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RESEARCH ARTICLE

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Clinicopathological and prognostic features of Epstein-Barr virus infection, microsatellite instability, and PD-L1 expression in gastric cancer

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Background and Objectives: Gastric cancer (GC) has recently been categorized in molecular subtypes, which include Epstein-Barr (EBV)-positive and microsatellite instability (MSI) tumors. This distinction may provide prognostic information and identifies therapeutic targets. The aim of this study was to evaluate EBV, MSI, and PD-L1 immunoexpression in GC and its relationship with clinicopathological characteristics and patient's prognosis.

Methods: We evaluated 287 GC patients who underwent D2-gastrectomy through immunohistochemistry for DNA mismatch repair proteins and PD-L1, and in situ hybridization for EBV detection utilizing tissue microarray.

Results: EBV-positive and MSI were identified in 10.5% and 27% of the GCs, respectively. EBV positivity was associated to male gender ($P = 0.032$), proximal location ($P < 0.001$), undetermined Lauren type ($P < 0.001$), poorly differentiated histology ($P = 0.043$) and severe inflammatory infiltrate ($P < 0.001$). MSI-tumors were associated to older age ($P = 0.002$), subtotal gastrectomy ($P = 0.004$), pN0 ($P = 0.024$) and earlier TNM stage ($P = 0.020$). PD-L1-positive was seen in 8.8% of cases, with predominant expression in EBV-positive GC ($P < 0.001$). MSI was associated to better survival outcomes.

Conclusion: EBV-positive GCs had increased PD-L1 expression, while MSI GC had better survival outcome. EBV and MSI subgroups are distinct GC entities, their recognition is feasible by conventional techniques, and it may help individualize follow-up and guide adjuvant therapy.

KEYWORDS
Epstein-Barr virus infection, microsatellite instability, molecular targeted therapy, programmed death-ligand 1, stomach neoplasms

1 | INTRODUCTION

Gastric cancer (GC) is the 4th most common cancer and the 2nd leading cause of cancer-related death worldwide.[1] In the current clinical practice, histological features including Lauren histological type and TNM stage system are used to predict prognosis and survival.[2] However, these parameters are insufficient to predict disease progression in an individual basis. Also, they do not reflect the heterogeneity of gastric tumors, or even assist in the development of targeted therapies. Thus, identifying subtypes of GC according to molecular characteristics is one of the current goals to better understand its heterogeneity and improve clinical outcome.[3]

Based on gene expression profile studies, GC was categorized into four molecular subtypes: Epstein-Barr virus (EBV)-positive tumors; microsatellite instability (MSI) tumors; genomically stable (GS) tumors; and chromosomal-instable (CIN) tumors.[4] In addition to defining subgroups biologically more homogeneous, the molecular classification has potential prognostic and therapeutic implications, since it may identify possible biomarkers and therapeutic targets of each subtype, particularly through the stratification according to EBV and MSI status.[5,6]

Currently, programmed death-ligand 1 (PD-L1) expression in tumor cells has been validated as a predictive marker for tumor response to anti-PD-1 or PD-L1 immunotherapy in different malignancies, including GC. According to The Cancer Genome Atlas (TCGA) study and recent trials, EBV and MSI GC subgroups may benefit from therapy with PD-1/PD-L1 antibodies.[4,7,8] However, the frequency and prognostic value of PD-L1 expression in GC remain controversial.

Although these studies represent a significant progress in the definition of GC from a biological perspective,[9] the spread of this classification is still limited by the lack of clinical scope. Also, the genomic technology for a complete molecular classification is not currently cost effective, preventing the widespread use of this diagnostic strategy into clinical practice.

Thus, the aim of this study was to investigate the EBV infection by in situ hybridization (ISH), and MSI status and PD-L1 expression using immunohistochemistry (IHC) in a western cohort of surgically treated GC patients. Additionally, we analyzed the clinicopathological and prognostic factors associated with IHC profiles.

2 | MATERIALS AND METHODS

2.1 | Patients

We reviewed all gastric adenocarcinoma patients who underwent potentially curative gastrectomy with lymphadenectomy at Instituto do Cancer do Estado de Sao Paulo—Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo (ICESP-HCFMUSP) between 2009 and 2016 from a prospective collected medical database. Inclusion criteria were: (1) gastric adenocarcinoma; (2) D2 lymphadenectomy; (3) R0 resection; and (4) formalin-fixed paraffin-embedded tissue (FFPE) blocks available for analysis. Gastric stump tumors, palliative resections and metastatic patients were not included.

All patients underwent total or subtotal gastrectomy with D2 lymph node (LN) dissection based on the guidelines of the Japanese Gastric Cancer Association.[10] Surgical specimens were fixed in 10% buffered formalin or Carnoýs Solution[11,12] and were evaluated according to the College of American Pathologists protocol.[13] IHC with cytokeratin were performed in some cases to detect lymph node micrometastases.[14] Tumor stage was defined according to the 7th edition of the TNM as proposed by the International Union Against Cancer (UICC).[2]

Clinical outcomes were overall survival (OS) and disease-free survival (DFS). Postoperative follow-up was performed on a quarterly basis in the first year and every 6 months in the following years. Clinical physical examinations and hematological tests were performed in all outpatients' visits. Imaging and upper gastrointestinal endoscopy were selectively performed. Absence in medical appointment for more than 12 months was considered as loss of follow-up.

The study was approved by the hospital ethics committee (NP771/2015) and is registered in the "Plataforma Brasil" (a National Research Council) under the CAAE number 43453515.6.0000.0065. Informed consent of patients was waived because of the retrospective nature.

