBV positive, $n=22$, 7.4%), C2 (aberrant MLH-1 expression or MSI, $n=29$, 9.8%), C3 (aberrant E-cadherin expression or EMT, $n=53$, 17.8%), C4 (aberrant p53 expression, $n=119$, 40.1%), and C5 (normal p53 expression, $n=74$, 24.9%). These subtypes were associated with tumor size ($P=0.007$), location ($P=0.049$), grade, and WHO classification ($P<0.001$), Lauren type ($P<0.001$), lymph node metastasis (pN stage) ($P=0.008$), perineural invasion ($P=0.004$), and MSI status ($P<0.001$) (Supplement Table 1). Herein, the overall survival based on the C-subtype was the same as that reported previously; MSI tumors revealed the best prognosis, followed by EBV-positive tumors, those with normal p53 expression, and those with aberrant p53 expression, with the C3 type showing the worst prognosis ($P<0.001$) (Fig. 3b).

Differences in immune contexture and PD-L1 expression were evaluated on the basis of the molecular subtype (Table 4). Among them, C1 and C2, being subtypes with better prognosis, were significantly associated with high levels of immune density, including CD3⁺, CD8⁺, and Foxp3⁺ TILs, regardless of location ($P=0.001$, $P<0.001$, $P=0.005$ in TC, $P=0.018$, $P=0.001$, $P<0.001$ in IM, respectively). However, C3 type with poor overall survival displayed low level of Foxp3⁺ TILs compared with other C-subtype (32 cases (60.4%) in TC, 36 cases (67.9%) in IM). PD-L1 expression displayed high positivity rates in the C1 and C2 types at any location (TC and IM) and in any cell type (tumor cells or TILs). In multivariable analyses using Cox proportional hazards regression models, C3 subtype was identified as an independent poor prognostic factor compared with the other subtypes (hazard ratio, 2.105; $P=0.001$) (Supplement Table 2). Moreover, the proportion of subtypes C1 and C2 with a high total PD-L1 status was significantly high ($P<0.001$, Table 4). The proportion of type I tumors was high in groups with a suitable prognosis, namely C1 and C2. The C4 subtype accounted for the greatest proportion of type II tumors (42.9%, Table 4).

Fig. 1 Overall survival according to the immune cell type in immune contexture. Kaplan–Meier survival curves for overall survival based on TIL level in tumor center and invasive margin; **a** visual estimate of TILs, **b** CD3⁺ TILs, **c** CD8⁺ TILs, **d** Foxp3⁺ TILs

Discussion

This study investigated the immune contexture including type, density, and location of TILs, PD-L1 on tumor cells, and TILs in accordance with the molecular subtypes of gastric cancer. The density of each TIL differed significantly in accordance with the molecular subtype. CD3⁺, CD8⁺, and Foxp3⁺ immune cells displayed the greatest density in subtypes C1 and C2, whereas the C3 and C4 subtypes showed a low density of all types of immune cells. Type II tumors (low level of TILs/low PD-L1 expression levels) were significantly correlated with poor overall survival and accounted for the greatest proportion in gastric cancers aberrantly

expressing p53 (subtype C4). These findings partly reveal the differences in the tumor microenvironment based on the molecular subtype of gastric cancer, thus revealing their potential therapeutic relevance.

With respect to immune contextures in gastric cancer, high levels of visually estimated TILs and Foxp3⁺ TILs were markedly associated with better overall survival, concurrent with previous reports [14–17]. TILs markedly contribute to the immune response against tumors. Several studies have reported an association between TILs and clinical outcomes in patients with various types of cancer [15–17]. Regarding gastric cancer, certain studies have reported that a high TIL density in gastric cancer is associated with favorable

Table 2 The relationship between PD-L1 expression and clinicopathologic characteristics of gastric cancer

