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Expression of PD-1 in Tumor Cells is Associated with Shorter Survival in Non-metastatic Intestinal-type Gastric Adenocarcinoma

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ABSTRACT

There is an urgent need to discover novel prognostic biomarkers and treatment strategies for gastric cancer (GC) patients. Several immune-related markers have been proposed as prognostic tools and immunotherapeutic targets to manage diseases. In this regard, we evaluated the expression pattern and prognostic significance of programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), $CD45RO^+$ tumor-infiltrating lymphocytes (TILs), and DNA mismatch repair (MMR) proteins (MLH1, MSH2, PMS2, and MSH6) in non-metastatic intestinal-type gastric adenocarcinoma.

Samples and data from 70 GC patients were collected. Immunohistochemistry staining was used to detect the markers. We then evaluated the prognosis significance of each marker and their intercorrelation.

Cytoplasmic PD-1 expressed by tumor cells was significantly associated with poorer survival. However, multivariate analysis indicated stronger prognostic values for TNM stage, tumor location, and extracellular mucin. A significant positive association was found between $CD45RO^{high}$ TILs and PD-1 expression on tumor-infiltrating cells (TICs). All GC patients with deficient MMR (d-MMR) had a high number of $CD45RO^+$ TILs and $PD\text{-}1^+$ TICs.

Despite the association of PD-1 overexpression in TCs with shorter overall survival, histopathological factors, including tumor location, TNM stage, and extracellular mucin, remain the most decisive prognostic factors in non-metastatic intestinal-type gastric adenocarcinoma. Additionally, our data support a prognostic role for d-MMR and CD45RO, but not PD-1 and PD-L1 expression on TICs.

Keywords: Immunohistochemistry; CD45RO antigens; DNA mismatch repair; PD-L1; PD-1; Stomach neoplasms

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INTRODUCTION

Gastric cancer (GC) remains the fifth cause of cancer morbidity and the third cause of cancer mortality worldwide.[1] GC is a heterogeneous disease, classified by histological characteristics; these include the intestinal (the most common type related to *Helicobacter pylori*) and diffuse types.[2] Despite significant improvements in science and medicine, GC still has one of the lowest five-year survival rates among all cancers.[3]

Recently, many studies have emphasized the vital role of host antitumor immune responses in cancer patients. Several immune-related markers are selected as prognostic tools.[4] The best example is Immunoscore, which classifies colorectal cancer patients according to their $CD3^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs).[5] Additionally, several immune-related markers have been used as immunotherapeutic targets, such as anti-programmed death-1 (anti-PD-1) and anti-programmed death-ligand 1 (anti-PD-L1) immune checkpoint inhibitors (ICI).[6] As T cells become activated in the tumor microenvironment (TME), they express PD-1 on their surfaces, which binds to PD-L1 on tumor cells (TCs). Through this mechanism, tumors escape immunosurveillance by attenuating T cell receptor signaling. PD-1 and PD-L1 blockade reinvigorate T cells and potentiate antitumor immunity.[7] However, ICIs lead to positive results in a small group of GC patients due to the heterogeneity in TME components and tumor cells' intrinsic features.[8]

One proposed explanation for the heterogeneity of tumors is tumor microsatellite instability (MSI). MSI is a condition of genetic hypermutability due to a defective DNA mismatch repair (MMR) system. MMR proteins, including human MutL homolog 1 (MLH1), PMS1 homolog 2 (PMS2), mutS homolog 2 (MSH2), and mutS homolog 6 (MSH6), have a unified function of identification and correction of spontaneous base-base mismatches and insertions/deletions occurring during DNA replication. A deficient DNA MMR system increases the MSI rates, leading to a higher tumor mutational burden (TMB) and increases neoantigens, resulting in increased immune infiltration.[9] MSI is a characteristic of TCs and creates a genetic fingerprint that is specific to each tumor.[10] Recent clinical trials have shown the importance of MSI in predicting the effectiveness of anti-PD-1 immunotherapy in some cancers, including GC.[11]

Among different subpopulations of lymphocytes present in TME including $CD3^+$, $CD8^+$ cytotoxic, and $CD45RO^+$ memory TILs have been shown to be strongly associated with good clinical outcomes in colorectal cancer patients.[12,13] $CD45RO^+$ TILs are memory cells that have lower activation thresholds and vigorously proliferate via low levels of immunostimulants. Additionally, due to their self-renewal ability, they have a long lifespan, increasing immunosurveillance.[14] However, their involvement in the survival of GC patients remains to become clear.