2.2 | Tissue microarray

All hematoxylin and eosin (HE)-stained slides were reviewed and representative tumor tissue samples were selected for each case. For tissue microarray (TMA) construction, the corresponding areas were punched out from individual FFPE tumor blocks and arrayed in a precision mechanized system (Beecher Instruments, Silver Springs, MD). A total of nine TMAs were constructed with three tumor and two adjacent non-tumoral mucosa tissue cores (2 mm in diameter) from each patient. TMA blocks were sectioned at 4 μm thick and histological sections were submitted to HE staining, IHC, and ISH.

2.3 | Immunohistochemistry

The iHC reactions to determine the MSI status were performed with Ventana BenchMark ULTRA automated staining system according to the manufacturer's instructions using monoclonal antibodies against the four mismatch repair (MMR) proteins: anti-MLH1 (clone M1–Ventana, Ref. 790–4535), anti-MSH2 (clone G219-1129–Cellmarque, Ref. 760–4265), anti-MSH6 (clone 44–Ventana, Ref. 790–4455), anti-PMS2 (clone EPR 3947–Cellmarque, Ref. 760–453)—all ready to use.

Briefly, for PD-L1 staining paraffin sections were de-paraffinized in xylene and rehydrated through graded ethanol. After blocking peroxidase activity with hydrogen peroxide and antigen retrieval by heat induced using citrate buffer (pH 9.0), sections were incubated at 4°C with the primary rabbit monoclonal anti-PD-L1 antibody (1:50, clone 28–8, Abcam, Cambridge, MA). Avidin-biotin free short polymer-based peroxidase amplification system (Novolink, Novocastra, UK) and diaminobenzydine (DAB) (Sigma, D-5637, EUA) as chromogen were

used for development of reaction products. After the reactions, all sections were counterstained with hematoxylin.

2.4 | Immunohistochemical evaluation

IHC reactions for MMR were qualitatively interpreted as positive, negative, or inconclusive, according to the deposition of the chromogen product on the nuclei of the cancer cells. Tumors were considered negative for MLH1, MSH2, PMS2, or MSH6 expression only if there was a complete absence of nuclear staining in the TCs. GCs lacking MLH1, MSH2, PMS2, or MSH6 expression were considered microsatellite instability (MSI status) or MMR deficient, whereas GCs that maintained expression of all markers were considered as microsatellite stable (MSS status) or MMR proficient. Lymphocytes and normal epithelium served as positive internal controls.

For PD-L1 analysis, specimens were scored on the basis of the percentage of stained tumor cells (TCs) and tumor infiltrating immune cells (TIICs). PD-L1-positive cases were defined by the presence of at least 1% of TCs or TIICs with membrane staining, regardless of the intensity. The highest score was selected if two or three cores from the same case exhibited different PD-L1 expression scores. Melanoma metastasis samples previously recognized as positive were used as positive controls. Tris-buffered saline was used instead of primary antibody for negative controls.

IHC analysis was carried out by two pathologists (ESM and SFF) in a blinded manner. If there was difference between these two observers, these slides were reanalyzed by both investigators using a multiheaded microscope.

2.5 | EBV in situ hybridization

EBV status was determined by ISH using probes against Epstein-Barr encoded RNA 1 (EBER1) (Y5200, DAKO, Carpinteria, CA) by avidin-biotin peroxidase method. Known EBV-positive pharyngeal tumor specimens were used as positive controls, and slides treated without the probe were used as negative controls. Samples with dark-blue staining in tumor cell nuclei were considered positive.

2.6 | Statistical analysis

Descriptive statistics included frequencies with percent for nominal variables and mean with ± standard deviation (SD) or median ± interquartile range (IQR) for continuous variables. The Chi-square tests were used for categorical data and t-test for continuous data to evaluate the differences between the variables. Disease-free survival (DFS) and overall survival (OS) were estimated using the Kaplan-Meier method, and differences in survival were examined using the Log Rank Test. To determine factors associated with DFS and OS, hazard ratios (HR) with 95% confidence intervals (95%CI) were calculated by univariate and multivariate Cox proportional hazard regression models. Variables that were significant on univariate analysis were included as co-variables a multivariate Cox regression to determine which variables independently affected prognosis.

DFS was calculated from the date of surgery to the date of relapse. OS was defined as the time between surgery and death of any cause or last follow-up. For the better comparative study of recurrence, postoperative mortality (defined as death within 30 days after surgery or until hospital discharge) was excluded from the DFS analysis. Statistical software package SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL) was used for analyses. All CIs were stated at the 95% confidence level and all P values were two-sided. Statistical significance was defined as $P < 0.05$.

3 | RESULTS

3.1 | Patient characteristics

A total of 287 patients meet the inclusion criteria. Of these, 58.5% were male and mean age was 61.5 years-old (SD ±12.1, range 26-87). According to the American Society of Anesthesiologists (ASA) classification, 256 (82.9%) patients were ASA I/II and 31 (10.8%) ASA III/IV. Subtotal gastrectomy was performed in 60.6% of cases, and 55.7% of patients had poorly differentiated adenocarcinoma. The mean number of LNs retrieved was 41.8 (SD ± 17.7), and 56.4% of patients had LN metastasis. TNM stage was I/II in 158 cases (55.1%) and III/IV in 129 cases (44.9%). Neoadjuvant and adjuvant therapy were administered in 34 (11.8%) and 158 (55%) patients, respectively. Overall 30-day mortality was 4.2% (12 patients).

