Programmed Death-Ligand 1 Expression Is Common in Gastric Cancer Associated With Epstein-Barr Virus or Microsatellite Instability

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Abstract: Blockade of the programmed death 1 (PD-1) pathway has emerged as a novel therapy for cancer. Therefore, development of biomarkers for response prediction, such as PD-ligand 1 (PD-L1) expression by immunohistochemistry, may help to stratify patients. Solid tumors with CD8 T-cell rich tumor microenvironment have been implicated to be associated with increased PD-L1 expression. We hypothesized that gastric cancers associated with Epstein-Barr virus infection (EBV+) or microsatellite instability (MSI), both of which are known to harbor such tumor microenvironment, are associated with increased PD-L1 expression. Forty-four resected gastric cancers including 7 EBV+, 16 MSI, and 21 microsatellite stable cancers without EBV (EBV−/MSS) were studied for PD-L1 expression and T-cell subpopulations by immunohistochemistry. Positive PD-L1 expression (PD-L1+), defined as membranous staining in either tumor cells or tumor immune infiltrates, was seen in 32 (72%) gastric cancers. EBV+ or MSI cancers showed significantly higher rates of PD-L1+ compared with EBV−/MSS cancers (7/7, 100%; 14/16, 87%; 11/21, 52%; $P = 0.013$). PD-L1+/EBV+ and PD-L1+/MSI cancers had significantly more CD8 T cells at tumor invasive front than PD-L1+/EBV−/MSS cancers ($P < 0.001$). PD-L1+ was not associated with the depth of invasion or nodal metastasis ($P = 0.534$, 0.288). Multivariate analysis showed PD-L1+ was not an independent predictor of disease-free survival while MSI was ($P = 0.548$, 0.043). In summary, EBV+ or MSI gastric cancers are more likely to express PD-L1 and have increased CD8 T cells at tumor invasive front than EBV−/MSS cancers. Our results suggest EBV infection and MSI should be investigated for predicting response to PD-1 blockade.

Key Words: medullary carcinoma, gastric carcinoma with lymphoid stroma, signet-ring cell carcinoma, tumor microenvironment, mismatch repair deficiency

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Gastric cancer remains one of the leading causes of cancer mortality worldwide.[1] It is often diagnosed at an advanced stage and thus systemic chemotherapy may be the only treatment option. However, apart from trastuzumab, the monoclonal antibody targeting gastric cancers that overexpress the human epidermal growth factor receptor 2 protein (ERBB2 also known as HER2/neu),[2] the ability to use an optimal treatment based upon biological or genetic features of a gastric cancer is still lacking.

The programmed death 1 (PD-1)/PD-ligand 1 (PD-L1) immune checkpoint functions in maintaining self-tolerance and regulating immune response in peripheral tissue to minimize unnecessary tissue damage.[3] It is one of the strategies used in solid tumors to evade T-cell–mediated, endogenous antitumor immune response.[4–7] Binding of PD-L1 expressed on tumor cells and/or tumor-associated immune cells to its receptor PD-1 on activated T cells can suppress T-cell function and restrict tumor killing.[3,8,9] PD-L1 expression on tumor cells can either be driven by constitutive oncogenic signaling pathways[10–12] or be induced by interferon-γ produced by CD8 T cells.[13] The latter mechanism is termed "adaptive immune resistance"[3,4]; it represents a response exerted by tumor in an attempt to protect itself from the endogenous antitumor immune reaction.

Therapeutic agents blocking the PD-1/PD-L1 checkpoint have shown durable clinical responses in patients with various cancer types.[5,6,14,15] Results from these studies suggest that positive PD-L1 expression in tumor microenvironments (including both tumor cells and immune cells) in pretreatment specimens may be used to predict clinical response.[5,14] Furthermore, PD-1/PD-L1 checkpoint inhibition is most effective in solid tumors with a preexisting antitumor response, especially preexisting CD8 T cells.[5,6,15]

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Gastric cancer associated with Epstein-Barr virus infection (EBV+) and gastric cancer with microsatellite instability (MSI) have rich lymphocytic infiltration in tumor stroma and thus can be classified as gastric carcinoma with prominent lymphoid stroma (medullary carcinoma).[16,17] The lymphoid stroma in these tumors has high number of CD8 T cells, capable of mounting a robust antitumor inflammatory response.[18–20]

Positive PD-L1 expression has been reported in gastric cancer.[21–27] However, most previous studies focused on investigating the role of PD-L1 expression in predicting outcomes of gastric cancer patients; only a few evaluated PD-L1 expression in gastric cancer in the context of tumor immune microenvironment and/or CD8 T cells. The study by Thompson et al[21] demonstrated concordant increase in PD-L1 expression and CD8 T cells in a cohort of 34 gastric/gastroesophageal junction adenocarcinomas. Herein, we hypothesized that gastric cancers associated with EBV infection or MSI are more likely to express PD-L1 and to have increased CD8 T cells in tumor microenvironment than gastric cancers that are microsatellite stable and without EBV infection (EBV−/MSS). We retrospectively characterized PD-L1 expression in tumor and T-cell subtypes at the tumor invasive front (tumor-stroma interface) by immunohistochemistry in a cohort of EBV+, MSI, and EBV−/MSS gastric cancers. We also determined whether PD-L1 expression correlates with patient survival. Our results demonstrate PD-L1 expression in gastric cancer is commonly seen with EBV infection, MSI, and increased T cells, especially CD8 T cells, in tumor microenvironment.

MATERIALS AND METHODS

Case Selection

This study was approved by the Institutional Review Boards at the University of Pittsburgh (IRB#: PRO14080122). Surgical pathology archives of the Department of Pathology at the University of Pittsburgh Medical Center between 2004 and 2015 were searched for gastric cancer resection cases that had been tested for EBV infection and/or MSI as components of the clinical workup. Resection cases with formalin-fixed paraffin-embedded blocks available were included in this study.