	PD-L1 expression on tumor cells			PD-L1 expression on TILs		
	Negative	Positive	<i>P</i> value	Negative	Positive	<i>P</i> value
<i>Number (%)</i>	279 (93.9)	18 (6.1)		273 (91.9)	24 (8.1)	
<i>Age</i>			0.120			0.531
Mean $\pm$ SD	62.13 $\pm$ 10.932	66.33 $\pm$ 13.266		62.26 $\pm$ 11.019	63.75 $\pm$ 12.231	
<i>Sex</i>			0.391			0.038
Male	190 (68.1)	14 (77.8)		183 (67.0)	21 (87.5)	
Female	89 (31.9)	4 (22.2)		90 (33.0)	3 (12.5)	
<i>Size (mm)</i>			0.024			0.482
Mean $\pm$ SD	54.11 $\pm$ 30.703	71.22 $\pm$ 36.127		54.77 $\pm$ 31.046	59.46 $\pm$ 33.974	
<i>Location</i>			0.462			0.729
Cardia, EGJ	14 (5.0)	1 (5.6)		13 (4.8)	2 (8.3)	
Body	123 (44.1)	11 (61.1)		124 (45.4)	10 (41.7)	
Antrum, pylorus	134 (48.0)	6 (33.3)		128 (46.9)	12 (50.0)	
N/A	8 (2.9)	0 (0.0)		8 (2.9)	0 (0.0)	
<i>Gross type</i>			0.259			0.152
Non-excavated	208 (74.6)	16 (88.9)		203 (74.4)	21 (87.5)	
Excavated	71 (25.4)	2 (11.1)		70 (25.6)	3 (12.5)	
<i>Grade & WHO classification</i>			0.902			0.156
W/D, M/D	139 (49.8)	10 (55.6)		133 (48.7)	16 (66.7)	
P/D	97 (34.8)	7 (38.9)		96 (35.2)	8 (33.3)	
Signet ring cell	35 (12.5)	1 (5.6)		36 (13.2)	0 (0.0)	
Mucinous	8 (2.9)	0 (0.0)		8 (2.9)	0 (0.0)	
<i>Lauren type</i>			0.783			0.032
Intestinal	123 (44.1)	7 (38.9)		119 (43.6)	11 (45.8)	
Diffuse	111 (39.8)	7 (38.9)		113 (41.4)	5 (20.8)	
Mixed	45 (16.1)	4 (22.2)		41 (15.0)	8 (33.3)	
<i>pT stage</i>			0.043			0.229
T1	71 (25.4)	1 (5.6)		68 (24.9)	4 (16.7)	
T2	53 (19.0)	3 (16.7)		50 (18.3)	6 (25.0)	
T3	52 (18.6)	8 (44.4)		52 (19.0)	8 (33.3)	
T4	103 (36.9)	6 (33.3)		103 (37.7)	6 (25.0)	
<i>pN stage</i>			0.852			0.080
N0	114 (40.9)	6 (33.3)		110 (40.3)	10 (41.7)	
N1	49 (17.6)	3 (16.7)		44 (16.1)	8 (33.3)	
N2	46 (16.5)	4 (22.2)		46 (16.8)	4 (16.7)	
N3	70 (25.1)	5 (27.8)		73 (26.7)	2 (8.3)	
<i>Lymphovascular invasion</i>			0.029			0.894
Positive	136 (48.7)	4 (22.2)		129 (47.3)	11 (45.8)	
Negative	143 (51.3)	14 (77.8)		144 (52.7)	13 (54.2)	
<i>Perineural invasion</i>			0.715			0.989
Positive	174 (62.4)	12 (66.7)		171 (62.6)	15 (62.5)	
Negative	105 (37.6)	6 (33.3)		102 (37.4)	9 (37.5)	
<i>MSI</i>			0.033			0.032
MSI-H	27 (9.7)	5 (27.8)		26 (9.5)	6 (25.0)	
MSI-I/L	252 (90.3)	13 (72.2)		24.7 (90.5)	18 (75.0)	

