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SHORT COMMUNICATION

Transcriptional analysis of immune genes in Epstein–Barr virus-associated gastric cancer and association with clinical outcomes

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Abstract

Background Epstein–Barr virus-associated gastric cancer (EBVaGC) has traditionally been associated with high expression of PD-L1 and immune infiltration. Correlations between PD-L1 and other immune-related gene (IRG) expressions in EBVaGC have not been previously described.

Methods We performed NanoString® transcriptomic profiling and PD-L1 immunohistochemistry (IHC) (using the FDA approved Dako PD-L1 IHC 22C3) on EBVaGC samples from gastric cancer patients undergoing primary tumor resections at Samsung Medical Centre, South Korea. For controls, EBV-negative samples from the previously reported Asian Cancer Research Group (EBVnegACRG) cohort were used. Genes tested included *PD-L1* and other IRGs related to intra-tumoral cytolytic activity, cytokines and immune checkpoints. Samples with *PD-L1* expression > 34th percentile were defined as PD-L1$_{high}$ and the remaining as PD-L1$_{low}$.

Results We identified 71 cases of EBVaGC and 193 EBV-negative ACRG samples as controls. EBVaGC showed higher expression of all queried immune genes compared to EBVnegACRG samples ($p < 0.01$). PD-L1 immunohistochemistry expression correlated with *PD-L1* transcript expression ($r = 0.63$, $p < 0.001$). Tumor-infiltrating lymphocyte patterns were also found to be different between PD-L1$_{low}$ and PD-L1$_{high}$ groups. PD-L1$_{low}$ EBVaGC samples ($n = 24$, 34%) had consistently decreased expression of all other immune genes, such as *CD8A, GZMA* and *PRF1* and *PD-1* ($p < 0.001$). PD-L1$_{low}$ EBVaGC samples were also associated with worse disease-free survival (HR 5.03, $p = 0.032$) compared to PD-L1$_{high}$ EBVaGC samples.

Conclusions A substantial proportion of EBVaGC does not express high levels of *PD-L1* and other immune genes. EBVaGCs which have lower transcriptomic expression of *PD-L1* tend to have a similarly low expression of other immune genes, IHC scores and a poorer prognosis.

Keywords Epstein–Barr virus-associated gastric cancer · Immune genes · PD-L1

Introduction

Epstein–Barr virus (EBV)-associated gastric cancer (EBVaGC), characterized by the presence of EBV in gastric cancer (GC) cells, is a distinct GC subtype found in ~ 10% of cases and with specific clinicopathologic and molecular features [1]. Although advances have been made in elucidating distinct molecular features of EBVaGC, no therapeutic modalities specific to EBVaGCs currently exist. Despite robust immune cell presence in EBVaGCs, few studies have explored immune subtyping of EBVaGCs and its association with survival [2–4]. Previous EBVaGC-specific studies have focused on histomorphological characteristics, such as tumor-infiltrating lymphocyte (TIL) patterns [4] or expression of PD-L1 through immunohistochemistry (IHC)

Raghav Sundar and Aditi Qamra have contributed equally and shared the first authorship.

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[5, 6]. Large genomic studies of gastric cancer conducted by The Cancer Genome Atlas group (TCGA) [1] and the Asian Cancer Research Group (ACRG) [7] only include a small sample size of EBVaGC. There is a strong rationale for additional characterization of the immune landscape of EBVaGC as these may highlight new prognostic markers and avenues for therapy. Here, we report results from one of the largest cohorts of EBVaGC primary samples with integrated analysis of transcriptomic expression of key immune-related genes (IRG), TIL subtyping and PD-L1 IHC expression using a recently Food and Drug Administration (FDA) approved antibody and scoring algorithm for gastric cancer.

Methods

EBVaGC cohort

EBV-encoded RNA in-situ hybridization (EBER-ISH) was performed on gastric cancer samples from patients undergoing primary tumor resections at Samsung Medical Centre, South Korea, from 1996 to 2016 (supplementary methods). Only cases with a strong signal within almost all tumor cell nuclei were considered to be positive and included in this cohort. Analysis of tumor-infiltrating lymphocytes was performed and classified according to previously established histologic subtypes based on host inflammatory immune response [lymphoepithelioma-like carcinoma (LELC), Crohn's disease-like lymphocytic reaction (CLR) and conventional adenocarcinoma (CA)] [4]. PD-L1 immunohistochemistry (IHC) was performed using the FDA approved PharmDx 22C3 Dako PD-L1 antibody and scored using the Combined Positive Score (CPS) (supplementary methods).