In situ hybridization or IHC analysis could not be performed for EBV, MSI, and PD-L1 in 1, 65, and 2 patients, respectively.

3.2 | Clinicopathologic characteristics of GC according to EBV status

Of 286 patients who were assessable by ISH, 30 (10.5%) patients had GC positive for EBV infection. All EBV-positive cases showed intense nuclear staining, with positivity observed in all tumor cells (Figure 1A). None of the cases were positive for the EBER/ISH reaction in the non-tumoral mucosa. Univariate analysis revealed that male gender ($P = 0.032$), proximal location ($P < 0.001$), total gastrectomy ($P < 0.001$), poorly differentiated histologic type ($P = 0.043$), undetermined type according to the Lauren classification ($P < 0.001$) and moderate/severe inflammatory infiltrate ($P < 0.001$) were associated with EBV-positive GC (Table 1).

3.3 | Clinicopathologic characteristics according to MMR deficiency

Among 222 studied GC, 60 (27%) had MSI. Isolated loss of MLH1, MSH2 and PMS2 expression occurred in 8, 1, and 13 cases, respectively (Figure 1B). One case showed simultaneous loss of expression of MSH2 and MSH6 and one of MLH1, MSH2, and PMS2. Most MSI cases had simultaneous loss of MLH1 and PMS2 (60%, 36 of 60 patients). The remaining 162 (73%) patients were classified as MMS, as they had normal expression of the four MMR proteins.

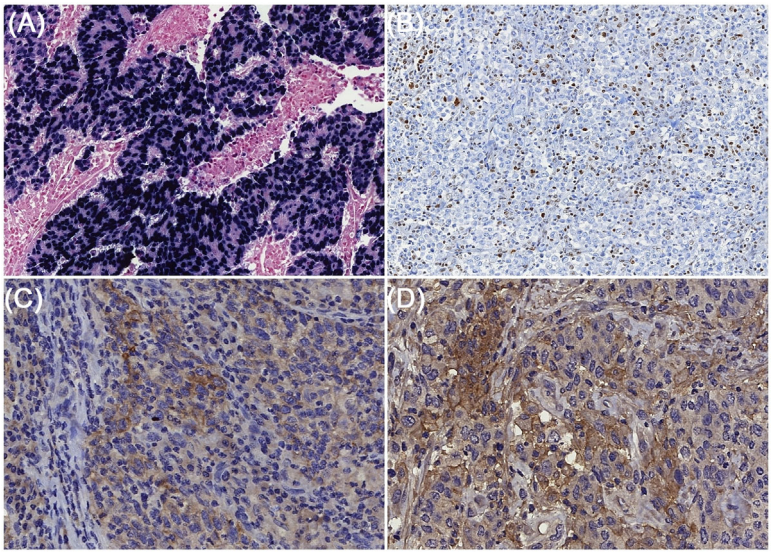

FIGURE 1 Representative images of in situ hybridization and immunohistochemical results. A, EBV-positive case by in situ hybridization; B, MSI gastric cancer, with loss of MLH-1 expression; and PD-L1-positive case, with (C) PD-L1 expression in tumor cells and (D) PD-L1 expression in tumor and immune cells of inflammatory infiltrate

The clinicopathologic characteristics according to MSI status are showed in Table 1. Older age ($P = 0.002$), subtotal resection ($P = 0.004$), absence of LN metastasis ($P = 0.016$), and lower TNM stage ($P = 0.018$) were related to MMR deficiency. Although the presence of moderate/severe inflammatory infiltrate was higher in MSI tumors than in MSS (40% vs 27.2%), this difference did not reach statistical significance ($P = 0.065$).

3.4 | Clinicopathologic characteristics according to PD-L1 expression

Among 285 cases, 25 cases (8.8%) were positive for PD-L1. PD-L1 expression was observed in tumor and/or immune cells, but not in non-neoplastic gastric epithelium (Figures 1C and 1D). According to the expression pattern, 18 of 25 cases (72%) showed a PD-L1 expression in TCs and 14 (56%) in TIICs. Simultaneous expression of PD-L1 (TCs and TIICs) was observed in seven cases. The percentage of stained TCs ranged from 1% to 80% (mean of 20.4%, SD ± 26.1), while stained TIICs ranged from 1% to 30% (mean of 13.7%, SD ± 11.0). Most of cases with PD-L1 positive expression showed a weak/moderate staining intensity. Only a few cases showed strong staining intensity in tumor/immune cells, in general these cases corresponded mainly to GC with solid-type histological features, than other histological subtypes.

PD-L1 expression was associated to macroscopic type I/II ($P = 0.002$) and undetermined Lauren classification ($P < 0.001$). No association was found between the PD-L1 immunoexpression and pN status ($P = 0.507$) or TNM stage ($P = 0.206$) (Table 2).

Considering the expression pattern of PD-L1 separately, PD-L1 TCs expression was more frequently observed in macroscopic type I/II ($P = 0.045$), undetermined Lauren classification ($P < 0.001$), poorly differentiated adenocarcinoma ($P = 0.015$), and moderate to severe lymphoid stroma ($P = 0.010$). Similar, PD-L1 expression in TIICs correlated significantly with macroscopic type I/II ($P = 0.017$) and undetermined classification ($P = 0.002$). There were no significant differences between the others clinicopathological characteristics and PD-L1 expression in TC or TIIC.