Due to the importance of the immune-related markers of the TME, this study was conducted to investigate the expression of PD-1 and PD-L1 (as potential immune-suppressing markers) and their association with $CD45RO^+$ TILs densities (as indicators of efficient antitumor immunity). Additionally, the significance of the MMR system, which is an indicator of TMB, was assayed in non-metastatic intestinal-type gastric adenocarcinoma. Moreover, the prognostic significance of the mentioned markers was evaluated.

MATERIALS AND METHODS

Patients

In this retrospective study, medical records of GC patients undergoing gastrectomy at Imam Reza Hospital (Mashhad, Iran) between 2004 and 2017 were reviewed. Finally, 70 primary intestinal-type GC patients without a history of preoperative chemotherapy or radiation therapy, who had complete clinicopathologic and follow-up data, were included in the study. Formalin-fixed paraffin-embedded (FFPE) tissue blocks of the included patients were used as clinical samples. The 8th edition of the Union of International Cancer Control/American Joint Committee on Cancer (AJCC) TNM stage for GC[15] was considered the reference to determine the patients' TNM stages. Written informed consent forms were signed by all study participants. Patients' clinicopathological characteristics were extracted from their pathology reports and are presented in Table 1.

Immunohistochemistry

The FFPE tumor tissue blocks were cut into sets of seven consecutive sections of 3-μm thickness for immunohistochemistry (IHC) staining of seven targets, including MLH1, PMS2, MSH2, MSH6, PD-1, PD-L1, and CD45RO (Table 2). Sections were then mounted on

positively charged glass slides. For deparaffinization, sections were placed at 60°C for 15 minutes and immersed in xylene for 30 minutes. Decreasing ethanol solutions (100% and 96%) were used for rehydration. Antigen retrieval was performed in a pressure cooker for 18 minutes using a high pH retrieval solution (Tris-EDTA pH 9). The sections were subsequently treated with 10% H_2O_2 and 10% goat serum to block the endogenous peroxidase and nonspecific hydrophobic interactions. Then, primary antibodies specific to the targets were incubated for 1 hour in a humid chamber at room temperature on slides. After washing with phosphate-buffered saline (PBS), they were incubated with an enhancer and poly-HRP for 20 and 25 minutes,

Table 1. Clinicopathological characteristics of patients

Characteristics		n (%)
Age (years)	Total	64 (34–83)
	≤64	34 (48.6)
	>64	36 (51.4)
Sex	Male	52 (74.3)
	Female	18 (25.7)
Tumor location	Cardia	24 (35.8)
	Non-cardia	43 (64.2)
Tumor size (cm)	≤5	43 (63.2)
	>5	25 (36.8)
T stage	T1	1 (1.4)
	T2	9 (12.9)
	T3	46 (65.7)
	T4	14 (20)
N stage	N0	23 (32.9)
	N1	14 (20)
	N2	18 (25.7)
	N3	15 (21.4)
TNM stage	I	6 (8.6)
	II	26 (37.1)
	III	38 (54.3)
Histological grade	I	22 (31.4)
	II	38 (54.3)
	III	10 (14.3)
LVI	Positive	36 (51.4)
	Negative	34 (48.6)
Neural Involvement	Positive	36 (51.4)
	Negative	34 (48.6)
Margin involvement	Free	53 (75.7)
	Involved	16 (22.9)
Extracellular mucin	Positive	14 (20.0)
	Negative	56 (80.0)
Intracellular mucin	Positive	6 (8.6)
	Negative	64 (91.4)
Surgery type	Total	51 (72.9)
	Distal-subtotal	19 (27.1)
Cancer death	Dead	43 (61.4)
	Alive	27 (38.6)

