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Intratumoural PD-L1 expression is associated with worse survival of patients with Epstein–Barr virus-associated gastric cancer

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Background: This study investigated the clinical relevance and prognostic impact of the overall expression of programmed cell death protein ligand-1 (PD-L1) and programmed cell death protein ligand-2 (PD-L2), in patients with Epstein–Barr virus-associated gastric cancer (EBVaGC).

Methods: After reviewing 1318 consecutive cases of surgically resected or endoscopic submucosal dissected gastric cancers, the expression status of PD-L1 and PD-L2 in 120 patients with EBVaGC identified by EBV-encoded RNA *in situ* hybridisation was retrospectively analysed using immunohistochemistry (IHC). For each IHC marker, positivity was separately in intraepithelial tumour cells (iTu-) and immune cells in the tumour stroma area (str-).

Results: Among 116 eligible patients, 57 (49.1%) and 66 patients (56.9%) were determined as iTu-PD-L1-positive and str-PD-L1-positive, respectively, whereas 23 (21.6%) and 45 patients (38.8%) were determined as iTu-PD-L2 positive and str-PD-L2 positive, respectively. Intraepithelial tumour cell PD-L1 positivity was found to be significantly associated with lymph node (LN) metastasis ($P=0.012$) and a poor disease-free survival (DFS) ($P=0.032$), yet not overall survival ($P=0.482$). In a multivariate analysis, iTu-PD-L1 positivity was independently associated with a poor DFS ($P=0.006$, hazard ratio = 12.085). In contrast, str-PD-L2-positivity was related to a lower T category ($P=0.003$), absence of LN metastasis ($P=0.032$) and perineural invasion ($P=0.028$). Intraepithelial tumour cell and str-PD-L2 positivity showed a trend towards an improved DFS, although not significant ($P=0.060$ and $P=0.073$, respectively).

Conclusions: Intraepithelial tumour cells PD-L1 expression can be used to predict a poor outcome in patients with EBVaGC and can represent a rational approach for PD-1/PD-L pathway-targeted immunotherapy.

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Immunotherapy has begun to revolutionise cancer treatment, by introducing therapies such as checkpoint inhibitors that target the host immune system instead of the tumour (Chen and Mellman, 2013). For an anticancer immune response, the programmed cell death protein-1 (PD-1) pathway is considered an important inhibitory mechanism regulating T-cell exhaustion (Jin and Yoon, 2016). PD-1, which belongs to the CD28 family of proteins, is a receptor expressed on a number of immune cells, including T cells, B cells, monocytes, NK cells, and dendritic cells (Park *et al*, 2016). PD-1 has two ligands, the programmed cell death protein ligand-1 (PD-L1) and programmed cell death protein-ligand-2 (PD-L2). PD-L1 is expressed on T cells, B cells, dendritic cells, macrophages, mesenchymal stem cells, bone marrow-derived mast cells, and some non-haematopoietic cells, whereas PD-L2 is mainly expressed on antigen-presenting cells, including macrophages, dendritic cells, and non-haematopoietic tissues (Jin and Yoon, 2016). Several studies have already demonstrated that PD-L1 or PD-1 is highly expressed on tumour cells in gastric cancers (GCs) (Wu *et al*, 2006; Takaya *et al*, 2015; Zhang *et al*, 2015; Böger *et al*, 2016; Derks *et al*, 2016; Dong *et al*, 2016a; Eto *et al*, 2016; Kim *et al*, 2016; Takano *et al*, 2016; Cho *et al*, 2017). Interestingly, Wang *et al* (2016) suggested that GC patients with positive PD-L1 expression had a significantly shorter survival than PD-L1-negative patients, although the prognostic impact remained inconsistent.

The Cancer Genome Atlas data revealed that Epstein–Barr virus (EBV)-associated GC (EBVaGC) closely associated with a PIK3CA mutation, PD-L1/2 overexpression, EBV-CIMP, CDKN2A silencing, and immune cell signalling activation among GC subtypes. As previous studies have suggested that high PD-L1 expression is related to a good responsiveness to anti-PD-1/PD-L1 therapy in human cancers, the EBVaGC subtype, in particular, may be a potential candidate for anti-PD-1/PD-L1 therapy among GC subtypes (Herbst *et al*, 2014; Taube *et al*, 2014). Furthermore, accumulating evidence suggests that tumour-infiltrating T cells may also be critical for the good responsiveness to anti-PD-1/PD-L1 treatment (Teng *et al*, 2015). As EBVaGC is known to be associated with a prominent lymphoid infiltration of the stroma and high density of tumour-infiltrating lymphocytes (TILs), evaluating the PD-1 pathway affecting EBV infection may also be helpful in selecting appropriate targets for therapeutic interventions against EBVaGC. Moreover, this close relationship points to the possibility of PD-L1 and PD-L2 as prognostic markers in EBVaGC patients, suggesting a pivotal role of the immune mechanism in the EBVaGC subset. Several studies have reported PD-L1 expression in 34.4–92.5% of EBVaGC patients (Derks *et al*, 2016; Dong *et al*, 2016a; Kawazoe *et al*, 2016; Ma *et al*, 2016). Notably, Saito *et al* (2016) recently reported that PD-L1 expression in tumour cells was associated with poor overall survival (OS) and disease-specific survival in 96 patients with EBVaGC. However, unfortunately, detailed clinicopathologic characteristics of EBVaGC showing PD-L1 have not been fully elucidated and no published study has yet investigated the clinical significance and prognostic impact of PD-L2 expression in EBVaGC.

