

Prognostic Value of PD-L1 Immunohistochemical Marker in Gastric Carcinoma and Its Correlation with HER2 Status

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Abstract

Objective: Programmed death-ligand 1 (PD-L1) and human epidermal growth factor receptor 2 (HER2) are currently considered as prognostic markers and therapeutic targets in many human cancers. This study aims to evaluate immunohistochemical (IHC) expression of PD-L1 in gastric cancer (GC) and explore its prognostic role in terms of association with HER2 expression, different clinico-pathological variables, in particular density and cluster designation (CD)8 positivity in tumor infiltrating lymphocytes (TILs) and with patients' disease-free and overall survival (DFS, OS). **Methods:** This retrospective cohort study included 111 diagnosed primary GC patients who underwent surgical resection at the Gastrointestinal Surgery Center (GISC), Faculty of Medicine, Mansoura University, Egypt. After demographic, clinicopathological and survival data collection, histopathological evaluation was done for GC typing, staging and assessment of the histopathological prognostic parameters. IHC was performed for PD-L1, HER2 and CD8. PDL-1 was scored using the Combined Positive Score (CPS). **Results:** PD-L1 was expressed in 43.2% of GCs at a CPS cut-off value ≥ 1 . PDL-1 positivity was significantly associated with high TILs and CD8+ TILs ($p=0.008$, 0.016 respectively), indicating its contribution to tumor microenvironment along with the TILs. Multivariate analysis spotted PD-L1 positivity as an independent prognostic predictor for shorter OS in GC ($p=0.013$), with a tendency toward shorter DFS. Only 9.9% GCs were HER2 positive (score +3) with no significant association with PD-L1. **Conclusion:** PDL-1 is a promising prognostic and therapeutic target in GC that may direct the selection of patients for immunotherapy and checkpoint-blockade (pembrolizumab) therapy.

Keywords: Programmed Death-Ligand 1- gastric cancer- immunohistochemistry- TILs- survival

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Introduction

Gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide (Ferlay et al., 2021). Despite advances in the recognition of new risk factors, prevention, diagnosis and treatment of GC, it remains a global health problem and carries poor prognosis, as most of patients present with inoperable, advanced or metastatic disease requiring palliative treatment (Arai and Nakajima, 2020). The reported five-year survival for advanced or metastatic GC ranges between 5 to 20%, with a median overall survival (OS) of about one year (Global cancer observatory, 2020).

HER2 and Vascular endothelial growth factor receptor 2 (VEGFR2), (Trastuzumab and Ramucirumab respectively) are the only targeted therapies approved so far. As up to 20% of gastric tumors overexpress HER2, trastuzumab was introduced for clinical use in patients with advanced GC (Van Cutsem et al., 2015). Although trastuzumab extends the survival of HER2 positive patients, those patients typically develop therapeutic

resistance (Beer et al., 2020).

Notably, cancer immunotherapy has caused a paradigm shift from conventional therapies that target cancer cells directly to innovative therapies that utilize the host immune system (Sanmamed and Chen, 2018). Immune checkpoint inhibitors (ICIs), which target inhibitory receptors on immune effector cells and reactivate the immune response, have been highlighted over the past several years. In particular, the Programmed death-1 (PD-1)/ Programmed death-ligand 1 (PD-L1) axis has been identified as a promising target for ICIs (Kono et al., 2020). Programmed cell death 1/programmed cell death ligand 1 is a negative modulatory signaling pathway for activation of T cell (Helmy et al., 2020).

On molecular basis, The Cancer Genome Atlas (TCGA) Program has classified GC into four molecular subtypes: Epstein-Barr Virus (EBV)-positive (9%), Microsatellite Instability (MSI)-high (22%), genomically stable (20%) and chromosomal instability (50%) subtypes. Within these subtypes, EBV-positive and MSI-high GC were found to be associated with PD-L1 over-expression,

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so that there is a rationale for a potential response to immunotherapy in this entity based on the expression of PD-L1 in some subgroups (Beer et al., 2020).

Aim

This study was designed to evaluate immunohistochemical (IHC) expression of PD-L1 in gastric cancer (GC) and explore its prognostic role in terms of association with HER2 expression, different clinico-pathological variables, in particular density and cluster designation (CD)8 positivity in tumor infiltrating lymphocytes (TILs) and with patients' disease-free and overall survival (DFS, OS).

Materials and Methods

Patients and clinical data

This cross-sectional retrospective cohort study was conducted on formalin-fixed, paraffin-embedded (FFPE) tissue blocks for primary GCs obtained from resection specimens of 111 GC patients who were naive to preoperative chemotherapy or radiotherapy. Patients were diagnosed and operated at the Gastrointestinal Surgery Center (GISC) at our institute during the period from January 2014 to December 2018.

The demographic and clinicopathological data of the included 111 patients were retrospectively retrieved from the pathologic database of the Surgical Pathology Laboratory at the GISC including patients' age and gender, tumor site; size; and shape; histological type; depth of tumor invasion; nodal metastases, distant metastases, tumor stage, lymphovascular and perineural invasion. The follow-up data were collected via accessing patients' medical records, and telephone-based patient or relative interviewing. The follow-up data of concern included: the follow-up duration registered in months; the presence or absence of relapse either local recurrence or distant metastases that was obtained from radiological or histopathological investigatory data; DFS that was considered as the period from the date of primary surgery to the date of a documented relapse; disease-related mortality, and finally the OS that was calculated from the date of primary surgery till the time of disease specific death or last follow up.

Histopathological Evaluation

Routine, hematoxylin and eosin (H&E)-stained, 3-4 micrometer-thick, microscopic slides were prepared from all retrieved tissue blocks and were re-evaluated independently by two pathologists to (1) ascertain the diagnosis, (2) classify the tumors histopathologically according to the most updated WHO classification of gastric neoplasms (Klimstra et al., 2019), and (3) assess the tumor infiltrating lymphocytes (TILs). Despite no current consensus exists on the morphologic evaluation of TILs in GC, TILs are globally defined as the mean percentage of the invasive tumor area (including the tumor bed and peri-tumoral stroma) occupied by lymphocytes and plasma cells (Zhang et al., 2019). Based on this definition, the tumors included in this study were divided into three grades: grade 1 (low, $\leq 10\%$) that considered TILs-low,

grade 2 (moderate, 10–50%), and grade 3 (high, $>50\%$), patients with grade 2 or 3 were considered with TILs-high (Cheng et al., 2021).

