Original articles

Clinicopathological features of program death ligand-1 (PD-L1) expression with tumor-infiltrating lymphocytes (TILs), mismatch repair (MMR) and Epstein-Barr virus (EBV) status in a large cohort of gastric cancer

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Abstract

Background: Recently, anti-programmed death 1 (PD-1) or its ligand (PD-L1) antibodies have shown promising results in gastric cancer (GC). PD-L1 expression and tumor-infiltrating lymphocytes (TILs), and mismatch-repair (MMR) deficiency (D-MMR) have been proposed as predictive biomarkers for anti-PD-1/PD-L1 antibodies. Moreover, it has been recently reported that PD-L1 gene was frequently amplified in Epstein-Barr virus (EBV)-positive GC. However, little is known about clinical relevance of PD-L1 expression with TILs, MMR and EBV status in GC.

Patients and Methods: We performed a tissue microarray analysis in 487 advanced GC patients who underwent a gastrectomy without preoperative chemotherapy. PD-L1 expression on tumor cells (TC) and tumor-infiltrating immune cells (IC), densities as well as expressions of lymphocyte-associated markers (CD3, CD4, CD8, and FOXP3) of TILs and MMR status were evaluated by immunohistochemistry. EBV status was evaluated by in situ hybridization.

Results: PD-L1 expression on TC and IC, D-MMR, and EBV were identified in 22.8, 61.4, 5.1, and 5.1% of all cases, respectively. PD-L1 expression was more frequently observed in elderly (TC; $P = 0.002$), male (TC; $P = 0.029$, IC; $P = 0.043$), poorly differentiated adenocarcinoma with solid-type histology (TC; $P < 0.001$, IC; $P < 0.001$), D-MMR (TC; $P < 0.001$, IC; $P < 0.001$), and EBV-positive status (TC; $P = 0.001$, IC; $P = 0.050$). A strong association was observed between PD-L1 expression and high densities of either CD3 (+), CD8 (+), or FOXP3 (+) TILs (TC; $P < 0.001$, IC; $P < 0.001$). In multivariate analysis, high density of CD8 (+) TILs was significantly associated with better survival, while PD-L1 expression, densities of other subtypes of TILs, MMR and EBV status were not independent prognostic factors.

Conclusions: In GC, PD-L1 expression was associated with distinct clinicopathological features including high density of TILs, D-MMR and EBV-positive status, but not a

prognostic factor.

Key words: gastric cancer, PD-L1, TILs, MMR status, EBV

Key message

In GC, PD-L1 expression was associated with distinct clinicopathological features including high density of TILs, D-MMR and EBV-positive status. PD-L1 expression was not a prognostic factor in GC. Impact of these characteristics on efficacy of anti-PD-1/PD-L1 antibodies warrants further evaluation.

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Introduction

Recently, blockade of immune checkpoint molecules with monoclonal antibodies has emerged as a promising strategy in several malignancies [1-6]. Programmed death 1 (PD-1), which belongs to the CD28 family of proteins, is a negative costimulatory receptor expressed on the surfaced of activated T cells [7]. The binding of PD-1 and its ligands, PD-L1 and PD-L2 on tumor or immune cells, can inhibit a cytotoxic T-cell response, which leads tumor cells to escape from immune system [7]. Accordingly, it has been considered that blockade of this interaction restores the antitumor activity of T cells [7]. Indeed, clinical trials of anti-PD-1/PD-L1 antibodies have shown high response rates and significantly longer overall survival (OS) in several malignancies [1-6]. On the basis of these trials, the US Food and Drug Administration have approved anti-PD-1 antibodies, nivolumab or pembrolizumab, for treating patients with advanced melanoma, non–small cell lung cancer (NSCLC) and renal-cell carcinoma. Pembrolizumab has shown promising results in phase I trial for patients with advanced gastric cancer (GC) [8]. Several phase III trials of anti-PD-1/PD-L1 antibodies are ongoing for advanced GC.

Although there have been no established biomarkers of anti-PD-1/PD-L1 antibodies, some reports have shown that PD-L1 expression on tumor cell membrane (TC) and tumor-infiltrating immune cells (IC) and tumor-infiltrating lymphocytes (TILs) were associated with better clinical outcomes of anti-PD-1/PD-L1 antibodies in several malignancies [1, 5, 9, 10]. For instance, PD-L1 expression in NSCLC and pre-existing CD8 (+) TILs in melanoma have been shown to be correlated with improved efficacy of pembrolizumab [5, 10]. In GC, there was also some correlation between PD-L1 expression on TC or IC and tumor response of pembrolizumab in Phase I trial [8]. Interestingly, The Cancer Genome Atlas (TCGA) reported that PD-L1/2 gene amplifications were elevated in Epstein-Barr virus (EBV)-positive gastric cancer [11]. Moreover, recent trial showed that

mismatch-repair status (MMR) predicted clinical benefit of pembrolizumab [12]. However, information on clinical relevance of PD-L1 expression with TILs, MMR and EBV status in GC has been limited.

In this study, we investigated clinicopathological features of PD-L1 expression on TC and IC in a large cohort of stage III and IV GC using tissue microarrays (TMAs), including the evaluation of TILs, MMR and EBV status.

Methods

Patients and tissue microarrays

Formalin-fixed, paraffin-embedded (FFPE) tumor samples from 487 patients with stage III and IV GC who underwent a gastrectomy at our hospital from January 2002 to December 2010 were examined in this study. None of patients had systematic chemotherapy before surgery. Construction of the tissue microarrays (TMAs) using these tumors have been previously described elsewhere [13]. Briefly, two representative tumor cores (2mm in diameter) were obtained from the same FFPE-tissue brock in each case. Serial 4-μm sections were prepared and used for hematoxylin and eosin stain, immunohistochemistry (IHC), and in situ hybridization (ISH). Clinicopathological characteristics, including age, sex, tumor location, histology, lymphatic invasion, venous invasion, depth of invasion, lymph node metastasis, residual tumor, and adjuvant chemotherapy were reviewed from medical records. Disease stage was classified using the TNM criteria of International Union Against Cancer (UICC), seventh edition. Histological types were classified according to the Japanese classification of gastric carcinoma: 3rd English edition. The study protocol was approved by the institutional review board at the National Cancer Center.