EBV-negative ACRG cohort

We have previously reported molecular characterization of a large cohort of gastric cancer samples as part of the Asian Cancer Research Group (ACRG) [7]. These samples were also from the same Samsung Medical Centre, South Korea, collected from 2004 to 2007. From this cohort, we selected samples of EBV-negative gastric cancer, with clinical data available as a comparative control for this study (EBVnegACRG cohort).

NanoString® analysis

To measure the transcriptomic expression of IRGs in EBVaGC samples, we used the NanoString platform, an FDA approved platform that has been shown to work with low input formalin-fixed paraffin-embedded RNA samples. Nanostring nCounter Reporter CodeSets were designed for 14 IRG corresponding to intra-tumoral cytolytic activity (CYT) [8], cytokines and immune checkpoints (supplementary methods, Supplementary Table 1). Analysis of older samples (> 10 years old) versus newer samples was performed to reduce sample-degradation bias (supplementary methods, Supplementary Fig. 1).

Statistical analysis

Associations of clinicopathologic features to histologic subclassification was performed using Fisher's exact test. Disease-free survival (DFS) was calculated from the time of surgery to the time of disease progression or death, and overall survival (OS) was calculated from time of surgery to time of death. Kaplan–Meier (KM) curves and log rank test were used for survival analysis. The hazard ratio (HR) and its 95% confidence interval (CI) were evaluated for each analysis using Cox proportional hazards regression. All analyses were done using R (3.4.1). Samples with *PD-L1* expression greater than the 34th percentile were defined as PD-L1$_{high}$ and remaining PD-L1$_{low}$.

Results

Patients' characteristics

We identified 71 cases of EBVaGC from a cohort study between 1996 and 2016 [4]. As a comparative control, 193 EBV-negative GC samples (of which 149 had clinical data available) were selected from the ACRG cohort [7]. Patients' characteristics of these cohorts are described in Table 1. For the EBVaGC cohort, the median age was 56 years, with 87% being male. In general, clinicopathological features predicting poor outcome were only found in a minority of EBVaGC samples: lymphovascular invasion (42%), perineural invasion (28%) and nodal metastases (37%), consistent with the good prognosis of this GC subgroup.

Immune gene expression profiles of EBVaGC

Unsupervised clustering of the expression data revealed clear separation between EBVaGC and EBVnegACRG GCs (Fig. 1a). When analyzed as a group, EBVaGC samples collectively showed higher expression of all queried IRGs (Fig. 1b, c) compared to EBVnegACRG samples ($p < 0.01$, Wilcoxon one-sided test), consistent with previous reports of increased immune infiltration in EBVaGC [1, 2]. EBVaGC samples showed significantly increased expression of *PD-L1* ($p < 0.001$, Fig. 1d) compared to EBVnegACRG samples. Notably however, not all EBVaGC samples showed higher PD-L1 expression compared to EBVnegACRG samples. We thus divided the EBVaGC samples into PD-L1$_{high}$ ($n = 47$, 66%) and PD-L1$_{low}$ ($n = 24$, 34%) expressing groups. We