SD indicates standard deviation, *EGJ* esophagogastric junction, *N/A* not available, *W/D* we differentiated tubular adenocarcinoma, *M/D* moderately differentiated tubular adenocarcinoma, *P/D* poorly differentiated tubular adenocarcinoma, *MSI* microsatellite instability, *EBV* Epstein–Barr virus, *EMT* epithelial–mesenchymal transition

Fig. 2 Overall survival in the cohort according to the PD-L1 expression of tumor cells and TILs in tumor center and invasive margin. Kaplan-Meier survival curves for overall survival following total PD-L1 expression on tumor cells (**a**) and TILs (**b**)

outcomes, especially EBV-associated gastric cancer [18, 19]. Among TILs, we individually enumerated CD3⁺, CD8⁺, and Foxp3⁺ T cells and determined the absolute numbers of labeled TILs at the TC and IM of tumors. Lee et al. reported that CD3⁺ and CD8⁺ TILs are independent favorable prognostic factors in gastric cancer [18], as opposed to the present findings revealing no association with prognosis. Furthermore, the Foxp3⁺ T cell density was associated with better prognosis of gastric cancer in our datasets. Foxp3 is the transcription factor for regulatory T (Treg) cells, which are physiologically present in the immune system and maintain self-tolerance [20]. The clinical significance of Foxp3⁺ T cells remains controversial among various cancer types. Regarding prognostic significance of Foxp3⁺ TILs in gastric cancer, different studies have reported conflicting results [21], probably because they comprise a different subpopulation of Foxp3⁺ T cells, potentially including a higher proportion of non-Treg cells [22]. Therefore, further studies are required to evaluate not only the absolute number of Foxp3⁺ T cells, but also the components of Foxp3⁺ subpopulations in gastric cancers.

PD-L1 expression in gastric cancer was not associated with the prognosis of gastric cancer. Diverse interpretations of PD-L1 expression have been previously reported [23–27]. In the current study, we assessed PD-L1 expression levels on tumor cells and TILs and separately estimated the intensity and proportion of labeled cells. The prognostic significance of PD-L1 expression is controversial in not only gastric cancer, but also other malignancies [22–26]. Furthermore, immune subtypes (I-subtypes) based on the total

TIL levels and the PD-L1 expression levels in tumor cells, consistent with Teng's classification [28], were associated with patient prognosis, i.e., type II tumors (low total TILs and low PD-L1 status) were significantly correlated with poor overall survival.

We evaluated differences in immune contexture and PD-L1 expression based on the molecular subtype of gastric cancer. Subtypes C1 (EBV) and C2 (MSI), being subtypes with better prognosis, were significantly associated with high levels of immune density, including CD3⁺, CD8⁺, and Foxp3⁺ TILs and PD-L1 upregulation. The proportions of visually estimated and Foxp3⁺ TILs in the C3 subtype (aberrant E-cadherin expression; EMT) were lower than those in other subtypes. Carcinogenesis induced by microbe-associated inflammation could be prevented by tumor-infiltrating Foxp3⁺ Tregs, since Foxp3⁺ Tregs suppress the immune function of cytotoxic T cells induced by *Helicobacter pylori*. [29, 30]. Thus, these results indicate low Foxp3⁺ TIL levels in diffuse-type gastric cancer (C3, aberrant E-cadherin expression) having less association with *H. pylori*. Moreover, the results in this study suggest the prognostic or predictive implication of Foxp3⁺ TILs related to molecular subtype of gastric cancer.

