Prognostic Perspectives of STING and PD-L1 Expression and Correlation with the Prognosis of Epstein-Barr Virus-Associated Gastric Cancers

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Background/Aims: Epstein-Barr virus-associated gastric cancers (EBVaGCs) have unique molecular and clinicopathological characteristics. The cyclic GMP-AMP synthase-stimulator of interferon genes (STING) pathway is recently recognized as the critical innate immunity against pathogens and tumors. STING is also a master regulator in the cancer-immunity cycle and targeting STING could synergize with existing immune-checkpoint therapies. However, the role of STING in GC, especially in EBVaGC, and its correlation with programmed death-ligand 1 (PD-L1) remain largely unclear.

Methods: We collected 78 cases of EBVaGCs and 210 cases of EBV-negative GC (EBVnGC) from a total of 1,443 cases of GC analyzed by EBV-encoded small RNA in situ hybridization. We investigated STING and PD-L1 expression and their concomitant prognostic value in EBVaGCs and EBVnGCs using tissue microarray and immunohistochemistry. The effects of STING and PD-L1 expression on the overall survival of patients with EBVaGC or EBVnGC were assessed by univariate and multivariate analysis.

Results: We found that both STING and PD-L1 exhibited significantly higher expression in the EBVaGCs than that in the EBVnGCs. The expression of STING was positively correlated with that of PD-L1 in EBVaGCs. Simultaneous negative expression of STING and PD-L1, and positive expression of STING were independent prognostic risk factors for EBVaGC and EBVnGC, respectively.

Conclusions: This is the first prognostic retrospective study of STING and PD-L1 expression and the prognosis among EBVaGC and EBVnGC. The expression and prognostic value of STING and PD-L1 are different in the two types of GCs. STING and PD-L1 are promising prognostic biomarkers and therapeutic targets for EBVaGC and EBVnGC. (Gut Liver 2022;16:875-891)

Key Words: PD-L1; STING; Epstein-Barr virus; Gastric cancer; Biomarkers

INTRODUCTION

Gastric cancer (GC) is the 6th most common and the 3rd most fatal cancer worldwide. GC is also recognized as a malignant tumor-associated with pathogen infection, such as Helicobacter pylori, Epstein-Barr virus (EBV), etc. Among these pathogens, EBV was detected in 2% to 18% of GCs, and EBV-associated GCs (EBVaGCs) caused by EBV infection have unique clinicopathological characteristics, including male predominance, proximal stomach dis-
tribution tendency, lower rates of lymph node metastasis, and better prognosis than EBV-negative GCs (EBVnGCs). Histologically, EBVaGC is particularly enriched in lymphoepithelioma-like carcinoma (LELC) (75.7% to 90.3%), also known as GC with lymphoid stroma or medullary carcinoma. Based on the pattern of host inflammatory immune responses, EBVaGCs can be further classified into three histological subtypes, including LELC, adenocarcinoma with Crohn’s disease-like lymphocytic reaction (CLR), and conventional adenocarcinoma (CA). Molecularily, EBVaGC is characterized by hypermethylation of tumor suppressor genes, mutations in PIK3CA and recurrent amplification at 9p24.1, a chromosomal region harboring CD274, JAK2 and PDCD1LG2 genes.

CD274 gene encodes for programmed death-ligand 1 (PD-L1), also known as B7-H1, which can interact with programmed death 1 (PD-1) receptor in T cells limiting its activity to attack tumor cells (TCs) by decreasing T cell proliferation and increasing T cell apoptosis. Overexpression of PD-L1 on the surface of TCs triggers the PD-1/PD-L1 molecular brakes to escape immune surveillance and promote tumor progression. Nevertheless, the relationship between PD-L1 expression and prognosis is still controversial in GC. In a recent meta-analysis of 15 studies from Europe and Asia, PD-L1 overexpression in TCs was associated with a poor prognosis of GC in 11 studies. On the contrary, three studies reported that the overexpression of PD-L1 was a protective factor for GC prognosis, while one study did not indicate any association. Although PD-L1 expression and EBV infection, together with microsatellite instability and tumor mutation burden, have been proposed as a potential biomarker in GC immunotherapy. The efficacy of a few clinical trials with immune checkpoint inhibitors (ICIs) in the treatment of EBVaGC patients are inconsistent. Therefore, it is necessary to further study the features of PD-L1 expression in different subtypes of EBVaGC and its relationship with clinical prognosis, so as to provide reliable biomarkers for screening targeted therapies and predicting prognosis.

Stimulator of interferon genes (STING) is a multifunctional adaptor protein encoded by the TMEM173 gene, and it is well known as an essential component of the cyclic GMP-AMP synthase-STING pathway. The central role of this pathway is to recognize endogenously and/or exogenously cytoplasmic DNA produced by bacteria, viruses and TCs, and mediate innate immunity against pathogens and tumors by inducing the production of type-I interferon and inflammatory chemokines. Recently, several studies have shown that suppression of STING expression can promote tumorigenesis in melanoma, colorectal adenocarcinoma, and lung cancer. Limited literature shows that the expression of STING in GC is significantly decreased, which was positively correlated with tumor size, depth of invasion, lymph node metastasis, TNM stage and short survival time. Depleting STING expression also promotes the ability of migration and invasion in GC cells. The increased expression of STING in tumor-associated macrophages is also associated with poor prognosis in GC patients. Silencing STING gene can promote tumor-associated macrophages polarizing into pro-inflammatory subtype and induce GC cells apoptosis through the IL6R-JAK-IL24 pathway. STING has been shown as a master regulator of cancer-immunity and activating cyclic GMP-AMP synthase-STING pathway may have optimal effects of immunotherapy. However, the role of STING in GC and its correlation with PD-L1 remain largely unclear. In the current study, we investigated STING and PD-L1 expression in both EBVaGC and EBVnGC, and their concomitant prognostic value. We aimed to define the interrelations and prognostic implications of STING and PD-L1 in these two entities, especially in EBVaGC.

**MATERIALS AND METHODS**

1. **Patient selection**

In situ hybridization for EBV-encoded small RNA (EBER) was performed in 1,443 surgically resected GCs at the Nanjing Drum Tower Hospital, Nanjing, Jiangsu Province, China, from January 2014 to December 2020. The patient’s principal clinical characteristics and preoperative treatment information were collected from medical records. Finally, 78 cases of EBVaGC were confirmed. Two hundred and forty-eight patients with curatively resected EBVnGC between January 2014 and January 2015 were selected as control group. The study inclusion criteria were as follows: (1) radical resection of primary gastric adenocarcinoma with negative excision margin; (2) EBER positivity was identified strong and diffused in tumor cell nuclei in EBVaGC group; (3) all tissue blocks (2) EBER positivity was identified strong and diffused in tumor cell nuclei in EBVaGC group; (3) all tissue blocks and histological slides of selected cases were available for analysis. Exclusion criteria were: (1) patients who received preoperative neoadjuvant chemotherapy, radiation therapy, or immunotherapy; (2) multiple tumors including two or more GC; (3) patients with insufficient clinicopathological or follow-up information; (4) patients with specific subtypes of GC were excluded from the EBVnGC group, including hepatoid adenocarcinoma, adenocarcinoma with enteroblastic differentiation, micropapillary adenocarcinoma, gastric adenocarcinoma of fundic gland type, adenosquamous carcinoma, mixed adenoneuroendocrine carcinoma, and undifferentiated carcinoma; or (5) GCs
with mismatch repair (MMR) deficiency. Overall survival (OS) was estimated from the date of surgery till the date of last follow-up or mortality. This study was approved by the Committee on Medical Ethics of Nanjing Tower Drum Hospital (IRB number: 2020(467)). Written informed consent was obtained from all patients.