Table 1 Patients' characteristics

EBVaGC		ACRG	
Clinical feature	*n* (%)	Clinical feature	*n* (%)
Age (years)		*Age (years)*	
Median (range)	56 (33–75)	Median (range)	64 (24–84)
Gender		*Gender*	
Male	62 (87.3)	Male	104 (69.8)
Female	9 (12.7)	Female	45 (30.2)
Tumor location		*Tumor location*	
Antrum	8 (11.2)	Antrum	79 (53.0)
Body	48 (67.6)	Body	15 (33.6)
Cardia	8 (11.2)	Cardia	16 (10.7)
Others	7 (9.9)	Whole	4 (2.7)
Lymphovascular invasion		*Lauren histology*	
Yes	30 (42.3)	Diffuse	76 (51.0)
No	41 (57.7)	Intestinal	62 (41.6)
Perineural invasion		Mixed	11 (7.4)
Yes	20 (28.2)	*WHO classification*	
No	51 (71.8)	Tubular (well/moderately differentiated)	52 (34.9)
Lymph nodes metastasis		Tubular (poorly differentiated)	70 (47.0)
Yes	26 (36.6)	Signet ring cell carcinoma	21 (14.1)
No	45 (63.4)	Papilliary	3 (2.0)
TNM stage		Hepatoid	1 (0.7)
I	35 (49.2)	Mucinous	1 (0.7)
II	19 (26.8)	Others	1 (0.7)
III	17 (23.9)	*TNM stage*	
Histology by host immune reaction		I	2 (1.3)
Lymphoepithelioma-like carcinoma (LELC)	30 (42.2)	II	70 (47.0)
Crohn's disease-like lymphoid reaction (CLR)	33 (46.4)	III	39 (26.2)
Conventional adenocarcinoma (CA)	8 (11.3)	IV	36 (24.2)
		Unknown	2 (1.3)

observed that PD-L1$_{low}$ samples had consistently decreased expression of all other IRG including markers of CYT, such as *CD8A, GZMA* and *PRF1* as well as immune checkpoints, such as *PD-1* ($p < 0.001$, Wilcoxon one-sided test) (Fig. 2a, b). When compared to the EBVnegACRG cohort, a similar trend was seen, with reduced IRG expression identified in those with reduced *PD-L1* expression (Fig. 2a).

PD-L1 immunohistochemical expression and tumor-infiltrating lymphocyte classification

PD-L1 IHC was performed on all 71 samples. Tumor and immune PD-L1 expression was scored independently and the CPS was calculated (Table 2). PD-L1 CPS scores correlated with PD-L1 transcriptomic expression ($r = 0.63$, $p < 0.001$) (Supplementary Fig. 2). PD-L1$_{low}$ tumors tended to have lower CPS scores compared to PD-L1$_{high}$ tumors (Table 2; $p = 0.004$). Analysis of tumor-infiltrating lymphocytes was also performed and classified according to host inflammatory immune response: LELC, CLR, CA [4]. We observed significant differences in the expression of PD-L1 within these subtypes representative of tumor-infiltrating lymphocyte status (Table 2). PD-L1$_{low}$ had a higher proportion of CA subtype (25 vs 4%), while PD-L1$_{high}$ had a higher proportion of LELC subtype (51 vs 25%). There were no significant differences in the other clinicopathological parameters, such as T stage, N stage, lymphatic and perivascular invasion between the two groups (Table 2).

PD-L1$_{low}$ is associated with worse survival

The EBVaGC cohort had a median follow-up of 66 months. Of the five deaths in the cohort, one of them was not cancer related. With limited OS events precluding meaningful statistical analyses, DFS was chosen as the survival outcome for further statistical and correlative analyses. PD-L1$_{low}$ samples were associated with worse DFS [HR 5.03 (0.97–25.92), $p = 0.032$, Fig. 2c]. We saw similar trends for