Immunohistochemistry (IHC)

Primary antibodies used for IHC were anti-PD-L1 (SP142) rabbit monoclonal antibody, CONFIRM anti-CD3 (2GV6) rabbit monoclonal antibody, CONFIRM anti-CD4 (SP35) rabbit monoclonal antibody, anti-CD8 (C8/144B) mouse monoclonal antibody, anti-FOXP3 (236A/E7) mouse monoclonal antibody (Abcam, Cambridge, MA, USA), anti-MLH1 (ES05) mouse monoclonal antibody, anti-MSH2 (FE11) mouse monoclonal antibody, anti-PMS2 (EP51) rabbit monoclonal antibody, and anti-MSH6 (EP49) rabbit monoclonal antibody. Antibodies against PD-L1, CD3, and CD4 were purchased from Ventana (Tucson, AZ), and all the other antibodies were from Dako (Copenhagen, Demmark). CD3, CD4, and CD8 IHC were stained using a fully automated Ventana Benchmark ULTRA automated slide processing system (Ventana) according to the manufacturer's instructions. MLH1, MSH2, PMS2, and MSH6 IHC were performed with Dako's autostainer according to the manufacturer's instructions. The IHC assay for PD-L1 and FOXP3 staining is described in supplementary material S1.

Evaluation of PD-L1 expression

All tissue cores immunohistochemically stained with anti-PD-L1 antibody were evaluated by two pathologists (AK and TK). Specimens were scored as IHC 0, 1+, 2+, 3+ if <1%, ≥1 % but <10 %, ≥10 % but <20 %, or ≥20 % of TC or IC were PD-L1 positive, respectively (Figure 1). PD-L1 positivity on TC or IC was defined by the presence of ≥1 % of TC or IC with membrane staining. A higher score was selected if cases showed different PD-L1 expression scores between two cores from the same case.

Measurement of TILs

Stained slides were digitized using the NanoZoomer HT Scan System (Hamamatsu Photonics, Japan). Densities of TILs were assessed according to a previous report with some modification [14]. Briefly, 0.30 mm^2 of representative tumor area including PD-L1 positive tumor cells in each core was selected, then numbers of positive cells were counted in a total of 0.60 mm2 area. The median values were employed for cut-off points for high densities of TILs (vs. low densities of TILs).

Evaluation of MMR status

Tumors were considered negative for MLH1, MSH2, PMS2, or MSH6 expression when there was a complete absence of nuclear staining of tumor cells, while positive stainings were confirmed in normal epithelial and lymphocytes as inner control. Tumors lacking either MLH1, MSH2, PMS2, or MSH6 expression were considered MMR defective (D-MMR), whereas tumors that maintained expression of MLH1, MSH2, PMS2, and MSH6 were considered MMR proficient (P-MMR).

EBV *in situ* hybridization

Chromogenic in situ hybridization for EBV-encoded RNA (EBER) was performed using fluorescein-labeled oligonucleotide probes (INFORM EBER Probe, Ventana). The visualization system used was the BenchMark ULTRA with enzymatic digestion (ISH Protease 3, Ventana) and the iViewBlue detection kit (Ventana).

Statistical analysis

Comparisons of categorical variables were tested using the chi-square test or Fisher's exact, as appropriate. We performed survival analyses in patients with R0 resection. OS was defined from the date of surgery until death from any cause. Patients who were alive were censored at the last follow-up date. OS rates were estimated using the Kaplan–Meier method, and differences among the groups according to PD-L1 expression, densities of TILs, MMR and EBV status were identified by univariate and multivariate analyses using Cox proportional hazards models and presented as hazard ratios (HR) with 95 % confidence intervals (CI). Confounders in univariate and multivariate analyses included age, histology, depth of invasion, lymphatic invasion, venous invasion, TNM stage, and adjuvant chemotherapy. Statistical analyses were performed using IBM® SPSS® Statistics version 21 (IBM Corporation, Armonk, NY, USA). All tests were two-sided, and differences were considered significant when $P < 0.05$.

Results

Prevalence of PD-L1 expression according to MMR and EBV status

Prevalence of PD-L1 expression including the evaluation of MMR and EBV status is shown in Table 1. The PD-L1 IHC scores on TC were 0 in 376 (77.2%), 1+ in 67 (13.8%), 2+ in 23 (4.7%), and 3+ in 21 (4.3%) patients, while those on IC were 0 in 188 (38.6%), 1+ in 278 (57.1%), 2+ in 19 (3.9%), and 3+ in 2 (0.4%) patients. Accordingly, PD-L1 expression on TC and IC (IHC 1+, 2+, and 3+) was positive in 22.8 and 61.4% of all cases, respectively. A total of 61.8% of patients had positive PD-L1 expression on either TC or IC. Concordance rate of PD-L1 IHC scores on TC for the two cores was 83.2 %, while that on IC was 65.3% (supplementary Table S1).

D-MMR and EBV was detected in 25 patients (5.1%) and 25 patients (5.1%) of all cases, respectively. PD-L1 positivity was higher in D-MMR and EBV-positive GC compared with

P-MMR (TC; 72.0 vs. 20.1%, P < 0.001, IC; 96.0 vs. 59.5%, P < 0.001) and EBV-negative GC (TC; 52.0 vs. 21.2%, P = 0.001, IC; 80.0 vs. 60.4%, P < 0.001) (Table 1; supplementary Figure S1). Moreover, the PD-L1 IHC score 2+ and 3+ was also more frequently observed in D-MMR and EBV-positive GC compared with P-MMR (TC; 36.0 vs. 7.6%, P < 0.001, IC; 24.0 vs. 3.2%, P < 0.001) and EBV-negative GC (TC; 24.0 vs. 8.2%, P = 0.022). Expression status of each MMR protein was shown in supplementary Table S2.

