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Tumor budding in gastric adenocarcinoma; reflections on tumor microenvironment and programmed death ligand 1 (PD-L1) expression

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Abstract

Introduction: The significance of tumor budding and programmed death ligand 1 (PD-L1) has not been established in gastric adenocarcinoma (GAC).
Objectives: This study evaluated tumor budding and PD-L1 expression with regard to tumor microenvironment, clinicopathologic parameters, and overall survival in GAC.
Patients and Methods: Totally, 102 GAC cases were assessed immunohistochemically. The associations of tumor budding and PD-L1 with clinicopathologic features, tumor-infiltrating lymphocytes (TILs), tumor stroma percentage (TSP), and overall survival were analyzed.
Results: High tumor budding (42.2% of cases) was correlated with distal tumor location, large tumor size, *Helicobacter pylori* infection, poor differentiation (P = 0.0008, 0.033, 0.011, and 0.005, respectively), lymphovascular invasion, high tumor and nodal stages, and TSP (all $P < 0.0001$). Tumor budding was highest in the low TILs/high TSP group. PD-L1 expression (43.1% of cases) was correlated with proximal location (p = 0.00021), poor differentiation (P = 0.036), N stage (P = 0.049), high TILs ($P < 0.0001$), and low tumor budding (P = 0.002). PD-L1 expression was highest in the low tumor budding / high TILs category ($P < 0.0001$). Cox regression showed that high tumor budding (hazard ratio [HR]: 15.282, P = 0.024, 95% confidence interval [CI]: 1.441–162.069) and positive PD-L1 (HR: 7.502, P = 0.015, 95% CI: 1.469–38.31) were independent prognostic factors for overall survival.
Conclusion: Tumor budding is correlated with poor prognostic parameters, whereas PD-L1 expression is inversely correlated with tumor budding. Both are independent predictors of short overall survival. Anti-PD-L1 immunotherapy could be effective in GAC with nodal metastasis, especially cases with high TILs and low tumor budding.

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Introduction

Stomach cancer is the fifth most commonly occurring cancer worldwide, comprising 5.6% of cases, and the fourth most common cause of cancer-related death, representing 7.7% of cases (1). Gastric adenocarcinoma (GAC) is a multifactorial disease that involves an interplay between environmental and genetic factors. In the latest World Health Organization (WHO) classification, the main histologic subtypes identified are papillary, tubular, poorly cohesive, mucinous, and mixed adenocarcinomas. Another commonly recognized and reproducible classification is Lauren's system, which classifies lesions as intestinal, intermediate, or diffuse type (1). Established prognostic factors include tumor stage, histology, and human epidermal growth factor receptor 2 (HER2) expression (1,2). However, prognosis is still poor, as most patients are diagnosed late, especially in developing countries. Recently, elements of the tumor microenvironment have been used as prognostic indices to assess tumor progression in many cancer types (2,3).

Tumor budding is identified as a sign of epithelial mesenchymal transition. Tumor stroma percentage (TSP) and immune response factors, such as tumor-infiltrating

Key point

Tumor budding and programmed death ligand 1 (PD-L1) indicate micro-environmental cross-talks and are emerging as promising prognostic markers for several solid tumors. In gastric adenocarcinoma (GAC), their roles have yet to be clarified. This study revealed that tumor budding is correlated with high tumor grade and stage, high nodal stage, and short overall survival. Furthermore, PD-L1 is inversely correlated with tumor budding and positively correlated with nodal stage and poor survival. PD-L1 expression is highest in tumors with low-budding/high tumor-infiltrating lymphocytes (TILs) introducing this category as potential candidate for immunotherapeutic treatment. Immunotherapy with anti-PD-L1 can be promising in GAC.

lymphocytes (TILs), are identified as signs of tumor progression. The interactions among these factors contributed to the development of immunotherapy through the identification of multiple immune checkpoints (4).

Tumor budding has been used in clinical practice as a complementary prognostic feature in reporting colorectal carcinoma, after being officially approved by the Union for International Cancer Control and the European Society for Medical Oncology (5). Several studies have been conducted on the correlation of tumor budding with the different clinicopathologic parameters to validate its significance as an independent prognostic feature in GAC. However, no consensus on its use in routine practice or standardized scoring system has been established yet (2).

Tumor stroma percentage may play a crucial role in the various processes in tumor progression, such as angiogenesis, metastasis, and immune evasion in different types of cancer cells (3). TILs have recently gained research attention in many cancers, including GAC, as a prognostic feature and indicator for immunotherapy. High TILs are generally associated with good prognosis (3, 4).

The function of T cells in attacking abnormal cells and pathogens but avoiding normal host tissue is regulated by many immune checkpoints. Among them is the programmed cell death 1/programmed death ligand 1 (PD-1/PD-L1) pathway. PD-L1 is a transmembrane surface glycoprotein encoded by the *CD274* gene and expressed by various immune system cells, such as lymphocytes and dendritic cells. The binding between PD-L1 and its receptor PD-1 in tumors can create an immunosuppressive microenvironment that enhances tumor growth (6). At present, pembrolizumab is the only approved immune checkpoint inhibitor treatment against PD-L1 in many solid tumors, such as those in lung, bladder, and colon cancers. However, to date, its approved use in GAC is limited to tumors with microsatellite instability, DNA mismatch repair deficiencies, or advanced cases after failure of initial treatment. Identifying potential additional immune checkpoint inhibitors will improve treatment decisions and, subsequently, patient prognosis (7).