2. Histological examination
Formalin-fixed, paraffin-embedded GC tissues were obtained from each surgical resection specimen for histology evaluation, and each tumor was sampled with at least four blocks or entirely submitted for histology evaluation, along with the adjacent uninvolved gastric mucosal tissues and regional lymph nodes. The tumor location was categorized into proximal (including cardia, corpus and fundus) and distal (antrum and pylorus). All slides were reviewed blindly by two pathologists (Q.S. and Y.F.) according to the 5th edition of World Health Organization digestive system tumors. All EBVaGCs were histopathologically subclassified into LELC, CA, and CLR (Fig. 1A) in accordance with the criteria described by Song et al. Briefly, LELC was defined as the following histologic features: (1) a relatively clear tumor border, (2) more lymphocytes than cancer cells, (3) a syncytial growth pattern with ill-defined tubules, and (4) no desmoplasia. Cases showing patchy lymphocyte infiltration with three or more lymphoid follicles with active germinal centers on each tissue section were classified as CLR. CA was defined by cancer cells with scattered lymphocyte infiltration but no lymphoid follicles and notable desmoplasia. All tumors were staged following the rules set in the cancer staging manual of the American Joint Cancer Committee in the 8th edition.

3. Tissue microarray construction
After a histological review of all hematoxylin and eosin stained tumor sections, representative tumor tissue samples were chosen from each patient. The selected areas were circled with a marker pen on the slides and the corresponding formalin-fixed, paraffin-embedded tissue blocks for tissue microarray (TMA) construction. Tissue cores (two tumors and two adjacent non-neoplastic mucosas from each case) with 2 mm in diameter were constructed using the TMA Grand Master (3DHISTECH Ltd., Budapest, Hungary). For the two tumor cores, one was punched out from the tumor center, the other was derived from the invasive front of the deepest tumor invasion portion, and the necrotic tumor areas were avoided. Each TMA block contained 30 tumor cores and 30 non-tumor mucosa tissue cores, and was then cut into 4-μm-thick sections for hematoxylin and eosin and immunohistochemical (IHC) staining.

4. Immunohistochemistry
PD-L1 IHC staining was carried out on Dako Auto-stainer Link 48 platform (Agilent Technologies, Santa Clara, CA, USA) using an automated staining protocol validated for the PD-L1 IHC 22C3 pharmDx kit (Agilent Technologies). Monoclonal antibodies against STING (A3575, dilution 1:200; ABclonal Technology, Wuhan, China), MLH1 (ES05, dilution 1:100; Novocastra, Newcastle, UK), PMS2 (EP51, dilution 1:100; Epitomics, Burlingame, CA, USA), MSH2 (FE11, dilution 1:100; Dako, Glostrup, Denmark), and MSH6 (EP49, dilution 1:150; Epitomics) were performed according to a previously described method. Appropriate positive and negative controls were employed in each run of staining.

Fig. 1. Representative features of EBVaGC and EBV-encoded RNA in situ hybridization (ISH). (A) lymphoepithelioma-like carcinoma (LELC), carcinoma with Crohn’s disease-like lymphocytic reaction (CLR), conventional adenocarcinoma (CA), and EBV-encoded RNA ISH (×40). (B) Representative immunohistochemical images of MLH1, PMS2, MSH2, and MSH6 expression in EBVaGC. All the cases in this study showed a MMR proficient immunophenotype with MLH1, PMS2, MSH2 and MSH6 retained in the tumor nucleus (×100). EBV, Epstein-Barr virus; EBVaGC, EBV-associated gastric cancer.
5. Evaluation of immunostaining

As a comprehensive method to evaluate the expression of PD-L1 in TCs and immune cells (ICs), the combined positive score (CPS) was used as the criterion to judge the expression status of PD-L1 in the current study. Positive immunostaining for PD-L1 was assessed by cell membrane staining, and TCs that were stained only in the cytoplasm were referred to as negative, whereas ICs were counted if there is any staining. The CPS score was calculated according to the following formula described previously:25 ([PD-L1 positive TCs+PD-L1 positive mononuclear ICs]/total viable TCs)×100. Finally, PD-L1 was considered positive if the score was ≥1 for CPS for tumor cores, whereas PD-L1 was determined as positive when ≥1% non-neoplastic gastric mucosa or inflammatory cells were stained in non-tumor group.

For STING staining, the signal intensity was scored as follows: 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow-brown), and 3 (strong staining, brown). The staining distribution was scored based on the percentage of positive staining cells as follows: score 0, ≤5%; score 1, 6% to 25%; score 2, 26% to 50%; score 3, 51% to 75%; score 4, 76% to 100%. The final score was equal to the staining distribution score multiply the signal intensity score, with a series of results ranging from 0 to 12. A score of ≥1 was considered as a positive expression.

To study where there is a correlation between the expression of PDL1 and STING, the positive expression level of PDL1 and STING was sub-divided into two groups, namely, low and high expression. According to the IHC score, STING expression was classified as low expression (IHC score 1–6), and high expression (IHC score 7–12). For PD-L1 staining, CPS scores of 1–9 were defined as the low expression and ≥10 as high expressions.

IHC staining against MMR proteins was performed to assess for MMR deficiency. Defective MMR (dMMR) was defined by lacking either MLH1, MSH2, MSH6, or PMS2 expression in tumor nuclei, with infiltrating lymphocytes as the internal positive control. On the contrary, positive nuclear staining for all four MMR proteins was defined as MMR proficient. IHC analysis was performed independently by two certified pathologists (Q.S. and Y.F.) in a blinded fashion. Discrepancies between evaluators were resolved by discussion.

6. EBER in situ hybridization

To verify EBV infection status of the selected GC tissues in TMA blocks, EBER in situ hybridization was performed in all TMA blocks with INFORM EBER probe (Ventana Medical Systems, Tucson, AZ, USA) following the manufacturer’s instructions. Diffuse and strong nuclear positive signals were found in all EBVaGC selected tissues in TMAs (Fig. 1A), whereas the EBVnGCs did not.

7. Statistical analysis

All Statistical analyses were performed using SPSS version 20.0 for Windows (IBM Corp., Armonk, NY, USA).