Accordingly, this study used a relatively large series of EBVaGC to determine the clinical and prognostic significance of PD-L1 and PD-L2 in both tumour cells and immune cells, and examine the association with the density of TILs.

MATERIALS AND METHODS

Patients and samples. A total of 120 EBVaGC cases were used in this study, all of which were obtained from our previous studies (Kang *et al*, 2016). The inclusion criteria for patient selection and results have already been reported (Kang *et al*, 2016). Briefly, all the patients were histologically diagnosed with gastric adenocarcinoma and underwent surgical resection or endoscopic mucosal dissection (ESD) at Kyungpook National University Medical Center (KNUMC) between January 2011 and November 2014. Their status as EBV positive was determined by EBV-encoded RNA *in situ* hybridisation conducted at our molecular pathology laboratory. The baseline characteristics of the 120 patients are presented in Supplementary Table 1. The clinical and pathologic information was collected based on a review of the patients' medical records and pathologic reports, respectively. The patients were all staged according to the seventh edition of the American Joint Committee on Cancer staging Manual for Stomach cancer (Edge *et al*, 2010).

The Institutional Review Boards of Kyungpook National University Medical Center (IRB No.: KNUMC 2016-05-012) approved this study. The Reporting Recommendations for Tumour marker Prognostic Studies criteria were followed when reporting the results of this study (McShane *et al*, 2005).

Pathologic quantification of TILs. The evaluation of the percentage of intratumoural TILs (iTu-TILs) and stromal TILs (str-TILs) used a modified version of the TIL scoring recommendations of the International TILs Working Group 2014 on breast cancer (Dieci *et al*, 2014). The detailed quantification definition has been previously described (Kang *et al*, 2016). Specifically, the iTu-TILs were defined as the percentage of mononuclear inflammatory cells within an intraepithelial tumour cell nest and in direct contact with tumour cells. Meanwhile, str-TILs were defined as the percentage of tumour stroma of invasive carcinoma to be occupied by mononuclear inflammatory cells. The percentage of TILs was evaluated in 10% increments; if the percentage of iTu-TILs and str-TILs was $<10\%$, 1%, or 5% criteria were used on full sections of haematoxylin and eosin-stained slides, and evaluated by two pathologists (ANS and HIB). The quantification of iTu-TILs and str-TILs was identified based on our previously published data (Kang *et al*, 2016).

Immunohistochemistry and interpretation. All the tissue slides were stained using an automatic immunohistochemistry (IHC) staining instrument (BenchMarkXT, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. The following primary antibodies were used: PD-L1 (E1L3N; 1 : 100; Cell Signaling Technology, Danvers, MA, USA) and PD-L2 (clone 176611; 1 : 150; R&D Systems, Minneapolis, MN, USA). In brief, a representative formalin-fixed, paraffin-embedded tissue sample for each case was cut into 4 μm-thick sections, transferred to poly-L-lysine-coated adhesive slides, and dried in a microwave at 60 °C for 1 h. Therefore, the sections were deparaffinised using an EZ Prep solution (Ventana Medical Systems). The cell-conditioning solution 1 (CC1) standard (pH 8.4 buffer containing Tris/borate/EDTA) was then used for antigen retrieval, followed by blocking with inhibitor D (3% H_2O_2) at 37 °C for 4 min. Next, the slides were incubated with the primary antibodies at 37 °C for 60 min. The staining was optimised using a positive and negative control for each marker according to the recommendation of the supplier's data sheet. All the sections were visualised with an UltraView Universal DAB detection kit (Ventana Medical Systems).

The expression of PD-L1 and PD-L2 was evaluated by two experienced gastrointestinal pathologists (ANS and HIB) with no prior knowledge of the clinical data. The stained slides were semi-quantitatively scored for the intensity and percentage of membranous and/or cytoplasmic staining of the tumour cells and tumour stoma area (immune cells), respectively. The interpretation of each marker was based on the following criteria: 0, no expression or expression in $<1\%$; 1+, weak/faint expression in $\geqslant 1\%$; 2+, moderate expression in $\geqslant 1\%$; 3+, strong expression $\geqslant 1\%$ of iTu- or immune cells in tumour str-. The cases were then classified

into dichotomous covariates: negative (0 and 1+) and positive (2+ and 3+).