Objectives

This study aimed (a) to evaluate the role of tumor budding in GAC using the International Tumor Budding Consensus Conference (ITBCC) 2016 scoring system for colorectal cancer (8); (b) to elucidate the relationship between tumor budding and the different clinicopathological parameters and tumor micro-environmental factors, including TILs and TSP; (c) to assess immunohistochemically the role of PD-L1 expression in GAC and its relationship with different clinicopathologic parameters; (d) to analyze the possible crosstalk between tumor budding and TILs as tumor micro-environmental elements and its reflection on and PD-L1 expression for better tumor characterization to maximize the benefit of anti-PD-L1 immunotherapeutic agents; and (e) to study the effect of tumor budding and PD-L1 expression on overall survival.

Patients and Methods

Sample selection and clinicopathologic characterization

This retrospective cohort study examined GAC specimens collected from the laboratories of the pathology department at Ain Shams university and specialized hospitals from 2015 to 2021. Data from patients' archival medical records and materials to their cases were collected, including the following: (a) age, gender, and tumor location as revealed by endoscopic and imaging reports; (b) overall survival reported and calculated from the available records at the clinical oncology department (estimated from the date of diagnosis until date of last follow-up or death; and (c) hematoxylin and eosin (H&E) slides and paraffin blocks. Re-assessment and confirmation of the histopathologic diagnosis were performed. Tumors were graded according to the WHO classification as well, moderately, or poorly differentiated (1). Other pathologic parameters were re-assessed and reported, including tumor size, presence of perineural invasion, lymphovascular invasion, T stage, N stage, presence of intestinal metaplasia, and positive *Helicobacter pylori* infection in adjacent non-neoplastic mucosa. All aforementioned parameters were evaluated according to the WHO classification (1). Histopathologic evaluation of H&E slides was conducted for TSP, TILs, and tumor budding, and immunohistochemical staining was performed to evaluate for PD-L1.

Assessment of TILs

No consensus on TILs scoring in gastric cancer has yet been reached. In this study, the scoring recommendations of the International TILs Working Group 2014 on breast cancer as modified by Kang et al (8) for gastric cancer were adopted. Briefly, one section representing the invasive tumor border is selected. On 200-400× magnification, the stromal TILs (str-TILs) score was evaluated as the percentage of str-TILs to the tumoral stromal area by semi-quantitative assessment. Only mononuclear cells, such as lymphocytes and plasma cells, were scored; granulocytic infiltrations were excluded. Subsequently, all cases were divided into high TIL and low TIL cases. Cutoff value was settled as the median of str-TIL percentage, which was revealed to be 15%.

Assessment of TSP

Representative H&E slides of the deepest invasive margin of the tumor were chosen. Further, at 100× magnification with tumor cells on all four sides of the slide, the TSP, which is the percentage of the stroma to the whole tumor, was calculated. Necrotic and mucinous areas were excluded. High and low TSP were defined as >50% and ≤50%, respectively (3). Based on the TSP and TILs, cases were further classified into four groups: group 1; low TSP/high TILs (i.e., TSP ≤ 50% and TILs > 15%); group 2; low

TSP/low TILs (i.e., TPS ≤ 50% and TILs ≤ 15%); group 3; high TSP/high TILs (i.e., high TSP > 50% and TILs > 15%); and group 4; high TSP/low TILs (i.e., high TSP > 50% and TILs ≤ 15%).

Assessment of tumor budding

Tumor budding was evaluated using the ITBCC 2016 scoring system for colorectal cancer (8). A tumor bud was defined as a single tumor cell or a non-gland-forming cluster of <5 tumor cells at the invasive tumor front. First, the field area of the 20× objective microscope lens was calculated based on the eyepiece field number diameter. Second, the hotspot areas with the highest tumor budding at the invasive front were selected. Moreover, tumor buds were counted and divided by the normalization factor to determine the budding (Bd) count per 0.785 mm^2. Immunohistochemical staining for pan-CK was performed in some problematic cases where tumor buds were masked by heavy inflammatory cellular infiltrate. Tumor budding was graded accordingly into the three-grade system as follows: low grade (Bd1, 0–4 tumor buds); intermediate grade (Bd2, 5–9 tumor buds); and high grade (Bd3, ≥10 tumor buds). Lastly, tumors with grades Bd1 and Bd2 were combined to form the low tumor budding group, whereas the grade Bd3 tumors comprised the high tumor budding group. Based on the cutoff for the low and high TILs and tumor budding groups, cases were further categorized into four categories: category 1: high tumor budding / high TILs (i.e., high budding + TILs > 15%); category 2: low tumor budding /high TILs (i.e., low budding + TILs > 15%); category 3: high tumor budding /low TILs (i.e., high budding + TILs ≤ 15%); category 4: low tumor budding / low TILs (i.e., low budding + TILs ≤ 15%); and

Exclusion criteria included patients with diffuse/poorly cohesive gastric cancers, as assessments of budding is not feasible (tumors composed only of discohesive individual malignant cells, which makes the identification of tumor buds difficult) (9-11). Cases that underwent neo-adjuvant therapy were also excluded.

Immunostaining procedure

Sections (4 µm thick) from paraffin-embedded tissues were obtained for the primary antibody, PD-L1 (clone 22C3 pharmDx, monoclonal mouse antihuman, dilution 1:50, Agilent, Santa Clara, CA, USA). Immunostaining was performed using the automated Benchmark Ventana (GX) (Arizona, USA) instrument according to the manufacturer's instructions. First, the slides, antibodies, and ultra-view detection kit dispenser were loaded into the Bench-Mark instrument. The standard CC1 protocol was selected, and the antibody was incubated for 32 minutes at 37°C. Furthermore, slides were removed, rinsed, and washed 10 times with a buffer from Ventana (Ref. 950-300, Lot G24035). Tonsillar tissue was used as positive control. Negative control was established by omitting the primary antibody. Both positive and negative controls were included in each run.