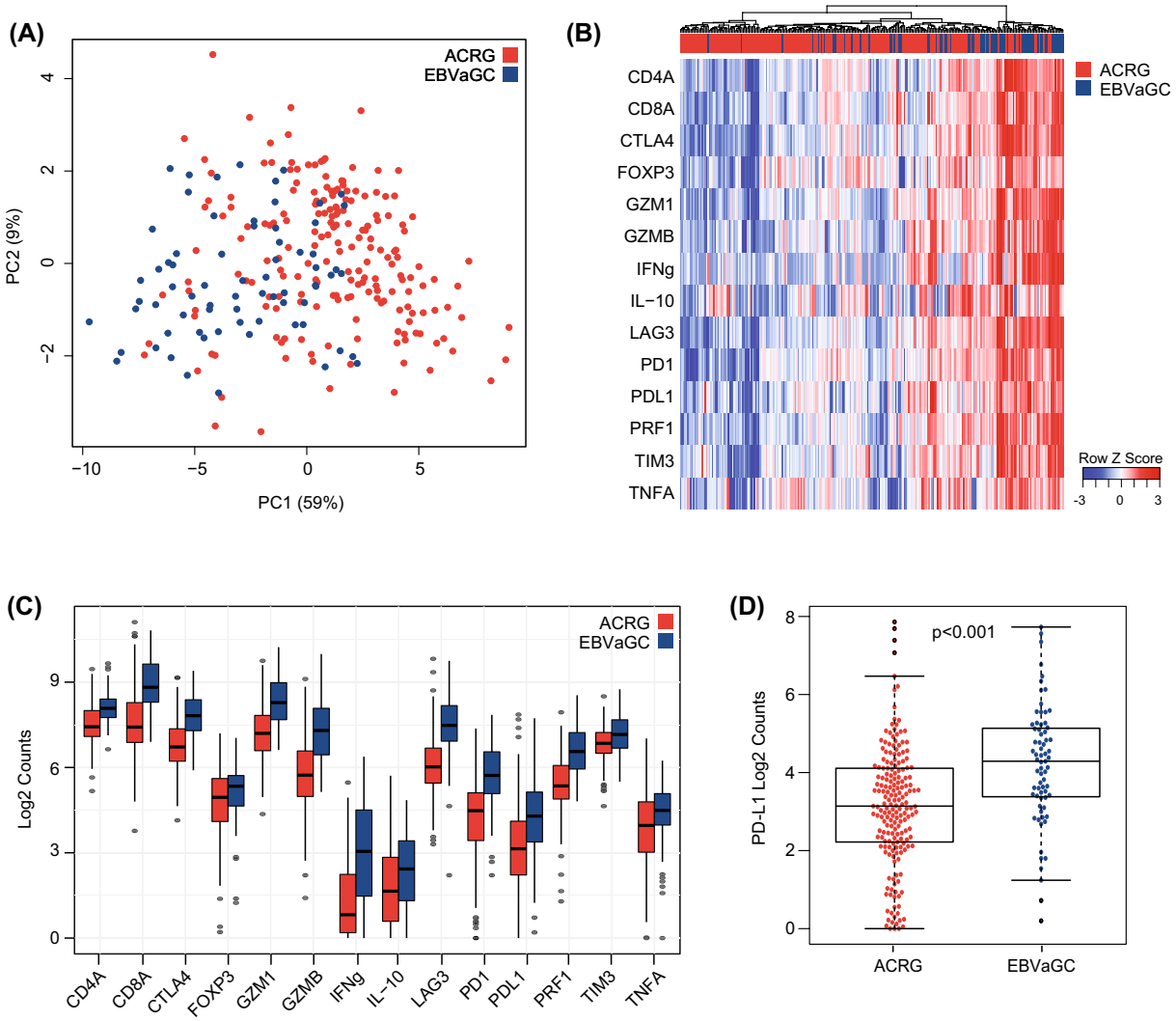

Fig. 1 Immune gene expression profiles of EBVaGC. **a** Principal component analysis of ACRG (red) and EBVaGC (blue) samples showing separation of EBV-positive samples from EBV-negative ones. **b** Unsupervised heatmap of immune genes for EBVaGC and ACRG samples. EBVaGC samples mostly cluster together and have increased immune gene expression compared to ACRG EBV-negative samples. **c** Boxplot of immune genes for EBVaGC and ACRG samples showing significantly increased expression in EBVaGC samples (p value < 0.01, Wilcoxon one-sided test). **d** Boxplot of *PD-L1* expression in EBVaGC and ACRG samples ($p < 0.001$, Wilcoxon one-sided test)

the EBVnegACRG cohort as well, where PD-L1$_{low}$ samples showed worse DFS [HR 1.44 (95% CI 0.92–2.25), $p = 0.11$, Fig. 2d].

Discussion

Here, we analyzed a cohort of EBV-positive gastric cancers, with an integrated analysis of PD-L1 IHC, TIL subtyping and transcriptomic expression of *PD-L1* and other key IRG. Through this analysis we made the following findings: While *PD-L1* expression in EBVaGC is higher than non-EBVaGC, there-in lies a spectrum, with a proportion of EBVaGC having lower transcriptomic expression of *PD-L1*. This group of *PD-L1* low expressors also have reduced expression of other IRG including immune checkpoints. This same group of *PD-L1* low expressors have a poorer prognosis compared to *PD-L1* high expressing EBVaGC. *PD-L1* transcriptomic expression correlates with PD-L1 protein expression as measured by immunohistochemistry. *PD-L1* low expressors also tend to have a different pattern of tumor-infiltrating lymphocytes.