Concerning IHC status, PD-L1 positivity was frequent in EBV-positive GC, presenting more intense and extensive immunostaining in these tumors. Among PD-L1-positive cases, 52% were EBV-positive, while in PD-L1 negative group only 6.5% had EBV-positivity ($P < 0.001$). A significantly higher incidence of EBV-positive cases were also observed when considering only PD-L1-positive TCs (66.7% vs 6.7%, $P < 0.001$) and PD-L1-positive TIICs (42.9% vs 8.9%, $P < 0.001$).

By contrast, the presence of MSI was similar between PD-L1-positive (26.1%) and PD-L1-negative (27.1%) GCs ($P = 0.915$). The same was observed regarding MSI-status when evaluated only TCs (31.2% for PD-L1-positive and 26.7% for PD-L1-negative, $P = 0.771$) and TIICs (21.4% for PD-L1-positive and 27.4% for PD-L1-negative, $P = 0.764$).

The MSI-status and EBV-positive GCs were mutually exclusive, except in two cases, which also showed positivity for PD-L1

3.5 | Prognosis and survival analysis

The median follow-up was 29 months (IQR 16-44 months). At the time of this study, 73 patients had disease recurrence (29 locoregional and 44 distant) and 84 patients died. The median follow-up was 36 months (mean of 38.3 months, SD ± 20.4) for surviving patients. The 5-year DFS and 5-year OS rate for the entire cohort was 73.5% and 70.7%, respectively.

According to the IHC results, GCs were divided in three different groups. Among the 222 cases available for the two status (EBV and MSI), 19 cases (9%) were classified as EBV-positive group, 58 cases

TABLE 1 Clinicopathological characteristics of gastric cancer patients according to Epstein-Barr virus infection and microsatellite instability status

	EBV infection			MMR expression		
Variables	**EBV-negative *n* = 256 (%)**	**EBV-positive *n* = 30 (%)**	***P***	**MSS *n* = 162 (73%)**	**MSI *n* = 60 (27%)**	***P***
Age (years)			0.973			**0.002**
Mean (±SD)	61.5 (±12.2)	61.4 (±11.2)		60.1 (±11.5)	65.8 (±12.5)	
Sex			**0.032**			0.236
Male	144 (56.2)	23 (76.7)		98 (60.5)	31 (51.7)	
Female	112 (43.8)	7 (23.3)		64 (39.5)	29 (48.3)	
Tumor site			**<0.001**			0.217
Upper	26 (10.2)	8 (26.7)		18 (11.1)	6 (10)	
Middle	45 (17.6)	7 (23.3)		33 (20.4)	9 (15)	
Lower	179 (69.9)	11 (36.7)		103 (63.6)	45 (75)	
Others	6 (2.3)	4 (13.3)		8 (4.9)	0 (0)	
Type of resection			**<0.001**			**0.004**
Subtotal	165 (65.5)	8 (26.7)		90 (55.6)	46 (76.7)	
Total	91 (35.5)	22 (73.3)		72 (44.4)	14 (23.3)	
Tumor size			0.075			0.118
Mean (±SD)	4.8 (3.0)	6.4 (±4.4)		4.8 (±3.3)	5.5 (±3.2)	
Grade of histological differentiation			**0.043**			0.531
Well/moderately	118 (46.1)	8 (26.7)		68 (42)	28 (46.7)	
Poorly	138 (53.9)	22 (73.3)		94 (58)	32 (53.3)	
Lauren type			**<0.001**			0.087
Intestinal	125 (48.8)	11 (36.7)		72 (44.4)	34 (56.7)	
Diffuse	101 (39.5)	8 (26.7)		68 (42)	15 (25)	
Mixed	24 (9.4)	4 (13.3)		12 (7.4)	8 (13.3)	
Undetermined	6 (2.3)	7 (23.3)		10 (6.2)	3 (5)	
Tumor invasion			0.440			0.127
pT1/pT2	104 (40.6)	10 (33.3)		60 (37)	29 (48.3)	
pT3/pT4	152 (59.4)	20 (66.7)		102 (63)	31 (51.7)	
pN status			0.699			**0.016**
pN negative	110 (43)	14 (46.7)		60 (37)	33 (55)	
pN positive	146 (57)	16 (53.3)		102 (63)	27 (45)	
Lymphatic invasion			0.783			0.104
Absent	125 (49)	15 (51.7)		81 (50.6)	23 (38.3)	
Present	130 (51)	14 (48.3)		79 (49.4)	37 (61.7)	
Venous invasion[a]			0.506			0.439
Absent	169 (66.3)	21 (72.4)		105 (65.6)	36 (60)	
Present	86 (33.7)	8 (27.6)		55 (34.4)	24 (40)	
Perineural invasion[a]			0.453			0.216
Absent	132 (52.2)	13 (44.8)		80 (50.6)	36 (60)	
Present	121 (47.8)	16 (55.2)		78 (49.4)	24 (40)	
Peritumoral inflammatory infiltrate			**<0.001**			0.065
Absent/weak	182 (71.1)	8 (26.7)		118 (72.8)	36 (60)	
Moderate/severe	74 (28.9)	22 (73.3)		44 (27.2)	24 (40)	
pTNM stage			0.553			**0.018**

(Continues)

TABLE 1 (Continued)

Variables	EBV infection			MMR expression		
	EBV-negative *n* = 256 (%)	EBV-positive *n* = 30 (%)	*P*	MSS *n* = 162 (73%)	MSI *n* = 60 (27%)	*P*
I/II	139 (54.3)	18 (60)		82 (50.6)	41 (68.3)	
III/IV	117 (45.7)	12 (40)		80 (49.4)	19 (31.7)	

EBV, Epstein-Barr virus; MMR, mismatch repair; MSS, microsatellite stable; MSI, microsatellite instability; SD, standard deviation.
The *P* values in bold are statistically significant.
[a]Some cases were not assessed for this status due to absence of the respective information in the original histopathological report.