Assessment of PD-L1 immunostaining

Tumor cells with either partial or complete membranous staining with any degree of intensity were considered positive. Positively stained immune cells were defined by membranous and/or cytoplasmic staining. The percentages of positive cells for both tumor cells and immune cells (including lymphocytes and macrophages) were calculated. A composite positive score (CPS) was obtained by dividing the positive cells by the total number of viable cells then multiplying by 100. At least 100 viable tumor cells should exist in the PD-L1–stained slide to be considered adequate for assessment. A cutoff CPS value ≥ 1% was considered positive for PD-L1 expression, whereas CPS < 1% was considered negative (6).

All histopathologic variables and immunohistochemical interpretation were evaluated independently by two pathologists blinded to the data, and any discrepancy was resolved by re-investigation on a multi-head microscope until a consensus was reached.

Statistical analysis

IBM SPSS (version 26.0, IBM Corp., Philadelphia, USA) was used to perform all statistical operations. Parametric numerical data were expressed as mean ± standard deviation, whereas nonparametric numerical data were expressed as median (interquartile range, IQR). Non-numerical data were expressed as frequency (percentage). Data categorization for determining the associations between tumor budding and the different clinicopathological parameters was performed by cross-tabulation using the Pearson's chi-square test. Overall survival was assessed using the Kaplan–Meier curve. Survival distribution among groups was analyzed using the log-rank test. Multivariate analysis for survival was performed using the Cox proportional hazards regression model. A P value <0.05 was considered significant, whereas $P < 0.001$ was considered highly significant.

Results

Clinicopathologic characteristics of GAC

This study included 102 GAC cases. The median age of the patients was 61.5 years (range, 23–90 years). The patients were 39.2% (40/102) female and 60.8% (62/102) male. Classified according to Lauren's classification, the cases were 89.2% (91/102) intestinal-type adenocarcinoma (well and moderately differentiated tumors), whereas 10.8% (11/102) were intermediate type (poorly differentiated tumors). The median size of the tumors was 4 cm at their widest dimension (range, 1 to 10 cm). Distally located tumors (antrum and pylorus) represented 54% of cases (55/102), whereas proximally located tumors (fundus and body) represented 46% (47/102).

Regarding elements of the tumor microenvironment, the median percentage of TILs was 15% (range, 1%–80%).

Low and high tumor TILs were observed in 58 (56.9%) and 44 cases (43.1%), respectively, whereas low and high TSP were observed in 60 (58.8%) and 42 cases (41.2%), respectively. The most frequent grouping was low TSP/low TIL, representing 33.3% (Figure 1). High and low tumor budding were found in 43 (42.2%) and 59 cases (57.8%), respectively. The most frequent tumor budding/TIL category was category 2, low tumor budding /high TILs at 29.4% (Figure 2). Details of the clinicopathological data are listed in Table 1. Out of 102 cases, only 74 cases had survival data. The median overall survival for the 74 cases was 48 months (range, 14.25–60 months) (Figure 3).

Relationships of tumor budding with clinicopathologic features, tumor microenvironmental elements, and survival

A significant relationship was found between high tumor budding and distal tumor location (antrum and pylorus;

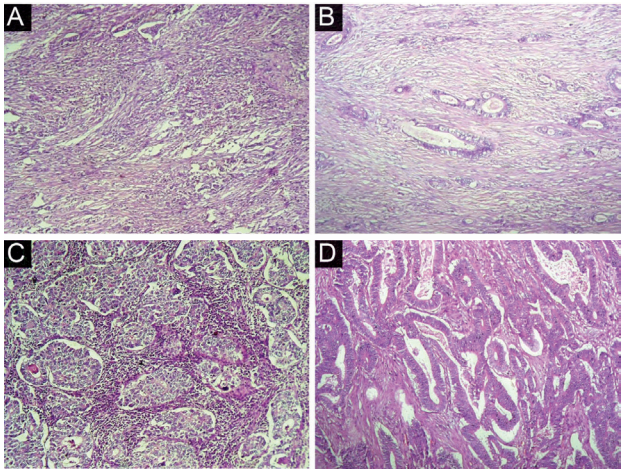

Figure 1. Groupings according to tumor stroma percentage (TSP) and tumor-infiltrating lymphocytes (TILs): A) group 1, high TSP/ high TILs; B) group 2, high TSP/low TILs; C) group 3, low TSP/high TILs; and D) group 4, low TSP/low TILs. (hematoxylin and eosin [H&E], 100×).

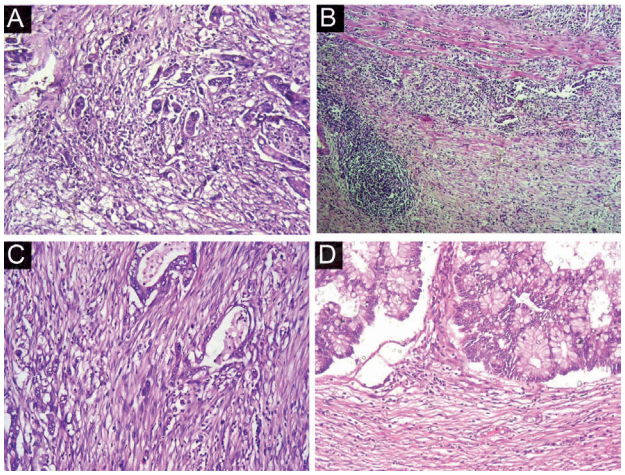

Figure 2. Categorization of cases using tumor budding (TB) and TILs. A) category 1, high budding and high TILs; B) category 2, low budding and high TILs; C) category 3, high budding and low TILs; and D) category 4: low budding and low TILs (H&E, 200×).