Table 1. Baseline Clinopathological Features of EBVaGC and EBVnGC Patients

|                | EBVaGC  | EBVnGC | p-value |
|----------------|---------|--------|---------|
| Age, yr        |         |        |         |
| Means±SD       | 60.4±10.7 | 62.1±10.8 | 0.246   |
| <60            | 29 (38.2) | 78 (37.1) | 0.875   |
| ≥60            | 47 (61.8) | 132 (62.9) |         |
| Gender         |         |        |         |
| Male           | 66 (86.8) | 149 (70.9) | 0.006*  |
| Female         | 10 (13.2) | 61 (29.1) |         |
| Tumor location |         |        |         |
| Proximal       | 59 (77.6) | 134 (63.8) | 0.028*  |
| Distal         | 17 (22.4) | 76 (36.2) |         |
| Tumor size, cm |         |        |         |
| Means±SD       | 4.7±2.5 | 4.8±2.4 | 0.688   |
| <5             | 45 (59.2) | 116 (55.2) | 0.550   |
| ≥5             | 31 (40.8) | 94 (44.8) |         |
| Differentiation|         |        |         |
| Well/moderate  | 22 (28.9) | 113 (53.8) | <0.001* |
| Poor           | 54 (71.1) | 97 (46.2) |         |
| Lauren’s type  |         |        |         |
| Intestinal     | 37 (48.7) | 123 (58.6) | 0.137   |
| Diffuse/mixed  | 39 (51.3) | 87 (41.4) |         |
| LVI            |         |        |         |
| Absent         | 50 (65.8) | 80 (38.1) | <0.001* |
| Present        | 26 (34.2) | 130 (61.9) |         |
| PI             |         |        |         |
| Absent         | 25 (32.9) | 54 (25.7) | 0.230   |
| Present        | 51 (67.1) | 156 (74.3) |         |
| pTumor depth   |         |        |         |
| pT1/2          | 30 (39.5) | 45 (21.4) | 0.002*  |
| pT3/4          | 46 (60.5) | 165 (78.6) |         |
| pLNM           |         |        |         |
| Absent         | 37 (48.7) | 40 (19.1) | <0.001* |
| Present        | 39 (51.3) | 170 (80.9) |         |
| pTNM           |         |        |         |
| I/II           | 46 (60.5) | 73 (34.8) | <0.001* |
| III/IV         | 30 (39.5) | 137 (65.2) |         |
| STING          |         |        |         |
| Negative       | 28 (36.8) | 106 (50.5) | 0.041*  |
| Positive       | 48 (63.2) | 104 (49.5) |         |
| PD-L1          |         |        |         |
| Negative       | 27 (35.5) | 175 (83.3) | <0.001* |
| Positive       | 49 (64.5) | 35 (16.7) |         |

Data are presented as number (%) unless otherwise indicated. EBV, Epstein–Barr virus; GC, gastric cancer; EBVaGC, EBV-associated GC; EBVnGC, EBV-negative GC; LVI, lymphatic vascular invasion; PI, perineural invasion; LNM, lymph node metastasis; STING, stimulator of interferon genes; PD-L1, programmed death-ligand 1. *Statistically significant, p<0.05.
and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The frequencies of categorical variables were compared using the chi-square test, the Fisher exact test or nonparametric tests, as appropriate, and comparisons of quantitative variables were performed by the Student t-test. The correlations between the expression levels of PD-L1 and STING were analyzed by the Pearson correlation analysis. OS was calculated using the Kaplan-Meier method. A multivariate Cox proportional hazards model with Bonferroni correction was fitted to identify independent risk factors. The hazard ratio (HR) of each factor and its 95% confidence interval (CI) were evaluated. All p-values represented were two-sided and p<0.05 was considered to indicate statistical significance.

### RESULTS

#### 1. Clinicopathological features

EBVaGC was found in 78 cases from a total of 1,443 GCs (5.4%) was analyzed. Two patients were excluded because of insufficient tissue for preparation of TMA and preoperative chemotherapy, respectively. Finally, 76 EBVaGCs were enrolled in this study. All GCs in these TMAs were reconfirmed to be EBER positive and without dMMR phenotype by in situ hybridization and IHC staining, respectively (Fig. 1B). In the EBVnGC group, nine patients with special histological subtype GC, 26 patients with dMMR GC, and three patients with limited tissues for TMA construction were excluded, and the remaining

| Table 2. Baseline Clinicopathological Features According to Histologic Subtypes among EBVaGCs |
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| Variable | LELC (n=29) | CLR (n=28) | CA (n=19) | p-value$^\dagger$ | p-value$^\ddagger$ | p-value$^\S$ |
| Age, yr | | | | | | |
| Mean±SD | 59.6±9.8 | 58.4±8.1 | 64.6±14.1 | 0.589 | 0.166 | 0.056 |
| <60 | 10 (34.5) | 14 (50.0) | 5 (26.3) | 0.236 | 0.551 | 0.104 |
| ≥60 | 19 (65.5) | 14 (50.0) | 14 (73.7) | | | |
| Gender | | | | | | |
| Male | 26 (89.7) | 25 (89.3) | 15 (78.9) | 0.699 | 0.542 | 0.576 |
| Female | 3 (10.3) | 3 (10.7) | 4 (21.1) | | | |
| Location | | | | | | |
| Proximal | 21 (72.4) | 23 (82.1) | 15 (78.9) | 0.381 | 0.865 | 0.917 |
| Distal | 8 (27.6) | 5 (17.9) | 4 (21.1) | | | |
| Tumor size, cm | | | | | | |
| Mean±SD | 4.1±2.7 | 4.6±2.4 | 5.7±2.2 | 0.421 | 0.027* | 0.108 |
| <5 | 21 (72.4) | 18 (64.3) | 6 (31.6) | 0.509 | 0.005* | 0.028* |
| ≥5 | 8 (27.6) | 10 (35.7) | 13 (68.4) | | | |
| Differentiation | | | | | | |
| Well/moderate | 5 (17.2) | 13 (46.4) | 4 (21.1) | 0.018* | 0.962 | 0.142 |
| Poor | 24 (82.8) | 15 (53.6) | 15 (78.9) | | | |
| Lauren’s type | | | | | | |
| Intestinal | 12 (41.4) | 15 (53.6) | 10 (52.6) | 0.357 | 0.444 | 0.949 |
| Diffuse/mixed | 17 (58.6) | 13 (46.4) | 9 (47.4) | | | |
| LVI | | | | | | |
| Absent | 22 (75.9) | 21 (75.0) | 7 (36.8) | 0.940 | 0.007* | 0.009* |
| Present | 7 (24.1) | 7 (25.0) | 12 (63.2) | | | |
| PI | | | | | | |
| Absent | 13 (44.8) | 9 (32.1) | 3 (15.8) | 0.325 | 0.076 | 0.357 |
| Present | 16 (55.2) | 19 (67.9) | 16 (84.2) | | | |
| pTumor depth | | | | | | |
| pT1/2 | 16 (55.2) | 12 (42.9) | 2 (10.5) | 0.352 | 0.005* | 0.040* |
| pT3/4 | 13 (44.8) | 16 (57.1) | 17 (89.5) | | | |
| pLNM | | | | | | |
| Absent | 19 (65.5) | 14 (50.0) | 4 (21.1) | 0.236 | 0.007* | 0.090 |
| Present | 10 (34.5) | 14 (50.0) | 15 (78.9) | | | |
| pTNM | | | | | | |
| I/II | 24 (82.8) | 19 (67.9) | 3 (15.8) | 0.191 | <0.001* | 0.001* |
| III/IV | 5 (17.2) | 9 (32.1) | 16 (84.2) | | | |

Data are presented as number (%) unless otherwise indicated.

EBV, Epstein-Barr virus; GC, gastric cancer; EBVaGCs, EBV-associated GCs; LELC, lymphoepithelioma-like carcinoma; CLR, adenocarcinoma with Crohn’s disease-like lymphocytic reaction; CA, conventional adenocarcinoma; LVI, lymphatic vascular invasion; PI, perineural invasion; LNM, lymph node metastasis.