Clinical and Histopathologic Features

Hematoxylin and eosin (H&E)–stained slides of each case were reviewed to confirm the cancer diagnosis and the corresponding pathologic findings were recorded. These included the histologic classification, histologic grade, anatomic location, the pathologic TNM staging (tumor, lymph node, and distant metastasis), and concurrent *Helicobacter pylori* infection or previous infection with eradication. For histologic typing, the World Health Organization's (WHO) classification[16] was used; the histologic grade was based on the WHO and American Joint Committee on Cancer Criteria recommendations.[16,28,29] When necessary, cases were restaged to the current American Joint Committee on Cancer Criteria recommendations.[28,30] Corresponding clinical data including patient age, sex, presurgical and postsurgical treatments, and follow-up information were collected.

Immunohistochemistry and EBV In Situ Hybridization

One representative formalin-fixed paraffin-embedded block of the cancer in each case was chosen for immunohistochemical analysis. Immunohistochemical labeling was performed using antibodies against PD-L1 (clone SP263; Ventana), PD-1 (clone NAT; Abcam), CD3 (polyclonal; Dako), CD4 (clone SP35; Dako), CD8 (clone 144B; Dako), CD163 (clone MRQ-26; Leica), E-cadherin (clone NCH-38; Dako), MLH1 (clone G168-728; Ventana), MSH2 (clone G219-1129; Ventana), PMS2 (clone EPR3947; Cell Marque), MSH6 (clone 44; Ventana), and *H. pylori* (polyclonal; Ventana), on 4 μM unstained tissue sections using automated stainers including the Ventana Benchmark Ultra platform (Ventana Medical Systems, Tucson, AZ) and the Leica BOND-III automated stainer (Leica Biosystems Inc., Buffalo Grove, IL) according to the manufacturers' recommendations.

Cases that were only tested by a 2-antibody DNA mismatch repair immunohistochemical panel using antibodies against PMS2 and MSH6 were stained with antibodies against MLH1 and MSH2. All showed staining results concordant with results derived from the 2-antibody panel. In another word, MSI cases remained MSI while all MSS cases were MSS when evaluated by results of the 4-antibody panel. All EBV-positive cases that had not been tested for MSI were stained for MLH1, MSH2, PMS2, and MSH6. All cases showed preserved nuclear expressions of all 4 proteins and thus all were MSS.

All MSS cases that had not been tested for EBV infection were tested by in situ hybridization using prediluted probes targeting EBV-encoded early RNA (*EBER*, Ventana) on unstained tissue sections in 4-μM thickness using the Ventana XT automated stainer (Ventana Medical Systems). All cases tested for *EBER* were also tested for the integrity of total RNA using RNA-positive control probe and all showed adequate total RNA preservation.

All H&E slides and immunohistochemical and in situ stains were reviewed and scored independently by 2 pathologists without knowledge of MSI and EBV infection status.

Characterization of T Cells in Immune Infiltrates

The tumor-associated immune infiltrates, defined as the population of peritumoral (immune cells in immediate contact with tumor cell nests) and intratumoral inflammatory cells, observed in gastric cancers were predominantly composed of lymphocytes and histiocytes. This was very similar to that reported in other solid tumors, such as melanoma and renal cell carcinoma.[4,5]

T cells in the tumor-associated immune infiltrates at the invasive front in each tumor were further quantified using methods modified from a previous study.[7] In particular, the number of CD3, CD4, CD8, or PD-1-positive

cells per high-power field was quantified by counting in 5 representative, high-power fields at the invasive front of the tumor. The mean number of positive cells per high-power field for each antibody was recorded. A high-power field was defined as the area seen through a 40× -objective lens of an Olympus BX46 microscope. The invasive front of the tumor (tumor-stroma interface) was defined as the leading edge of the tumor cell nests that was invading into the gastric wall and juxtaposed with uninvolved stomach in a given tissue section.

CD4 to CD8 ratio was calculated by dividing the mean number of CD4 T cells per high-power field by the mean number of CD8 T cells per high-power field in each gastric tumor. A ratio <0.90 was defined as CD4 < CD8; a ratio >1.10 was defined as CD4 > CD8; a ratio between 0.90 and 1.10 was defined as CD4 = CD8. The percentage of PD-1 T cells in CD3 T cells was calculated as well.

Characterization of PD-L1 Staining

PD-L1 staining was scored similar to those reported previously.[4,5] A membranous PD-L1 staining pattern was seen in tumor cells and tumor-associated immune cells and was considered as specific staining. As preliminary results from ongoing clinical studies on PD-1 blockade in gastric cancer used either distinct stromal or 1% tumor cell PD-L1 staining as enrollment criteria,[31] positive PD-L1 expression in this study was defined as any membranous staining in either tumor cells or tumor immune infiltrates to maximize the number of PD-L1-positive cases. The percentage of tumor cells demonstrating membranous PD-L1 staining was also scored in 5% increments.

The pattern of PD-L1 staining in gastric cancer was classified as: "diffuse" when there was contiguous membranous staining in a large focus involving at least 10% of the tumor in the representative tissue section (Fig. 1A); predominantly at the "invasive front" when PD-L1 staining was seen in tumor cells and the infiltrating immune cells at the invasive front of the tumor (Fig. 1B); predominantly in "immune infiltrates" when PD-L1 stain was seen predominantly in the tumor infiltrating immune cells (Fig. 1C); "negative" when minimal (rare, single cells with membranous PD-L1 staining) or no PD-L1 staining was detected in a tumor (Fig. 1D). In all cases with "negative" PD-L1 staining in cancer, PD-L1 staining in lymphoid aggregates in tissue uninvolved by cancer was present and served as the internal control for PD-L1 immunohistochemistry.

Statistical Analysis

The GraphPad Prism 6 for Windows, Version 6.00 (GraphPad Software Inc., La Jolla, CA) and IBM SPSS (Release 23.0.0.0) were used for statistical analyses. A P-value <0.05 was considered statistically significant. Tests used included Fisher exact test, χ^2 test, Kruskal-Wallis 1-way analysis of variance, Mann-Whitney Wilcoxon test, Student t test, Kaplan-Meier survival analysis, and Cox regression analysis. For survival analysis, overall survival (OS) was the duration between diagnosis and either death or the latest clinical follow-up time; disease-free survival (DFS) was the duration between complete surgical resection and disease progression documented by imaging and/or pathologic evaluations of metastasis. Univariate and multivariate analyses for DFS were performed using data from gastric cancer cases without distant metastasis at the time of surgery (ie, M0 or stage I & II & III, N = 40).