Table 1. Summary of clinicopathological characteristics of cases (n=102)

Feature	No. (%)
Age	
<50	22 (21.7)
≥50	80 (78.4)
Gender	
Female	40 (39.2)
Male	62 (60.8)
Site of tumor	
Antrum	36 (35.3)
Body	42 (41.2)
Fundus	5 (4.9)
Pylorus	19 (18.6)
Size of tumor	
<2 (15)	15 (14.7)
2-5 (60)	60 (58.8)
>5 (27)	27 (26.5)
Intestinal metaplasia in adjacent mucosa	
Present	21 (20.6)
Absent	81 (79.4)
Helicobacter bacilli in adjacent mucosa	
Present	23 (22.5)
Absent	79 (77.5)
Tumor differentiation	
Well	14 (13.7)
Moderate	77 (75.5)
Poor	11 (10.8)
Perineural invasion (n=102)	
Present	11 (10.8)
Absent	91 (89.2)
Lymphovascular invasion (n=102)	
Present	45 (44.1)
Absent	57 (55.9)
Tumor stage	
T1	15 (14.7)
T 2	34 (33.3)
T3	48 (47.1)
T4	5 (4.9)
Nodal stage	
N00	58 (56.9)
N1	28 (27.5)
N2	13 (12.7)
N3	3 (2.9)
TSP	
Low	60 (58.8)
High	42 (41.2)
TILs	**TILs**
Low	58 (56.9)
High	44 (43.1)
Groups	
Group 1: High TSP/ High TILs	18 (17.6)
Group 2: High TSP/ Low TILs	24 (23.5)
Group 3: Low TSP/ High TILs	26 (25.5)
Group 4: Low TSP/ Low TILs	34 (33.3)
Tumor budding	
Low	59 (57.8)
High	43 (42.2)
Categories	
Category 1: High budding/ High TIL	14 (13.7)
Category 2: Low budding/ High TIL	30 (29.4)
Category 3: High budding/ Low TIL	29 (28.4)
Category 4: Low budding/ Low TIL	29 (28.4)

TSP; tumor stroma percentage; TILs, tumor infiltrating lymphocytes.

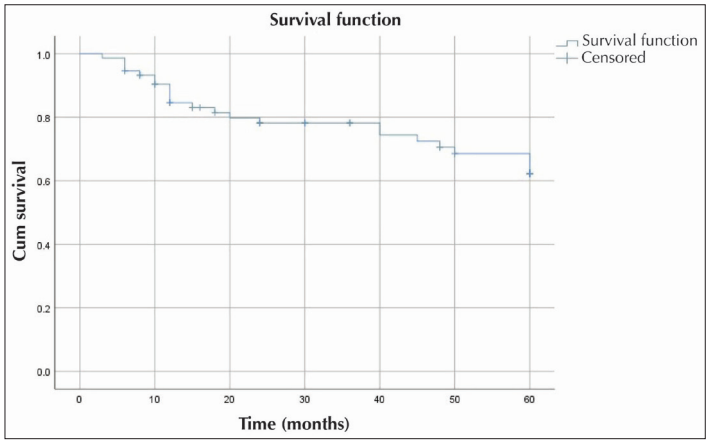

Figure 3. Kaplan–Meier curve showing overall survival for the cases studied.

$P = 0.0008$), large tumor size ($P = 0.033$), *H. pylori* infection ($P = 0.011$), poorly differentiated tumors ($P = 0.005$), positive lymphovascular invasion (LVI), and higher tumor stage (T3 and T4) and higher N stage (all $P < 0.0001$). No significant relationship was found between tumor budding and age, gender, intestinal metaplasia, perineural invasion, or TIL.

Regarding the relationships between tumor budding and elements of the tumor microenvironment, a high TSP was detected in 76.2% and 18.3% of high and low tumor budding cases with significant difference ($P < 0.0001$). No significant relationship was found between TILs and tumor budding. However, low TILs/high TSP cases most frequently had high tumor budding (87.5%), whereas the low TIL/low TSP cases least frequently had high tumor budding at 23.5% ($P < 0.0001$). All details are listed in Table 2.

A significantly shorter overall survival was observed in cases with high tumor budding than those with low tumor budding on the long-rank test ($P < 0.0001$), with a median overall survival of 12 months (95% confidence interval [CI], 0.871–13.707) and 60 months (95% CI, 0), respectively (Figure 4).

PD-L1 expression in GAC

The immune composite score showed that positive PD-L1 expression was detected in 44 (43.1%) cases comparing to 58 (56.9%) of cases with negative expression for PD-L1 expression (Figure 5).

Relationships of PD-L1 expression with clinicopathological features and elements of the tumor microenvironment

Significant relationships were found between positive PDL1 expression and each of proximal tumor location (body and fundus; $P = 0.00021$), poorly differentiated tumors ($P = 0.036$), positive perineural invasion ($P = 0.036$), and nodal stage ($P = 0.049$). No significant relationship was found between PD-L1 expression and each of age, gender, tumor size, intestinal metaplasia, *H. pylori* infection, LVI, tumor stage, or nodal stage.

Significant relationships were found between positive PD-L1 expression and each of high TILs ($P < 0.0001$) and low tumor budding ($P = 0.002$). No significant relationship was observed between TSP and PD-L1 expression.

Positive PD-L1 expression was significantly the highest in category 2 (low tumor budding/high TIL), representing 86.7% of cases ($P < 0.0001$). All details are listed in Table 3.