LVI: lymphovascular invasion, T: Depth of invasion ; N: Lymph Nodes; M: Metastases

Table 2. Antibodies were used for analysis with immunohistochemistry

Antibody	Category Number/Clone	Dilution	Source	Cell	Type of positivity
Anti-PD-1	Mouse monoclonal antibody, CAT. NO. IHC001-100	1/150	GenomeMe Richmond, Canada	TCs/TICs	Cytoplasmic staining Membrane staining
Anti-PD-L1	Rabbit monoclonal antibody, CAT. NO. IHC441-7	1/100	GenomeMe Richmond, Canada	TCs/TICs	Membrane staining
Anti-CD45RO	Mouse monoclonal antibody, Clone UCHL1	1/400	Dako, Denmark	TILs	Membrane staining
Anti -MLH1	Mouse monoclonal antibody, CAT. NO. IHC409-100	1/200	GenomeMe Richmond, Canada	TME cells	Nuclear staining
Anti-PMS2	Mouse monoclonal antibody, CAT. NO. IHC422-7	1/200	GenomeMe Richmond, Canada	TME cells	Nuclear staining
Anti-MSH2	Mouse monoclonal antibody, CAT. NO. BMS034	Ready-to-use	Zytomed, Germany	TME cells	Nuclear staining
Anti-MSH6	Mouse monoclonal antibody, CAT. NO. IHC026-7	Ready-to-use	GenomeMe Richmond, Canada	TME cells	Nuclear staining

PD-1: programmed death-1, PD-L1: programmed death-ligand 1, TCs: tumor cells, TICs: tumor-infiltrating cells, TILs: tumor-infiltrating lymphocytes, TME: tumor microenvironment cells

respectively. As instructed by the detection kit [Meda View™ Two-step Polymer-HRP Anti-Mouse&Rabbit System, Medaysis], 3,3'-diaminobenzidine (DAB) was used to visualize the reaction. Then, tissues were counterstained with hematoxylin, dehydrated in increasing graded ethanol, and immersed in xylene for cleaning the slides. Finally, the sections were mounted with a permanent mounting medium. The IHC procedure was validated using appropriate controls, including immunostaining of human tonsils for PD-1, PD-L1, and CD45RO and normal colons for MMR proteins and negative controls (PBS instead of primary antibody). An expert pathologist evaluated the IHC-stained slides.

IHC Interpretation

Evaluation of PD-1 and PD-L1 Expression

PD-1 and PD-L1 expressions were measured semi-quantitively on both TCs and tumor-infiltrating cells (TICs). PD-L1 expression on TCs and TICs was categorized as positive and negative according to a 1% cutoff value.[16] A 10% cutoff point was used to determine PD-1 expression on TICs. In other words, PD-1 was considered positive when more than 10% of TICs expressed PD-1.[17] Also, according to a previous study for evaluating the PD-1 expression in TCs, a 50% cutoff point was determined.[18]

Evaluation of CD45RO$^+$ TILs

CD45RO$^+$ TILs were counted in five high-power fields, and their mean was considered as the number of CD45RO$^+$ TILs. Patients were stratified into high and low CD45RO$^+$ TILs according to the receiver operating characteristic (ROC) curve. GC samples with ≤135 CD45RO$^+$ TILs were considered CD45RO^{low}, and those with a higher number of CD45RO$^+$ TILs were categorized as CD45RO^{high}.

Evaluation of MMR System

If the expression of all four MMR proteins in TCs were at least 10%, the MMR system was considered intact (p-MMR). Whereas, if at least one of the MMR proteins was expressed in fewer than 10% of TCs while non-tumoral cells normally expressed MMR proteins, the MMR system was considered deficient (d-MMR).[20]

Statistical Analysis

Statistical analysis was performed using SPSS version 21. Pearson's chi-squared test and Fisher's exact test were used to determine the association between

PD-1, PD-L1 expression, infiltrating $CD45RO^+$ TILs, MMR status, and other clinicopathological characteristics. ROC curves were used to determine the optimal cutoff points for the $CD45RO^+$ TILs as our continuous IHC marker. Overall survival (OS) was measured as the period between the operation date and the date of cancer-related death. Univariable and multivariable Cox regressions were performed to evaluate the prognostic significance of the markers. Survival curves were obtained using the Kaplan–Meier (K-M) method. A $p<0.05$ was considered significant.