*Statistically significant, p<0.05; $^\dagger$LELC vs CLR; $^\ddagger$LELC vs CA; $^\S$CLR vs CA.
210 patients were enrolled. Compared with the EBVnGCs group, the patients with EBVaGC had a male predisposition ($p=0.006$), proximal locations ($p=0.028$). Although EBVaGC was more poorly differentiated ($p<0.001$), it was superior to EBVnGC in lymphatic vascular invasion (LVI) ($p<0.001$), tumor invasion depth ($p=0.002$), lymph node metastasis (LNM) ($p<0.001$), and pTNM stage ($p<0.001$). The clinicopathological features of patients with EBVaGC and EBVnGC are described in Table 1.

Subgroup analysis showed that LELC accounted for 38.2% of EBVaGC, followed by CLR and CA, which accounted for 36.8% and 25%, respectively. Overall, there was no significant difference in clinicopathological characteristics between LELC and CLR groups. Except for the special histological morphology of LELC, that is, dense lymphocytes wrapped to ill-defined cancerous glands, the

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**Fig. 2.** Representative immunohistochemical images of PD-L1 and STING expression in EBVaGC and its adjacent tissue. PD-L1 positive expression in tumor cells (A), immune cells (B), or both counterparts (C). PD-L1 negative expression in non-tumor adjacent tissue (D). High expression (E), low (F), or negative STING expression (G) in tumor cells. Positive STING expression in normal adjacent tissue (H) ($\times 400$). STING, stimulator of interferon genes; PD-L1, programmed death-ligand 1.
histological differentiation of LELC was poorer than that of CLR (p=0.018). Compared with the LELC (p=0.005) and CLR groups (p=0.028), most of the tumors in the CA group were ≥5 cm in size, and the mean maximal tumor size in the CA group was significantly larger than that in the LELC group (p=0.027). LVI (CA vs LELC, p=0.007; CA vs CLR, p=0.009), deeper gastric wall infiltration (CA vs LELC, p=0.005; CA vs CLR, p=0.040), LNM (CA vs LELC, p=0.007), and higher tumor stage (CA vs LELC, p=0.005; CA vs CLR, p=0.040) were significantly more prevalent in CA group. There were no statistically significant differences among three histologic subtypes of EBVaGC in age, gender, location of the tumor, Lauren classification, and perineural invasion (Table 2).

2. Expression of STING and PD-L1 in GC and its corresponding non-neoplastic gastric mucosa

The positive expression rates of PD-L1 in EBVaGC and EBVnGC were 64.5% and 16.7% respectively, whereas PD-L1 was negative in all corresponding non-neoplastic gastric mucosa cores in the two groups (p<0.001) (Fig. 2A-D, Supplementary Table 1). Actually, PD-L1 was only scattered cytoplasmic stained by very few stroma infiltrating lymphocytes with a positive rate of less than 1%, but not in the non-neoplastic gastric epitheliums in some cases. In contrast, the expression rate of STING was significantly lower in both EBVaGC (63.2% vs 90.8%, p<0.001) and EBVnGC (49.5% vs 68.6%, p<0.001) than in non-tumorous gastric mucosa (Fig. 2E-H, Supplementary Table 1).

The correlation between PD-L1 or STING expression and clinicopathological variables is summarized in Table 3. In PD-L1 positive EBVaGC, the proportion of Lauren intestinal-type GC was higher than that in the PD-L1 negative group (p=0.014). In addition, although there was no significant difference in statistics, EBVaGC with positive PD-L1 expression showed a lower LVI rate (p=0.057). Compared with STING negative EBVaGC, tumors with positive expression of STING had a lower incidence of LVI (p=0.027). As mentioned above, the LELC group and the CLR group have similar clinicopathological features (Table 2, Supplementary Fig. 1A), so these two groups were combined as the non-CA group. Compared with the CA group, the prevalence of positive PD-L1 expression was significantly lower in the CA group than that in the non-CA group (p=0.019). There was no significant difference in the expression of STING between the CA group and the non-CA group (Table 2).

In the EBVnGC control group, the patients with PD-L1 positive expression showed a higher proportion of poorly differentiated tumors (p<0.001) and Lauren diffuse /mixed-type GCs (p<0.001), while STING-positive EB-VnGCs were more likely to have LNM (p<0.001).

3. Correlation between the expression of STING and PD-L1

PD-L1 expression in EBVaGCs was significantly correlated with STING expression (p<0.001) (Supplementary Table 2). There was a general tendency for STING and PD-L1 in EBVaGC to be both negative, both low expression, or both high expression. However, there was no significant correlation between PD-L1 and STING expression in the EBVnGC group.

In our combined analysis of STING and PD-L1 expression in EBVaGC (Table 4), co-expression of STING and PD-L1 was more frequent in Lauren intestinal-type EBVaGC patients (p=0.016) and patients with absence of LVI (p=0.014) than in those with no PD-L1/STING positive expression. Less frequent LVI was also found in the EBVaGCs with either PD-L1 or STING expression, but without statistical significance when compared to that in both negative PD-L1/STING expression groups (p=0.086). No significant difference in clinicopathological characteristics was found between the PD-L1/STING co-expression group and the group with one of PD-L1/STING expressions in EBVaGC group. However, the PD-L1/STING co-expression EBVnGCs showed a positive correlation with more poorly differentiated tumors (p<0.001) and Lauren diffuse /mixed-type GCs (p<0.001) than PD-L1/STING alone or co-negative expressing cases.

4. Prognostic significance of STING and PD-L1

Follow-up data were available in all 76 EBVaGC patients and 210 EBVnGC patients, and the median follow-up period for the survival analyses was 38 months (range, 3 to 86 months) and 46 months (range, 5 to 82 months), respectively. In the EBVaGC group, 12 patients (15.8%) died due to disease, and the estimated 5-year OS rate of patients was 76.1%. In the EBVnGC group, 112 patients died of disease, with an estimated 5-year OS rate of 47.6%.

The Kaplan-Meier curves for OS are shown in Fig. 3. As expected, EBVaGC patients had a significantly better OS than patients with EBVnGC (p=0.011), but there was no statistical difference in prognosis between CA and EBVnGC patients (p=0.785) (Supplementary Fig. 1B). In the EBVaGC group, CA patients had the worst OS compared to the patients with LELC and CLR (p<0.001) (Supplementary Fig. 1C). EBVaGC patients with positive expression of PD-L1 had a significantly better OS than those with PD-L1 negative expression (p=0.002). Similarly, patients with STING positive expression showed a better OS rate (p=0.012). In our combined analysis of the expression of STING and PD-L1, patients with none of STING and PD-L1 negative expression showed a lower proportion of Lauren diffuse type GCs (p=0.039), lower incidence of LVI (p=0.007), deeper gastric wall infiltration (CA vs LELC, p=0.005; CA vs CLR, p=0.040), and higher tumor stage (CA vs LELC, p=0.005; CA vs CLR, p=0.040) were significantly more prevalent in CA group. There were no statistically significant differences among three histologic subtypes of EBVaGC in age, gender, location of the tumor, Lauren classification, and perineural invasion (Table 2).