Immune checkpoint inhibitors have recently received approval for the management of advanced gastric cancer [9, 10]. PD-L1 has emerged as one of the leading biomarkers for selecting patients who might benefit from anti-PD-(L)1

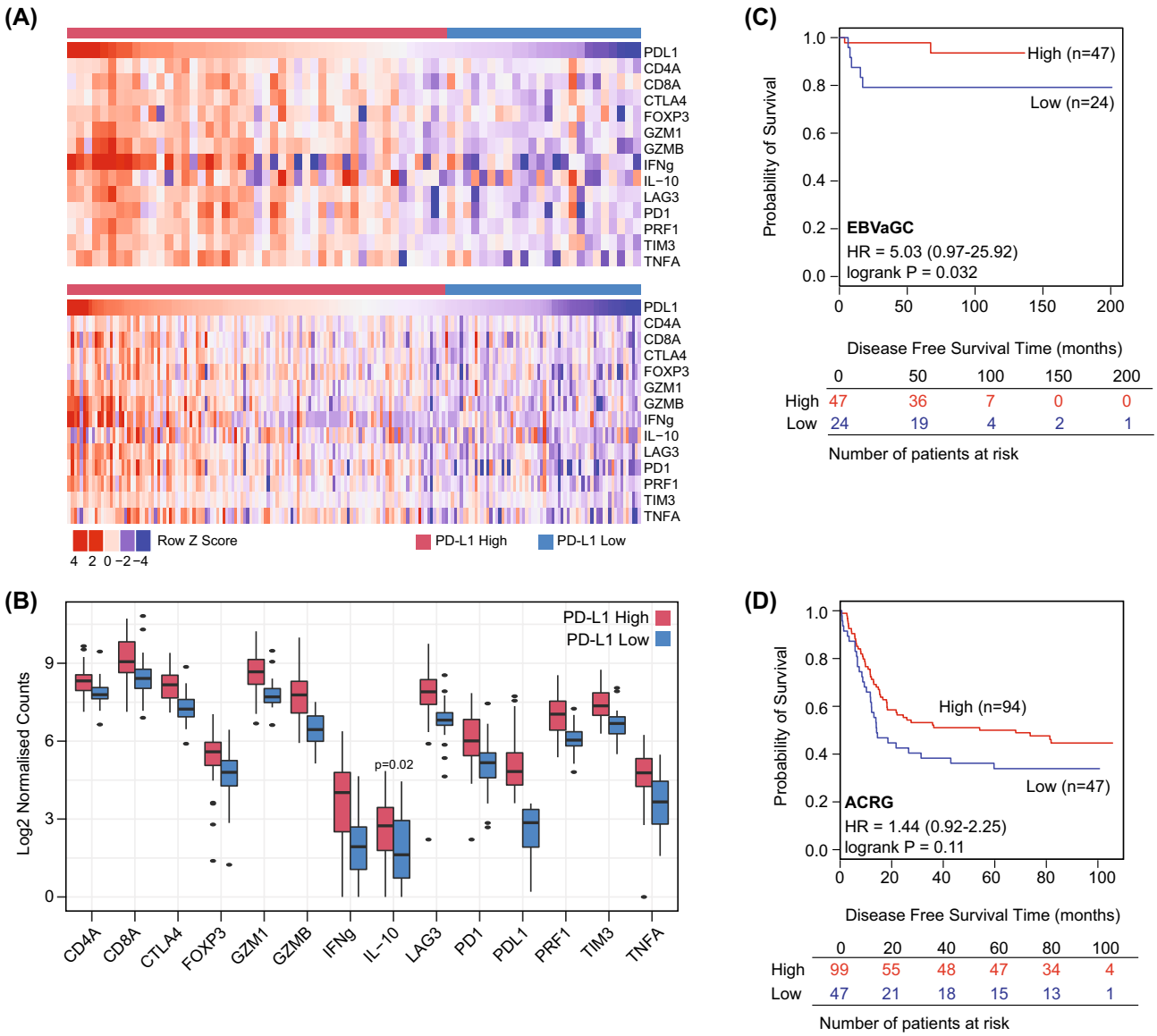

Fig. 2 Prognostic value of PD-L1 expression in EBVaGCs. **a** Heatmap of immune gene expression in EBVaGC and ACRG samples sorted by *PD-L1* expression. **b** Boxplot of immune genes grouped by PD-L1 expression [p value < 0.001 for all genes except *IL-10* ($p = 0.02$), Wilcoxon one-sided test]. Kaplan–Meier (KM) survival curves for disease-free survival of patients grouped by PD-L1 expression in **c** EBVaGC samples, **d** ACRG samples

therapy [11]. However, several discrepant results from large clinical trials using immunohistochemical expression of PD-L1 have brought into question its utility and reliability as a predictive biomarker for immune checkpoint inhibition [10, 12]. Our study has analyzed PD-L1 at the transcriptomic level and correlated this with protein expression levels (IHC). Furthermore, previous studies have used various antibodies and algorithms for assessing PD-L1 IHC [5, 6, 13]. Discordance between various PD-L1 antibodies has been described [14]. Thus, it is of major clinical and scientific relevance that our study used the FDA approved PD-L1 antibody and algorithm to study PD-L1 IHC scores in our EBVaGC cohort. Notably, in both EBV-positive and EBV-negative patients, we found higher transcriptomic expression of *PD-L1* to predict for better prognosis. Two previous studies have reported a tendency towards a poorer prognosis in EBVaGC with tumoral expression of PD-L1 with anti-PD-L1 antibody using clone E1L3N (Cell Signaling Technology, Danvers, MA) [5, 6]. However, in our previous studies on PD-L1 IHC in microsatellite instability-high and EBVaGC, distinct molecular subtypes of gastric cancer with high immune cell infiltration, PD-L1 expression was a good prognosticator using clone SP142 (Ventana, Tucson, AZ) [15, 16]. From these data, it is unlikely