The median overall survival of cases with positive PD-L1 was 52 months (95% CI, 44.894–58.068), whereas that of cases with negative PD-L1 expression was 45 months (95%CI, 37.7337–51.931). The log-rank test showed no significant difference in the overall survival between cases with positive and negative PD-L1 expression ($P = 0.099$; Figure 6).

Multivariate Cox regression survival analysis

On multivariate analysis of overall survival using Cox regression models, higher tumor stages (T3/T4) (hazard ratio [HR] 10.169, 95% CI 1.223–84.575; $P = 0.032$), high tumor budding (HR 15.282, 95% CI 1.441–162.096; $P = 0.024$), and positive PD-L1 with the lowest hazard ratio (HR 7.502, 95% CI 1.469–38.310; $P = 0.015$) were associated with a short overall survival, independent of the other clinicopathologic and environmental variables. Thus, the increased risk of mortality with positive PD-L1 expression was 7.5 folds, whereas that with high tumor budding was 15.3 folds. Positive lymph node status and short overall survival were also associated with near significant results (HR 11.059, 95% CI 0.898–136.154; $P = 0.061$) (Table 4, Model 1).

Cox regression analysis revealed high TSP (HR 10.439, 95% CI 3.068–35.524; $P < 0.0001$) and high tumor budding (HR 23.638, 95% CI 6.930–80.629; $P < 0.0001$) as the top two independent factors associated with short overall survival (Table 4, Model 2).

Discussion

Gastric adenocarcinomas are among the most common gastrointestinal tumors worldwide after colorectal carcinoma. However, unlike colorectal carcinoma, risk

Table 2. Relationship between tumor budding and other clinicopathological features (102 cases)

Feature (n)	High TB (n=43) (n, %)	Low TB (n=59) (n, %)	χ^2	*P* value
Age				
<50 (22)	8 (36.4)	14 (63.6)	0.386	0.534 (NS)
≥50 (n=80)	35 (43.8)	45 (56.2)		
Gender				
Female (40)	17 (42.5)	23 (57.5)	0.003	0.955 (NS)
Male (62)	26 (41.9)	36 (58.1)		
Site				
Distal (55)	32 (58.2)	23 (41.8)	11.148	0.0008 (HS)
Proximal (47)	11 (23.4)	36 (76.6)		
Size				
<2 (15)	3 (20.0)	12 (80.0)	4.515	0.033 (S)
2-5 (60)	24 (40.0)	36 (60.0)		
>5 (27)	16 (59.3)	11 (40.7)		
Adjacent intestinal metaplasia				
Present (21)	10 (47.6)	11 (52.4)	0.324	0.569 (NS)
Absent (81)	33 (40.7)	48 (59.3)		
Adjacent Helicobacter bacilli				
Present (23)	15 (65.2)	8 (34.8)	6.476	0.011(S)
Absent (79)	28 (35.4)	51 (64.6)		
Differentiation				
Well/moderate (91)	34 (73.4)	57 (62.6)	7.654	0.005 (S)
Poor (n=11)	9 (81.8)	2 (18.2)		
Perineural invasion				
Present (n=11)	4 (36.4)	7 (63.6)	0.170	0.680 (NS)
Absent (n=91)	39 (42.9)	52 (57.1)		
Lymphovascular invasion				
Present (n=45)	35 (77.8)	10 (22.2)	39.328	0.001(HS)
Absent (n=57)	8 (14.0)	49 (86.0)		
Tumor stage				
T1/2 (49)	4 (8.2)	45 (91.8)	44.688	0.001(HS)
T3/4 (53)	39 (73.6)	14 (26.4)		
Nodal stage				
N 0 (58)	8 (13.8)	50 (86.2)		
N1 (28)	21 (75.0)	7 (25.0)	45.248	0.001 (HS)
N2 (13)	11 (84.6)	2 (15.4)		
N3 (3)	3 (100.0)	0 (0.0)		
TSP				
Low (n=60)	11 (18.3)	49 (81.7)	33.915	0.001(HS)
High (n=42)	32 (76.2)	10 (23.8)		
TILs				
Low (n=58)	29 (50.0)	29 (50.0)	3.392	0.066 (NS)
High (n=44)	14 (31.8)	30 (68.2)		
Groups				
Gr1: High TSP/ High TIL (n=18)	11 (61.1)	7 (38.9)		
Gr 2: High TSP/ Low TIL (n=24)	21 (87.5)	3 (12.5)	37.721	0.001(HS)
Gr 3: Low TSP/ High TIL (n=26)	3 (11.5)	23 (88.5)		
Gr 4: Low TSP/ Low TIL (n=34)	8 (23.5)	26 (76.5)		

Chi-square test.

TB, tumor budding; TSP, tumor stroma percentage; TILs, tumor infiltrating lymphocytes; Gr, group; S, significant; HS; highly significant; NS, non-significant.

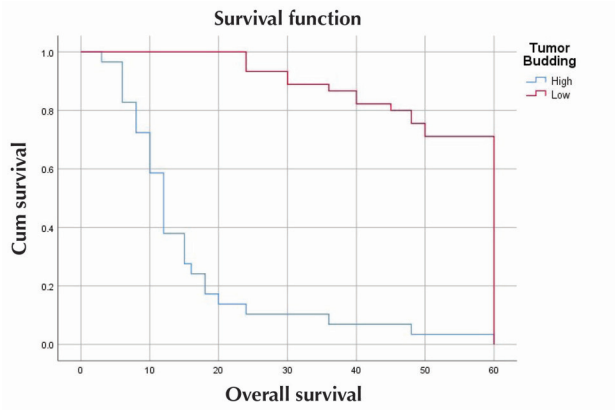

Figure 4. Kaplan–Meier curve showing overall survival for cases with high and low budding (log-rank test, $P < 0.0001$).