RESULTS

Patient Demographics

The clinicopathological characteristics of the study population (n=70) are reported in Table 1. The patients' mean age and tumor sizes were 64.9 years and 5.35 centimeters, respectively. According to Lauren's classification[21], all tumors were classified as intestinal subtype, of which 35.8% were cardia and 64.2% were non-cardia based on tumor anatomical location. Most of the patients were classified as stage III (54.3%). In most cases, the type of gastrectomy was total (72.9%). The average follow-up period was 29.35 months (0.5-81.5). During this time, 61.4% patients died, and 38.6% patients survived. Table 3 represents the frequency of stained markers, including CD45RO (Figure 1), PD-1, PD-L1 (Figures 2 and 3), and the MMR system (Figure 4). Interestingly, we observed PD-1 expression in TCs with a cytoplasmic pattern in 24.3% of cases, with about half of them expressing PD-1 in more than 50% of TCs. Additionally, 75.7% of patients had TICs expressing PD-1. Among all patients, 52.9% had $CD45RO^{high}$ TILs, 20% had $PD\text{-}L1^+$ TCs, and MMR status for 7.1% was deficient.

Table 3. Frequency of PD-1, PD-L1, CD45RO, and MMR status of intestinal-type gastric adenocarcinoma

Characteristics		n (%)
TICs-PD-1	<10%	17 (24.3)
	≥10%	53 (75.7)
TCs-PD-1	Negative	53 (75.7)
	Positive (1-50%)	9 (12.9)
	Positive (>50%)	8 (11.4)
TILs-CD45RO	≤135	33 (47.1)
	>135	37 (52.9)
TICs-PD-L1	<1%	26 (37.1)
	≥1%	44 (62.9)
TCs-PD-L1	<1%	56 (80.0)
	≥1%	14 (20.0)
MMR status	Deficient	5 (7.1)
	Proficient	65 (92.1)

PD-1: programmed death-1, PD-L1: programmed death-ligand 1, TCs: tumor cells, TICs: tumor-infiltrating cells, MMR: mismatch repair

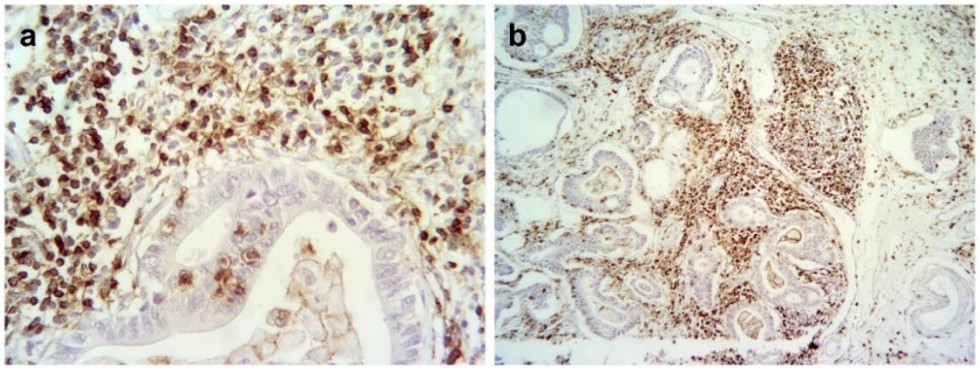

Figure 1. Immunohistochemical staining of CD45RO; membranous expression of CD45RO on tumor-infiltrating lymphocytes with (a) ×40 and (b) ×10 magnification.