MSI group (27%) and 143 cases (64%) EBV-negative/MSS group. Two of the patients presenting MSI and positivity for EBV infection were not classified in any of the groups.

Using EBV-negative/MSS group as reference, MSI GCs patients showed significant better survival, with 3-year DFS rate of 86.2% ($P = 0.006$) and 5-year OS of 77.6% ($P = 0.049$). Three-year DFS and 5-year OS rates were similar between EBV-positive GC and reference group ($P = 0.598$ and $P = 0.118$, respectively) (Figure 2).

Also, when evaluating survival considering only EBV status, there was no significant difference in 3-year DFS rate between EBV-positive and EBV-negative patients (70% vs 73.8%, respectively, $P = 0.610$) and in 5-year OS rate (76.7% vs 69.9%, respectively, $P = 0.432$). Likewise, considering only the instability profile, MSI patients had longer 3-year DFS rate compared to MSS patients (85% vs 68.8%, $P = 0.011$) and 5-year OS was superior in the MSI group but this did not achieve statistical significance (76.7% vs 69.1%, $P = 0.116$).

Regarding PD-L1 expression, survival rates were similar for PD-L1-positive and PD-L1-negative patients: 3-year DFS of 73.9% and 73.2% ($P = 0.974$), respectively; and 5-year OS of 72% and 70.4% ($P = 0.908$), respectively.

Considering the different expression patterns, no difference in 5-year OS was observed regarding PD-L1 expression in TCs (63.6%, $P = 0.717$), TIICs (57.1%, $P = 0.182$), or both (100%, $P = 0.164$) when compared to the PD-L1-negative (70.5%) (determined as reference group). Similarly, no differences were observed in 3-year DFS with respect to PD-L1 expression in TCs (63.6%, $P = 0.364$), TIICs (80%, $P = 0.722$) or both (85.7%, $P = 0.500$) when compared to the PD-L1-negative (73.3%) (reference group). When adjusted for the presence of LN metastasis, 3-year DFS was significantly worse for PDL1-positive TCs pN+ group compared to the reference group (PDL1-negative TCs with pN+) (20% vs 60.8%, $P = 0.001$).

3.6 | Analysis of prognostic factors

Univariate and multivariate analysis were performed to evaluate the prognostic factors affecting 3-year DFS and 5-year OS (Table 3).

Multivariate analysis identified pT3/pT4 and LNM as independent factors associated with significantly lower 3-year DFS. Furthermore, in the cox regression multivariate analysis the presence of MSI was significantly associated with longer 3-year DFS in GC patients, while EBV and PD-L1 expression were not significant prognostic factors for recurrence.

Regarding OS, multivariate analysis revealed that total gastrectomy and pT3/pT4 status were associated to worse 5-year OS. There was no significant association between EBV, MSI, and PD-L1 expression and patient survival.

4 | DISCUSSION

In this study we investigated the GC subtypes EBV-positive and MSI, as well as the PD-L1 immunoexpression in surgically resected specimens of GC using techniques available in the routine histological diagnosis. The results demonstrated that IHC and ISH methodologies can identify GC subtypes comparable to the molecular classification.[4] PD-L1 expression was significantly associated to EBV-positive GC, but not with MSI GCs. Furthermore, MSI status was found to be an independent factor associated to improved prognosis and survival.

Recent data suggests that GC is a complex disease comprised of four main molecular subtypes. The addition of molecular classification is a promising strategy in the clinical management of patients with GC, allowing not only better understanding of the disease and more precise prognosis, but also opening the possibility to develop and perform genome-guided personalized therapy, especially with regard to EBV and MSI status.[15]

In this research, 10.5% of patients were EBV-positive and 27% had MSI, which is comparable to the rates seen in TCGA, (9% and 22% of GCs, respectively).[4] The simultaneous loss of MLH1 and PMS2 expression was the most common type of instability (60% in our study), as also described by Kim et al[16] in 71.4% of their MSI GC cases.

In particular, EBV-positive and MSI tumors have been reported as distinct clinicopathological entities. Similar to literature data, EBV-positive GC was predominant in male patients, in lesions of the proximal stomach, and poorly differentiated histologic type with prominent inflammatory infiltrate.[17,18] Although some authors reported that both EBV and MSI GC usually are less aggressive, with lower depth of invasion in the gastric wall and infrequent LN metastasis,[19–22] only MSI CG was associated with pN0 status and lower TNM stage. Additionally, as expected MSI GC occurred more frequently in older patients than MSS GC, since epigenetic changes including methylation intensifies as age increases.[23]

Recent studies have suggested that EBV-positive and MSI GC may be prime candidates for immunotherapeutic agents targeting T-cell immune checkpoints, particularly PD-1/PD-L1 directed therapy.[4,7,24]

TABLE 2 Clinicopathological characteristics of gastric cancer patients according to programmed death ligand 1 (PD-L1) expression