Statistical analyses. The descriptive statistics are reported as proportions and medians. The categorical variables were evaluated using a χ^2-test, Fisher's exact test, or Pearson's correlation (R) test, as appropriate. Disease-free survival was measured from the time of surgery or ESD to the time of tumour recurrence or death from any cause. Overall survival was calculated from the date of surgery or ESD to death from any cause. Data were censored if patients were free of recurrence or alive at the last follow-up. The patient subgroups were compared with respect to disease-free survival (DFS) and OS with the use of Kaplan–Meier curves, log-rank test, and multivariate survival analysis based on the Cox proportional hazard regression model. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated for each factor. All the tests were two-sided and statistical significance was set at $P<0.05$. All the statistical analyses were performed using SPSS for Windows (version 19.0, SPSS Inc., Chicago, IL, USA).

RESULTS

PD-L1 and PD-L2 expression. PD-L1 and PD-L2 IHC results were available for 116 of the 120 cases (4 cases were excluded due to loss of tissue or poor IHC staining). Immunohistochemical expression of PD-L1 and PD-L2 was observed in tumour cells (iTu-) and/or the immune cells in tumour stromal area (str-) in EBVaGC tissues (Figure 1). Intraepithelial tumour cell PD-L1 positivity was detected in 57 of the 116 EBVaGC cases (49.1%) (2+ in 39 and 3+ in 18 cases), whereas str-PD-L1 positivity was observed in 66 cases (56.9%) (2+ in 65 cases and 3+ in 1 case). In addition, EBVaGC exhibiting both iTu-PD-L1 and str-PD-L1 was observed in 26 cases (22.4%). Meanwhile, iTu-PD-L2 positivity was detected in 25 cases (21.6%) (2+ in all cases), whereas str-PD-L2 positivity was observed in 45 cases (38.8%) (2+ in all cases). In addition, EBVaGC exhibiting both iTu-PD-L2 and str-PD-L2 was observed in 14 cases (12.1%).

Association with clinicopathologic features and TILs. As described in Table 1, iTu-PD-L1 positivity was significantly associated with the presence of lymph node (LN) metastasis ($P=0.012$), whereas str-PD-L1 positivity was related to categories associated with dense TILs including str-TIL positivity ($P=0.003$), iTu-TIL positivity ($P=0.004$), and gastric carcinoma with lymphoid stroma (GCLS) ($P=0.024$). In contrast, str-PD-L2 positivity were correlated with a lower pT category ($P=0.003$) and the absence of LN metastasis ($P=0.0032$) and perineural invasion ($P=0.028$), str-TIL positivity ($P<0.001$), iTu-TIL positivity ($P<0.001$), GCLS ($P<0.001$), and a non-conventional adenocarcinoma subclassification ($P<0.001$). However, iTu-PD-L2 positivity showed no association with any clinicopathologic characteristics.

To determine the relationship between PD-L1 and PD-L2 expression in EBVaGC, we analysed the correlation between the levels of PD-L1 and PD-L2 expression (Table 2 and Supplementary Figure 1). The iTu expression of PD-L1 and PD-L2 was correlated with the str expression of PD-L1 and PD-L2 with each other, respectively (iTu-PD-L1 positivity and str-PD-L1: $R=-0.224$, $P=0.016$; iTu-PD-L1 positivity and str-PD-L1: $R=0.185$, $P=0.047$).

Patient outcomes. At the time of the last follow-up (April 2016), the median follow-up duration for the survival analyses was 36.2 months (range 6.4–66.6). The probability of OS and DFS at 3 years was 92.2% and 88.5%, respectively. During the analyses, 13 (10.8%) patients experienced recurrence and 13 (10.8%) died. In the univariate analysis, iTu-PD-L1 positivity was significantly associated with a worse DFS ($P=0.032$) (Figure 2A), yet not with OS ($P=0.482$). In the multivariate analysis using the Cox proportional hazard model adjusted for conventional clinical covariates, iTu-PD-L1 positivity was an independent worse prognostic factor for DFS (HR = 12.085, 95% CI = 2.013–72.559, $P=0.006$) (Table 3). Meanwhile, both iTu-PD-L2 positivity and str-PD-L2 positivity tended to show a favourable DFS (iTu-PD-L2: $P=0.060$; str-PDL-2: $P=0.073$), yet without statistical significance in the univariate analysis (Figure 2B and C).