RESULTS

Clinical and Histopathologic Features of Gastric Cancers

Forty-four gastric cancer resection cases were identified, including 7 EBV+, 16 MSI, and 21 EBV−/MSS cancers (Table 1). Most EBV+ cancers were found in the proximal stomach (5/7, 71%; $P = 0.085$) and were diagnosed in men (6/7, 86%; $P = 0.240$). Patients with MSI cancers were significantly older than patients with EBV+ and EBV−/MSS cancers (mean age [y], 79 vs. 68 vs. 69; $P = 0.004$). Histologically, 71% (5/7) of EBV+ and 63% (10/16) of MSI gastric cancers were classified as carcinoma with lymphoid stroma (medullary carcinoma) according to the WHO classification. However, this histologic subtype was only seen in 5% (1/21) of the EBV−/MSS cancers ($P = 0.001$). In addition, 1 EBV−/MSS tumor was adenosquamous carcinoma and 2 were mixed adenoneuroendocrine carcinomas.

The mean total follow-up time was 43.1 months (range, 0.3 to 124.1 mo) and mean disease-free interval was 34.1 months (range, 0.0 to 116.8 mo). No significant associations were found between either total follow-up time or disease-free interval and the status of EBV infection and MSI ($P = 0.412$ and 0.121, respectively). However, significantly fewer patients with either EBV+ or MSI cancers had disease recurrence compared with patients with EBV−/MSS tumors (2/7 [29%], 2/16 [13%], 9/21 [43%]; $P = 0.045$).

EBV+ or MSI Gastric Cancers Tend to Show Positive PD-L1 Expression

Membranous PD-L1 stain was seen in both tumor cells and tumor-associated immune infiltrates. Four PD-L1 staining patterns were observed. These included: (1) diffuse (contiguous membranous staining involving 10% or more tumor; Fig. 1A), (2) invasive front (staining seen predominantly in tumor cells and the associated immune cells at the tumor-stroma interface; Fig. 1B), (3) immune infiltrates (staining only seen in tumor-associated immune cells; Fig. 1C), and (4) negative (no staining in either tumor cells or tumor-associated immune cells; Fig. 1D). Gastric cancers demonstrating any of the first 3 staining patterns were considered positive for PD-L1 expression.

Of the 10 gastric cancers with diffuse staining pattern ($\geq 10\%$ of cells in tumor), 3 cases showed contiguous PD-L1 staining involving 10% to 20% cells of the tumor, 3 cases showed contiguous staining involving 20% to 30% cells in the tumor, 1 had contiguous staining in 30%