Tissue Microarray Construction

The tissue microarray blocks (TMA) were constructed using a completely manual validated technique (Shebl et al., 2011). Four cores were taken from each case; two cores from the tumor's center and two cores from the tumor's invasive front to evaluate both tumor tissue and TILs respectively. Cores from tonsillar tissue were inserted in each block to be set as positive and negative control for PD-L1 and to verify its specificity (kluger et al., 2015). Similarly, cores of HER2-positive breast carcinoma were used as a positive control for HER2 and cores of splenic tissue and nodal tissue were used as a tissue control for CD8.

Immunohistochemistry

IHC was performed with Autostainer Link 48, using its optimized reagents with pharmDx kits EnVision™ FLEX Visualization Systems (Link code K8000) and EnVision FLEX Hematoxylin (Link code K8008) according to the user's-guide standardized procedure pre-programmed into the autostainer software. Pre-treatment (dewaxing and dehydration) of FFPE sections with heat-induced epitope retrieval (HIER) using the 3-in-1 specimen preparation procedure was done with these parameters: pre-heat temperature: 65°C; epitope retrieval: 97°C for 20 minutes; cool down to 65°C. The automated protocol is based on an indirect biotin-avidin system and uses a universal biotinylated immunoglobulin secondary antibody and diaminobenzidine (DAB) substrate. After the staining procedure has been completed, the sections were dehydrated, cleared and mounted.

For all the stained immunohistochemical antibodies, the interpretation of IHC was done semiquantitatively and independently by two examining pathologists using an ordinary light microscope, then scoring was done for each antibody based on its most appropriate specific scoring technique/ system. Anti-PD-L1 (QR001) Rabbit Monoclonal primary antibody (Quartett, Berlin, Germany, 1:100, Ready to use) was used and the CPS was applied for PD-L1 final scoring that was calculated by dividing the number of PD-L1 positive tumor cells, lymphocytes and histiocytes by the total number of vital tumor cells and then multiplying the result by 100. (Kulangara et al., 2019). A CPS ≥ 1 is considered positive (Shitara et al., 2018). Anti-HER2/neu (4B5) Rabbit Monoclonal primary antibody kit (Ventana/Roche Tissue Diagnostics) was used and staining reaction interpretation was based on the study of Hofmann et al. (2008), considering +3 as positive staining when moderate to strong complete or basolateral membranous reactivity in more than 10% of cancer cells is detected. For evaluation of cytotoxic T-lymphocytes, Anti-CD8 (Mouse Monoclonal primary antibody, clone C8/144B, ready to use, catalog number: IR62361-2) was used, the number of CD8 positive cells was assessed in the intra-tumoral stroma and at the invasive tumor front, then categorized into CD8 (low, negative at the cutoff of $\leq 10\%$) and CD8 (high, positive at the cut off of $>10\%$)

groups (Choi et al., 2020; Cheng et al., 2021).

Statistical analysis:

Statistical analyses were done using SPSS 20.0 (IBM Corporation, New York, USA). The associations of PD-L1 and HER2 with clinicopathological variables were assessed by the Pearson chi-Square (χ^2) test and Fischer Exact test (FET) that was used as correction for (χ^2) test when more than 20% of cells have count less than 5. To estimate PD-L1 and HER2 association with patients' survival, Kaplan–Meier curves were constructed, and the log-rank test was performed for the statistical comparison of two groups. For multivariate analysis, Cox regression analysis was used to calculate predictors affecting OS and DFS with calculation of hazard ratio. P-value was considered as significant if ≤ 0.05 .

Ethical considerations

This study was conducted upon approval of the committed Institutional Research Board (IRB) at Faculty of Medicine, Mansoura University, Egypt (Code Number: MD.19.06.191, 2019). Pathology code numbers of paraffin blocks were used instead of patients' names to ensure confidentiality and anonymity. All procedures followed the current revision of Helsinki Declaration of

medical research involving human subjects (The World Medical Association, 2013). Finally, the donor blocks were returned to archive for any additional patient's or investigative use.

Results

According to the aforementioned criteria for PDL-1, and HER2 IHC evaluation, 65.8% GCs were PD-L1 negative (CPS < 1), while 43.2% GCs were positive (CPS ≥ 1), 16 cases showed PD-L1 staining in the tumor cells only (Figures 3 and 4), 12 cases showed staining reaction involving both tumor cells and TILs (Figure 5) and 20 cases showed staining reaction in TILs only (Figure 6), 9.9% GCs were HER2 positive with score +3 (Figure 8), while 4.5% GCs were equivocal with score +2 and the remainder was negative (score 0 and +1) (Figure 9). Regarding TILs density, 32.4% GCs were TILs- high (Figure 1) while 67.6% GCs were TILs-low (Figure 2), and 70.3% GCs were CD8-low, while 29.7% GCs were CD8-high within TILs (Figure 7). As demonstrated in table 1, PD-L1 showed positive significant association with patient age, as the mean age of patients with PD-L1 positive carcinomas was significantly higher than that for patients with PD-L1 negative carcinomas (60.58 ± 12.828

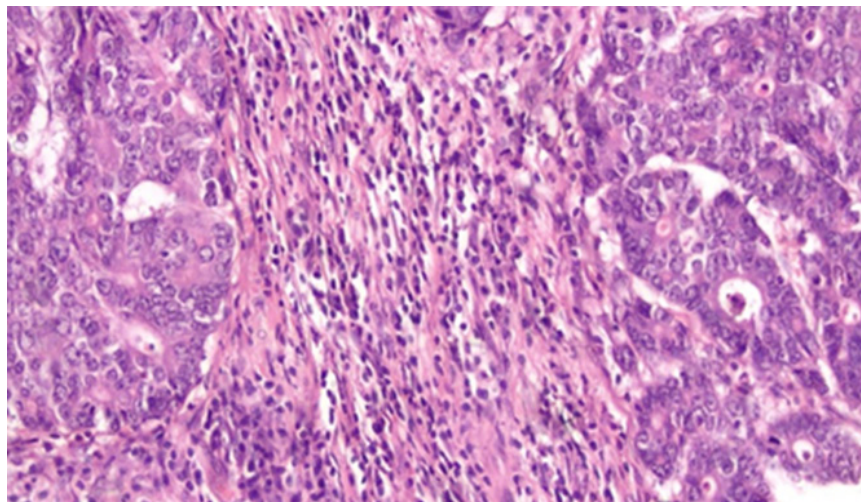


Figure 1. Tumor Invasive front with High TILs (H&E X100).