Variables	PD-L1-negative *n* = 260 (%)	PD-L1-positive *n* = 25 (%)	*P*
Age (years)			0.502
Mean (±SD)	61.4 (±12.0)	63.1 (±12.6)	
Sex			0.318
Male	150 (57.7)	17 (68)	
Female	110 (42.3)	8 (32)	
Tumor site			0.981
Upper	31 (11.9)	3 (12)	
Middle	48 (18.5)	4 (16)	
Lower	172 (66.2)	17 (68)	
Others	9 (3.5)	1 (4)	
Type of resection			0.641
Subtotal	158 (60.8)	14 (56)	
Total	102 (39.2)	11 (44)	
Macroscopic type[a]			**0.002**
I/II	57 (23.8)	12 (54.5)	
III/IV	183 (76.2)	10 (45.5)	
Tumor size			0.056
Mean (±SD)	4.8 (±3.1)	6.4 (±4.0)	
Grade of histological differentiation			0.880
Well/moderately	119 (45.8)	7 (28)	
Poorly	141 (54.2)	18 (72)	
Lauren type			**<0.001**
Intestinal	128 (49.2)	8 (32)	
Diffuse	103 (39.6)	5 (20)	
Mixed	24 (9.2)	4 (16)	
Undetermined	5 (1.9)	8 (32)	
Tumor invasion			0.696
pT1/2	104 (40)	9 (36)	
pT3/4	156 (60)	16 (64)	
pN status			0.175
pN negative	109 (41.9)	14 (56)	
pN positive	151 (58.1)	11 (44)	
Lymphatic invasion[a]			0.928
Absent	127 (49)	12 (50)	
Present	132 (51)	12 (50)	
Venous invasion[a]			0.170
Absent	176 (68)	13 (54.2)	
Present	83 (32)	11 (45.8)	
Perineural invasion[a]			0.249
Absent	129 (50.2)	15 (62.5)	
Present	128 (49.8)	9 (37.5)	
Peritumoral inflammatory infiltrate			0.103
Absent/weak	177 (68.1)	13 (52)	
Mod/severe	83 (31.9)	12 (48)	
pTNM stage			0.163
I/II	139 (53.5)	17 (68)	
III/IV	121 (46.5)	8 (32)	

SD, standard deviation.
The *P* values in bold are statistically significant.
[a]Some cases were not assessed for this status due to absence of the respective information in the original histopathological report.

PD-L1 is a transmembrane surface glycoprotein encoded by the CD274 gene expressed by several cell types of the immune system (as lymphocytes and dendritic cells), but can also be expressed in tumor cells. Although PD-L1 has been reported in several solid tumors, only few studies investigated its expression in GC.[25] A meta-analysis showed that PD-L1 positivity in GC ranges from 14.3-69.4%.[26] In this patient cohort only 8.8% of patients had PD-L1 expression on either TCs or TIICs, this is much lower than the 40% rate of PD-L1 overexpression seen in the phase I trial of pembrolizumab for advanced GC.[7]

The binding of PD-L1 to its receptor PD-1 (expressed on activated T cells) can induce the suppression of T-cell receptor signaling resulting in down regulation of the immune response, which enables tumor cells to escape immune destruction. Accordingly, therapy with anti-PD-L1/PD-1 may prevent this interaction, restoring cancer cell-directed immune response.[27]

In general, PD-L1 is expressed on TCs by innate immune resistance and adaptive immune resistance. In the innate immune resistance, constitutive oncogenic signaling induces PD-L1 expression, whereas in cases of adaptive immune resistance, the PD-L1 upregulation is induced by tumor infiltrating T-cells.[27] Our study demonstrated that GC with PD-L1-positive TCs were associated with an intense inflammatory infiltrate (61.1% of cases), which may suggest that the adaptive immune resistance contributes more to PD-L1 expression in GC than innate immune resistance.

Several researchers have demonstrated that PD-L1 expression in GC was significantly higher in EBV and MMR-deficient tumors.[4,5,28] A recent study showed that PD-L1 positivity was more frequent in MSI than MSS GCs on both TCs (72.0% vs 20.1%, $P < 0.001$) and TIICs (96.0% vs 59.5%, $P < 0.001$). Similarly, PD-L1 positivity on TCs and TIICs was more frequent in EBV-positive than in EBV-negative GC (52.0% vs 21.2%, $P = 0.001$; and 80.0% vs 60.4%, $P < 0.001$; respectively).[5]

This study also observed that PD-L1 expression was enriched in EBV-positive GC (52% of PD-L1-positive cases had EBV infection, while only 6.5% in PD-L1-negative group had EBV-positive). However, no differences were observed in PD-L1 expression between MSI and MSS GCs. Thus, our findings suggest that anti-PD-1/PD-L1 antibodies may have more therapeutic efficacy in EBV-positive GC. It is worth mentioning that EBV status was not collected in phase 1b

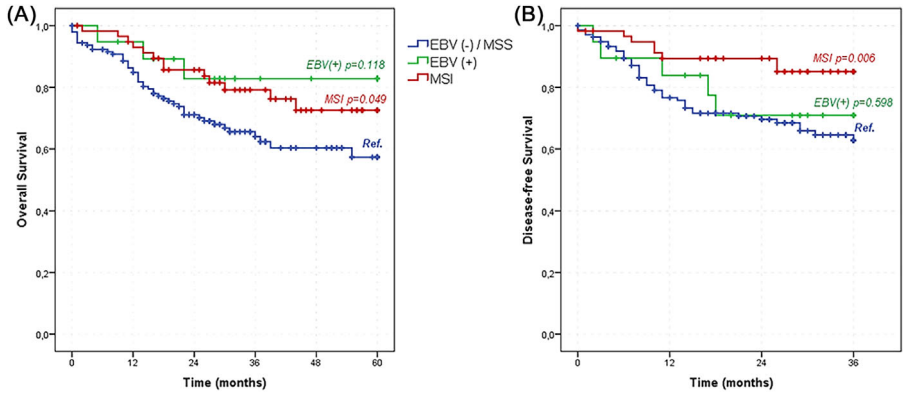

FIGURE 2 Kaplan-Meier survival curve analysis. A, Overall survival and (B) disease-free survival of GC patients according to the IHC profile. *EBV-negative and microsatellite stable (MSS) cases were considered as reference group