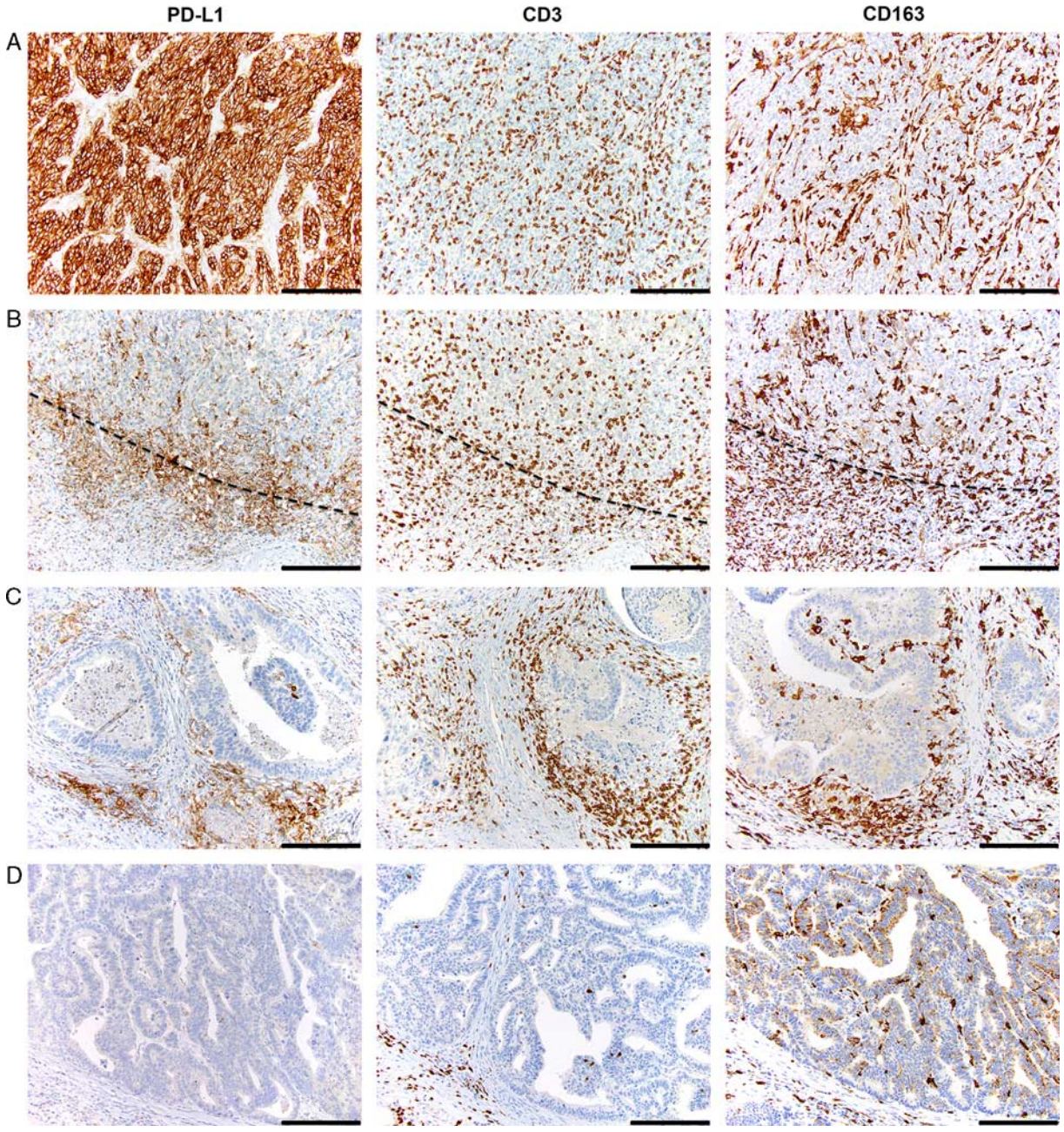

FIGURE 1. The 4 patterns of PD-L1 expression in gastric cancers. PD-L1 stain of a tumor section and the corresponding CD3 and CD163 stains are shown from left to right in each panel. A, Diffuse membranous PD-L1 staining in an MSI cancer. B, Membranous PD-L1 staining at the invasive front in an MSI cancer. The dashed line in each photomicrograph indicates the invasive front of the tumor/tumor-stroma interface. C, Membranous PD-L1 staining in immune infiltrates in an EBV−/MSS cancer. D, Negative/no PD-L1 staining in tumor cells and immune infiltrates in an EBV−/MSS cancer. Scale bar: 200 μm.

to 40% cells, 1 case 60% to 70% tumor cells (EBV+), and 2 cases 80% to 90% tumor cells (1 MSI and 1 EBV−/MSS) (Table 2).

Thirty-two of the 44 cases (72%) were PD-L1 positive (PD-L1+). Both EBV+ caners and MSI cancers were more likely to be PD-L1+ compared with EBV−/MSS cancers (7/7 [100%], 14/16 [87%], 11/21 [52%]; $P = 0.013$) (Table 2). The majority of PD-L1+/EBV+ and PD-L1+/MSI gastric caners showed diffuse (4/7 [57%], 5/14 [36%]) or invasive front staining patterns (2/7 [29%], 9/14 [64%]). In contrast, PD-L1+/EBV−/MSS cancers often had PD-L1 staining only in immune infiltrates (7/11, 64%). Of note, PD-L1 expression was negative (PD-L1−) in the 2 (100%) mucinous adenocarcinomas and in 4 of 5 (80%) signet-ring cell carcinomas (including 1 MSI and 3 EBV−/MSS cases).

TABLE 1. Clinical and Histopathologic Features (N = 44)

	Total (N = 44)	EBV + (N = 7)	MSI (N = 16)	EBV −/MSS (N = 21)	*P*
Sex (n [%])					
Male	25 (57)	6 (86)	8 (50)	11 (52)	0.240
Female	19 (43)	1 (14)	8 (50)	10 (48)	
Mean age (y, range)	73 (29-89)	68 (51-89)	79 (68-87)	69 (29-83)	**0.004**
Active *H. pylori* infection (n [%])	11 (25)	2 (29)	3 (19)	6 (29)	0.800
Anatomic location (n [%])					
Proximal stomach	19 (43)	5 (71)	6 (37)	8 (38)	0.085
Antrum	19 (43)	1 (14)	10 (63)	8 (38)	
Proximal & antrum	6 (14)	1 (14)	0	5 (24)	
Histologic type, WHO classification (n [%])					
Carcinoma with lymphoid stroma (medullary carcinoma)	16 (36)	5 (71)	10 (63)	1 (5)	**0.001**
Adenocarcinoma	25 (57)	2 (29)	6 (37)	17 (81)	
Signet-ring cell	5	1	1	3	
Tubular	1	0	0	1	
Papillary	1	0	0	1	
Mucinous	2	0	1	1	
Mixed	16	1	4	11	
Other types	3 (7)	0	0	3 (14)	
Histologic grade (n [%])					
G1	3 (7)	1 (14)	0	2 (10)	0.520
G2	6 (14)	0	3 (19)	3 (14)	
G3	35 (79)	6 (86)	13 (81)	16 (76)	
pT (n [%])					
T1	9 (20)	2 (29)	2 (13)	5 (24)	0.495
T2	8 (18)	2 (29)	4 (25)	2 (10)	
T3	17 (39)	2 (29)	8 (50)	7 (33)	
T4	10 (23)	1 (14)	2 (13)	7 (33)	
pN (n [%])					
N0	16 (36)	4 (57)	7 (44)	5 (24)	0.623
N1	14 (32)	1 (14)	6 (37)	7 (33)	
N2	5 (11)	1 (14)	1 (6)	3 (14)	
N3	9 (20)	1 (14)	2 (13)	6 (29)	
pM (n [%])					
M0	40 (91)	7 (100)	16 (100)	17 (81)	0.090
M1	4 (9)	0	0	4 (19)	
Stage (n [%])					
Stage I	10 (23)	3 (43)	4 (25)	3 (14)	0.379
Stage II	17 (39)	2 (29)	8 (50)	7 (33)	
Stage III	13 (30)	2 (29)	4 (25)	7 (33)	
Stage IV	4 (9)	0	0	4 (19)	
OS					
Mean total follow-up time (mo, range)	43.1 (0.3-124.1)	58.0 (16.0-99.5)	41.9 (0.5-118.1)	39.1 (0.3-124.1)	0.412
Death (n [%])	20 (45)	3 (43)	6 (37)	11 (52)	0.659
Alive (n [%])	24 (55)	4 (57)	10 (63)	10 (48)	
DFS					
Mean disease-free interval after surgery (mo, range)	34.1 (0-116.8)	52.5 (3.9-99.0)	39.7 (0.2-116.8)	23.7 (0.0-106.9)	0.121
No recurrence (n [%])	27 (61)	5 (71)	14 (87)	8 (38)	**0.045***
Recurrence (n [%])	13 (30)	2 (29)	2 (13)	9 (43)	
Never disease-free (n [%])	4 (9)	0	0	4 (19)	
Systemic treatment (n [%])					
None	18 (41)	2 (29)	8 (50)	8 (38)	0.183
Neoadjuvant treatment only	7 (16)	3 (43)	3 (19)	1 (5)	
Adjuvant treatment only	10 (23)	2 (29)	2 (13)	6 (29)	
Both	9 (20)	0	3 (19)	6 (29)	

**P*-value was calculated by comparing patients with or without recurrence between the 3 groups.
P-values in bold are statistically significant

Positive PD-L1 Expression in EBV+ or MSI Gastric Cancers is Associated With Increased CD8 T Cells at Tumor Invasive Front

In EBV −/MSS gastric cancers, tumor-associated immune infiltrates were predominantly located at the invasive front (Fig. 2A). In contrast, the immune infiltrates in EBV + or MSI cancers were seen not only at invasive front but also in the tumor stroma and in tumor cell nests (Figs. 2B, C). As immune infiltrates were observed at the invasive front in all 3 groups, the quantity and subtypes of T cells at the invasive front were further characterized.