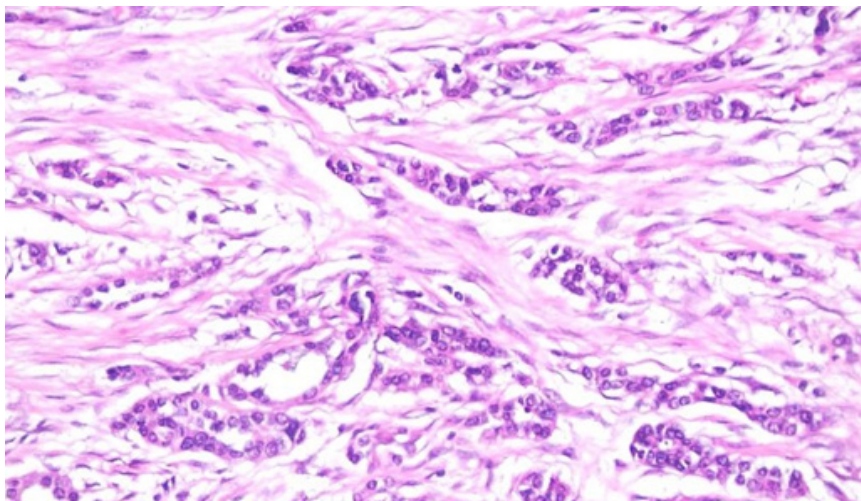


Figure 2. Tumor Invasive front with Low TILs (H&E x200).

Table 1. The Association between PD-L1 Expression and Different Clinicopathological Variables, CD8, and HER2 in Gastric Carcinoma

Variables	PD-L1 expression		Test of significance
	Positive CPS ≥ 1 n=48 (%)	Negative CPS < 1 n=63 (%)	
Age			$\chi^2 = 7.646$
≤ 58 Y (n=56; 50.5%)	17 (30.4)	39 (69.6)	p = 0.006*
>58 Y (n=55; 49.5%)	31 (56.4)	24 (43.6)	
Sex			$\chi^2 = 0.812$
Male (n=70; 63.1%)	28(40.0)	42 (60.0)	p = 0.367
Female (n= 41; 36.9%)	20 (48.8)	21 (51.2)	
Tumor site			$\chi^2 = 3.732$
Upper third (N= 26; 23.4%)	7 (26.9)	19 (73.1)	P = 0.155
Middle third (N= 26; 23.4%)	13 (50.0)	13 (50.0)	
Lower third (N=59; 53.2%)	28 (47.5)	31 (52.5)	
Tumor size			$\chi^2 = 0.054$
≤ 6 cm (N=75; 67.6%)	33 (44.0)	42 (56.0)	P = 0.816
> 6 cm (N=36; 32.4%)	15 (41.7)	21 (58.3)	
Histological type			FET = 0.807
Tubular adenocarcinoma (N=55; 49.5%)	22 (40.0)	33 (60.0)	P = 0.908
Mucinous adenocarcinoma (N=4; 3.6%)	2 (50.0)	2 (50.0)	
Poorly cohesive carcinoma (N=47; 42.3%)	22 (46.8)	25 (53.2)	
Undifferentiated carcinoma (N=5; 4.5%)	2 (40.0)	3 (60.0)	
Lauren classification			$\chi^2 = 0.338$
Intestinal type (N=59; 53.2%)	24 (40.7)	35 (59.3)	P = 0.561
Diffuse type (N=52; 46.8%)	24 (46.2)	28 (53.8)	
TILs			$\chi^2 = 6.931$
Low (N=75; 67.6%)	26 (34.7)	49 (65.3)	P = 0.008*
High (N=36; 32.4%)	22 (61.1)	14 (38.9)	
CD8+ TILs			$\chi^2 = 5.768$
Low (N=78; 70.3%)	28 (35.9)	50 (64.1)	P = 0.016*
High (N=33; 29.7%)	20 (60.6)	13 (39.4)	
Tumor depth of invasion			$\chi^2 = .632$
PT1-T2 (N=14; 12.6%)	7 (50.0)	7 (50.0)	P = 0.729
PT3 (N=85; 76.6%)	35 (41.2)	50 (58.8)	
PT4 (N=12; 10.8%)	6 (50.0)	6 (50.0)	
Lymph node metastasis			$\chi^2 = 0.632$
Negative (N=26; 23.4%)	13 (50.0)	13 (50.0)	P = 0.427
Positive=85; 76.6%)	35 (41.2)	50 (58.8)	
Distant metastasis			$\chi^2 = .055$
Negative (N=89; 80.2%)	38 (42.7)	51 (57.3)	P = 0.815
Positive (N=22; 19.8%)	10 (45.5)	12 (54.5)	
Tumor stage			$\chi^2 = 0.394$
I-II (N=46; 41.4%)	21 (45.7)	25 (54.3)	P = 0.821
III (N=43; 38.7%)	17(39.5)	26 (60.5)	
IV (N=22; 19.8%)	10 (45.5)	12 (54.5)	
Lymphovascular invasion			$\chi^2 = 2.406$
Negative (N=39; 35.1%)	13 (33.3)	26 (66.7)	P = 0.121
Positive (N=72; 46.9%)	35 (48.6)	37 (51.4)	
Perineural invasion			$\chi^2 = 0.120$
Negative (N=72; 64.9%)	32 (44.4)	40 (55.6)	P = 0.729
Positive (N=39; 35.1%)	16 (41.0)	23 (59.0)	
HER2			FET = 0.182
Negative (0, +1) (N=95; 85.6%)	41 (43.2)	54 (56.8)	P = 1.000
Equivocal (+2) (5; 4.5%)	2 (40.0)	3 (60.0)	
Positive (+3) (N=11; 9.9%)	5 (45.5)	6 (54.5)	

χ^2 , Chi-Square test; FET, Fisher's Exact Test; P, Probability value; *, statistically significant (P<0.05).