Regarding the potential therapeutic relevance of gastric cancer, we assume that six cases (27.3%) of gastric cancer of the C1 subtype also included type I tumors related to inflammatory signaling during adaptive immune resistance mechanisms. Since these tumors contain preexisting TILs, which were turned off via PD-L1, this group may comprise candidates for single-agent anti-PD-1/L1 blockade. Regarding the

Table 3 Clinicopathologic characteristics according to the immune subtype (I-subtype)

	Type I	Type II	Type III	Type IV	P value
<i>Age</i>					0.065
Mean $\pm$ SD	66.24 $\pm$ 13.723	62.69 $\pm$ 11.047	69.63 $\pm$ 7.501	61.54 $\pm$ 10.880	
<i>Sex</i>					0.452
Male	10 (58.8)	69 (74.2)	6 (75.0)	119 (66.5)	
Female	7 (41.2)	24 (25.8)	2 (25.0)	60 (33.5)	
<i>Size (mm)</i>					0.013
Mean	58.29 $\pm$ 31.096	59.08 $\pm$ 31.270	83.88 $\pm$ 35.126	51.53 $\pm$ 30.408	
<i>Location</i>					0.430
Cardia, EGJ	1 (5.9)	4 (4.3)	0 (0.0)	10 (5.6)	
Body	9 (52.9)	33 (35.5)	3 (37.5)	89 (49.7)	
Antrum, pylorus	7 (41.2)	54 (58.1)	5 (62.5)	74 (41.3)	
N/A	0 (0.0)	2 (2.2)	0 (0.0)	6 (3.4)	
<i>Gross type</i>					0.000
Non-excavated	15 (88.2)	82 (88.2)	7 (87.5)	120 (67.0)	
Excavated	2 (11.8)	11 (11.8)	1 (12.5)	59 (33.0)	
<i>Grade & WHO classification</i>					0.094
W/D, M/D	9 (52.9)	55 (59.1)	4 (50.0)	81 (45.3)	
P/D	8 (47.1)	29 (31.2)	3 (37.5)	64 (35.8)	
Signet ring cell	0 (0.0)	5 (5.4)	1 (12.5)	30 (16.8)	
Mucinous	0 (0.0)	4 (4.3)	0 (0.0)	4 (2.2)	
<i>Lauren type</i>					0.178
Intestinal	6 (35.3)	51 (54.8)	3 (37.5)	70 (39.1)	
Diffuse	6 (35.3)	30 (32.3)	4 (50.0)	78 (43.6)	
Mixed	5 (29.4)	12 (12.9)	1 (12.5)	31 (17.3)	
<i>pT stage</i>					0.000
T1	2 (11.8)	6 (6.5)	0 (0.0)	64 (35.8)	
T2	6 (35.3)	19 (20.4)	0 (0.0)	31 (17.3)	
T3	5 (29.4)	20 (21.5)	3 (37.5)	32 (17.9)	
T4	4 (23.5)	48 (51.6)	5 (62.5)	52 (29.1)	
<i>pN stage</i>					0.000
N0	7 (41.2)	19 (20.4)	4 (50.0)	90 (50.3)	
N1	3 (17.6)	18 (19.4)	2 (25.0)	29 (16.2)	
N2	3 (17.6)	24 (25.8)	1 (12.5)	22 (12.3)	
N3	4 (23.5)	32 (34.4)	1 (12.5)	38 (21.2)	
<i>Lymphovascular invasion</i>					0.000
Positive	5 (29.4)	60 (64.5)	2 (25.0)	73 (40.8)	
Negative	12 (70.6)	33 (35.5)	6 (75.0)	106 (59.2)	
<i>Perineural invasion</i>					0.000
Positive	7 (41.2)	72 (77.4)	8 (100.0)	99 (55.3)	
Negative	10 (58.8)	21 (22.6)	0 (0.0)	80 (44.7)	
<i>MSI</i>					0.000
MSI-H	6 (35.3)	5 (5.4)	4 (50.0)	17 (9.5)	
MSI-I/L	11 (64.7)	88 (94.6)	4 (50.0)	162 (90.5)	

SD indicates standard deviation, EGJ esophagogastric junction, N/A not available, W/D we differentiated tubular adenocarcinoma, M/D moderately differentiated tubular adenocarcinoma, P/D poorly differentiated tubular adenocarcinoma, MSI microsatellite instability