EBV + or MSI gastric cancers had significantly more CD3, CD4, CD8, and PD-1 T cells at tumor invasive front compared with EBV −/MSS cancers

TABLE 2. PD-L1 Expression and Tumor Immune Microenvironment Stratified by EBV and MSI Status

	All (N = 44)	EBV+ (N = 7)	MSI (N = 16)	EBV−/MSS (N = 21)	*P*
Pattern of PD-L1 staining (n [%])					
Diffuse	10 (23)	4 (57)	5 (31)	1 (5)	< 0.001
Invasive front	14 (32)	2 (29)	9 (56)	3 (14)	
Immune infiltrates	8 (18)	1 (14)	0	7 (33)	
Negative	12 (27)	0	2 (13)	10 (48)	
Portion of cells in tumor with PD-L1 staining (n [%])					
0% (PD-L1−)	12 (27)	0	2 (13)	10 (48)	0.013
> 0% (PD-L1+)	32 (72)	7 (100)	14 (87)	11 (52)	
> 0, < 5%	16	2	5	9	
≥ 5%, < 10%	6	1	4	1	
≥ 10%, < 20%	3	1	2	0	
≥ 20%, < 30%	3	1	2	0	
≥ 30%, < 40%	1	1	0	0	
≥ 40%	3	1	1	1	
T cells at the invasive front (mean/high-power field [range])					
CD3	436 (112-865)	580 (265-865)	527 (149-780)	318 (112-654)	< 0.001
CD4	249 (51-516)	310 (164-516)	287 (51-489)	200 (52-514)	0.011
CD8	246 (35-640)	366 (129-512)	306 (63-640)	161 (35-338)	< 0.001
PD-1	97 (5-331)	137 (48-230)	127 (5-331)	60 (17-178)	0.002
CD4/CD8 ratio (n [%])					
CD4 ≥ CD8	29 (66)	3 (43)	8 (50)	18 (86)	0.028
CD4 < CD8	15 (34)	4 (57)	8 (50)	3 (14)	
Portion of immune cells expressing PD-1 (n [%])					
< 5%, > 0%	3 (7)	0	2 (13)	1 (5)	0.481
≥ 5%	41 (93)	7 (100)	14 (87)	20 (95)	

(*P*-values: CD3: < 0.001, CD4: 0.011, CD8: < 0.001, PD-1: 0.002) (Table 2). When stratified by PD-L1 expression, PD-L1+ cancers had significantly more T cells at the invasive front than PD-L1− cancers (Fig. 3A). Subgroup analysis showed that PD-L1+/EBV+ and PD-L1+/MSI gastric cancers (N = 21) had significantly more CD3, CD8, and PD-1 T cells than PD-L1+/EBV−/MSS cancers ($P = 0.0016$, 0.0006, 0.016) (Fig. 3B). In contrast, CD4 T-cell counts between these 2 groups of tumors were comparable (mean [range]: 308 [164 to 516] and 240 [142 to 514], respectively; $P = 0.1198$). "PD-L1 expression and tumor-associated immune infiltrates at the invasive front in EBV−/MSS gastric cancers were additionally correlated with E-cadherin expression by immunohistochemistry (Supplementary Figs. 1, 2). Supplemental Digital Content 1, http://links.lww.com/PAS/A401"

Positive PD-L1 Expression is Not Associated With Depth of Invasion, Nodal Metastasis or Survival

When stratified by PD-L1 expression, PD-L1+ was not associated with depth of invasion, positive lymph node metastasis, or distant metastasis ($P = 0.534$, 0.104, and 0.056, respectively). However, significantly higher percentage of PD-L1+ cases were stage I and stage II tumors than those of PD-L1− cases (stages I and II: 23/32, 72% vs. 4/12, 33%; $P = 0.035$) (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/PAS/A401).

Univariate analysis showed that neither PD-L1 expression nor the status of EBV infection and MSI was associated with OS ($P = 0.390$ and 0.833, respectively) (Figs. 4A, C). In contrast, both PD-L1+ and MSI were positive prognostic factors for DFS (Figs. 4B, D), whereas positive lymph node metastasis and high stage were associated with significant decreased DFS ($P = 0.026$, 0.017, 0.028, 0.004) (Table 3). EBV+ gastric cancers were trending toward longer DFS; however, the difference was not statistically significant ($P = 0.126$), likely due to the small number of EBV+ cases included in this study. Cox regression multivariate analysis, however, showed that PD-L1 expression was not associated with DFS after adjusting for stage and status of EBV infection and MSI ($P = 0.548$). In contrast, high stage and MSI both were independent prognostic factors for DFS ($P = 0.009$, 0.043).

As MSI was an independent prognostic factor of DFS, subgroup analyses were performed in MSI gastric cancers and in EBV−/MSS cancers to investigate the role of PD-L1 expression in predicting patient survival. Univariate analysis showed that, in each group, PD-L1 expression was not a significant prognostic factor for either OS (in MSI group: hazard ratio [95% confidence interval]: 0.21 [0.03-1.27], $P = 0.089$; in EBV−/MSS group: 0.96 [0.28-3.35], $P = 0.953$) or DFS (MSI group: 0.11 [0.07-1.87], $P = 0.128$; EBV−/MSS group: 0.72 [0.21-2.54], $P = 0.615$). These results suggest that MSI is a confounding factor for PD-L1 expression and outcome correlation studies in gastric cancer.