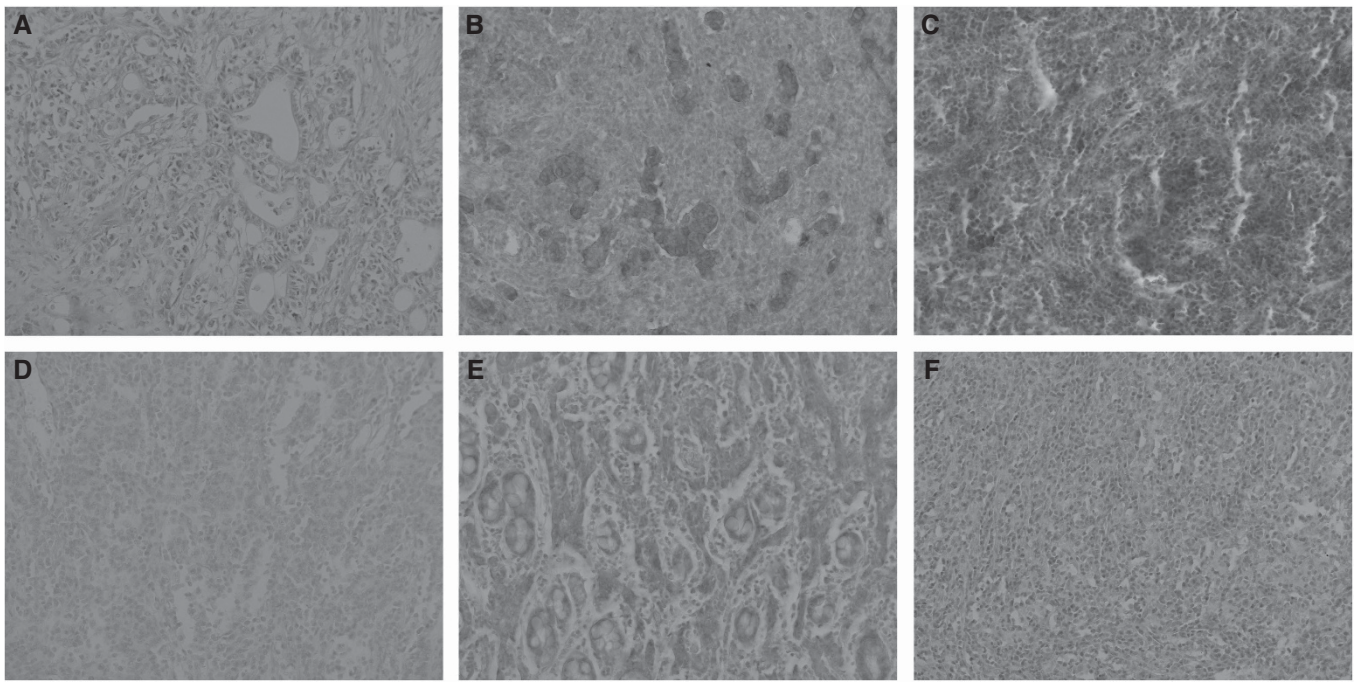

Figure 1. Representative features of immunohistochemical expression of PD-L1 and PD-L2 in EBV-associated gastric cancers. (**A**) Representative photographs of intraepithelial tumour cells (iTu-) or immune cells in tumour stroma area (str-)-PD-L1 negativity; (**B**) iTu-PD-L1 positivity; (**C**) str-PD-L1 positivity. (**D**) Representative picture of iTu- or str-PD-L2 negativity; (**E**) iTu-PD-L2 positivity; (**F**) str-PD-L2 positivity.

Table 1. The association between PD-L1/PD-L2 expression and clinicopathologic features