Clinicopathological features according to PD-L1 expression

Baseline patient characteristics are shown in Table 2. PD-L1 expression was more frequently observed in patients aged ≥65 years than in those aged <65 years (TC; 28.1 vs. 16.4%, P=0.002), male than in female (TC; 25.7 vs. 16.9%, P=0.029, IC; 64.5 vs. 55.0%, P=0.043), and poorly differentiated adenocarcinoma with solid-type (por1) histology than in other histological subtypes (TC; P < 0.001, IC; P < 0.001) (Table 2).

Associations between PD-L1 expression and TILs

Median densities of CD3 (+), CD4 (+), CD8 (+), and FOXP3 (+) TILs were 725/mm^2, 303/mm^2, 384/mm^2, 53/mm^2, respectively. As shown in supplementary Table S3 and Figure S2, 78% of PD-L1 positive TC tumors were associated with high density of CD8 (+) TILs while 42% of PD-L1 negative tumors were (P < 0.001). 65% of PD-L1 positive IC tumors were associated with high density of CD8 (+) TILs while 27% of PD-L1 negative tumors were (P < 0.001). A strong association was also observed between PD-L1 expression and high densities of CD3 (+) and FOXP3 (+) TILs (TC; P < 0.001, IC; P < 0.001). In addition, there was association between PD-L1 expression and high densities of CD4 (+) TILs (TC; P = 0.004, IC; P = 0.008), although it was not as strong as that of CD3 (+), CD8 (+), and FOXP3 (+) TILs.

Survival analysis

Survival analyses of 383 patients with R0 resection were shown in supplementary Figure S3. In multivariate analysis, high density of CD8 (+) TILs was significantly associated with better survival (HR 0.63; 0.39-0.99; P = 0.050), while PD-L1 expression on TC and IC, densities of other subtypes of TILs, MMR and EBV status were not independent prognostic factors (supplementary Table S4).

Discussion

In this study, we investigated PD-L1 expression on TC and IC with TILs, MMR, and EBV status in 487 surgically resected specimens of GC. The date from a large cohort of GC in this study revealed higher PD-L1 positivity in elderly, male, poorly differentiated adenocarcinoma with solid-type (por1) histology, D-MMR, and EBV-positive GC. Furthermore, we elucidated a strong association between PD-L1 expression and high densities of TILs. To our knowledge, this study is the first to provide information on clinicopathological features of PD-L1 expression in a large cohort of GC including its association with TILs, MMR and EBV status.

In our patient cohort, a total of 61.8% of patients had positive PD-L1 expression on either TC or IC, comparable to the rate seen in Phase I trial of pembrolizumab for advanced GC [8]. In this trial, a trend toward an association between higher levels of PD-L1 expression on TC or IC and objective response rate, progression-free survival, and OS was observed. Importantly, concordance rate of PD-L1 IHC scores on TC for the two cores was 83.2 %, while that on IC was 65.3% in our analysis. It has been recently reported that PD-L1 expression in NSCLC was frequently discordant between surgical resected and matched biopsy specimens (the overall discordance rate = 48%), mainly due to the lack of a PD-L1-positive IC component in matched biopsies [15]. Our study also showed that

concordance rate of PD-L1 IHC scores on IC was relatively lower than that of TC. In order to avoid underestimate PD-L1 positive tumors in GC, it might be important for the IHC analysis to be carried out on multiple biopsies.

Recently, pembrolizumab has shown promising efficacy in a phase II trial for patients with D-MMR tumors [12]. It has been shown that D-MMR colorectal cancers had higher mutation loads compared with P-MMR, leading to high infiltration of CD8 (+) T cells presumably due to recognition of a high number of tumor neoantigens and its corresponding expression of immune checkpoints in the tumor microenvironment [16]. Our study also showed that PD-L1 positivity on TC and IC was significantly higher in GC cases with D-MMR compared with P-MMR. Moreover, 72% of cases with D-MMR exhibited high density of CD8 (+) TILs (date not shown).

In agreement with TCGA report showing elevation of PD-L1 gene amplification in EBV-positive GC [11], PD-L1 protein expression on TC and IC was more frequently observed in EBV-positive GC than in EBV-negative in our analysis. Furthermore, high infiltration of CD8 (+) T cells is one of the characteristic features of EBV-positive GC [17]. Indeed, 24 of 25 cases (96 %) with EBV-positive GC in this study were associated with high density of CD8 (+) TILs (date not shown). Recent study showed that clinical efficacy of pembrolizumab for melanoma correlated with increased frequencies of pre-existing CD8 (+) T cells [10]. Considering these findings, anti-PD-1/PD-L1 antibodies might have more therapeutic efficacy on EBV-positive GC.

It has been considered that there are two general mechanism of expression of PD-L1 in tumor cells [7]. One is innate immune resistance and the other is adaptive immune resistance. Innate immune resistance means that constitutive oncogenic signaling induces PD-L1 expression in tumor cells. Meanwhile, adaptive immune resistance means that T cells induced PD-L1 upregulation, as shown in the previous report in melanoma [18]. Our study

demonstrated that approximately 80 % of PD-L1 positive on TC cases were associated with high densities of CD3 (+) and CD8 (+) TILs, suggesting that an adaption to endogenous anti-tumor immune response might have a more important role in PD-L1 expression in GC than constitutive oncogenic signaling. Interestingly, we found that PD-L1 expression was also strongly associated with high densities of FOXP3 (+) TILs, which is a representative marker of regulatory T cells (Tregs) suppressing the activity of cytotoxic T cells. Although we did not analyze the function of Tregs in this study, targeting Tregs might enhance the activity of anti-PD-1/PD-L1 antibodies in GC.

Impact of PD-L1 expression on prognosis remains controversial in several malignancies [18-23]. In GC, two previous studies showed that high PD-L1 expression in tumors was associated with poor prognosis [19, 20], while another one showed better prognosis [21]. In our study, no association between PD-L1 expression and prognosis was observed. These wide ranges of reported outcomes might be influenced by patient cohort (clinical stages) examined and evaluation criteria of PD-L1 expression.

In conclusion, PD-L1 expression in GC had distinct clinicopathological features including high density of TILs, D-MMR and EBV-positive status. We also showed that PD-L1 expression was not a prognostic factor in GC. Impact of these characteristics on efficacy of anti-PD-1/PD-L1 antibodies warrants further evaluation.