PD-L1 staining in 5% or more tumor cells has been used to define PD-L1 positivity in retrospective studies[4,21] and clinical studies on PD-1/PD-L1 blockade.[5,14,15] In our cohort, when PD-L1+ was defined by 5% or more tumor cells with membranous staining, 16 of 44 gastric cancers (36%) were PD-L1+ and 14 of the 16 cases (88%) were EBV+ or MSI (5 and 9, respectively,

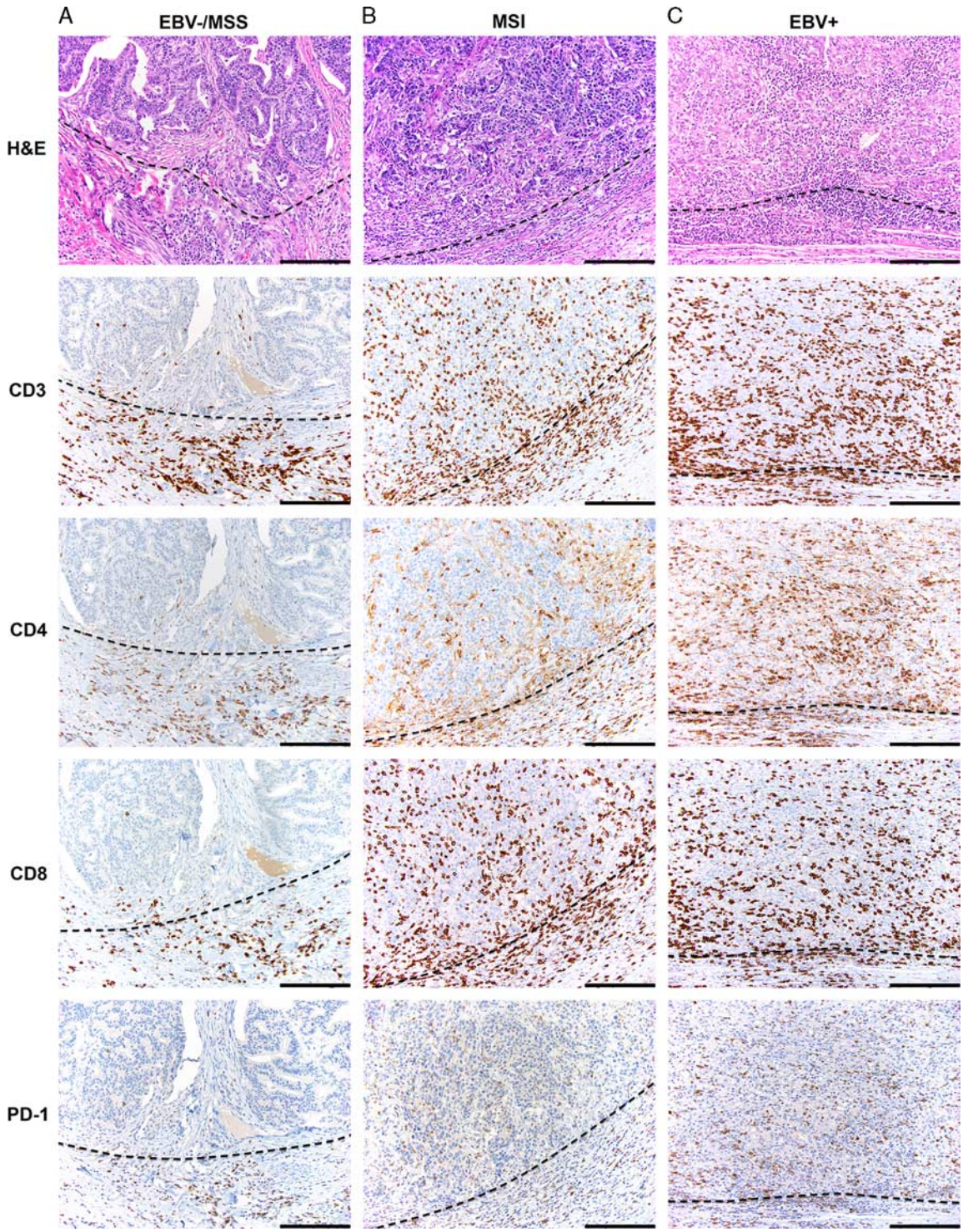

FIGURE 2. T-cell distribution in tumor-associated immune infiltrates in EBV−/MSS, MSI, and EBV+ gastric cancers. An H&E-stained tumor section and the corresponding CD3, CD4, CD8, and PD-1 stained sections are shown from top to bottom in each panel. The dashed line in each photomicrograph indicates the invasive front of the tumor/tumor-stroma interface. A, An EBV−/MSS gastric cancer. The tumor-associated immune infiltrates are located predominantly at the invasive front. B and C, MSI and EBV+ gastric cancers, respectively. The tumor-associated immune infiltrates are located at the invasive front, in stroma between tumor cells and infiltrating in tumor cell nests. Scale bar: 200 μm.

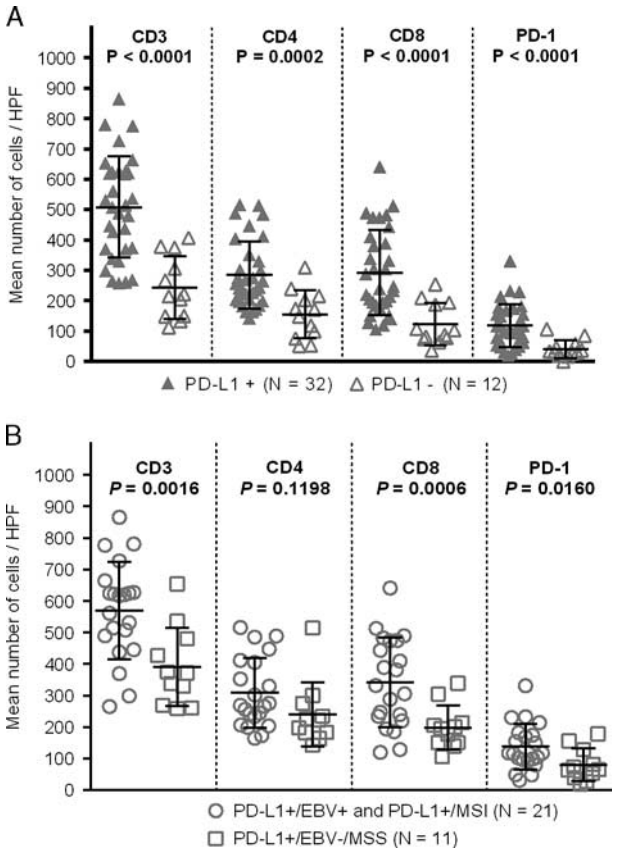

FIGURE 3. Characterization of T cells in tumor-associated immune infiltrates at tumor invasive front of each tumor. A, The mean number of CD3, CD4, CD8, and PD-1-positive T cells per high-power field (/HPF) stratified by PD-L1 expression (PD-L1+: >0%, positive; PD-L1−: 0%, negative). B, The mean number of CD3, CD4, CD8, and PD-1-positive T cells/HPF in PD-L1+ cases stratified by status of EBV infection and MSI. The bar in the middle represents group mean; the error bars represent SD.