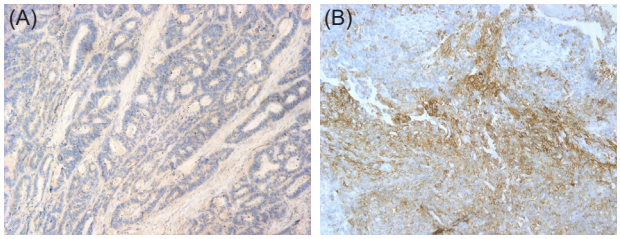

Figure 5. Immunohistochemical staining for PDL-1 expression. A) negative PDL-1 expression, no appreciable membranous staining. B) positive PDL-1 expression, considerable membranous staining is observed (200×).

stratification using histopathologic parameters of tumor budding is not well established (2).

In addition, in GAC, the relationship between tumor budding and other micro-environmental factors, especially TILs, an emerging marker for tumor development and progression, are not well understood (3). Studying the tumor microenvironment and tumor immunology could identify more candidates for immune checkpoint inhibitors with priority to anti-PD-L1 in the aim of improving patient survival. The administration of pembrolizumab (an anti-PD-L1 inhibitor) in the immunotherapeutic treatment of GAC is still restricted, hence necessitating further investigation to explore the benefit of its use and expand its possible candidates (7).

This study aimed to evaluate the role of tumor budding in GAC and its relationship with different clinicopathologic and micro-environmental variables, including its possible reflection on PD-L1 immunohistochemical expression and overall survival.

The ITBCC 2016 definition of high tumor budding (8) adopted in the current study was significantly associated with distal tumor location, large tumor size, *H. pylori* infection, poorly differentiated tumors, positive lymphovascular invasion (LVI), and higher tumor and nodal stages.

Similarly, previous studies reported a positive association of high tumor budding with tumor size, poorly differentiated tumors (5,10), LVI, lymph node metastasis (2), and TNM stages (5,10,11). While some found no significant association between tumor budding and tumor location (10), a study reported, in agreement to our results, higher budding in antral and pyloric tumors (distal location) (2). In fact, this could explain the positive relationship between positive *H. pylori* infection in adjacent mucosa and high tumor budding observed in our study, as the former commonly occurs in distal stomach sites (1). While this relationship is not well studied in the literature, Zhou et al (11) found a significant positive correlation between *H. pylori* infection and MMP-7 expression, another known marker of epithelial mesenchymal transition.

On the correlation of tumor budding with other micro-environmental factors, the current study found a positive correlation between high tumor budding and high TSP ($P<0.0001$). In GAC, the relationship between tumor budding and tumor stroma has not been established. However, a positive association between high tumor budding and high TSP was reported in other gastrointestinal tumors, such as colorectal cancer (12,13) and gall bladder carcinoma (14), and in breast carcinomas (15).

Regarding TILs, Zhang et al (15) reported an inverse relationship between TILs and tumor budding along different areas in the same tumor with $p < 0.001$ highlighting the weak immune surveillance around tumor buds. Indeed, no significant relationship was found between tumor budding and TILs in this study. However, the co-occurrence of low TILs and high TSP showed the highest tumor budding ($P<0.0001$) among all other groups including, low TILs/low TSP, high TILs/high TSP, and high TILs/low TSP. These results collectively highlight the diverse cross-talks between different micro-environmental elements.

The molecular profile of tumors associated stromal fibroblasts have been implicated in many processes as facilitating epithelial mesenchymal transition, increasing extracellular matrix, providing suitable metabolic substrates, and facilitating angiogenesis for the concurrent formation of tumor budding, tumor dissociation, dedifferentiation, and dissemination (13,15). These processes are likely exaggerated in the absence of immune surveillance, as reflected by reduced tumor inflammatory cells (i.e., high TSP/low TILs), thereby creating an immunosuppressed environment for tumor dissemination and progression (13,15). A deeper understanding of the molecular and functional features of TILs in the context of tumor budding is still required to gain new insights into the pathophysiology, progression, and prognosis of GAC with the development of new targeting therapeutic options.

Programmed death ligand 1 expression was observed in 43.1% of the studied cases. Regarding the relationship between PD-L1 expression and different clinicopathologic parameters, the current study found a significant

Table 3. Relationship between PD-L1 with other clinicopathological and micro-environmental features (102 cases)