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Disclosure

The authors have declared no conflicts of interest.

Acknowledgments

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Figure legends

Figure 1 PD-L1 expression in GC

PDL-1 tumor positivity was defined by the presence of ≥1 % of tumor cells with membrane staining. (A) IHC 0 (<1 %), (B) IHC 1+ (≥1 %, <10 %), (C) IHC 2+ (≥10 %, <20 %), (D) IHC 3+ (≥20 %)

Figure 2 PD-L1 expression in D-MMR and EBV-positive GC

PD-L1 expression was more frequently observed in D-MMR ($P < 0.001$) and EBV-positive GC ($P = 0.001$). Representative image of case with (A) D-MMR and (B) EBV-positive status with (C) PD-L1 IHC 3+.

Figure 3 Associations between PD-L1 expression and TILs

A strong association was observed between PD-L1 expression and high densities of CD3 (+), CD8 (+), and FOXP3 (+) TILs ($P < 0.001$). Representative image of case with (A) PD-L1 IHC 3+ and high densities of (B) CD3 (+), (C) CD8 (+), and (D) FOXP3 (+).

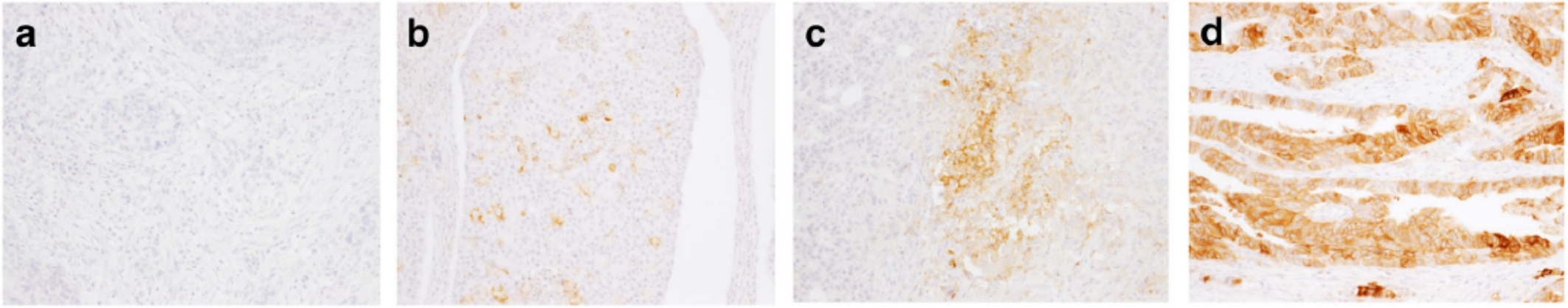
Figure 4 Kaplan–Meier plots of overall survival of according to PD-L1 expression and densities of TILs

(A) PD-L1 (+) vs. PD-L1 (-), (B) High/low CD3 (+), (C) High/low CD4 (+), (D) High/low CD8 (+), (E) High/low FOXP3 (+)

Supplementary material S1

For PD-L1 and FOXP3 staining, the sections were deparaffinized in xylene, dehydrated with graded ethanol and then immersed in methanol with 0.3 % hydrogen peroxidase for 20 min to block endogenous peroxidase. Antigen retrieval was performed at 121° C for 10 minutes in a pressure cooker (Pascal Pressurized Heating Chamber) with placed in Dako target retrieval solution for PD-L1 or citrate buffer for FOXP3, respectively. The sections were immersed in 2 % normal swine serum in PBS to block nonspecific binding for 30 min at room temperature. The Slides were then incubated overnight at 4° C with primary antibody. After being washed five times with PBS, the slides were incubated with labeled polymer horseradish peroxidase rabbit/mouse antibody for 30 minutes (Envision Plus Detection System; Dako). After extensive washing with PBS, the color reaction was developed in 2 % 3,3'-diaminobenzidine in 50 mmol/l Tris-buffer (pH7.6) containing 0.3 % hydrogen peroxide for 4 min. Background staining was performed with Mayers hematoxylin and sections then dehydrated through ascending alcohols to xylene and mounted.