$P = 0.001$) (Supplementary Table 2, Supplemental Digital Content 1, http://links.lww.com/PAS/A401). Univariate analysis showed PD-L1 expression was not associated with OS ($P = 0.302$) or DFS ($P = 0.242$; Table 3). MSI, after adjusting for stage, remained an independent positive prognostic factor for DFS (hazard ratio: 0.15, 95% confidence interval: 0.32-0.72, $P = 0.018$).

DISCUSSION

The aim of this study was to determine whether gastric cancers with either EBV infection or MSI, both of which have abundant CD8 T cells in tumor microenvironment, would be more likely to express PD-L1 than EBV−/MSS gastric cancers, and its implication in prognosis prediction. Herein we report that, EBV+ cancers and MSI cancers were more likely to show PD-L1 expression than EBV−/MSS gastric cancers. In these tumors, positive PD-L1 expression was associated with a concomitant, significant increase in the number of CD8 T cells at tumor invasive front. By multivariate analysis, PD-L1 expression in gastric cancer was not independently associated with prognosis, whereas MSI was.

This study is the first to demonstrate frequent PD-L1 expression in gastric cancers with MSI. Similar findings have been demonstrated in MSI colorectal cancers in which an adaptive immune resistance characterized by PD-L1 expression induced by CD8 T cells in tumor microenvironment was activated to protect cancer from removal.[7] The mechanism of action was associated with neoantigens due to the high somatic mutation rates in MSI colorectal cancers.[7,32] MSI gastric cancers also have elevated somatic mutation rates and preexisting, CD8 T-cell rich immune infiltrates.[18,20,33] Taken together, our findings suggest that an adaptive immune resistance may be induced in MSI gastric cancers.

Positive PD-L1 expression has been reported in 1 of 2 and 4 of 7 (57%) EBV+ gastric cancers by Thompson et al[21] and Setia et al,[27] respectively. *PD-L1* gene amplification and mRNA overexpression have been reported in a subset (15%) of EBV+ gastric cancers.[33] In these cancers, we suspect, positive PD-L1 expression would likely be the result of constitutive *PD-L1* gene overexpression. In EBV+ gastric cancers without *PD-L1* gene amplification, the mechanism for PD-L1 expression remains unclear. However, in other EBV-associated malignancies, PD-L1 expression can be induced through either constitutive oncogenic activation mediated by EBV-encoded latent-membrane protein 1 or by the adaptive immune resistance via interferon-γ as a result of active antiviral/antitumor response.[10,11]

Regardless of the mechanisms, positive PD-L1 expression and an increase in CD8 and PD-1 T cells were detected in the majority of the EBV+ and MSI gastric cancers in our study. These results suggest the presence of an endogenous antitumor immune response in EBV+ and MSI gastric cancers that has been suppressed by PD-L1. As indicated by recent clinical studies in other cancer types, solid tumors with T cells that are "turned off" by PD-L1 engagement are most likely to benefit from PD-1/PD-L1 blockade therapies.[5,6,15,32,34] Taken together, our findings suggest that EBV+ and MSI gastric cancers would be more likely to respond to anti-PD-1/anti-PD-L1 therapies than EBV−/MSS cancers. For this reason, the status of EBV infection and MSI, in addition to PD-L1 expression, in gastric cancer should be further investigated as potential biomarkers for predicting clinical response to PD-1/PD-L1 immune checkpoint blockade therapies.

In our cohort, PD-L1 expression was not associated with survival, whereas MSI was an independent positive prognostic factor for DFS. Although our cohort was enriched with EBV+ and MSI cancers, after adjusting these factors, in multivariate analysis, PD-L1 expression was not associated with DFS. These results are in contradiction with most studies in the literature in which positive or increased PD-L1 expression in gastric cancer has been associated with positive lymph node metastasis, depth of invasion, and reduced overall and/or progression-free survival.[21–25] None of these PD-L1