	iTu-PD-L1			str-PD-L1			iTu-PD-L2			str-PD-L2		
Variables	Positive $n=57$	Negative $n=59$	*P*-value	Positive $n=66$	Negative $n=50$	*P*-value	Positive $n=25$	Negative $n=91$	*P*-value	Positive $n=45$	Negative $n=71$	*P*-value
Age, years												
<62	25 (43.9)	30 (50.8)	0.451	31 (47.0)	24 (48.0)	0.912	14 (56.0)	41 (45.1)	0.372	21 (46.7)	34 (47.9)	0.100
⩾62	32 (56.1)	29 (49.2)		35 (53.0)	26 (52.0)		11 (44.0)	50 (54.9)		24 (53.3)	37 (52.1)	
Gender												
Male	43 (75.4)	50 (84.7)	0.209	53 (80.3)	40 (80.0)	0.968	17 (68.0)	76 (83.5)	0.096	34 (75.6)	59 (83.1)	0.347
Female	14 (24.6)	9 (15.3)		13 (19.7)	10 (20.0)		8 (32.0)	15 (16.5)		11 (24.4)	12 (16.9)	
Lauren classification												
Intestinal	9 (15.8)	15 (25.4)	0.434	11 (16.7)	13 (26.0)	0.438	7 (28.0)	17 (18.7)	0.237	10 (22.2)	14 (19.7)	0.365
Diffuse	42 (73.7)	39 (66.1)		49 (74.2)	32 (64.0)		14 (56.0)	67 (73.6)		33 (73.3)	48 (67.6)	
Mixed	6 (10.5)	5 (8.5)		6 (9.1)	5 (10.0)		4 (16.0)	7 (7.7)		2 (4.4)	9 (12.7)	
Tumour depth												
T1/2	35 (61.4)	41 (61.9)	0.360	45 (68.2)	31 (62.0)	0.488	20 (80.0)	56 (61.5)	0.100	37 (82.2)	39 (54.9)	0.003
T3/4	22 (38.6)	18 (30.5)		21 (31.8)	19 (38.0)		5 (20.0)	35 (38.5)		8 (17.8)	32 (45.1)	
Lymph node metastasis												
Absent	35 (61.4)	49 (83.1)	0.012	50 (75.8)	34 (68.0)	0.355	21 (84.0)	63 (69.2)	0.207	38 (84.4)	46 (64.8)	0.032
Present	22 (38.6)	10 (16.9)		16 (24.2)	16 (32.0)		4 (16.0)	28 (30.8)		7 (15.6)	25 (35.2)	
Lymphatic invasion												
Absent	34 (59.6)	42 (71.2)	0.191	44 (66.7)	32 (64.0)	0.765	17 (68.0)	59 (64.8)	0.817	33 (73.3)	43 (60.6)	0.168
Present	23 (40.4)	17 (28.8)		22 (33.3)	18 (36.0)		8 (32.0)	32 (35.2)		12 (26.7)	28 (39.4)	
Perineural invasion												
Absent	37 (64.9)	38 (64.4)	0.955	44 (66.7)	31 (62.0)	0.603	19 (76.0)	55 (61.5)	0.239	35 (77.8)	40 (56.3)	0.028
Present	20 (35.1)	21 (35.6)		22 (33.3)	19 (38.0)		6 (24.0)	35 (38.5)		10 (22.2)	31 (43.7)	
Venous invasion												
Absent	55 (96.5)	55 (93.2)	0.426	63 (95.5)	47 (94.0)	0.726	25 (100.0)	85 (93.4)	0.338	45 (100.0)	65 (91.5)	0.080
Present	2 (3.5)	4 (6.6)		3 (4.5)	3 (6.0)		0	6 (6.6)		0	6 (8.5)	
str-TILs (cutoff, 25%)												
Negative	22 (38.6)	23 (39.0)	1.000	18 (27.3)	27 (54.0)	0.004	10 (40.0)	35 (38.5)	1.000	8 (17.6)	37 (52.1)	<0.001
Positive	35 (61.4)	36 (61.0)		48 (72.7)	23 (46.0)		15 (50.0)	56 (61.5)		37 (82.2)	34 (47.9)	
iTu-TILs (cutoff, 27.5%)												
Negative	28 (49.1)	29 (29.2)	1.000	24 (36.4)	33 (66.0)	0.003	16 (64.0)	41 (45.1)	0.116	12 (26.7)	45 (63.4)	<0.001
Positive	29 (50.9)	30 (50.8)		42 (63.6)	17 (34.0)		9 (36.0)	50 (54.9)		33 (73.3)	26 (36.6)	
WHO classification												
Non-GCLS	27 (47.4)	30 (50.8)	0.715	26 (39.4)	31 (62.0)	0.024	12 (48.0)	45 (49.5)	1.000	9 (20.0)	48 (67.6)	<0.001
GCLS	30 (52.6)	29 (49.2)		40 (60.6)	19 (38.0)		13 (52.0)	46 (50.5)		36 (80.0)	23 (32.4)	
Three histologic subclassification												
LELC	16 (28.1)	19 (32.2)	0.294	22 (33.3)	13 (26.0)	0.314	6 (24.0)	29 (31.9)	0.329	22 (48.9)	13 (18.3)	<0.001
CLR	22 (38.6)	28 (47.5)		30 (45.5)	20 (40.0)		14 (56.0)	36 (39.6)		19 (42.2)	31 (43.7)	
CA	19 (33.)	12 (20.3)		14 (21.2)	17 (34.0)		5 (20.0)	26 (28.6)		4 (8.9)	27 (38.0)	

Abbreviations: CA = conventional adenocarcinoma; CLR = Crohn's disease-like lymphoid reaction; GCLS = gastric carcinoma with lymphoid stroma; iTu = intratumoural; LELC = lymphoepithelioma-like carcinoma; PD-L1 = programmed cell death protein ligand-1; PD-L2 = programmed cell death protein ligand-2; str = stromal; TILs = tumour-infiltrating lymphocytes; WHO = World Health Organization.

As str-TILs were also associated with DFS, the patients were further categorised into three groups (iTu-PD-Ll negativity and str-TIL positivity, and iTu-PD-L1 positivity and str-TIL negativity, and others). In univariate analysis, the patient groups with iTu-PD-L1 positivity and str-TIL negativity showed a shorter DFS than the other groups ($P=0.006$) (Figure 2D).

DISCUSSION

Accumulating data have revealed that PD-L1 expression can be a potential prognostic and therapeutic predictors for anti-PD-1/PD-L1 immunotherapy, therefore, understand the implications of PD-L1 expression in patients with EBVaGC is of critical importance (Jin and Yoon, 2016). However, as the detailed implications of PD-L1 and PD-L2 expression, two main ligands of PD-1, remain unclear in patients with EBVaGC, the present study investigated the clinicopathologic significance and prognostic value. As a result, the following key points were identified: iTu-PD-L1 positivity was significantly correlated with LN metastases and a poor prognosis, whereas str-PD-L2 positivity was inversely related to tumour invasion and LN metastasis. Notably, iTu-PD-L1 positivity was also identified as an independent prognostic factor of worse DFS. Furthermore, when combining iTu-PD-L1 and str-TILs, the patient with iTu-PD-L1 positivity and str-TIL negativity was associated with worse DFS when compared with the other patients with EBVaGC. Therefore, these findings support the concept that increased PD-L1 expression in tumours and intense lymphocytic infiltration around tumours can act as an immune modulator, promoting escape from immune surveillance in patients with EBVaGC.