KEYNOTE-012 study, so we have no evidence if there is an association between EBV-positivity and outcome in the context of pembrolizumab therapy.[7] In contrast, pembrolizumab has shown promising efficacy in for patients with MSI.[24]

Some characteristics have been described to support the conjecture that EBV and MSI tumors are subtypes that would potentially benefit from anti-PD-1/PD-L1 therapy. TCGA study demonstrated that the interferon (IFN)-γ gene, an additional marker of sensitivity to PD-1 therapy, was enriched in EBV-positive and MSI GCs.[4] IFN-γ plays an important role in the adaptive immune response against EBV infection and MSI immunogenic peptides, and its release by T-cells can directly induce PD-L1 expression.[29] Furthermore, PD-1/PD-L1 inhibition appears to be more effective in tumors with preexisting antitumor response—especially CD8 T-cells. As widely known, EBV and MSI GC are often associated with a rich lymphocytic infiltration in tumor stroma, which has a high number of CD8 T cells able to produce a marked antitumor inflammatory response.[30–32]

One of the main highlights of molecular classification refers to the best prognostic definition based on the division of subgroups. Both EBV and MSI tumors have been related to better survival.[4,22,33] Host immune response to EBV infection and against immunogenic antigens due to mismatch-repair defects appear to influence the prognosis and can be an explanation for the distinct survival pattern in these subtypes.[17,30] However, in the present study only MSI showed better survival rates and was an independent prognostic factor for DFS in GC patients. Perhaps other factors related to EBV infection, including the proportion of tumor-infiltrating lymphocytes, also play a role in determining the prognosis rather than EBV infection alone. Since the best prognosis is associated mainly when CD3+ and CD8+ T-lymphocytes are predominant in the inflammatory infiltrate, which had antitumor activity.[5,30,34]

Regarding PD-L1, the prognostic impact of its expression in patients with GC remains controversial to date. In general, studies have small populations and few meta-analyses have shown a correlation between PD-L1 and prognosis.[25,26,35,36] Some authors showed that PD-L1-positive expression was associated to poor prognosis in advanced GC,[37,38] whereas other studies proposed that increased PD-L1 expression are related to a better prognosis.[34,39] Still, some authors have shown no impact on survival.[5] In this investigation, no association between PD-L1 expression and the prognosis was observed. Also, we found that PD-L1 protein expression was more frequently in TCs than TIICs, different from previous studies.[5,39] Our results showed that only PD-L1 expression in tumor cell of pN+ GC patients was associated to poor prognosis, which requires a careful interpretation and further exploration in a large population. As only 25 patients were PD-L1-positive, these may have limited the survival analysis. Furthermore, this variability in reported outcomes might be influenced in part by the patient cohort, since we had 55.1% of GCs staged as I/II and high PD-L1 levels seems to be related to more advanced stages.[26,37,39]

Recently, with the approval of PD-1 monoclonal antibody pembrolizumab and nivolumab by US Food and Drug Administration (FDA) and the currently undergoing trials of anti-PD-1/PD-L1 antibodies for the treatment of advanced GC, that have shown promising clinical efficacies for molecular subgroups,[7,24,40] the evaluation of predictive biomarkers that can serve as therapeutic indication to more specific treatments becomes increasingly important in the diagnostic routine.

We categorized EBV-positive GC and MSI GC in two distinct histologic subsets using well-defined and easily reproducible methodologies to assess whether such a histologic sub-classification may be helpful in predicting prognosis. In this sense, we managed to overcome two major obstacles of addressing molecular signatures in practice: the cost effectiveness and technical complexity. Our IHC/ISH results were consistent with previous comprehensive molecular studies, and the methods are simple and cost-effective manner with practical application on a day-to-day basis.

Nonetheless, an accurate interpretation of IHC stains is still crucial for the establishment of a valid predictive biomarker based on histology, particularly with regard to PD-L1. The discrepancies in result may be explained, in part, by absence of a well-established evaluation criterion for PD-L1 expression and the lack of homogeneity among the studies. The cutoff values for positivity varied (ranges from >1% to 50%, we used ≥2%) and there is no definition of which pattern of expression should be evaluated (tumor cells/inflammatory infiltrate cells) or the antibody clones used. Moreover, in the preoperative

TABLE 3 Univariate and multivariate analyses of risk factors affecting survival in patients with gastric cancer