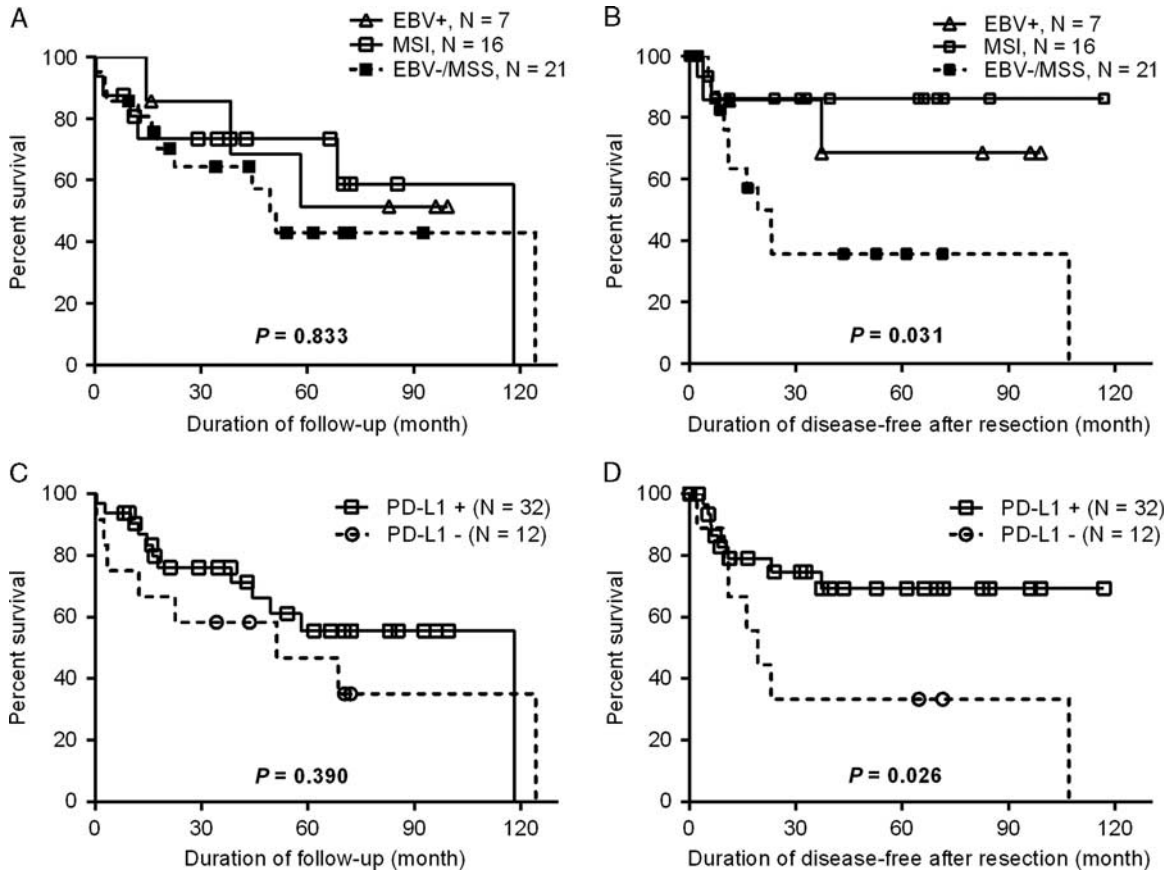

FIGURE 4. OS (A and C) and DFS (B and D) stratified by status of EBV infection and MSI (A and B) and PD-L1 expression (PD-L1+: >0%, positive; PD-L1 −: 0%, negative) (C and D).

expression and outcome correlation studies[21–25] has taken into consideration the status of either EBV infection or MSI. EBV+ and MSI gastric cancers together can account for a substantial proportion (roughly 20% to 30%) of all sporadic gastric cancers[27,33,35,36] and both have been shown to have better survival compared with

TABLE 3. Cox Regression DFS Analysis (N = 40)

	Univariate Analysis			Multivariate Analysis		
	P	HR	95% CI	*P*	HR	95% CI
PD-L1+ (> 0%)	**0.026**	0.30	0.10-0.87	0.548	0.68	0.20-2.38
PD-L1+ (≥ 5% tumor cells)	0.242	0.50	0.16-1.60	NA		
CD4 < CD8	0.103	0.29	0.06-1.29	NA		
Male sex	0.280	0.56	0.20-1.60	NA		
EBV and MSI status						
EBV−/MSS	Reference	1.00		Reference	1.00	
EBV+	0.126	0.30	0.07-1.40	0.239	0.35	0.06-2.00
MSI	**0.017**	0.16	0.03-0.72	**0.043**	0.18	0.03-0.95
pT1-2 vs. pT3-4	0.272	1.92	0.60-6.13	NA		
pN0 vs. pN1-3	**0.028**	5.39	1.20-24.11	NA		
Stage I-II vs. stage III	**0.004**	4.73	1.62-13.81	**0.009**	4.53	1.46-14.08
Systemic treatment						
None	Reference	1.00		NA		
Neoadjuvant	0.239	2.62	0.53-13.03	NA		
Adjuvant	0.572	1.59	0.32-7.87	NA		
Both	0.080	3.64	0.86-15.52	NA		

P-values in bold are statistically significant.
CI indicates confidence interval; HR, hazard ratio; NA, not applicable.

EBV−/MSS cancers.[27,36–38] Therefore, we believe, correlations between PD-L1 expression and patient outcome in gastric cancer reported in the literature should be interpreted with caution. These discrepancies highlight the need
for patient stratification based on EBV infection and MSI status when performing correlation studies. In addition, it is important to note that the PD-L1 monoclonal antibody used in our study is different from antibodies used in previous reports. These studies have used a number of monoclonal[21–23,27] and polyclonal PD-L1 antibodies[24,26] for immunohistochemistry and a variety of different scoring schemes/criteria to define positive PD-L1 expression. Therefore, it can be difficult to compare and contrast our results with those reported in literature.

Limitations of this study include its retrospective nature and its relatively small sample size. In addition, cases included in this study are not consecutive gastric cancer resections and therefore, the percentages of EBV+, MSI, and EBV−/MSS cases may not be representative of the prevalences of these tumors in sporadic gastric cancers. However, our cases were diagnosed in patients at a wide age range, in both male and female patients and across all clinical stages (stages I to IV). The distributions in age, sex, clinical stage, overall follow-up time and survival are similar to other studies of similar sample sizes on PD-L1 expression in gastric cancer, such as the study by Thompson et al.[21] We believe our cohort is representative at least to some extent of a consecutive case collection. Nonetheless, prospective studies with a large number of gastric cancer patients are needed to validate the findings described in our study. In addition, as none of the patients in this study received PD-1/PD-L1 blockade treatment, the value of either PD-L1 expression or the status of EBV infection and MSI in predicting clinical response to such therapy can only be examined in future studies.

In summary, this study is the first to investigate PD-L1 expression and its correlation with tumor immune microenvironment in MSI gastric cancers; it is the largest series to date that evaluates such correlation in EBV+ gastric cancers. Our study demonstrates that positive PD-L1 expression in gastric cancer is associated with EBV infection, MSI and abundant CD8 T cells at tumor invasive front. PD-L1 expression is not predictive of patient survival, whereas MSI is an independent predictor of better DFS. Despite a relatively small sample size, our findings suggest EBV infection and MSI should be further evaluated for their power to predict clinical response to anti-PD-1/anti-PD-L1 therapy. Future studies in PD-L1 expression and patient outcome correlation should stratify patients by EBV infection and MSI status.

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