C4 (aberrant p53) subtype, 51 cases (42.9%) also included type II tumors showing a lack of detectable immune reactions—so-called immunologic ignorance. However,

single-agent checkpoint blockade would be ineffective in this group. Alternatively, combinatorial therapy comprising an agent to generate an immunogenic tumor environment and

Fig. 3 Overall survival according to immune subtype and molecular subtype. Kaplan–Meier survival curves for overall survival following as immune subtype (I-subtype) based on the level of TILs and the sta-

tus of PD-L1 (**a**) and the molecular subtype (C-subtype) (**b**). *P* value was calculated from log-rank test

an immune checkpoint agent could be considered. On using a combination of anti-CTLA-4 and anti-PD-1 antibodies, CTLA-4 blockade induces tumor infiltration by activated T cells and a concomitant increase in IFN- γ . Finally, PD-L1 was induced in the tumor environment [31]. In this context, C4 with aberrant p53 expression can show improved clinical outcomes through combinatorial therapy. A recent phase 1b clinical trial of the anti-PD-1 antibody pembrolizumab in gastric cancer demonstrated significant antitumor effects [31]. More than 60% of C3 (EMT) and C5 (normal p53) gastric cancers showed a type IV (immune tolerance) tumor microenvironment (high TILs level and low PD-L1 status). However, although type IV tumors contain high levels of TILs, they comprise a heterogeneous population of lymphoid cells. Moreover, type IV tumors may undergo another suppressive pathway, rather than the PD-1/PD-L1 immune checkpoint pathway. With respect to type IV tumors, immunotherapeutic strategies are yet incomplete. Therefore, appropriate selection of patients potentially benefiting from immunotherapy is important, and a consensus regarding the immunohistochemical determination of TILs level and PD-L1 is mandatory.

In conclusion, this study shows that the tumor microenvironment including immune contexts and PD-L1 expression levels differs depending on the molecular subtypes, and these immunologic signatures are significantly associated with overall survival. Differences in the tumor microenvironment account for differences in tumor development and progression in accordance with the molecular subtypes and

can also provide additional treatment alternatives including immunotherapy. Ultimately, an understanding of the molecular and immunological characteristics in gastric cancers is expected to contribute to improved patient survival. Therefore, evaluation of the immune contexture and a consistent interpretation of PD-L1 expression status are essential to select an appropriate immunotherapeutic candidate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no association with or financial interest in any commercial company relevant to this study.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Table 4 Immune contexture and PD-L1 expression based on the molecular subtype of gastric cancer (C-subtype)