The positive expression rates of PD-L1 in EBVaGC and EBVnGC were 64.5% and 16.7% respectively, whereas PD-L1 was negative in all corresponding non-neoplastic gastric mucosa cores in the two groups (p<0.001) (Fig. 2A-D, Supplementary Table 1). Actually, PD-L1 was only scattered cytoplasmic stained by very few stroma infiltrating lymphocytes with a positive rate of less than 1%, but not in the non-neoplastic gastric epitheliums in some cases. In contrast, the expression rate of STING was significantly lower in both EBVaGC (63.2% vs 90.8%, p<0.001) and EBVnGC (49.5% vs 68.6%, p<0.001) than in non-tumorous gastric mucosa (Fig. 2E-H, Supplementary Table 1).

The correlation between PD-L1 or STING expression and clinicopathological variables is summarized in Table 3. In PD-L1 positive EBVaGC, the proportion of Lauren intestinal-type GC was higher than that in the PD-L1 negative group (p=0.014). In addition, although there was no significant difference in statistics, EBVaGC with positive PD-L1 expression showed a lower LVI rate (p=0.057). Compared with STING negative EBVaGC, tumors with positive expression of STING had a lower incidence of LVI (p=0.027). As mentioned above, the LELC group and the CLR group have similar clinicopathological features (Table 2, Supplementary Fig. 1A), so these two groups were combined as the non-CA group. Compared with the CA group, the prevalence of positive PD-L1 expression was significantly lower in the CA group than that in the non-CA group (p=0.019). There was no significant difference in the expression of STING between the CA group and the non-CA group (Table 2).

In the EBVnGC control group, the patients with PD-L1 positive expression showed a higher proportion of poorly differentiated tumors (p<0.001) and Lauren diffuse /mixed-type GCs (p<0.001), while STING-positive EB-VnGCs were more likely to have LNM (p<0.001).
Table 3. Clinicopathological Characteristics among EBVaGCs and EBVnGCs According to the Expression of PD-L1 and STING

| Characteristics       | PD-L1                  |                 | STING                  |                 |
|-----------------------|------------------------|-----------------|------------------------|-----------------|
|                       | EBVaGC (n=49)          | EBVnGC (n=35)   | EBVaGC (n=48)          | EBVnGC (n=28)   |
| Age, yr               |                        |                 |                        |                 |
| Mean±SD               | 59.2±10.0              | 62.6±11.6       | 60.3±12.4              | 62.5±10.5       |
| <60                   | 20 (40.8)              | 9 (25.7)        | 13 (37.1)              | 6 (21.4)        |
| ≥60                   | 29 (59.2)              | 18 (66.7)       | 22 (62.9)              | 11 (66.7)       |
| Gender                |                        |                 |                        |                 |
| Male                  | 42 (85.7)              | 24 (88.9)       | 27 (77.1)              | 12 (70.6)       |
| Female                | 7 (14.3)               | 3 (11.1)        | 8 (22.9)               | 5 (33.3)        |
| Location              |                        |                 |                        |                 |
| Proximal              | 38 (77.6)              | 21 (77.8)       | 35 (72.9)              | 24 (85.7)       |
| Distal                | 11 (22.4)              | 6 (22.2)        | 13 (27.1)              | 4 (14.3)        |
| Tumor size, cm        |                        |                 |                        |                 |
| Mean±SD               | 4.9±2.6                | 4.2±2.4         | 5.4±2.4                | 4.7±2.4         |
| <5                    | 29 (59.2)              | 16 (59.3)       | 16 (45.7)              | 100 (57.1)      |
| ≥5                    | 20 (40.8)              | 11 (40.7)       | 19 (54.3)              | 75 (42.9)       |
| Differentiation       |                        |                 |                        |                 |
| Well/moderate         | 15 (30.6)              | 7 (25.9)        | 14 (40.0)              | 99 (56.6)       |
| Poor                  | 34 (69.4)              | 20 (74.1)       | 21 (60.0)              | 76 (43.4)       |
| Lauren’s type         |                        |                 |                        |                 |
| Intestinal            | 29 (59.2)              | 8 (29.6)        | 11 (31.4)              | 12 (40.7)       |
| Diffuse/mixed         | 20 (40.8)              | 19 (70.4)       | 24 (68.6)              | 63 (36.0)       |
| LVI                   |                        |                 |                        |                 |
| Absent                | 36 (73.5)              | 14 (51.9)       | 14 (40.0)              | 66 (37.7)       |
| Present               | 13 (26.5)              | 13 (48.1)       | 21 (60.0)              | 109 (62.3)      |
| PI                    |                        |                 |                        |                 |
|Absent                 | 19 (38.8)              | 6 (22.2)        | 10 (28.6)              | 44 (25.1)       |
| Present               | 30 (61.2)              | 21 (77.8)       | 25 (71.4)              | 131 (74.9)      |
| pTumor depth          |                        |                 |                        |                 |
| pT1/2                 | 21 (42.9)              | 9 (33.3)        | 6 (17.1)               | 39 (22.3)       |
| pT3/4                 | 28 (57.1)              | 18 (66.7)       | 29 (82.9)              | 136 (77.7)      |
| pLNM                  |                        |                 | 30 (91.4)              | 136 (77.7)      |
| Absent                | 27 (55.1)              | 10 (37.1)       | 5 (14.3)               | 35 (20.0)       |
| Present               | 22 (44.9)              | 17 (62.9)       | 30 (85.7)              | 140 (80.0)      |
| pTNM                  |                        |                 | 30 (15.3)              | 140 (80.0)      |
| I/II                  | 32 (65.3)              | 14 (51.9)       | 10 (28.6)              | 63 (36.0)       |
| III/IV                | 17 (34.7)              | 13 (48.1)       | 25 (71.4)              | 112 (64.0)      |
| Histologic subtypes   |                        |                 | 18 (37.5)              | 12 (42.9)       |
| LELC                  | 24 (49.0)              | 5 (18.6)        | 18 (37.5)              | 7 (25.0)        |
| CA                    | 8 (16.3)               | 11 (40.7)       | 11 (22.9)              | 8 (28.6)        |

Data are presented as number (%) unless otherwise indicated.

EBV, Epstein-Barr virus; GC, gastric cancer; EBVaGC, EBV-associated GC; EBVnGC, EBV-negative GC; PD-L1, programmed death-ligand 1; STING, stimulator of interferon genes; LVI, lymphatic vascular invasion; PI, perineural invasion; LNM, lymph node metastasis; LELC, lymphoepithelioma-like carcinoma; CLR, adenocarcinoma with Crohn’s disease-like lymphocytic reaction; CA, conventional adenocarcinoma; NA, not available.