Table 2. Univariate and Multivariate Survival Analysis of the Disease-Free Survival (DFS) and Overall Survival (OS) in Gastric Carcinoma

Variables	Overall survival				Disease free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Histopathological parameters/ predictors	Median OS time / months	Log rank test	Hazard ratio 95.0% CI	P value	Median DFS time / months	Log rank test	Hazard ratio 95.0% CI	P-value
Age								
≤58 (r)	19.00 (14.29 - 23.71)	$\chi^2=6.623$	1.04 (1.01 - 1.06)	0.001*	21.00 (11.35-30.65)	$\chi^2=0.157$	-	0.021
>58	12.00 (10.90-13.09)	$P=0.010^*$			19.00 (10.17-27.28)	$P=0.692$		0.719
Sex								
Male	14.00 (8.96 - 19.04)	$\chi^2=0.853$			40.00 (14.52-65.48)	$\chi^2=6.42$		0.085
Female (r)	14.00 (11.13 - 16.87)	$P=0.356$			12.00 (8.77-15.23)	$P=0.011^*$	0.53 (0.26 - 1.09)	
Tumor site								
Upper third	13.00 (9.86 - 16.14)	$\chi^2=0.996$			10.00 (6.79-13.21)	$\chi^2=4.621$		
Middle third	13.00 (10.64-15.36)	$p=0.608$			18.00 (11.18-24.81)	$P=0.099$		
Lower third	15.00 (11.69-18.31)							
Tumor size								
≤6 cm	15.00 (12.36 - 17.64)	$\chi^2=0.096$			40.00 (14.08-65.92)	$\chi^2=0.466$		0.061
>6 cm	14.00 (12.29 - 15.70)	$P=0.756$			18.00 (11.23-24.77)	$P=0.495$		
Histological type								
Tubular adenocarcinoma (r)	20.00 (12.16 - 27.84)	$\chi^2=8.554$	-	0.055	40.00 (10.70-69.29)	$\chi^2=44.555$	-	0.002*
Mucinous adenocarcinoma	9.00 (0.00 - 18.80)	$P=0.036^*$	2.09 (0.61 - 7.21)	0.239	18.00 (3.59-32.40)	$P<0.001^*$	1.47 (0.18 - 11.95)	0.061
Poorly cohesive carcinoma	13.00 (10.30 - 15.70)		2.06 (1.21 - 3.49)	0.088*	10.00 (7.61 - 12.38)		17.44 (2.80-108.44)	0.29
Undifferentiated carcinoma	10.00 (5.19 - 14.80)		2.29 (0.66 - 8.02)	0.192	8		10.92 (0.90 - 132.53)	
Lauren classification								
Intestinal type (r)	15.00 (9.35 - 20.65)	$\chi^2=2.024$			40.00 (10.62-69.38)	$\chi^2=31.045$	0.39 (0.07 - 2.25)	0.164
Diffuse type	13.00 (10.53 - 15.46)	$P=0.155$			10.00 (8.15-11.85)	$P<0.001^*$		0.496
Tumor depth of invasion								
pT2-T1 (r)	13.00(5.28- 20.72)	$\chi^2=2.453$			26.00 (16.79 - 35.21)	$\chi^2=9.575$	-	0.091
pT3	14.00 (12.17-15.83)	$P=0.293$			9.00 (7.73 - 10.27)	$P=0.008^*$	1.58 (0.42 - 5.88)	0.744
pT4	10.00 (7.31 - 12.69)						3.85(0.81 - 18.35)	
Lymph node metastasis								
Negative (N0) (r)	19.00 (13.76 - 24.24)	$\chi^2=1.020$			40.00 (10.31 - 69.69)	$\chi^2=7.819$	0.79 (0.19 - 3.21)	0.021
Positive (N1, N2, N3)	14.00 (11.79 - 16.21)	$P=0.313$			17.00 (11.92 - 22.08)	$P=0.005^*$		
Distant metastasis								
Negative (M0) (r)	15.00 (10.96 - 19.041)	$\chi^2=2.963$			40.00 (20.35 - 59.65)	$\chi^2=49.595$		
Positive (M1)	12.00 (9.81-14.19)	$P=0.085$			9.00 (7.85 - 10.15)	$P<0.001^*$		

Table 2. Continued

Variables	Overall survival				Disease free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Histopathological parameters/ predictors	Median OS time / months	Log rank test	Hazard ratio 95.0% CI	P value	Median DFS time / months	Log rank test	Hazard ratio 95.0% CI	P-value
Tumor stage								
I-II (r)	14.00 (12.25 - 15.75)				40.00 (12.89 - 67.10)	$\chi^2 = 50.187$		
III	17.00 (12.08 - 21.92)	$\chi^2 = 4.038$			29.00 (10.08 - 47.92)	$P < 0.001^*$		
IV	12.00 (9.81 - 14.19)	$P = 0.133$			40.00 (10.31 - 69.69)			
Lymphovascular invasion					17.00 (11.92 - 22.0)			
Negative (r)	15.00 (12.24 - 17.76)	$\chi^2 = 1.003$				$\chi^2 = 6.561$		
Positive	13.00 (10.18 - 15.82)	$P = 0.317$			40.00 (20.35 - 59.65)	$P = 0.01^*$		
Perineural invasion								
Negative	18.00 (12.79 - 23.20)	$\chi^2 = 3.293$			29.00 (12.94 - 45.06)	$\chi^2 = 2.849$		
Positive	12.00 (10.10-13.89)	$P = 0.070$			12.00 (4.55 - 19.45)	$P = 0.091$		
TILs								
Low (r)	12.00 (10.88 - 13.12)	$\chi^2 = 9.661$	0.29 (.09 - .86)	0.026*	12.00 (10.85 - 13.15)	$\chi^2 = 16.269$	0.12 (.02 - .77)	0.025*
High	24.00 (16.95 - 31.05)	$P = 0.002^*$	-	-	29	$P < 0.001^*$		
CD8+ TILs								
Low (r)	13.00 (11.49 - 14.51)	$\chi^2 = 4.467$	1.17 (.39 - 3.50)	0.772	14.00 (9.30 - 18.70)	$\chi^2 = 6.757$	1.46 (.33 - 6.50)	0.622
High	20.00 (12.73 - 27.27)	$P = 0.035^*$	-	-	29.00 (24.58 - 33.42)	$P = 0.009^*$.12 (.02 - .77)	0.025*
PD-L1								
Negative (r)	19.00 (13.39 - 24.60)	$\chi^2 = 8.168$	1.92 (1.15 - 3.20)	0.013*		$\chi^2 = 2.797$		
Positive	13.00 (10.18-15.82)	$P = 0.004^*$	-	-	18.00 (8.93 - 27.07)	$P = 0.094$		
HER2								
Negative (0, +1)	14.00 (12.15 - 15.85)	$\chi^2 = 2.732$			21.00 (10.59 - 31.40)			
Equivocal (+2)	19.00 (3.97 - 34.03)	$P = 0.255$			19.00 (1.823 - 36.18)	$\chi^2 = 2.498$		
Positive (+3)	24.00 (10.47 - 37.53)				40.00 (7.78 - 72.22)	$P = 0.287$		