The PD-1 pathway is considered a critical inhibitory mechanism regulating T-cell exhaustion (Jin and Yoon, 2016). In tumour beds, T cells must pass through numerous barriers with immune checkpoints, such as PD-1 and PD-L1/PD-L2, as intrinsic regulators (Palucka and Coussens, 2016). The PD-1 pathway is involved in suppressing autoimmunity during T-cell activation,

allowing for immune tolerance of PD-L1- or PD-L2-expressed cells, with a blockade leading to T-cell rescue in subsequent response activation (Pardoll, 2012). Interestingly, PD-L1 mRNA and protein can be upregulated by cytokines produced by infiltrating immune cells (Dong *et al*, 2016b). Plus, oncogenic signalling pathways in tumour cells can activate PD-L1 expression (Dong *et al*, 2016b). Fang *et al* (2014) already reported that PD-L1 expression could be increased by exogenous and endogenous induction of Latent membrane protein 1 (LMP1)-induced PD-L1 and was suppressed by knocking down LMP1 in EBV-positive cell lines. Therefore, these mechanisms influence the regulation of virus immunity, as well as the cancer immune reaction in EBVaGC. To date, several studies have already shown that PD-L1 expression is associated with a worse prognosis in GC. According to a recent meta-analysis by Zhang *et al* (2016) of 1901 GC patients based on 10 studies, PD-L1 expression was identified as a valuable predictor of poor OS with a final HR for OS of 1.64. Recently, low PD-L1 expression and high CD8+ TILs were associated with better OS and DFS in 392 patients with surgically resected GC. Although this study included only 25 patients with EBVaGC, group with increased PD-L1 expression and high CD8+ TILs was closely correlated with EBV infection (Koh *et al*, 2017). However, there is still insufficient evaluation of PD-L1 expression in patients with EBVaGC. Furthermore, to the best of our knowledge, there is no previously reported evaluation of PD-L2 in EBVaGC. So far, the association between PD-L1 expression and prognosis has been inconsistent across various studies (Table 4). These conflicting results could be due to different patient ethnicities and different antibody clones and interpretation. Indeed, previous studies have only focused on the frequency of PD-L1 expression and not the impact on the survival outcomes of EBVaGC. In consistent with the current study, Saito *et al* (2016) reported that the PD-L1-positive EBVaGC patients exhibited a deeper invasion of the tumour and poorer prognosis than the PD-L1-negative patient. These authors also demonstrated that PD-L1-amplified cells corresponded to PD-L1-positive cells, showing high-intensity immunohistochemical cells. In contrast, another recent study found no association between PD-L1 expression and patient outcomes (Dong *et al*, 2016a). Consequently, the present findings need to be validated though further studies, in order to clarify the

Table 2. The correlation between the levels of PD-L1 and PD-L2 expression

	iTu-PD-L1	Str-PD-L1	iTu-PD-L2	Str-PD-L2
	R (*P*-value)	*R* (*P*-value)	*R* (*P*-value)	*R* (*P*-value)
iTu-PD-L1	—	−0.224 (0.016)	0.114 (0.224)	−0.146 (0.119)
Str-PD-L1	−0.224 (0.016)	—	−0.094 (0.315)	0.086 (0.361)
iTu-PD-L2	0.114 (0.224)	−0.094 (0.315)	—	0.185 (0.047)
Str-PD-L2	−0.146 (0.119)	0.086 (0.361)	0.185 (0.047)	—

Abbreviations: iTu = intratumoural; PD-L1 = programmed cell death protein ligand-1; PD-L2 = programmed cell death protein ligand-2; *R* = Pearson's correlation; str = stromal.