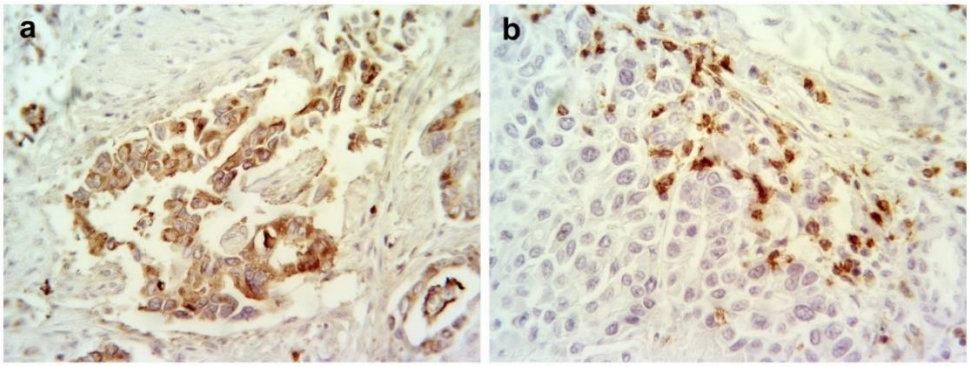

Figure 2. Immunohistochemical staining of programmed death-1 (PD-1); (a) cytoplasmic expression of PD-1 in tumor cells, and (b) membranous expression of PD-1 on tumor infiltrating cells with ×40 magnification.

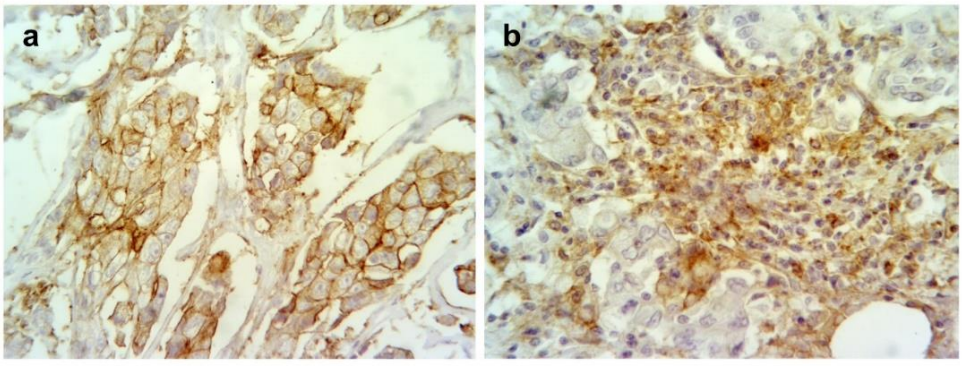

Figure 3. Immunohistochemical staining of programmed death-ligand 1 (PD-L1); membranous expression of PD-L1 on (a) tumor cells and (b) tumor infiltrating cells with ×40 magnification.