χ^2 , Chi-Square P: Probability value *statistically significant (if $P \leq 0.05$), statistically highly significant (if $P \leq .001$) N.B. Median is not reported because the survival step function does not cross the line $y=.5$. If the survival function does not reach .45, you will not be able to obtain a standard error or CI bounds for the median

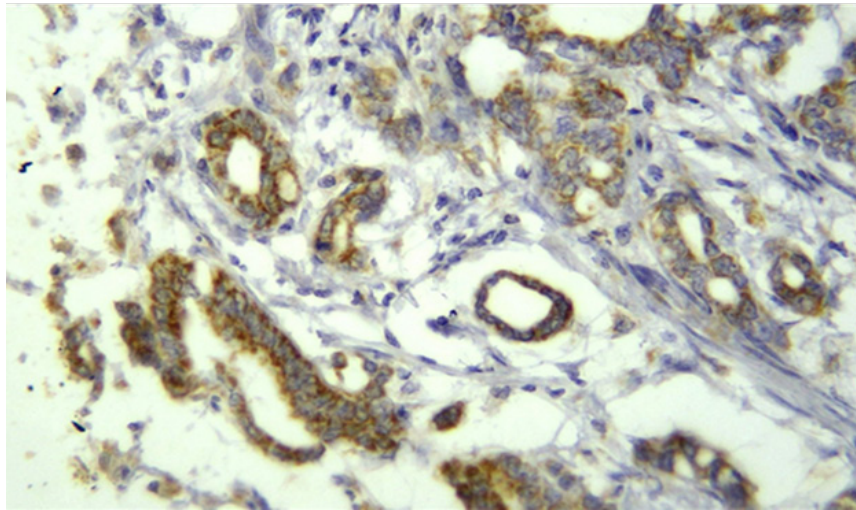


Figure 3. Moderately-Differentiated GC with PD-L1 Strong Membranous Staining in Tumor Cells (IHC, DAB x200)

versus 55.33 ± 11.769 , $p = 0.027$), and most of the patients aging less than 58 years (69.6%) had PD-L1 negative carcinomas compared to those aging above 58 years who had more frequently (56.4%) PD-L1 positive carcinomas ($P = 0.006$). There was a significant association between PD-L1 expression and TILs score. For emphasis, 22 out of the 36 carcinomas (61.1%) that showed lymphocytic-rich stroma were PD-L1 positive ($CPS > 1$) with a statistically significant P value of 0.008. A significant association was also noted between PD-L1 and high CD8 expression that was noted in 60.6% of PD-L1 positive carcinomas. While no observed significant association between PD-L1 and HER2 expression. Yet, HER2/neu tended to be more frequently positive (score +3) in larger size carcinomas ($P = 0.065$), fungating carcinomas ($P = 0.061$), and in Lauren's intestinal type carcinomas ($P = 0.066$).

The median period for DFS was 12 (2-63) months. Disease relapse occurred in 46 (41.4%) of patients. As shown in Table 2, Univariate analysis showed significant association between patient's DFS and female gender ($p = 0.011$), undifferentiated type GC ($p < 0.001$), diffuse Laurén type GC ($p < 0.001$), pT4 tumor depth of invasion ($p = 0.008$), positive nodal and distant metastases ($p = 0.005$;

< 0.001), tumor stage IV ($p < 0.001$), lymphovascular invasion ($p = 0.01$), low TILs ($p < 0.001$), and low CD8+TILs ($p = 0.009$) (Figure 10). There was a tendency of PD-L1-positive carcinomas and HER2/neu equivocal and negative carcinomas to occur in patients with lower DFS compared to the PD-L1-negative and HER2/neu score +3 carcinomas respectively, however this tendency has not reached the level of statistical significance ($p = 0.094$ and 0.287). Multivariate Cox regression analysis was applied to investigate the effect of the DFS-significantly associated parameters (in the univariate analysis) on the occurrence of earlier relapse in gastric carcinoma patients. Based on the abovementioned analysis, poorly-cohesive type gastric carcinoma was found to be an independent prognostic predictor for lower DFS (Hazard ratio [HR] = 17.441, 95.0 % Confidence interval [CI]: from 2.805 to 108.437 with $P = 0.002$).

The median OS was 13 (3-63) months and 78 patients (70.3%) died during follow-up period. Univariate survival analysis showed significant association between shorter OS and older patients' age ($p = 0.01$), poorly-cohesive type GC ($p = 0.036$), low-density TILs ($p = 0.002$), and low CD8+ TILs ($p = 0.035$) (Figure 10). Moreover, PD-L1

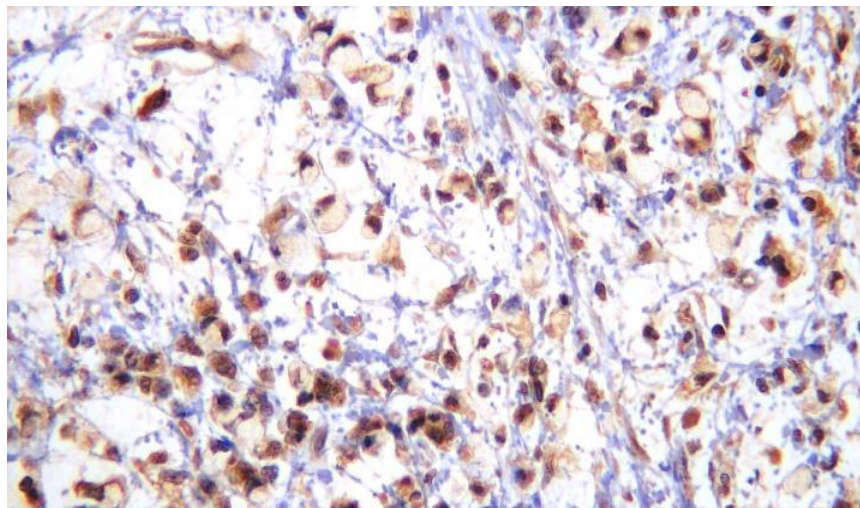


Figure 4. A Case of Signet Ring Cell Carcinoma with PD-L1 Membranous and Cytoplasmic Reaction in Tumor Cells (IHC, DAB x 400).