*Statistically significant, p<0.05; †p=0.003 vs CA, ‡p=0.019 vs LELC+CLR, §p=0.583 vs LELC+CLR.
Table 4. Clinicopathological Features of EBVaGC and EBVnGC Grouped According to the Combining Expression PD-L1 and STING

| Characteristics | EBVaGC | EBVnGC |
|-----------------|--------|--------|
|                 | STING & PD-L1+ (n=40) | STING or PD-L1+ (n=17) | STING & PD-L1– (n=19) |
| p-value†        |        |        |        |
| Age, yr         | Mean±SD | 60.0±9.9 | 60.3±11.4 | 61.4±12.1 | 0.929 | 0.652 | 0.786 |
| Gender          |         | 15 (37.5) | 7 (41.2) | 16 (36.8) | 13 (61.9) | 13 (67.6) | 13 (68.4) | 1.000 | 0.929 | 0.893 |
| Location (proximal vs distal) | 29 (72.5) | 15 (78.9) | 11 (57.9) | 0.342 | 0.142 | 0.786 |
| Tumor size, cm  | Mean±SD | 5.1±2.6 | 4.3±2.3 | 4.1±2.5 | 0.251 | 0.142 | 0.786 |
| Differentiation | Well/Moderate | 12 (30.0) | 6 (35.3) | 4 (26.3) | 0.694 | 0.683 | 0.562 |
| Histologic subtype | LELC/CLR | 33 (82.5) | 12 (70.6) | 12 (63.2) | 0.313 | 0.103 | 0.637 |
| PI              | Absent  | 17 (42.5) | 4 (23.5) | 4 (21.1) | 0.290 | 0.188 | 0.823 |
| LNM             | Absent  | 23 (57.5) | 13 (76.5) | 15 (78.9) | 0.368 | 0.016* | 0.042 |
| LNM stage       | pT1/2   | 26 (60.0) | 17 (42.5) | 8 (21.1) | 0.142 | 0.086 | 0.529 | 0.000* | 0.000* |
| LNM stage       | pT3/4   | 23 (57.5) | 12 (36.8) | 11 (57.9) | 0.204 | 0.142 | 0.786 |
| LNM stage       | pTNM   | 26 (60.0) | 17 (42.5) | 12 (63.2) | 0.368 | 0.016* | 0.042 |

EBV, Epstein-Barr virus; GC, gastric cancer; EBVaGC, EBV-associated GC; EBVnGC, EBV-negative GC; PD-L1, programmed death-ligand 1; STING, stimulator of interferon genes; LELC, lymphoepithelioma-like carcinoma; CLR, adenocarcinoma with Crohn's disease-like lymphocytic reaction; CA, conventional adenocarcinoma; LVI, lymphatic vascular invasion; PI, perineural invasion; LNM, lymph node metastasis; p-value, probability value; †STING & PD-L1+ vs STING or PD-L1+; ‡STING & PD-L1+ vs STING & PD-L1–; §STING or PD-L1+ vs STING & PD-L1–.

*Statistically significant, p<0.05; NA, not available.
L1 expression were associated with a worse OS (p=0.003). In the EBVnGC group, STING expression showed an opposite prognostic effect, with OS significantly worse in STING positive patients than in STING negative patients (p=0.010), while PD-L1 and PD-L1/STING combined expression had no significant prognostic effect (Fig. 4).

In the univariate analysis for OS (Table 5), EBVaGC tumor size greater than 5 cm (p=0.044; HR, 3.845; 95% CI, 1.040 to 14.213), patients with CA (p<0.001; HR, 11.429; 95% CI, 3.046 to 42.880), LVI (p=0.019; HR, 4.220; 95% CI, 1.269 to 14.033), LNM (p=0.016; HR, 12.635; 95% CI, 1.612 to 97.469), pTNM stage (p=0.005; HR, 19.174; 95% CI, 2.460 to 149.446), and the simultaneous loss of STING and PD-L1 expression (p=0.003; HR, 5.932; 95% CI, 1.830 to 19.228) were independent prognostic factors and significantly associated with a worse prognosis. whereas PD-L1 (p=0.005; HR, 0.167; 95% CI, 0.048 to 0.581) and STING expression (p=0.021; HR, 0.240; 95% CI, 0.072 to 0.804) were positively correlated with OS. Similarly, in the EBVnGC group, large tumor size (p=0.026; HR, 1.523; 95% CI, 1.051 to 2.207), more aggressive clinical features such as LVI (p=0.000; HR, 2.850; 95% CI, 1.834 to 4.428), perineural invasion (p=0.000; HR, 3.318; 95% CI, 1.893 to 5.816), greater invasion depth (p=0.000; HR, 4.589; 95% CI, 2.319 to 9.080), LNM (p=0.000; HR, 3.835; 95% CI, 1.938 to 7.588), and higher pTNM stage (p=0.000; HR, 4.687; 95% CI, 2.790 to 7.875) were significantly associated with worse prognosis, whereas STING positive expression (p=0.011; HR, 1.629; 95% CI, 1.118 to 2.373) were inversely associated with OS.

The multivariate Cox regression analysis identified advanced pTNM stage (p=0.008; HR, 16.294; 95% CI, 2.087 to 127.210) and PD-L1/STING double negative expression (p=0.01; HR, 4.57; 95% CI, 1.430 to 14.608) were independently associated with an unfavorable prognosis in patients with EBVaGC. For EBVnGC patients, LVI, greater pTumor depth, higher pTNM stage, and STING positive expression were independently associated with a poor prognosis, while distal gastric location was an independent protective factor for prognosis (Table 5).

### DISCUSSION

In this study, we evaluated the clinical relevance of STING and PD-L1 expression in both EBVaGC and EBVnGC. To our knowledge, this study is the first to provide information on STING and PD-L1 expression in the histological subtypes of EBVaGC classified based on the pattern of host inflammatory immune responses, as well as the correlation between the expression of the two proteins and their prognostic value.

EBVaGC accounts for 8.7% (range, 1.3% to 30.9%) of all GCs worldwide with variable prevalence between geographic regions,5,26 and with an incidence of 5.4% in this study. Consistent with the previous findings,3,5 EBVaGC in the present study was characterized by male dominance, non-distal stomach predilection, and higher morphohistologic association with LELC. The proportion of LELC in EBVaGC shows great discrepancies in different studies with a range from 25.6% to 72.3%,5,27,28 partly due to lack of standardized criteria regarding LELC.3,5 EBVaGC includes not only lymphoepithelioma-like histology, but also GCs with less or seldom intratumoral and peritumoral lymphatic infiltration. These infiltrating ICs reflect intraindividual differences in the host immune defenses
| Factor                        | Objective | EBVaGC Univariate analysis | EBVaGC Multivariate analysis | EBVnGC Univariate analysis | EBVnGC Multivariate analysis |
|------------------------------|-----------|----------------------------|------------------------------|----------------------------|------------------------------|
|                              |           | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value |
| Age                          | ≥60 yr    | 0.448 [0.121–1.660] | 0.229 | 1.421 [0.956–2.114] | 0.083 | Reference | Reference | Reference | Reference |
|                              | <60 yr    | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Gender                       | Female    | 0.653 [0.175–2.430] | 0.524 | 0.920 [0.608–1.392] | 0.694 | Reference | Reference | Reference | Reference |
|                              | Male      | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Location                     | Distal    | 1.686 [0.368–7.726] | 0.501 | 0.492 [0.322–0.752] | 0.001* | Reference | Reference | 0.600 [0.389–0.926] | 0.021* |
|                              | Proximal  | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Tumor size                   | ≥5 cm     | 3.845 [1.040–14.213] | 0.044* | 1.523 [1.051–2.207] | 0.026* | Reference | Reference | Reference | Reference |
|                              | <5 cm     | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Differentiation              | Poor      | 2.342 [0.513–10.700] | 0.272 | 0.701 [0.483–1.015] | 0.060 | Reference | Reference | Reference | Reference |
|                              | Well/moderate | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Histologic subtypes          | CA        | 11.429 [3.046–42.880] | <0.001* | 0.595 [3.000–1.000] | 1.132 | Reference | Reference | Reference | Reference |
|                              | LELC/CLR  | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Lauren’s type                | Diffuse/mixed | 0.595 [0.183–1.935] | 0.388 | 1.332 [0.917–1.934] | 0.132 | Reference | Reference | Reference | Reference |
|                              | Intestinal | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| LVI                          | Present   | 4.220 [1.269–14.033] | 0.019* | 1.764 [1.081–2.874] | 0.023* | Reference | Reference | Reference | Reference |
|                              | Absent    | 35.466 [5.195–46.488] | 0.179 | Reference | Reference | Reference | Reference | Reference | Reference |
| PI                           | Present   | 3.466 [5.195–46.488] | 0.088 | 4.589 [3.319–9.080] | 0.000* | Reference | Reference | Reference | Reference |
|                              | Absent    | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| pTumor depth                 | pT3/4     | 5.962 [0.768–46.273] | 0.088 | 4.589 [3.319–9.080] | 0.000* | Reference | Reference | Reference | Reference |
|                              | pT1/2     | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| pLNM                         | Present   | 12.635 [6.12–97.469] | 0.016* | 4.589 [3.319–9.080] | 0.000* | Reference | Reference | Reference | Reference |
|                              | Absent    | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| pTNM stage                   | III/IV    | 19.174 [2.460–149.446] | 0.005* | 4.589 [3.319–9.080] | 0.000* | Reference | Reference | Reference | Reference |
|                              | VII       | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| PD-L1                        | Positive  | 0.167 [0.048–0.581] | 0.005* | 4.589 [3.319–9.080] | 0.000* | Reference | Reference | Reference | Reference |
|                              | Negative  | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| STING                        | Positive  | 0.240 [0.072–0.804] | 0.021* | 1.629 [1.118–2.373] | 0.011* | Reference | Reference | Reference | Reference |
|                              | Negative  | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| PD-L1 & STING                | Double negative | 5.932 [1.830–19.228] | 0.003* | 4.570 [1.430–14.608] | 0.010* | Reference | Reference | Reference | Reference |
|                              | Other     | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |

EBV, Epstein-Barr virus; GC, gastric cancer; EBVaGC, EBV-associated GC; EBVnGC, EBV-negative GC; HR, hazard ratio; CI, confidence interval; LVI, lymphatic vascular invasion; PI, perineural invasion; LNM, lymph node metastasis; PD-L1, programmed death-ligand 1; STING, stimulator of interferon genes; CA, conventional adenocarcinoma; LELC, lymphoepithelioma-like carcinoma; CLR, adenocarcinoma with Crohn’s disease-like lymphocytic reaction; NA, not available.

*Statistically significant, p<0.05.
Some studies subclassified EBVaGC into LELC, CLR and CA according to the microscopic characteristics of host cellular immune response. In this study, the same classification criteria were used, and the results showed that there were significant differences in clinicopathological features and prognosis between the CA group and the other two subtypes. Patients with CA exhibited more aggressive biological behavior than patients with LELC and CLR, and were in a more advanced tumor stage, resulting in the worst 5-year OS among the three groups.

Fig. 4. Survival curves of EBVaGC and EBVnGC patients according to PD-L1 expression (A, B). Survival curves of EBVaGC and EBVnGC patients according to STING expression (C, D) and the simultaneous expression of both STING and PD-L1 (E, F). The log-rank test was used to calculate the p-value.

EBV, Epstein-Barr virus; GC, gastric cancer; EBVaGC, EBV-associated GC; EBVnGCs, EBV-negative GCs; PD-L1, programmed death-ligand 1; STING, stimulator of interferon genes.
Similar prognostic trends of CA patients were also seen in two Korean studies.\textsuperscript{2,29} CA was stratified into non-LELC or non-GC with lymphoid stroma subgroups in the majority of previous research on EBVaGC, but these subgroups actually comprise CA and different proportions of CLR.\textsuperscript{2,28,29} Our results showed that the clinicopathological features of CLR were similar to those of LELC rather than CA, and the survival of CLR patients was better than that of CA patients.\textsuperscript{2,29}

In this study, the prognosis of CA in EBVaGC was closer to that of EBVnGC, and there was no significant difference between the two groups, which was inconsistent with previous reports that the prognosis of EBVaGC was better than that of the other GC subtypes.\textsuperscript{30} In fact, the host inflammatory immune response is actively involved in the progression of GCs.\textsuperscript{31} LELC and CLR with more active inflammatory immune response have a better clinical outcome, while CA lack of immune response is associated with a worse prognosis. Therefore, it is of prognostic value to strictly distinguish the subtypes of EBVaGC, especially for CA patients. Histologically, dMMR GCs can also exhibit a prominent lymphoid infiltrated growth pattern.\textsuperscript{2,4} However, EBV positive and dMMR status are mutually exclusive in GC,\textsuperscript{32} which is consistent with the MMR proficient phenotype for all EBVaGCs in our study. In addition, the special histological subtypes of GC described in the World Health Organization classification and dMMR GC were excluded in the EBVnGC group to avoid their impact on the prognosis.\textsuperscript{7}

EBVaGC can establish an immunologically privileged microenvironment for TCs to evade host immune control by up-regulating PD-L1, so targeting the PD-1/PD-L1 signaling pathway to block the immunosuppressive state may be an important therapeutic strategy for EBVaGC patients.\textsuperscript{8} Since PD-L1 expression GC patients are more likely to benefit from PD1 blockade, the IHC expression of PD-L1 is used as one of the criteria for predicting the efficacy of PD-1/PD-L1 blockade therapy.\textsuperscript{33} In phase II clinical trial (NCT02589496), six patients with metastatic EBVaGC showed an overall response rate of 100% when treated with pembrolizumab, and all of the above EBVaGC cases showed positive IHC staining for PD-L1 in this study.\textsuperscript{11} Subsequently, in another phase Ib/II, multicenter clinical study of advanced GC (NCT02915432), only one among four EBVaGC patients achieved a partial response to toripalimab (an anti-PD-1 agent), with two and one patients showed stable disease and progressive disease in the remaining three cases.\textsuperscript{34} Interestingly, the patient who obtained partial response in this trial had a positive PD-L1 expression, while the rest three cases were negative for PD-L1, and similar findings were also seen in a recent prospective study of immunootherapy for EBVaGC.\textsuperscript{12} These results suggest that the status of PD-L1 may affect the efficacy of ICI therapy in patients with EBVaGC. The positive rate of PD-L1 in EBVaGC varies greatly in different studies, ranging from 34.4% to 94%.\textsuperscript{27,32,35-41} The discrepancy might be due to different antibodies and evaluating criteria used to assess PD-L1 expression.\textsuperscript{36-40} Therefore, the Food and Drug Administration-approved PD-L1 antibody and evaluating system to assess the PD-L1 IHC scores were used in our cohort, which could make the results more repeatable.