	Univariate analysis			Multivariate analysis		
Variables	**HR**	**95%CI**	***P***	**HR**	**95%CI**	***P***
Disease-free survival						
Male (vs female)	1.12	0.70-1.80	0.636	-	-	-
Age >65 (vs <65 years)	0.64	0.39-1.05	0.078	-	-	-
Diffuse/mixed Lauren type (vs. intestinal/indeterminate)	1.64	1.03-2.62	**0.038**	0.84	0.41-1.77	0.651
Poorly differentiated (vs well/moderately)	2.25	1.34-3.75	**0.002**	1.97	0.89-4.37	0.097
pT3/pT4 status (vs pT1/pT2)	11.82	4.76-29.33	**<0.001**	6.32	2.08-19.18	**0.001**
Presence of LNM (vs absence)	6.70	3.33-13.48	**<0.001**	2.48	1.02-6.07	**0.046**
Presence of lymphatic invasion (vs absence)	2,98	1,78-4.98	**<0.001**	1.32	0.71-2.45	0.369
EBV-positive (vs EBV-negative)	1.20	0.60-2.41	0.613	-	-	-
MSI (vs MSS)	0.41	0.20-0.84	**0.015**	0.48	0.23-0.99	**0.049**
PD-L1 positive (vs PD-L1 negative)	1.01	0.44-2.34	0.975	-	-	-
Neoadjuvant therapy (vs absence)	1.39	0.73-2.64	0.313	-	-	-
Adjuvant therapy (vs absence)	0.21	0.11-0.40	<0.001	1.01	0.45-2.27	0.976
Overall survival						
Male (vs female)	1.05	0.68-1.63	0.817	-	-	-
Age>65 (vs <65 years)	1.22	0.80-1.88	0.357	-	-	-
Total gastrectomy (vs subtotal)	2.22	1.44-3.41	**<0.001**	1.85	1.20-2.85	**0.006**
Diffuse/mixed Lauren type (vs intestinal/indeterminate)	1.58	1.02-2.44	**0.039**	0.95	0.53-1.72	0.873
Poorly differentiated (vs well/moderately)	2.03	1.27-3.26	**0.003**	1.58	0.83-2.99	0.164
pT3/pT4 status (vs pT1/pT2)	4.46	2.46-8.05	**<0.001**	3.09	1.60-5.96	**0.001**
Presence of LNM (vs absence)	2.90	1.75-4.80	**<0.001**	1.52	8.86-2.68	0.147
EBV-positive (vs EBV-negative)	0.73	0.34-1.59	0.436	-	-	-
MSI (vs MSS)	0.63	0.35-1.32	0.121	-	-	-
PD-L1 positive (vs PD-L1 negative)	1.05	0.48-2.27	0.909	-	-	-
Neoadjuvant therapy (vs absence)	1.02	0.53-1.98	0.947	-	-	-
Adjuvant therapy (vs absence)	0.71	0.45-1.11	0.136	-	-	-

HR, hazard ratio; 95CI, 95% confidence interval; LNM, lymph node metastasis; EBV, Epstein-Barr virus; MSS, microsatellite stable; MSI, microsatellite instability.
The *P* values in bold are statistically significant.

context, the diagnosis of PD-L1 expression by biopsy must be reliable. Remarkable, we observed that PD-L1-positive tumor cells were relatively lower and assessment of different percentages of three different staining intensities was indiscernible and not feasible in practice. Thus, it is advisable that IHC analysis to be performed on multiple biopsy specimens to avoid underestimation of PD-L1-positive GCs. Besides PD-L1, EBV and MSI status, it can be important to determine which others clinicopathological characteristics may have important clinical implications and may serve as a surrogate marker for PD-L1/PD-1-expression, in order to optimize the therapeutic indication.

Some limitations in the present study should be considered. This was a single-center study, some of the groups had a small number of cases (particularly the PD-L1-positive) and some patients have a relatively short follow-up. Regarding the technical limitations, we cannot exclude the risk of false-negative results for PD-L1 (due to its heterogeneous expression and the fact that TMA technique only evaluates small samples of the tumor).[41] In addition, as few GCs had PD-L1 expression, we dichotomized our results into negative and positive staining for PD-L1. Thus, we were not able to measure if differences in percentage of stained cells may have different impact on tumor biology and can be associated to patient survival.

Among the strengths, the study was conducted in a public high-volume center, reference in cancer treatment. The cohort represents western patients treated with potentially curative intent by high volume surgeons. To ensure a reliable assessment of prognosis and other surgically related variables, only standard D2-gastrectomy was considered. The mean number of LNs retrieved was high, attesting the high quality surgery and pathology, and the low risk of understaging. This risk was even reduced, since IHC for cytokeratin was performed for the detection of occult tumor cells in selected cases.[14] To avoid loss and provide a representation of different tumor areas, we used a large

number of cores per case. A standardized definition of IHC/ISH analysis was used and the final results were presented categorically, without disagreement between the cores of tissue. Moreover, previously incompletely addressed by other investigators, we explored PD-L1 expression in tumor and immune cells, which may be clinically relevant in the context of response to therapy in the future.

Therefore, the current investigation may have relevant clinical implications for GC. In addition to confirming MSI status as a predictive factor for better prognostic and showing that the association of PD-L1 expression with EBV may guide the use of immunotherapy for these patients, our data provide evidence that the definition of these categories is a strategy of easy implementation in the routine pathological examination.

5 | CONCLUSION

EBV-positive GC associates to increased PD-L1 expression and shows to be a potential target for specific antibody therapies. MSI GC have better survival outcome. Recognition of these subgroups by routine diagnostic techniques is feasible, allows for more meticulous staging and may guide neo/adjuvant treatment.

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CONFLICTS OF INTEREST

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors declare that they have no conflict of interest.

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SYNOPSIS

This study investigates the *Epstein-Barr* virus (EBV), microsatellite instability (MSI) and PD-L1 immunoexpression in a large western cohort of gastric cancer (GC) patients who underwent surgical resection with potentially curative intent. This is a topic of great interest since molecular classification of GC may serve as a prognostic stratification and assist in the development of genome-guided personalized therapy, especially with regard to EBV and MSI status. This manuscript presents GC profiles in a western population using feasible methodological strategies to define EBV and MSI categories.