Table 2 Differences in characteristics between PD-L1$_{high}$ and PD-L1$_{low}$ groups

Characteristics	PD-L1 groups		*p* value
	High	Low	
	n (%)	*n* (%)	
Age (years)			
< 55.5	22 (46.8)	13 (54.2)	0.62
≥ 55.5	25 (53.2)	11 (45.8)	
Gender			
Female	4 (8.5)	5 (20.8)	0.26
Male	43 (91.5)	19 (79.2)	
Lymph node metastasis			
Absent	30 (63.8)	15 (62.5)	1
Present	17 (36.2)	9 (37.5)	
Lymphatic invasion			
Absent	27 (57.5)	14 (58.3)	1
Present	20 (42.5)	10 (41.7)	
Perineural invasion			
Absent	37 (78.7)	14 (58.3)	0.10
Present	10 (21.3)	10 (41.7)	
T stage			
1	14 (29.8)	6 (25.0)	0.39
2	9 (19.1)	9 (37.5)	
3	18 (38.3)	6 (25.0)	
4	6 (12.8)	3 (12.5)	
N stage			
0	30 (63.8)	15 (62.5)	0.25
I	7 (14.9)	1 (4.2)	
II	8 (17.0)	4 (16.7)	
III	2 (4.3)	4 (16.7)	
Histology			
CA	2 (4.3)	6 (25.0)	0.01*
CLR	21 (44.7)	12 (50.0)	
LELC	24 (51.0)	6 (25.0)	
PD-L1 CPS score			
0	4 (8.5)	7 (29.2)	0.004*
1–5	12 (25.5)	11 (45.8)	
5–50	23 (48.9)	6 (25.0)	
> 50	8 (17.0)	0 (0)	

*Fisher test $p < 0.05$

that PD-L1 either measured by IHC or other methods, such as transcriptomic analyses would be a reliable stand-alone predictive or prognostic biomarker for gastric cancer. In view of the conflicting data of using a single gene/protein (PD-L1) as an immune-biomarker, we chose to analyze a group of important IRGs instead. Of significant note, we found that those tumors with low PD-L1 expression tended to have a similarly reduced expression of all other IRGs. In another study of 12 EBVaGC samples, expression of major histocompatibility complex class II genes and chemokine activity regulating genes were found to be deregulated more frequently [2]. These findings support the hypothesis that the better prognosis of EBVaGC is likely immune-related. In the era of immunotherapy, this is of particular significance in both EBVaGC and EBV-negative tumors, and GCs that display low levels of IRG may have to possibly be treated differently to those with high IRG expression.