Prognostic impact of PD-L1 expression remains controversial in GC. For EBVaGCs, favorable,\textsuperscript{39,42,43} neutral,\textsuperscript{35-37} and adverse\textsuperscript{32,35,36,40} prognosis have been reported. In addition to different PD-L1 detection methods, sample size, ethnic or geographical differences among these studies, the expression of PD-L1 in different cellular components of the tumor microenvironment may also lead to different prognosis for EBVaGC patients.\textsuperscript{32,35,36,39,41} In a meta-analysis of 3,291 patients, overexpression of PD-L1 was reported to be a significant poor prognostic factor in GC.\textsuperscript{10} However, the studies included in this meta-analysis only evaluated PD-L1 expression in TCs. It is known that the tumoral expression of PD-L1 could foster the TCS survival\textsuperscript{42} and help them to escape from the host immune surveillance,\textsuperscript{9} thus probably contributing to a worse prognosis in EBVaGC patients. EBVaGC is an inflammation-related disease, usually accompanied by massive inflammatory cell infiltration consisting primarily of CD8-positive T lymphocytes in and around the TCs.\textsuperscript{44} Different from non-small cell lung cancer in which PD-L1 is mainly expressed in TCs, it has been reported that the expression of PD-L1 in GC is more frequently seen in ICs than in TCs.\textsuperscript{32,45} In the Cancer Genome Atlas database, 94% of EBVaGCs showed PD-L1 expression in ICs, while only 50% of cases expressed PD-L1 in TCs.\textsuperscript{41} CPS, as a scoring method, incorporates PD-L1 expression by both TCs and ICs. In this cohort, the frequency of PD-L1 expression in TCs and ICs was 39.5% and 63.2% based on the scoring method described by Kudanagara \textit{et al.},\textsuperscript{25} respectively. Among 49 CPS positive cases, 30 cases (61.2%) showed a PD-L1 expression in TCs, 48 cases (97.8%) in ICs, and 30 cases (61.2%) with simultaneous expression of PD-L1 in both TCs and ICs (data not shown). As mentioned above, PD-L1 is more frequently expressed in ICs than in TCs in EBVaGC. Thus, the positive PD-L1 CPS expression in this study was affected more by ICs. Studies have shown that the expression of PD-L1 in ICs is associated with a favorable outcome in EBVaGCs.\textsuperscript{32,42,43} Consistent with these previous studies, our data showed that PD-L1 CPS positive predicting a better prognosis. A similar result was also reported by Sundar \textit{et al.}\textsuperscript{39} In this Korean study, both EBV-positive and -negative patients with a
higher-level transcriptomic expression of PD-L1 showed a favorable prognosis. The transcriptional expression was correlated with PD-L1 IHC CPS scores. The expression of PD-L1 in ICs results from their responses to tumor environmental signals. In the tumor microenvironment, the dead TCs release tumor antigens and inflammatory factors, which attract ICs to attack the tumor. In the process, tumor antigens and inflammatory cytokines could activate T cells to express PD-L1. In the mice model, PD-L1 expressed by activated CD8+ T cells could enhance cytotoxic T cells survival and maintain T cell immunity in anti-tumor immune responses. Thus PD-L1 expressed in ICs reflects a pre-existent boosted anti-tumor adaptive immunity, which may promote anti-tumor host defense and prolong the survival of EBVaGC patients. However, other studies also indicated that the CPS scores were not a prognosis factor for EBVaGC patients. Further studies are warranted to clarify the mechanism and clinical significance of PD-L1 expression in the immune microenvironment of EBVaGC.

However, there is still insufficient evaluation of STING expression in GC, and to the best of our knowledge, there is no previously reported evaluation of STING in EBVaGC. The expression of STING in GC was significantly lower than that in adjacent non-neoplastic mucosa, but the positive rate of STING in EBVaGC was higher than that in EBVnGC in this study. STING plays an important role in host defenses against pathogens such as viruses and bacteria by regulating type-I interferon signal transduction and innate immunity. In nasopharyngeal carcinoma, EBV can trigger STING-mediated type-I interferon production, so EBV may be responsible for the persistent expression of STING in EBVaGC. In addition, although there was no statistical difference in STING expression between EBVaGC subtypes, STING expression rate was lower in CA than that in LELC and CLR. The same was true for PD-L1 expression, which was significantly lower in CA than that in LELC and CLR. Activation of cyclic GMP-AMP synthase-STING signaling has been reported to stimulate PD-L1 expression in cancer cells. For the positive correlation between STING and PD-L1 expression in EBVaGC group, STING may be involved in the regulation of PD-L1 expression in EBVaGC. Recent studies have shown that activation of the STING-mediated pathway in small cell lung cancer is responsible for the production of chemokines in response to DNA damage in vitro, leading to increased immunogenicity of immunosuppressed tumors and concomitant upregulation of PD-L1 expression in TCs. Also, STING-deficient mice are less responsive to anti-PD-1/ PD-L1 blockers and radiation. And co-administration of ICI and STING agonist reduced and delayed tumor growth in the B16 melanoma mouse model. As previously noted, the efficacy of ICls for EBVaGC has varied in clinical trials, and since PD-L1-expressing GC patients are more likely to benefit from PD1/PD-L1 inhibitors, the combined application of STING agonists and ICls for PD-L1 negative EBVaGC patients is promising.

A previous study showed that decreased expression of STING predicts poor prognosis in GC patients, which is consistent with the observation in EBVaGC in our study. Moreover, when combining analyzed the expression of STING and PD-L1, the patients with both STING and PD-L1 negativity had a worse prognosis, with more LVI in this group compared with other EBVaGC patients. However, in the EBVnGC group, STING negative patients have better prognosis, which may be related to the specific clinicopathological and molecular characteristics of different GC subtypes. However, in this previous study, the authors did not strictly distinguish and exclude the special subtypes of GC, including EBVaGC and dMMR GC, which may lead to bias in prognosis observation. In addition, the function of STING in tumor migration and metastasis is divergent in different tumor types. Liang et al. found that activated STING promotes the recruitment of regulatory T cells in tongue squamous cell carcinoma by up-regulating CCL22, which mediates the immune escape of TCs, and is associated with the poor prognosis of tongue squamous cell carcinoma patients. Upregulation of STING expression has been reported to be associated with increased tumor growth in the mouse model of non-inflammatory Lewis lung cancer. In breast cancer, however, STING activation effectively limits migration and metastasis through nuclear factor kappa B signaling pathway-induced cell death. Taken together, the role of STING in tumor progression is still controversial, and its prognostic value and molecular mechanism in different subtypes of GC need to be further clarified.

Finally, our study has some limitations. First, this is a single-institution retrospective study with a limited sample size due to the relatively low incidence of EBV infection in GCs, further multicenter studies are needed to generalize the impact of STING and PD-L1 on the prognosis of different subtypes of GC, especially for EBVaGC. Second, TMA methodology was applied to IHC evaluation. Although each tumor has two cores from the tumor center and deepest tumor invasion portion, the heterogeneity of STING and PD-L1 expression within the tumor is inevitable. However, several previous studies have shown that IHC results from TMA and whole sections are largely comparable.

In conclusion, EBV infection is not a predictor of good prognosis in GC as traditionally thought. EBVaGC subtypes based on host immune response can be used to...
stratify the prognostic risk of related patients. CA is the worst prognostic subtype of EBVaGC, and its prognosis is similar to that of non-specific type EBVnGC. This is the first prognostic retrospective study of STING and PD-L1 expression and the prognosis among EBVaGC and EBVnGC. The expression and prognostic value of STING and PD-L1 are different in the two types of GCs, suggesting that the molecular mechanisms of tumorigenesis and development varied in distinct types of GCs. STING and PD-L1 are promising prognosis biomarkers and therapeutic targets for EBVaGC and non-specific type EBVnGC.

CONFLICTS OF INTEREST

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

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SUPPLEMENTARY MATERIALS

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