	C1 (EBV)	C2 (MSI)	C3 (EMT)	C4 (aberrant p53)	C5 (normal p53)	P value
<i>Number (%)</i>	22 (7.4)	29 (9.8)	53 (17.8)	119 (40.1)	74 (24.9)	
[Tumor center]						
<i>Visual estimate of TIL level</i>						0.000
Low	0 (0.0)	15 (51.7)	27 (50.9)	74 (62.2)	35 (47.3)	
High	22 (100.0)	14 (48.3)	26 (49.1)	45 (37.8)	39 (52.7)	
<i>CD3 level</i>						0.001
Low	3 (13.6)	11 (37.9)	30 (56.6)	71 (59.7)	35 (47.3)	
High	19 (86.4)	18 (62.1)	23 (43.4)	48 (40.3)	39 (52.7)	
<i>CD8 level</i>						0.000
Low	2 (9.1)	10 (34.5)	30 (56.6)	67 (56.3)	40 (54.1)	
High	20 (90.9)	19 (65.5)	23 (43.4)	52 (43.7)	34 (45.9)	
<i>Foxp3 level</i>						0.005
Low	4 (18.2)	12 (41.4)	32 (60.4)	69 (58.0)	37 (50.0)	
High	18 (81.8)	17 (58.6)	21 (39.6)	50 (42.0)	37 (50.0)	
<i>PD-L1 of tumor cells</i>						0.007
Negative	17 (77.3)	25 (86.2)	51 (96.2)	115 (96.6)	71 (95.9)	
Positive	5 (22.7)	4 (13.8)	2 (3.8)	4 (3.4)	3 (4.1)	
<i>PD-L1 of TILs</i>						0.001
Negative	15 (68.2)	24 (82.8)	51 (96.2)	112 (94.1)	71 (95.9)	
Positive	7 (31.8)	5 (17.2)	2 (3.8)	7 (5.9)	3 (4.1)	
[Invasive margin]						
<i>Visual estimate of TIL level</i>						0.016
Low	4 (18.2)	14 (48.3)	25 (47.2)	69 (58.0)	38 (51.4)	
High	18 (81.8)	15 (51.7)	28 (52.8)	50 (42.0)	36 (48.6)	
<i>CD3 level</i>						0.018
Low	5 (22.7)	10 (34.5)	28 (52.8)	67 (56.3)	40 (54.1)	
High	17 (77.3)	19 (65.5)	25 (47.2)	52 (43.7)	34 (45.9)	
<i>CD8 level</i>						0.001
Low	3 (13.6)	10 (34.5)	31 (58.5)	64 (53.8)	44 (59.5)	
High	19 (86.4)	19 (65.5)	22 (41.5)	55 (46.2)	30 (40.5)	
<i>Foxp3 level</i>						0.000
Low	2 (9.1)	10 (34.5)	36 (67.9)	68 (57.1)	39 (52.7)	
High	20 (90.9)	19 (65.5)	17 (32.1)	51 (42.9)	35 (47.3)	
<i>PD-L1 of tumor cells</i>						0.025
Negative	18 (81.8)	25 (86.2)	51 (96.2)	115 (96.6)	71 (95.9)	
Positive	4 (18.2)	4 (13.8)	2 (3.8)	4 (3.4)	3 (4.1)	
<i>PD-L1 of TILs</i>						0.000
Negative	16 (72.7)	24 (82.8)	51 (96.2)	117 (98.3)	73 (98.6)	
Positive	6 (27.3)	5 (17.2)	2 (3.8)	2 (1.7)	1 (1.4)	
[PD-L1 level]						
<i>PD-L1 level of tumor cells</i>						0.000
Low	16 (72.7)	22 (75.9)	50 (94.3)	114 (95.8)	70 (94.6)	
High	6 (27.3)	7 (24.1)	3 (5.7)	5 (4.2)	4 (5.4)	
<i>PD-L1 level of TILs</i>						0.000
Low	13 (59.1)	21 (72.4)	51 (96.2)	112 (94.1)	70 (94.6)	
High	9 (40.9)	8 (27.6)	2 (3.8)	7 (5.9)	4 (5.4)	
[Total PD-L1 status]						0.000
Low	11 (50.0)	19 (65.5)	50 (94.3)	108 (90.8)	69 (93.2)	
High	11 (50.0)	10 (34.5)	3 (5.7)	11 (9.2)	5 (6.8)	
[I subtype]						N/A
Type I (high TILs/high PD-L1)	6 (27.3)	5 (17.2)	0 (0.0)	3 (2.5)	3 (4.1)	
Type II (low TILs/low PD-L1)	0 (0.0)	6 (20.7)	14 (26.4)	51 (42.9)	22 (29.7)	
Type III (low TILs/high PD-L1)	0 (0.0)	2 (6.9)	3 (5.7)	2 (1.7)	1 (1.4)	
Type IV (high TILs/low PD-L1)	16 (72.7)	16 (55.2)	36 (67.9)	63 (52.9)	48 (64.9)	

Table 4 (continued)

EBV indicates Epstein–Barr virus, *MSI* microsatellite instability, *EMT* epithelial–mesenchymal transition, *TILs* tumor-infiltrating lymphocytes, *Foxp3* forkhead box protein 3, *PD-L1* programmed death-ligand 1

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