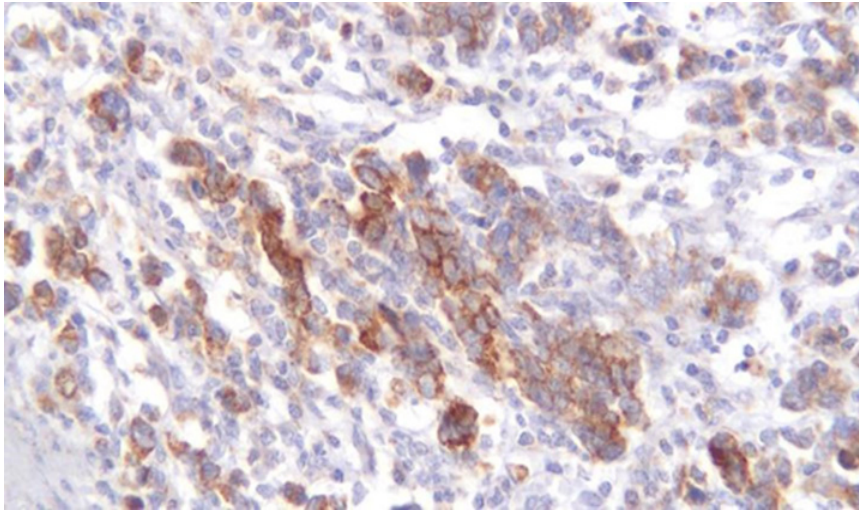


Figure 5. Poorly Differentiated Gastric Carcinoma with PD-L1 Membranous Staining in Tumor Cells & Cytoplasmic Staining in TILs (IHC, DAB x200)

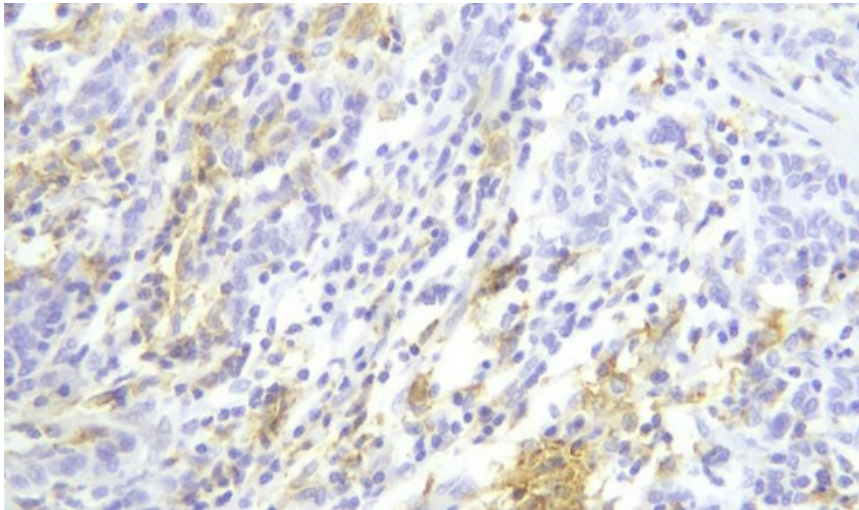


Figure 6. Poorly-Differentiated GC with PD-L1 Cytoplasmic Staining in TILs and Negative Tumor Cells (IHC, DAB x400).

positivity was significantly associated with shorter OS period ($p=0.004$), but no significant association was identified between HER2 expression and OS. Multivariate analysis reported that increased patients' age ($p=0.001$),

poorly-cohesive type gastric carcinoma ($p=0.008$), low TILs ($p=0.026$) and PD-L1 positive expression ($p=0.013$) are considered independent prognostic predictors for lower OS in GC patients (Table 2).

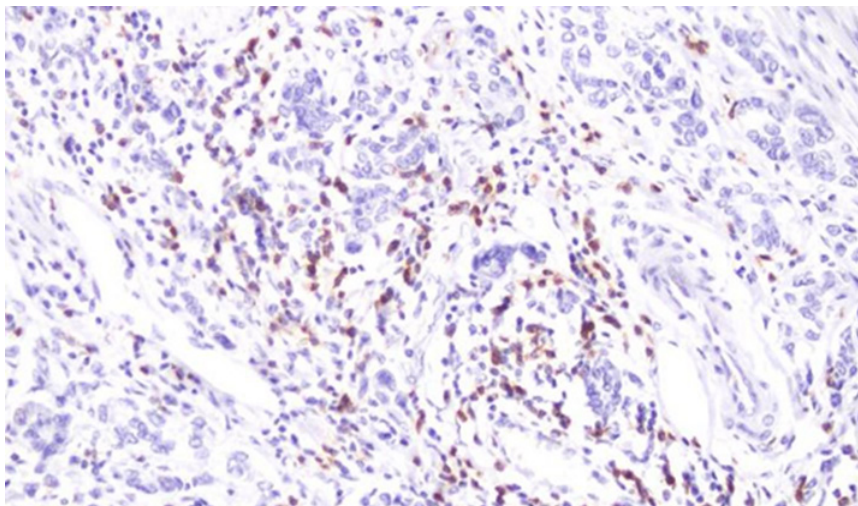


Figure 7. The Previous Case with Positive / high CD8 TILs (IHC, DAB x400).