Feature (n)	Negative PDL1 (n=58) (n, %)	Positive PDL1 (n=44) (n, %)	χ^2	*P* value
Age			0.057	0.812 (NS)
<50 (22)	13 (59.1))	9 (40.9)		
≥50 (80)	35 (43.8	45 (56.2)		
Gender				
Female (40)	24 (60.0)	16 (40.0)	0.264	0.607 (NS)
Male (62)	34 (54.8)	28 (45.2)		
Site				
Antrum (55)	41 (74.5)	14 (25.5)	13.691	0.00021 (HS)
Body (47)	17 (36.2)	30 (63.8)		
Size				
<2 (15)	9 (60.0)	6 (40.0)	0.321	0.852 (NS)
2-5 (60)	35 (58.3)	25 (41.7)		
>5 (27)	14 (51.9)	13 (48.1)		
Adjacent intestinal metaplasia				
Present (n= 21)	9 (42.9)	12 (57.1)	2.115	0.146 (NS)
Absent (n=81)	49 (60.5)	32 (39.5)		
Adjacent *Helicobacter* bacilli				
Present (23)	12 (52.2)	11 (47.8)	0.266	0.606 (NS)
Absent (79)	46 (58.2)	33 (41.8)		
Differentiation				
Well/moderate (n=91)	55 (60.4)	36 (39.6)	4.401	0.036 (HS)
Poor (n=11)	3 (27.3)	8 (72.7)		
Perineural invasion				
Present (n=11)	3 (27.3)	8 (72.7)	4.401	0.036 (S)
Absent (n=91)	55 (60.4)	36 (39.6)		
Lymphovascular invasion				
Present (n=45)	29 (64.4)	16 (35.6)	1.375	0.241 (NS)
Absent (n=57)	29 (50.9)	28 (49.1)		
Tumor stage			3.389	0.122 (NS)
T1/2 (49)	24 (49)	25 (51)		
T3/4 (n=53)	34 (64.2)	19 (35.8)		
Nodal stage				
0 (n=58)	32 (55.2)	26 (44.8)		
1 (n=28)	11(39.3)	17 (60.7)	3.845	0.049 (NS)
2 (n=13)	4 (30.8)	9 (69.2)		
3 (n=3)	0 (0.0)	3 (100.0)		
TSP (n=102)				
Low (n=60)	34 (56.7)	26 (43.3)	0.002	0.962 (NS)
High (n=42)	24 (57.1)	18 (42.9)		
TILs				
Low (n=58)	47 (81.1)	11 (18.9)	32.027	0.000 (HS)
High (n=44)	11 (25.0)	33 (75.0)		
TB				
Low (n=59)	26 (44.1)	33 (55.9)	9.341	0.002 (HS)
High (n=43)	32 (74.4)	11 (25.6)		
Categories				
Cg 1: High budding/ High TIL (14)	7 (50.0)	7 (50.0)		
Cg 2: Low budding/ High TIL (30)	4 (13.3)	26 (86.7)	37.891	0.0001 (HS)
Cg 3: High budding/ Low TIL (29)	25 (86.2)	4 (13.8)		
Cg 4: Low budding/ Low TIL (29)	22 (75.9)	7 (24.1)		

Chi-square test. TB, tumor budding; TSP, tumor stroma percentage; TILs, tumor infiltrating lymphocytes; Cg, category; S, significant; HS; highly significant; NS, non-significant.

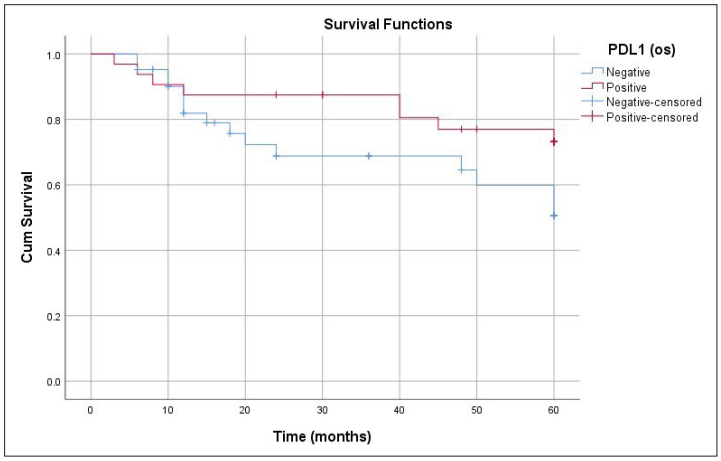

Figure 6. Kaplan–Meier analysis showing overall survival for PDL-1–positive and negative cases (log-rank test, $P = 0.099$).

correlation between positive PD-L1 expression with poorly differentiated tumors and perineural invasion (both $P=0.036$). In addition, PD-L1 expression was correlated with advanced nodal stage ($P=0.049$), although with a P value lower than that for tumor budding ($P<0.0001$). In agreement to our results, numerous studies have shown a positive association between PD-L1 expression and poor tumor differentiation, (6,16-20) and an advanced nodal stage (18,20,21). In addition, our study found a positive relationship between PD-L1 expression and proximal tumor location ($P=0.001$). However, studies that assessed this relationship showed variable results between nonsignificant (6) and a positive (20) relationships

The present study showed a non-significant relationship between PD-L1 expression and each of age, gender, tumor size, LVI, and T stage. Nevertheless, a wide variation of results have been reported in the literature. While some studies found a positive relationship between PD-L1 positivity and old age, male sex (17), higher tumor stage, and lymphovascular invasion (18), other studies found nonsignificant relationships of PD-L1 expression with T stage (20,21), lymphovascular invasion (20), and N stage (21). Interestingly, one study reported a positive correlation with a lower tumor stage (19).

This great diversity in results could be explained by the different clones used to detect PD-L1 and the different scoring methods used. For example, Böger et al (19) revealed that PD-L1 expression correlates with poor prognostic parameters of gastric carcinoma when the tumor cells were positive for PD-L1 expression. However, this significance was lost when stromal cells were evaluated for PD-L1 expression. In addition, Gu et al (17) reviewed a wide variety of studies, each using a different scoring method with different cutoff values, ranging from

Table 4. Multivariate cox regression analysis for factors affecting overall survival

	B	SE	P value	HR	95.0% CI for HR	
					Lower	Upper
Model 1*						
Site (distal stomach)	0.900	0.674	0.182	2.460	0.657	9.218
Size	-0.163	0.166	0.326	0.850	0.614	1.176
Positive perineural invasion	-0.520	0.952	0.585	0.594	0.092	3.844
Positive LVI	-0.109	0.625	0.862	0.897	0.264	3.052
Poor differentiation	0.557	1.124	0.620	1.745	0.193	15.805
Positive *Helicobacter pylori*	-0.465	0.571	0.416	0.628	0.205	1.925
T stage (T3/T4)	2.319	1.081	0.032	10.169	1.223	84.575
Positive LN	2.403	1.281	0.061	11.059	0.898	136.154
High TSP	1.210	1.146	0.291	3.355	0.355	31.701
High TILs	-1.589	0.903	0.079	0.204	0.035	1.199
Positive PD-L1	2.015	0.832	0.015	7.502	1.469	38.310
High TB	2.727	1.205	0.024	15.282	1.441	162.069
Model 2**						
High TSP	2.346	0.625	0.000	10.439	3.068	35.524
High TILs	-0.117	0.489	0.811	0.890	0.341	2.318
High TB	3.163	0.626	0.000	23.638	6.930	80.629

* Model 1 includes all studied clinic-pathologic and micro-environmental parameters.