In conclusion, we studied transcriptomic expression of IRG in a large cohort of EBVaGC. We have demonstrated that a substantial proportion of tumors have a low transcriptomic expression of *PD-L1* and tend to show a poorer survival outcome through low expression of other IRG. While large GCs landscaping groups and studies, such as the TCGA and ACRG have focused largely on tumoral genomic data, future studies must incorporate analyses of the interplay between the tumor and the immune system in order to better understand and treat this disease.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent to be included in the study, or the equivalent, was obtained from all patients.

References

1. The Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature 2014;513:202–9.
2. Kim SY, Park C, Kim HJ, Park J, Hwang J, Kim JI, et al. Deregulation of immune response genes in patients with Epstein–Barr virus-associated gastric cancer and outcomes. Gastroenterology. 2015;148:137 e9–47 e9.
3. Naidoo J, Page DB, Wolchok JD. Immune modulation for cancer therapy. Br J Cancer. 2014;111:2214–9.
4. Song HJ, Srivastava A, Lee J, Kim YS, Kim KM, Ki Kang W, et al. Host inflammatory response predicts survival of patients with Epstein–Barr virus-associated gastric carcinoma. Gastroenterology. 2010;139:84 e2–92 e2.
5. Saito R, Abe H, Kunita A, Yamashita H, Seto Y, Fukayama M. Overexpression and gene amplification of PD-L1 in cancer cells and PD-L1(+) immune cells in Epstein–Barr

virus-associated gastric cancer: the prognostic implications. Mod Pathol. 2017;30:427–39.
6. Seo AN, Kang BW, Kwon OK, Park KB, Lee SS, Chung HY, et al. Intratumoural PD-L1 expression is associated with worse survival of patients with Epstein–Barr virus-associated gastric cancer. Br J Cancer. 2017;117:1753–60.
7. Cristescu R, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, et al. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. Nat Med. 2015;21:449–56.
8. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell. 2015;160:48–61.
9. Kang YK, Boku N, Satoh T, Ryu MH, Chao Y, Kato K, et al. Nivolumab in patients with advanced gastric or gastro-oesophageal junction cancer refractory to, or intolerant of, at least two previous chemotherapy regimens (ONO-4538-12, ATTRACTION-2): a randomised, double-blind, placebo-controlled, phase 3 trial. Lancet. 2017;390:2461–71.
10. Muro K, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, et al. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. Lancet Oncol. 2016;17:717–26.
11. Brody R, Zhang Y, Ballas M, Siddiqui MK, Gupta P, Barker C, et al. PD-L1 expression in advanced NSCLC: insights into risk stratification and treatment selection from a systematic literature review. Lung Cancer (Amsterdam, Netherlands). 2017;112:200–15.
12. Lim JS, Sundar R, Chénard-Poirier M, Lopez J, Yap TA. Emerging biomarkers for PD-1 pathway cancer therapy. Biomark Med. 2017;11(1):53–67.
13. Derks S, Liao X, Chiaravalli AM, Xu X, Camargo MC, Solcia E, et al. Abundant PD-L1 expression in Epstein–Barr Virus-infected gastric cancers. Oncotarget. 2016;7:32925–32.
14. McLaughlin J, Han G, Schalper KA, Carvajal-Hausdorf D, Pelekanou V, Rehman J, et al. Quantitative assessment of the heterogeneity of PD-L1 expression in non-small-cell lung cancer. JAMA Oncol. 2016;2:46–54.
15. Cho J, Lee J, Bang H, Kim ST, Park SH, An JY, et al. Programmed cell death-ligand 1 expression predicts survival in patients with gastric carcinoma with microsatellite instability. Oncotarget. 2017;8:13320–8.
16. Park C, Cho J, Lee J, Kang SY, An JY, Choi MG, et al. Host immune response index in gastric cancer identified by comprehensive analyses of tumor immunity. Oncoimmunology. 2017;6:e1356150.

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