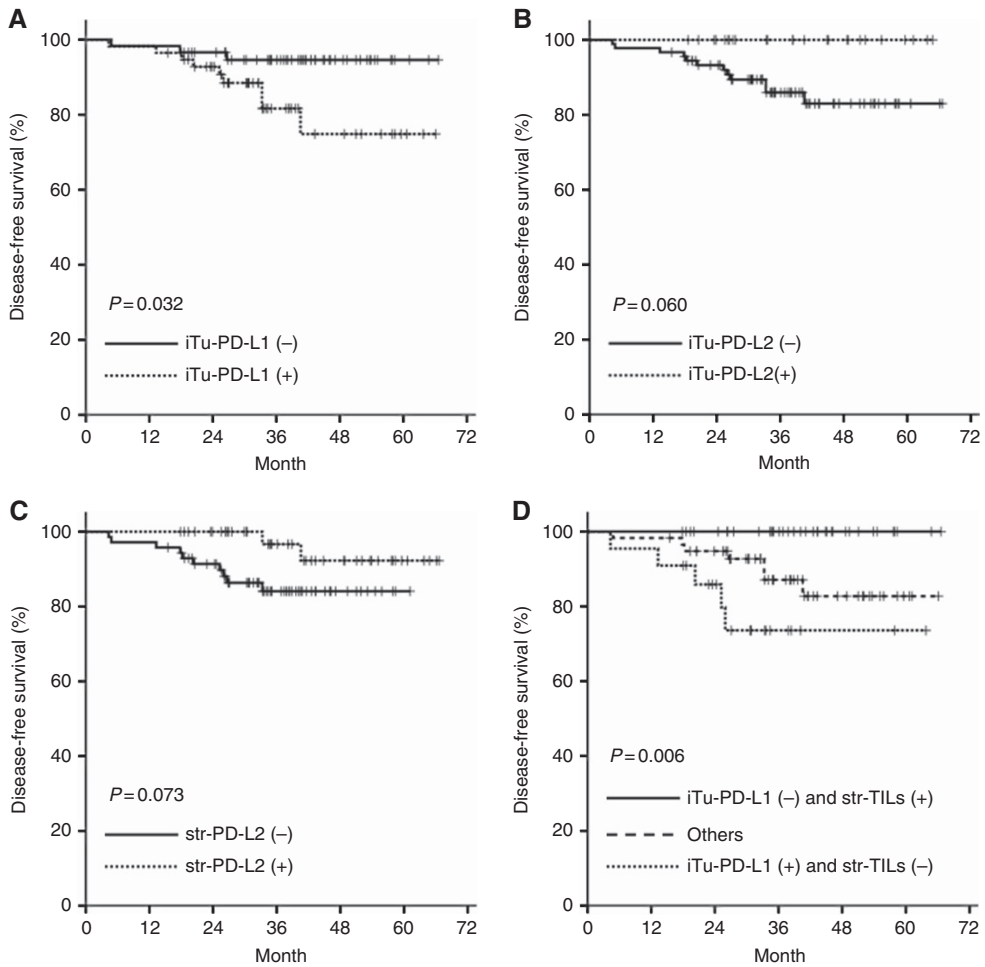

Figure 2. Survival analysis. (**A**) Kaplan–Meier survival curves for disease-free survival of patients stratified as iTu-PD-L1 positivity *vs* iTu-PD-L1 negativity, (**B**) iTu-PD-L2 positivity *vs* iTu-PD-L2 negativity, and (**C**) str-PD-L2 positivity *vs* str-PD-L2 negativity. (**D**) Kaplan–Meier survival curves for disease-free survival according to the three groups (iTu-PD-Ll negativity and str-TIL positivity, and iTu-PD-L1 positivity and str-TIL negativity, and others).

Table 3. Multivariate analysis for disease-free survival

		Disease-free survival in all patients		
		Multivariate analysis		
Variables	**Category**	***P***	**HR**	**95% CI**
Age, years	<62 *vs* ⩾62	0.584	0.613	0.106–3.534
Gender	Female *vs* male	0.250	3.879	0.385–39.115
pTNM stage	I *vs* II and III	0.014	13.863	1.688–113.847
WHO classification	GCLS *vs* non-GCLS	0.695	0.551	0.028–10.859
Lymphatic invasion	Negative *vs* positive	0.026	0.139	0.024–0.788
Venous invasion	Negative *vs* positive	0.027	0.055	0.004–0.715
Perineural invasion	Negative *vs* positive	0.096	4.702	0.762–29.034
Str-TILs	⩾25 *vs* <25	0.647	1.982	0.106–36.975
iTu-PD-L1	Negative *vs* positive	0.006	12.085	2.013–72.559

Abbreviations: CI = confidence interval; GCLS = gastric carcinoma with lymphoid stroma; R = hazard ratio; iTu = intratumoural; PD-L1 = programmed cell death protein ligand-1; str = stromal; TILs = tumour-infiltrating lymphocytes; WHO = World Health Organization.

Table 4. Frequency and prognostic value of PD-L1 expression in EBVaGC

References	Tumour type	Sample size	Country	PD-L1 expression	Criteria (cut-off)	Antibody	Prognostic role
Saito *et al* (2016)	EBVaGC	96	Japan	34.4%[a]	>5%	Clone E1L3N, Cell Signaling	OS and DSS[b]
Derks *et al* (2016)	EBVaGC	32	USA	50%[a]	>5%	Clone 405.9A11	Not reported[c]
Dong *et al* (2016a)	EBVaGC	59	China	92.5%[a]	>9.0[d]	Ab58810, Abcam	No significant
Kawazoe *et al* (2016)	EBVaGC	25	Japan	52.0%[a]	⩾1%	SP142, Ventana	Not reported
Ma *et al* (2016)	EBVaGC	7	USA	71.4%[a]	⩾5%	SP263, Ventana	Not reported
Current study	EBVaGC	116	South Korea	47.5%[a]	⩾1%	Clone E1L3N, Cell Signaling	DFS

Abbreviations: DFS = disease-free survival; DSS = disease-specific survival; D-L1 = programmed cell death protein ligand-1; EBVaGC = Epstein–Barr virus-associated gastric cancer; IFN-γ = interferon-γ; IHC = immunohistochemistry; OS = overall survival.
[a]PD-L1 expression evaluated in tumour epithelial cells.
[b]Univariate analysis.
[c]The study demonstrated that EBVaGC had high IFN-γ response gene expression.
[d]IHC staining was evaluated using the immunoreactive score (0–12) obtained by multiplying the staining intensity and percentage of positive tumour cells.

association between PD-L1 expression and the prognosis of EBVaGC.