Panel 1



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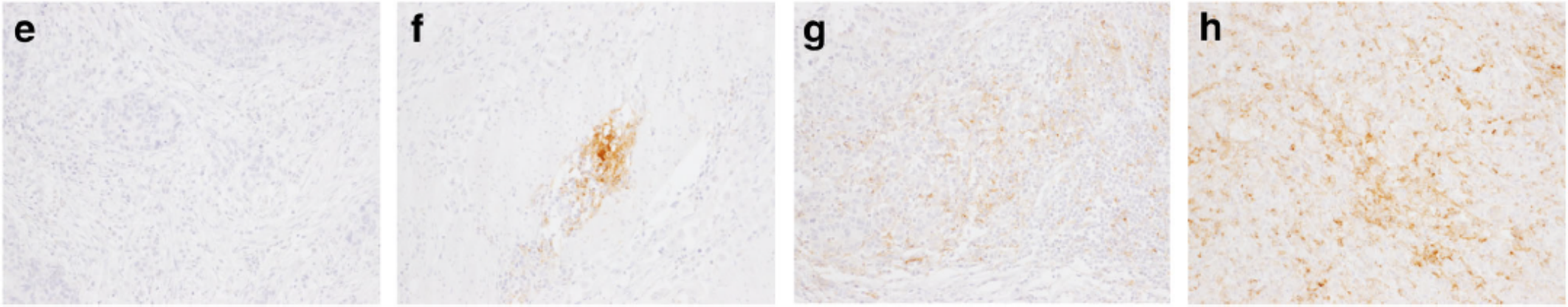
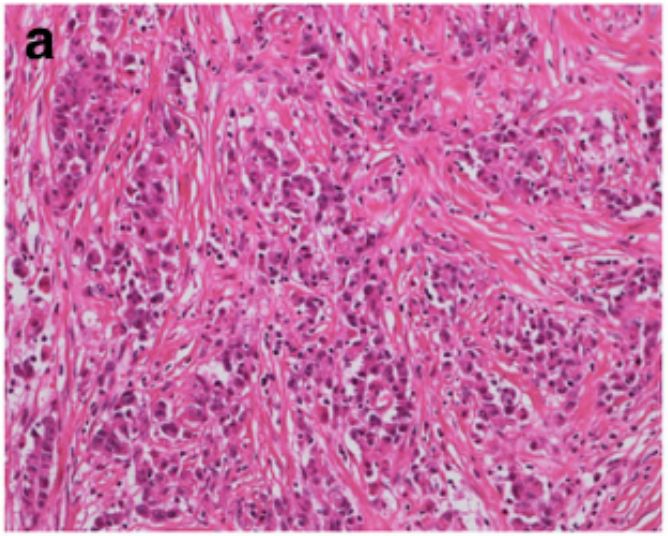
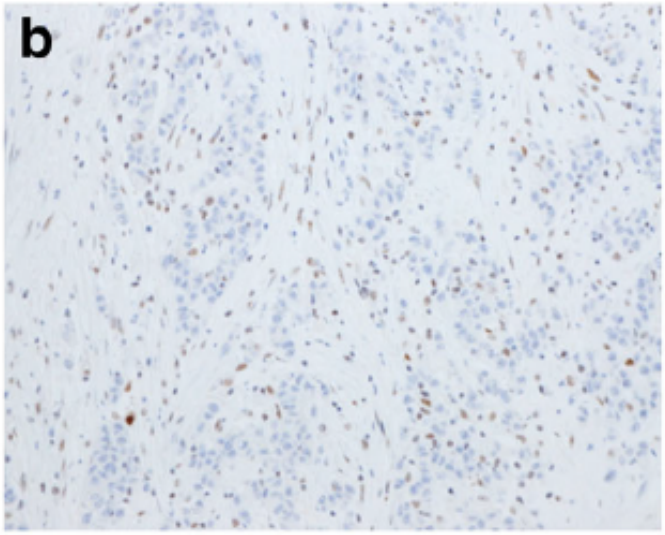
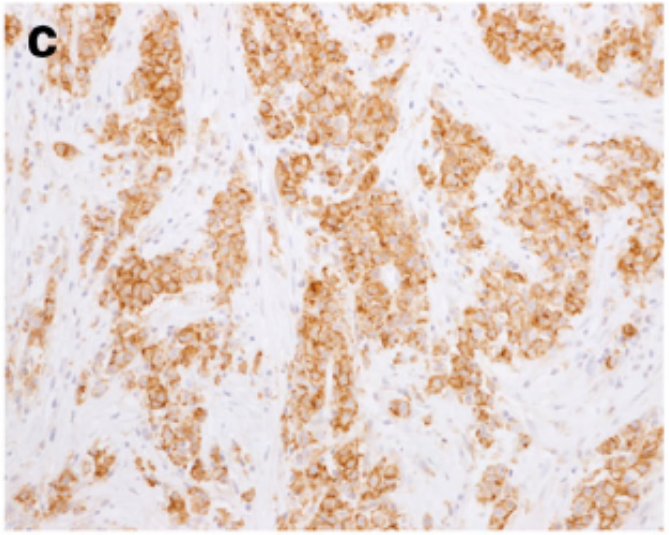