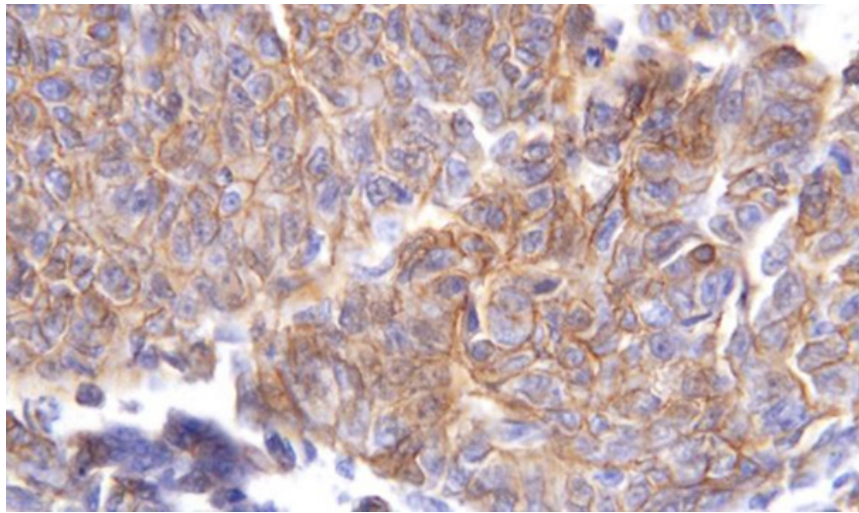


Figure 8. HER2 score +3 Membranous Staining (IHC, DAB x200)

Discussion

PD-L1 and HER2 are currently considered as prognostic markers and therapeutic targets in many human cancers. However, the prognostic role of PD-L1 in GC is still a subject of controversy and the relationships between PDL-1 expression and the clinicopathological features, tumor microenvironment, and HER2 status are still under investigation (Wang et al., 2018). Therefore, this cohort study aimed to evaluate PD-L1 and HER2 IHC expression and their possible association with the prognostic factors and survival in 111 GC patients.

In agreement a previous report (Kim et al.2016; Gao et al., 2017), PD-L1 was expressed in 43.2% of GC in the present study using the CPS at the cut-off ≥ 1 . Other studies described frequencies of PD-L1 positivity that ranges from 15.3% (Kang et al., 2016) up to 69.4% (Chang et al., 2016; Cho et al., 2017; Gu et al., 2017). Moreover, this study disclosed HER2 positive expression in 9.9% of GC. Yet, the frequency of HER positive GC ranges from 4% to 64% (Chua and Merrett, 2012; Chan et al., 2012). Such variations in PDL-1 and HER2 expression are attributable to the differences in: the interpretations of the

staining pattern, the scoring methods, the adopted cut-off value, as well as the different monoclonal antibodies used by the investigators.

The present study showed a statistically significant association between PD-L1 expression and the increasing patient's age, that matched with the findings of Wang et al., (2018) but contrasted to that of Oki et al., (2017). Furthermore, this study disclosed a significant association between positive PD-L1 and high both TILs ($p=0.008$) and CD8+ TILs ($p=0.016$). In the same vein, Ju et al. (2017) and Wang et al., (2018) reported PD-L1 expression in tumor cells and immune cells to be positively associated with the density of CD3+ and CD8+ TILs. Thus, the evaluation of tumor microenvironment in GCs seems imperative, as TILs-density may direct the selection of cases that could get more benefit from immunotherapeutic agents, in particular anti-PD-L1 therapies. In support to our observations, Sughayer et al., (2020) found that PD-L1 is not associated with any of the assessed clinico-pathological variables in their study.

Combined detection of the HER2 gene and PD-1/PD-L1 in gastric cancer provides an important reference index for the prognosis of GC and the benefit of both

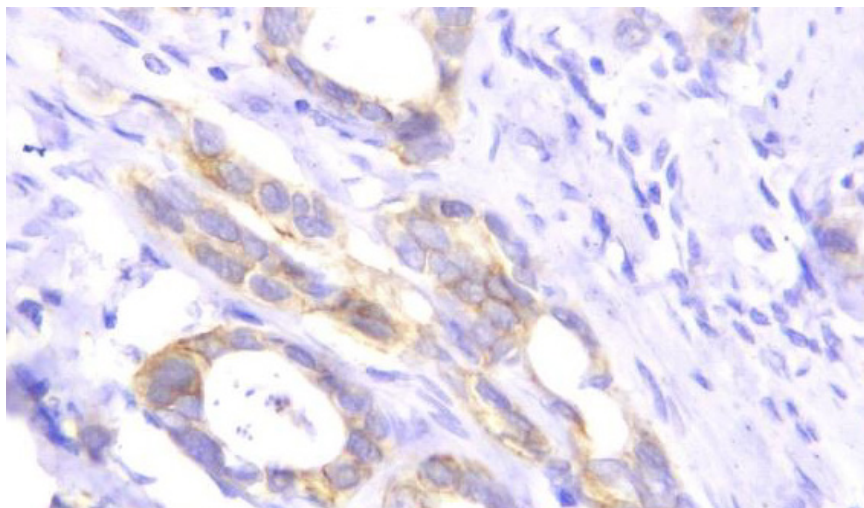


Figure 9. HER2 score +2 Lateral and Basolateral Membranous Staining (IHC, DAB x200) in GC Tumor Cells