** Model 2 includes only the studied micro-environmental parameters.

HR, hazards ratio; CI, confidence interval; Sig, significant; LVI, lympho-vascular invasion; T stage, tumor stage; TSP, tumor stroma percentage; TILs; tumor infiltrating lymphocytes; TB, tumor budding.

1% to 50%, reflecting variations in results among studies. This raises the urgent need for standardization in scoring methods and the used PD-L1 clone. This further will help to validate results especially when it comes to treatment decisions.

Despite these controversial results, a common agreement on the positive relationship between PD-L1 expression and nodal stage indicating poor survival was reported in previous meta-analysis (18,22).

programmed death ligand 1 expression and elements of tumor microenvironment were found inversely correlated with tumor budding ($P=0.002$) in the present study. Interestingly, the low tumor budding / high TILs category showed the highest percentage of positive PD-L1 expression (86.7%, $P=0.0001$). This category, in particular, should be interpreted with caution. While low tumor budding is correlated with favorable clinicopathologic parameters, increased PD-L1 expression with either TILs or tumor cells could indicate an increased risk for nodal metastasis. In the context of the tumor microenvironment, a low tumor budding /high TILs status could be a potential candidate for anti-PD-L1 treatment. While the relationship between tumor budding and PD-L1 expression is still not clear, Lang-Schwarz et al (4) reported an inverse relationship between tumor budding and PD-L1 expression in colorectal cancer. Furthermore, they found that a low tumor budding/high TILs status showed higher PD-L1 expression levels than other combinations. These results are similar to our study of GAC cases.

Our study also showed a positive correlation between PD-L1 expression and high TILs ($P=0.0001$). Accordingly, a positive correlation between PD-L1 expression and TILs was reported in some studies (21, 23). This positive correlation between high TILs and high nodal stage noted earlier, as demonstrated by Gu et al (17), could be due to the function of PD-L1 (B7-H1) as a member of the B7 family of immune-regulatory ligands in regulating T cell functions by binding to PD-1, a member of the CD28 family and a regulatory T cell receptor (T_{reg}). The PD-L1/ PD-1 interaction can suppress the effector T cells (T_{eff}) and maintain peripheral tissue tolerance. In tumors, PD-L1 is highly expressed on induced T reg cells (iT_{reg}) that in turn express molecules, such as CD25 and CTLA-4, which suppress T_{eff} cells that are anti-tumor T cells. Even at T_{reg} low levels, PD-L1 enhances and maintains Foxp3 expression on iT_{reg} cells and augments tumor immune suppression. The suppression of anti-tumor T cell responses is further magnified by increased PD-L1 expression on tumor cells, thereby upregulating tumor progression. This also could explain why good prognosis of gastric cancer cases with high TILs levels as reported by some studies is countered by expression of PD-1 expression (24).

This study showed that positive PD-L1 was an independent predictor for short overall survival. However, the effect of PD-L1 on overall survival is still controversial. Some studies reported PD-L1 as a poor prognostic factor (18, 24), whereas, others either found it was a good indicator (20) or it had a non-significant relationship with overall survival (17).

However, the HR for PD-L1 was the lowest (HR 7.502) in this study compared with tumor stage (HR 10.196) and tumor budding (HR 15.282).

The poor prognostic impact of high tumor budding on overall survival in GAC is also reported by several studies (2,5,10,11). This means that tumor budding is an effective histopathologic parameter for risk stratification in GAC, just as it is in colorectal carcinoma. Furthermore, the present study showed that a high tumor budding indicates the highest risk of mortality among all tumor micro-environmental elements.

Conclusion

High tumor budding is correlated with poor prognostic parameters and short overall survival and can be used for risk stratification in GAC, whereas PD-L1 expression is inversely correlated with tumor budding. However, PD-L1 expression is positively correlated with nodal stage and poor overall survival. Patients with node metastasis could benefit from anti-PDL-1 immunotherapeutic treatment, such as pembrolizumab. The 2016 ITBCC scoring system for colorectal cancer is also applicable in gastric cancer. However, the standardization of PD-L1 scoring is still required to obtain conclusive results. Tumors that express low tumor budding / high TILs showed the highest levels of PD-L1 positivity, which indicates a potential new target for pembrolizumab. Further studies should be conducted to elucidate the effect of tumor budding on PD-L1 expression and provide insights for better understanding of tumor immunohistochemical behaviors.

Limitations of the study

Data on overall survival were available for only 74 out of the 102 GAC cases included in the study.

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Authors' contribution

SMMA and MIH were the principal investigators of the study. Both prepared the concept and design. Both authors participated in preparing the final draft of the manuscript, revised the manuscript, and critically evaluated the intellectual contents. All authors have read and approved the content of the manuscript and confirmed the accuracy or integrity of any part of the work.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The institutional ethical committee at Faculty of medicine, Ain Shams University of Medicine approved all study protocols (Ref#FMASU R182/2021) in accordance with the 1964 Helsinki Declaration and

its later amendments. Accordingly, written informed consents were taken from all participants before any intervention. Ethical issues (including plagiarism, data fabrication, double publication) have been also completely observed by the authors.

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