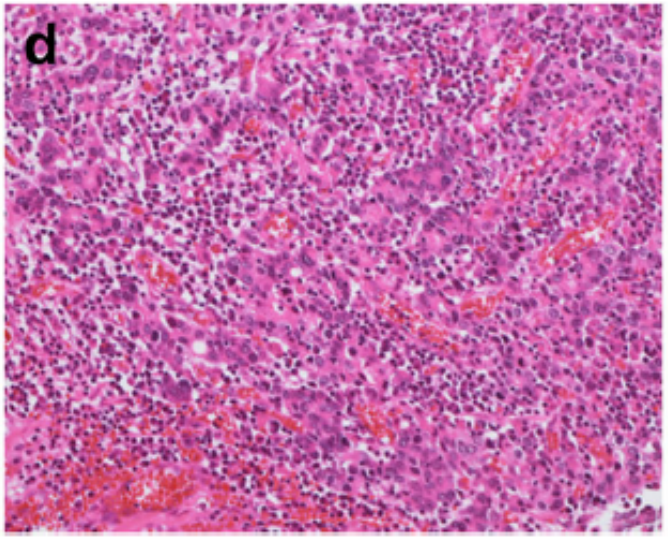
Panel 1

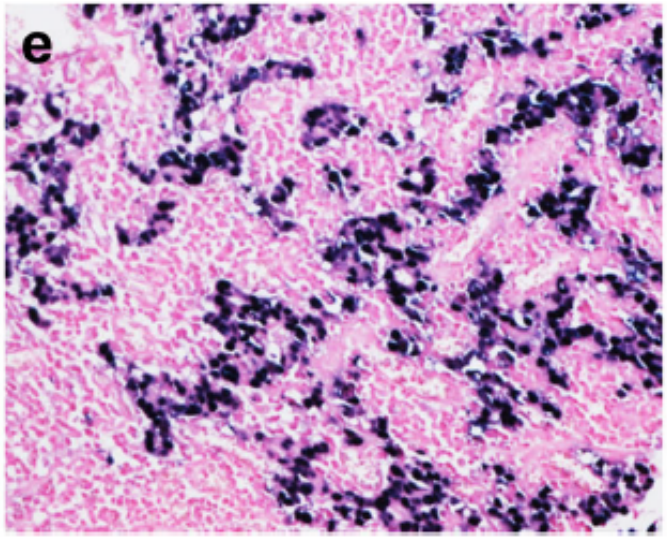


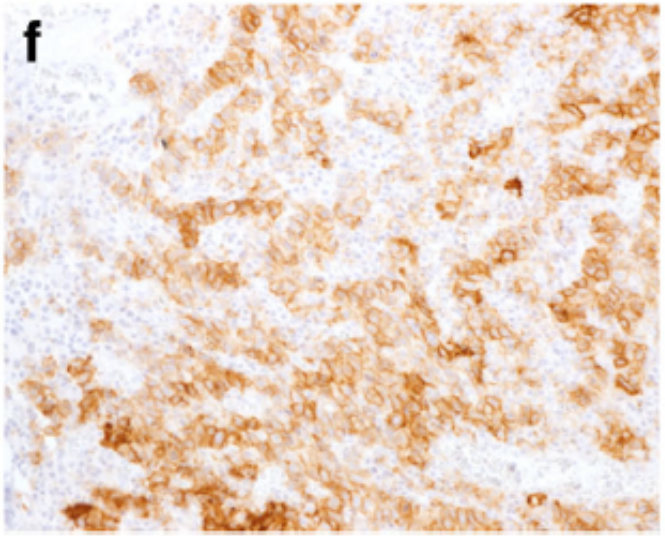


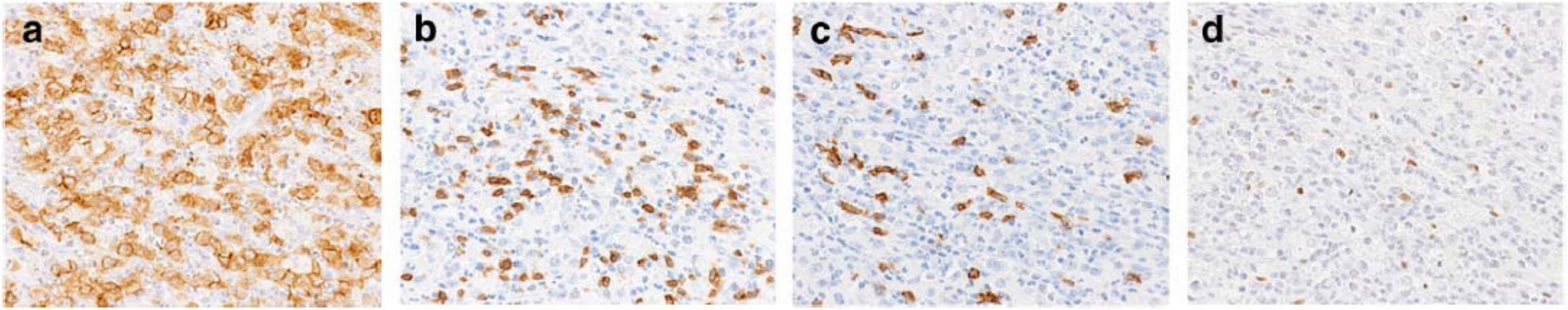


Panel 2








a
b
c
d

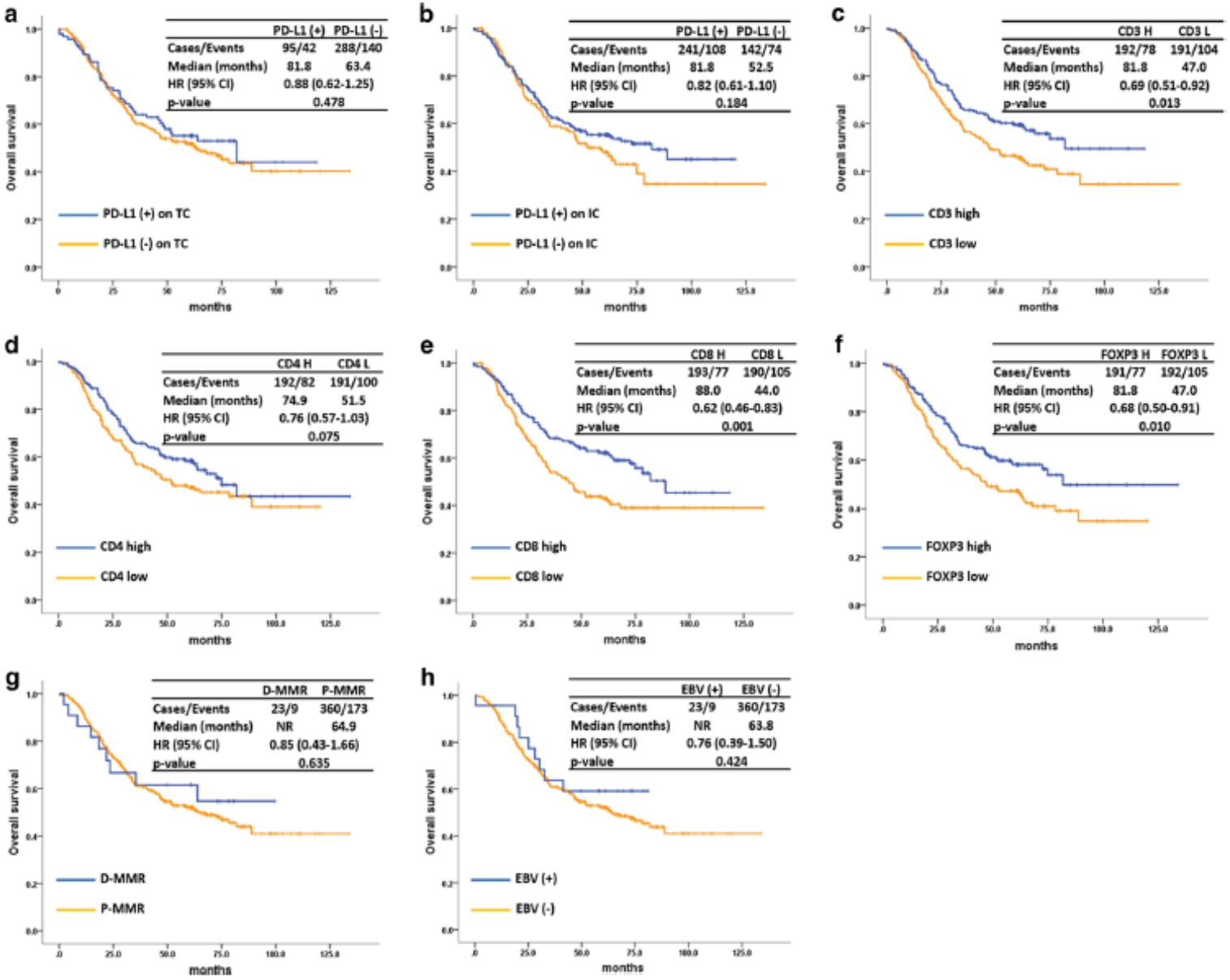

a
PD-L1 (+) PD-L1 (-)
Cases/Events 95/42 288/140
Median (months) 81.8 63.4
HR (95% CI) 0.88 (0.62-1.25)
p-value 0.478
PD-L1 (+) on TC
PD-L1 (-) on TC
Overall survival
months
b
PD-L1 (+) PD-L1 (-)
Cases/Events 241/108 142/74
Median (months) 81.8 52.5
HR (95% CI) 0.82 (0.61-1.10)
p-value 0.184
PD-L1 (+) on IC
PD-L1 (-) on IC
Overall survival
months
c
CD3 H CD3 L
Cases/Events 192/78 191/104
Median (months) 81.8 47.0
HR (95% CI) 0.69 (0.51-0.92)
p-value 0.013
CD3 high
CD3 low
Overall survival
months
d
CD4 H CD4 L
Cases/Events 192/82 191/100
Median (months) 74.9 51.5
HR (95% CI) 0.76 (0.57-1.03)
p-value 0.075
CD4 high
CD4 low
Overall survival
months
e
CD8 H CD8 L
Cases/Events 193/77 190/105
Median (months) 88.0 44.0
HR (95% CI) 0.62 (0.46-0.83)
p-value 0.001
CD8 high
CD8 low
Overall survival
months
f
FOXP3 H FOXP3 L
Cases/Events 191/77 192/105
Median (months) 81.8 47.0
HR (95% CI) 0.68 (0.50-0.91)
p-value 0.010
FOXP3 high
FOXP3 low
Overall survival
months
g
D-MMR P-MMR
Cases/Events 23/9 360/173
Median (months) NR 64.9
HR (95% CI) 0.85 (0.43-1.66)
p-value 0.635
D-MMR
P-MMR
Overall survival
months
h
EBV (+) EBV (-)
Cases/Events 23/9 360/173
Median (months) NR 63.8
HR (95% CI) 0.76 (0.39-1.50)
p-value 0.424
EBV (+)
EBV (-)
Overall survival
months

Table 1 Prevalence of PD-L1 expression according to MMR and EBV status

n (%)	All (n = 487)	P-MMR (n = 462)	D-MMR (n = 25)	P-value	EBV (-) (n = 462)	EBV (+) (n = 25)	P-value
PD-L1 (-) in TC	376 (77.2)	369 (79.9)	7 (28.0)	< 0.001	364 (78.8)	12 (48.0)	0.001
PD-L1 (+) in TC	111 (22.8)	93 (20.1)	18 (72.0)		98 (21.2)	13 (52.0)	
IHC 1+	67 (13.8)	58 (12.5)	9 (36.0)		60 (13.0)	7 (28.0)	
IHC 2+	23 (4.7)	19 (4.1)	4 (16.0)	< 0.001	20 (4.3)	3 (12.0)	0.022
IHC 3+	21 (4.3)	16 (3.5)	5 (20.0)		18 (3.9)	3 (12.0)	
PD-L1 (-) in IC	188 (38.6)	187 (40.5)	1 (4.0)	< 0.001	183 (39.6)	5 (20.0)	0.050
PD-L1 (+) in IC	299 (61.4)	275 (59.5)	24 (96.0)		279 (60.4)	20 (80.0)	
IHC 1+	278 (57.1)	260 (56.3)	18 (72.0)		259 (56.1)	19 (76.0)	
IHC 2+	19 (3.9)	14 (3.0)	5 (20.0)	< 0.001	18 (3.9)	1 (4.0)	1.00