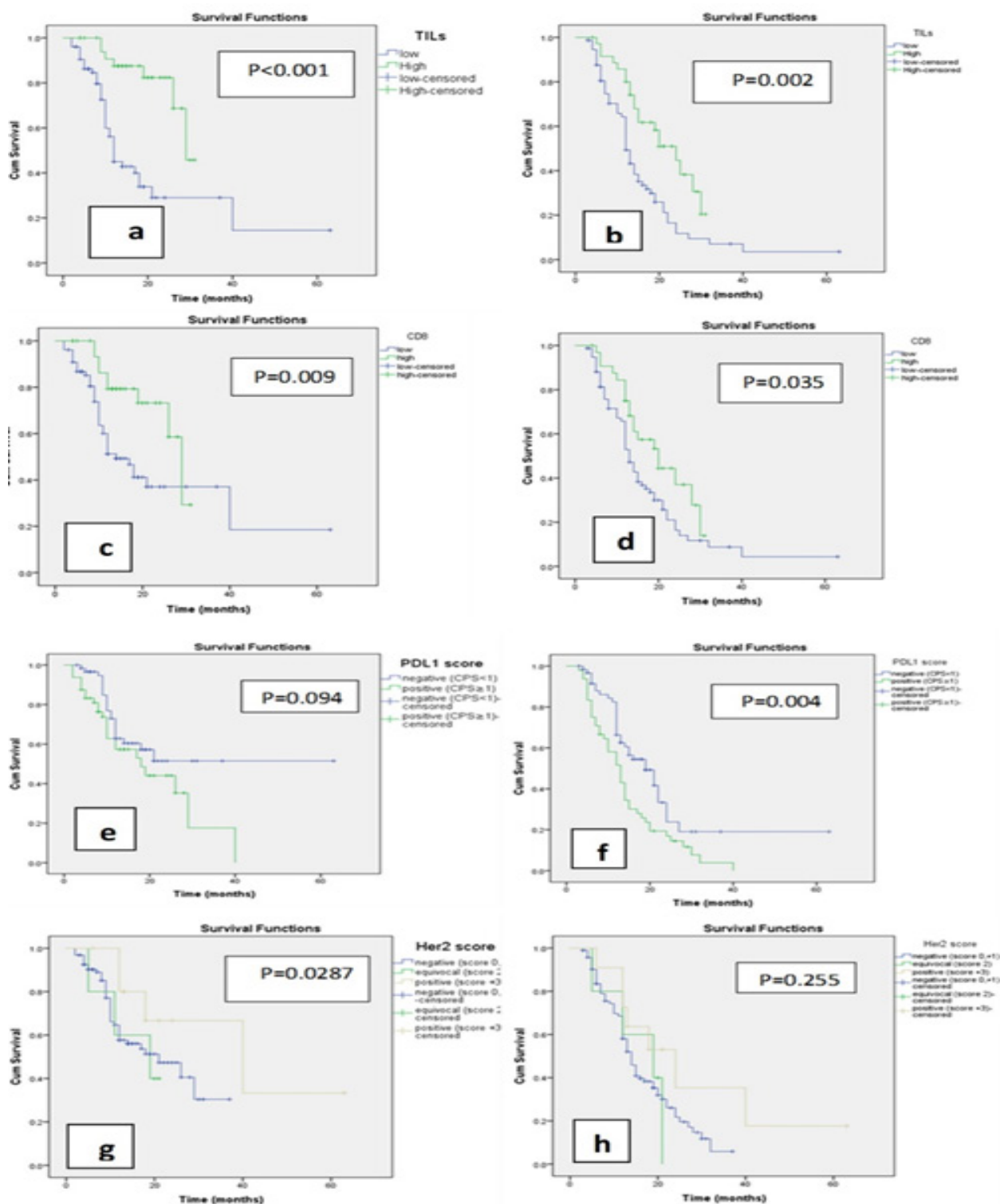


Figure 10. Kaplan-Meier Survival Curves for Patient with Gastric Carcinoma (GC) Stratified by Different Variables. Significantly lower disease-free survival (DFS) (a; log-rank; $p < 0.001$) and overall survival (OS) (b; log-rank; $p = 0.002$) in patients with low-density tumor infiltrating lymphocytes (TILs) compared to those with high density TILs GCs. Significantly lower DFS (c; log-rank; $p = 0.009$) and OS (d; log-rank; $p = 0.035$) in patients with low CD8-expression compared to those with high-expression GCs. No statistically significant association between PD-L1 and DFS (e; log rank; $p = 0.094$). Significantly lower OS in patients with PD-L1 positive compared to PD-L1 negative GCs (f; log-rank; $p = 0.004$). No statistically significant association between HER2 and both DFS and OS (g and H; log rank; $p = 0.0287$ and 0.0255).

HER2-targeting (Trastuzumab) and immunotherapy-based (nivolumab and pembrolizumab) drugs (Yun et al., 2020). Some studies reported a significant association between HER2 negativity and PD-L1 positivity (Wang et al., 2018),

meanwhile, others reported a high frequency of PD-L1 expression in the HER2 positive GCs (Oki et al., 2017). However, no association was noted between PD-L1 and HER2 expression in the present study ($p = 1.00$). This

discrepancy may be due to different studied population, different scoring methods or different monoclonal antibodies used.

By Multivariate analysis, the present work confirmed that increased patient's age, poorly-cohesive type GC, and low TILs ($p=0.001$, 0.008 and 0.026 respectively) are considered as independent prognostic factors for lower OS. These results accord with that of Kao et al. (2019) and Tian et al. (2021) who reported the same factors as independent predictors for shorter OS in advanced GC. As TILs contribute for inhibiting cancer progression, leading implications for the success of active cancer immunotherapy are warranted.

Regarding the association between PD-L1 expression and patient outcomes in this work, PD-L1 was found to predict poor OS despite lacking significant association with DFS. PD-L1 positivity associated with a shorter OS period ($p=0.004$), and PD-L1 positive expression was considered an independent poor prognostic factor for OS in GC patients ($p=0.013$). These findings conform to that of Shigemori et al., (2019) and fits to the theory of Cancer- Immunity Cycle (Chen and Mellman, 2013). However, contradictory data arise from the studies of Sughayer et al., (2020) who found that PD-L1 is an independent favorable prognostic factor for OS ($p=0.05$) and, Kawazoe et al., (2016) who indicated that PD-L1 has no prognostic role in GC.

As reported in the study by Janjigian et al., (2012), HER2 expression was not associated with either DFS or OS in our cohort. Several other studies suggested that HER2 overexpression associates significantly with worse prognosis (Dang et al., 2012). Thus far, the prognostic value of HER2 status in GC remains controversial, some studies reported HER2 positivity as an adverse prognostic factor, while some linked HER2 to better survival, and other studies even denied the association between HER2 and patients' survival (Kataoka et al., 2013). This discrepancy may be explained by different primary antibody used, the method of IHC scoring, different follow-up periods and the protocol of follow-up.

In conclusion, PD-L1 was spotted to be an independent prognostic predictor for survival of GC patients, being associated with the TILs level. Therefore, PDL-1 could be considered as a promising prognostic and therapeutic target in GC that may direct the selection of patients for immunotherapy and checkpoint-blockade therapy (Kawazoe et al., 2017).

Author Contribution Statement

All authors contributes equally, all authors reviewed the results and approved the final version of the manuscript.

Acknowledgments

None.

Compliance with Ethical Standards

This study was conducted upon approval of the committed Institutional Research Board (IRB) at Faculty

of Medicine, Mansoura University, Egypt (Code Number: MD.19.06.191, 2019). The study was processed under the ethical standards of the Helsinki declaration.

Conflict of interest statement

The authors declare no relevant financial affiliations or conflicts of interest.

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