Another interesting finding from the present study was that iTu-PD-L2- and str-PD-L2 positivity was tentatively associated with a favourable DFS and OS, although not significant in the EBVaGC patients. In contrast to PD-L1, PD-L2 expression is regulated by IL-4 and STAT6 on dendritic cells and macrophages, and PD-L2 have different functions under different Th1/Th2 inflammatory situations, even though PD-L1 and PD-L2 bind PD-1 together (Loke and Allison, 2003). However, despite increasing evidence that PD-L2 has important roles in several aspects of cancer, it is still unclear whether PD-L2 itself alters the function of the PD-1 pathway in cancer (Zhang *et al*, 2006). Some studies have shown a stimulatory role for PD-L2, whereas others suggest that PD-L2 can inhibit T-cell activation (Latchman *et al*, 2001; Wang *et al*, 2003; Jin and Yoon, 2016). In the present study, str-PD-L2 expression was positively correlated with dense iTu- and/or str-TILs, yet not iTu-PD-L2 expression. Ohigashi *et al* (2005) suggested that PD-L2 expression inversely correlated with tumour-infiltrating CD8+ T cells in 41 patients with oesophageal cancer. Thus, whether PD-L1 and PD-L2 are influenced by specific markers of TILs, such as CD4, CD8, and FOXP3, and interferon-γ would be an interesting focus for further studies of the tumour immunity of EBVaGC. Meanwhile, for the correlation between PD-L1 and PD-L2 expression, PD-L1 was found to be expressed independently of PD-L2 expression. This finding supports previous report, where iTu-PD-L2 expression was induced in the absence of PD-L1 co-expression tumours, in which case PD-L2 expression was less likely to possess PD-L1+ immune cells (Derks *et al*, 2015). These observations can also help to understand the exact role of PD-L2 on the natural outcomes of EBVaGC.

TILs could be used to identify a subgroup of patients with excellent outcomes (Chang *et al*, 2014) and our previous study also showed an independent association between a high density of TILs and a favourable recurrence-free survival or DFS in 120 patients with EBVaGC (Kang *et al*, 2016). This finding supports that TILs exhibit a host cellular immune response against tumours, indicating that immunotherapy may have a potential role in patients with EBVaGC (Kang *et al*, 2015). Several studies have already demonstrated a correlation between TILs and PD-L1 expression in EBVaGC (Kawazoe *et al*, 2016; Ma *et al*, 2016; Saito *et al*, 2016). Interestingly, when combining the iTu-PD-L1 expression and TIL score, the patient group with iTu-PD-L1 negativity and str-TIL positivity was associated with a better DFS than the other EBVaGC patients. This result creates a very meaningful subgroup that can provide more information for tailored therapy for each group of patients. In particular, this finding can provide a novel strategy for subgroups with decreased immunoediting via the PD-1 pathway and a strengthened immune response by TILs, and *vice versa*, considering that the state of the

immune equilibrium is a critical factor for therapeutic success (Chen and Mellman, 2013; Palucka and Coussens, 2016). In fact, alterations of TILs have already been implicated in immune homeostasis, as well as immune response in the PD-1 pathway (Menon *et al*, 2016).

Although the present data identified a significant prognostic role of PD-L1 expression in operable EBVaGC, these results should be cautiously interpreted due to potential limitations. The current study is a retrospective evaluation, and the evaluation and interpretation of PD-L1 and PD-L2 within tumours and/or a tumour microenvironment have not yet been standardised in GCs (Manson *et al*, 2016). Moreover, a standardised cutoff value for PD-L1- and PD-L2 positivity has not yet been clearly established (Festino *et al*, 2016), plus PD-L1 and PD-L2 expression cannot be used to predict treatment response to anti-PD-1/PD-L1 therapy in patients with EBVaGC. Nevertheless, the present study has several important differences with previous studies as follows: (1) a relatively large-scale cohort of EBVaGC patients with a Korean homogeneous ethnic identity (Jin and Yoon, 2016), (2) equivalent treatment application, (3) minimal loss to follow-up, (4) assessment of TILs, and (5) comprehensive interpretation of expression in cancer cells and immune cells as separate microenvironments of cancer cells.

CONCLUSION

In conclusion, str-PD-L1- and str-PD-L2 positivity was shown to be associated with dense iTu- and str-TILs in patients with EBVaGC. The current study also revealed significant prognostic impact of iTu-PD-L1 positivity by demonstrating an independent association with a poor DFS. Therefore, these findings support the concept that PD-L1 can be a prognostic indicator for predicting patient outcomes and a rationale for therapeutic targeting in EBVaGC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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