IHC 3+	2 (0.4)	1 (0.2)	1 (4.0)		2 (0.4)	0 (0)	

Table 2 Patient characteristics according to PD-L1 expression

n (%)			PD-L1 expression					
		All (n = 487)	TC (-) (n = 376)	TC (+) (n = 111)	P-value	IC (-) (n = 188)	IC (+) (n = 299)	P-value
Age	<65	220 (45.2)	184 (48.9)	36 (32.4)	0.002	95 (50.5)	125 (41.8)	0.060
(median, range 26-92)	≥65	267 (54.8)	192 (51.1)	75 (67.6)		93 (49.5)	174 (58.2)	
Sex	Male	327 (67.1)	243 (64.6)	84 (75.7)	0.029	116 (61.7)	211 (70.6)	0.043
	Female	160 (32.9)	133 (35.4)	27 (24.3)		72 (38.3)	88 (29.4)	
Tumor location	EGJ	33 (6.8)	26 (6.9)	7 (6.3)	0.737	17 (9.0)	16 (5.4)	0.220
	Upper third	103 (21.1)	80 (21.3)	23 (20.7)		41 (21.8)	62 (20.7)	
	Middle third	195 (40.1)	153 (40.6)	42 (37.8)		80 (42.6)	115 (38.5)	
	Lower third	149 (30.6)	113 (30.1)	36 (32.5)		48 (25.5)	101 (33.8)	
	Remnant	7 (1.4)	4 (1.1)	3 (2.7)		2 (1.1)	5 (1.7)	
Histology*	pap	15 (3.1)	12 (3.2)	3 (2.7)	< 0.001	7 (3.7)	8 (2.7)	< 0.001
	tub1	18 (3.7)	16 (4.3)	2 (1.8)		11 (5.9)	7 (2.3)	
	tub2	151 (31.0)	114 (30.3)	37 (33.3)		44 (23.4)	107 (35.8)	
	por1	46 (9.4)	22 (5.9)	24 (21.6)		5 (2.7)	41 (13.7)	
	por2	214 (43.9)	170 (45.1)	44 (39.6)		92 (48.9)	122 (40.8)	
	sig	13 (2.7)	13 (3.5)	0 (0)		5 (2.7)	8 (2.7)	
	muc	30 (6.2)	29 (7.7)	1 (0.9)		24 (12.8)	6 (2.0)	
Lymphatic invasion	Absent	38 (7.8)	26 (6.9)	12 (10.8)	0.179	12 (6.4)	26 (8.7)	0.354
	Present	449 (92.2)	350 (93.1)	99 (89.2)		176 (93.6)	273 (91.3)	
Venous invasion	Absent	55 (11.3)	48 (12.8)	7 (6.3)	0.059	20 (10.6)	35 (11.7)	0.717
	Present	432 (88.7)	328 (87.2)	104 (93.7)		168 (89.4)	264 (88.3)	
Depth of invasion	T1	1 (0.2)	1 (0.3)	0 (0)	0.635	0 (0)	1 (0.3)	0.143
	T2	21 (4.3)	14 (3.7)	7 (6.3)		5 (2.7)	16 (5.4)	
	T3	166 (34.1)	128 (34.0)	38 (34.2)		57 (30.3)	109 (36.5)	
	T4	299 (61.4)	233 (62.0)	66 (59.5)		126 (67.0)	173 (57.9)	
Regional lymph node	Absent	7 (1.4)	4 (1.1)	3 (2.7)	0.202	0 (0)	7 (2.3)	0.035
invasion	Present	480 (98.6)	372 (98.9)	108 (97.3)		188 (100)	292 (97.7)	
TNM stage	III	358 (73.5)	274 (72.9)	84 (75.7)	0.556	138 (73.4)	220 (73.6)	0.966
	IV	129 (26.5)	102 (27.1)	27 (24.3)		50 (26.6)	79 (26.4)	
Residual tumor	R0	383 (78.6)	-	-		-	-	
	R1, R2	104 (21.4)	-	-		-	-	
Adjuvant Chemotherapy	Yes	261 (53.6)	-	-		-	-	
	No	226 (46.4)	-	-		-	-	

pap, papillary adenocarcinoma; tub1, tubular adenocarcinoma,well-differentiated; tub2, tubular adenocarcinoma, moderately differentiated; por1, poorly differentiated adenocarcinoma, solid type; por2, poorly differentiated adenocarcinoma, non-solid type; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma; EGJ,

esophagogastric junction
*Histological classification is described according to the Japanese classification of gastric carcinoma: 3rd English edition

Table S1 Concordance of PD-L1 IHC scores for the two core

Core A	Core B				
TC/IC	0	1+	2+	3+	Total
0	376/188	26/80	4/2	1/0	407/270
1+	25/74	16/124	4/6	3/0	48/204
2+	7/0	5/5	3/6	2/0	17/11
3+	3/0	0/2	2/0	10/0	15/2
Total	411/262	47/211	13/14	16/0	487

Table S2 Expression of MMR protein

	All (n = 487)	%
MLH1 loss	25	5.1
MSH2 loss	0	0
PMS2 loss	17	3.5
MSH6 loss	0	0
MLH1 loss/PMS2 loss	17	3.5
MLH1 loss only	8	1.6

Table S3 Associations between PD-L1 expression and TILs

		CD3		CD4		CD8		FOXP3	
Median density		725/mm^2		303/mm^2		384/mm^2		53/mm^2	
n (%)		High	Low	High	Low	High	Low	High	Low
PD-L1 in TC	positive	86 (77)	25 (23)	69 (62)	42 (38)	87 (78)	24 (22)	84 (76)	27 (24)
	negative	157 (42)	219 (58)	175 (47)	201 (53)	157 (42)	219 (58)	158 (42)	218 (58)
P-value		< 0.001		0.004		< 0.001		< 0.001	
PD-L1 in IC	positive	188 (63)	111 (37)	164 (55)	135 (45)	193 (65)	106 (35)	179 (60)	120 (40)
	negative	55 (29)	133 (71)	80 (43)	108 (57)	51 (27)	137 (73)	63 (33)	125 (67)
P-value		< 0.001		0.008		< 0.001		< 0.001	

Table S4 Univariate and multivariate analysis for overall survival

Variables		Uuivariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
Age	<65	ref	-	ref	-
	≥65	1.57 (1.16-2.11)	0.003	1.39 (1.01-1.91)	0.041
Histology	Differentiated	ref	-	ref	-
	undifferentiated	1.35 (1.00-1.81)	0.051	1.48 (1.08-2.03)	0.014
Depth of invasion	pT1-3	ref	-	ref	-
	pT4	1.56 (1.16-2.10)	0.003	1.56 (1.14-2.15)	0.006
Lymphatic invasion	Absent	ref	-	ref	-
	Present	1.57 (0.87-2.82)	0.131	2.15 (1.16-3.97)	0.015
Venous invasion	Absent	ref	-	ref	-
	Present	1.49 (0.92-2.40)	0.102	1.47 (0.89-2.43)	0.131
TNM Stage	III	ref	-	ref	-
	IV	2.24 (1.50-3.35)	< 0.001	1.934 (1.26-2.97)	0.003
Adjuvant chemotherapy	No	ref	-	ref	-
	Yes	0.53 (0.40-0.71)	< 0.001	0.48 (0.36-0.66)	< 0.001
PD-L1 in TC	Negative	ref	-	ref	-
	Positive	0.88 (0.62-1.25)	0.478	1.05 (0.68-1.61)	0.836
PD-L1 in IC	Negative	ref	-	ref	-
	Positive	0.82 (0.61-1.10)	0.184	1.03 (0.72-1.47)	0.887
CD3	Low	ref	-	ref	-
	High	0.69 (0.51-0.92)	0.013	1.06 (0.62-1.84)	0.821
CD4	Low	ref	-	ref	-
	High	0.76 (0.57-1.03)	0.075	0.91 (0.62-1.32)	0.606
CD8	Low	ref	-	ref	-
	High	0.62 (0.46-0.83)	0.001	0.63 (0.39-0.99)	0.050
FOXP3	Low	ref	-	ref	-
	High	0.68 (0.50-0.91)	0.010	0.83 (0.58-1.12)	0.317
MMR	Proficient	ref	-	ref	-
	Deficient	0.85 (0.43-1.66)	0.635	0.66 (0.32-1.37)	0.265
EBV	Negative	ref	-	ref	-
	Positive	0.76 (0.39-1.50)	0.424	0.89 (0.44-1.61)	0.748