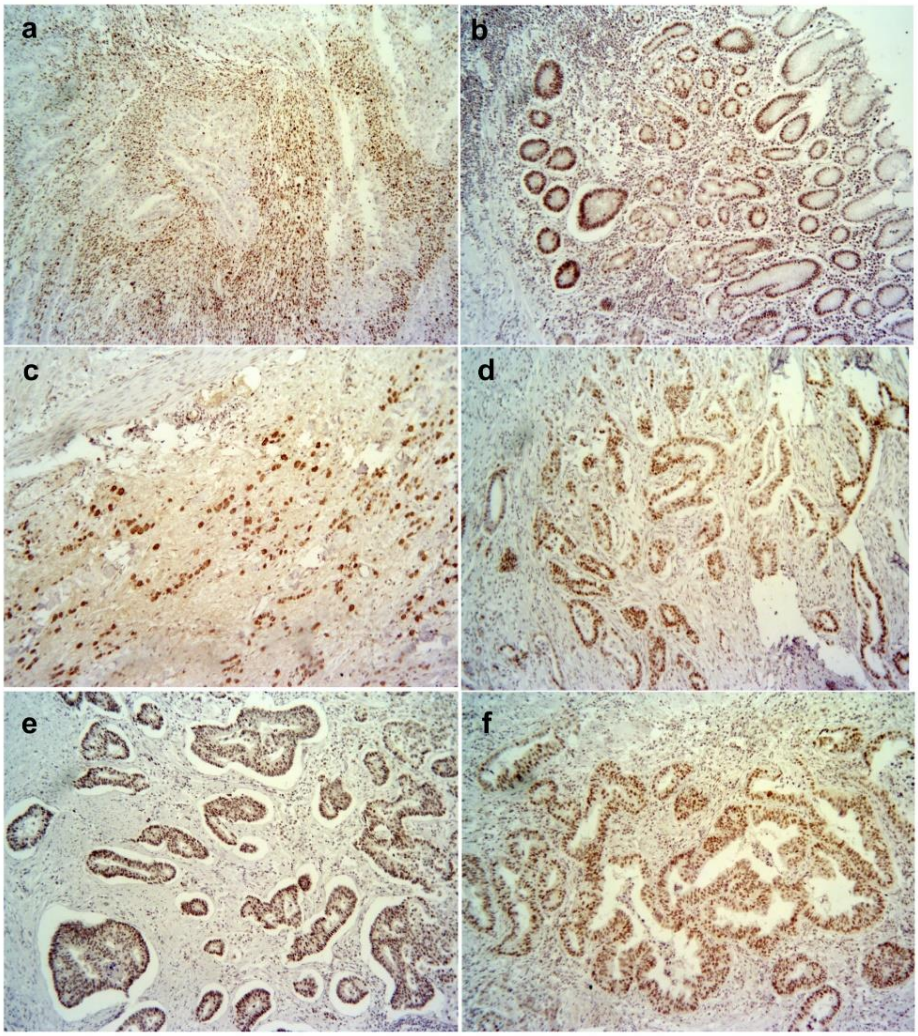

Figure 4. Immunohistochemical staining of mismatch repair proteins with the nuclear expression of MLH1, PMS2, MSH2, and MSH6 molecules representing (a) deficient MutL homolog 1 (MLH1), (b) normal mismatch repair, (c) positive MLH1, (d) positive PMS1 homolog 2 (PMS2), (e) positive mutS homolog 2 (MSH2), and (f) positive mutS homolog 6 (MSH6) on tumor cells with ×10 magnification.

Association between PD-1, PD-L1 Expression, CD45RO, and MMR Status

The associations between PD-1 and PD-L1 expression, CD45RO and MMR status are presented in Table 4, and the associations of the mentioned marker with patients' clinicopathological data are presented in Supplementary Table. The increased number of $CD45RO^+$ TILs was significantly associated with $PD\text{-}1^+$ TICs (p=0.026). Although not statistically significant, all d-MMR GC patients exhibited $PD\text{-}1^+$ TICs, higher proportions of $CD45RO^+$ TILs and $PD\text{-}L1^+$ TCs. In comparison, proficient MMR (p-MMR) GCs exhibited lower proportions of $CD45RO^+$ TILs, most of which had $PD\text{-}L1^-$ TCs; none of these associations reached statistical significance (p=0.325, p=0.056, and p=0.051, respectively). Additionally, the majority of $PD\text{-}1^+$ TCs were observed in females (p=0.007), and the rate of total gastrectomy was higher in patients with $PD\text{-}L1^-$ TCs (p=0.045). $PD\text{-}L1^+$ TCs and $PD\text{-}L1^+$ TICs were more frequent in patients with no history of neural invasion (p<0.001 and p=0.022, respectively), and the expression of PD-L1 on TCs was inversely associated with TNM stage (p=0.006) (Supplementary Table).

Table 4. The association between PD-1, PD-L1, and CD45RO expression, as well as MMR status of intestinal-type gastric adenocarcinoma

Variables	*TICs-PD-1*		*TCs-PD-1*			*TILs-CD45RO*		*TICs-PD-L1*		*TCs-PD-L1*		*MMR status*	
	<10%	**≥10%**	**Negative**	**1-50%**	**>50%**	**≤135**	**>135**	**<1%**	**≥1%**	**<1%**	**≥1%**	**Deficient**	**Proficient**
	p-value			***p-value***		***p-value***		***p-value***		***p-value***		***p-value***	
TICs-PD-1				0.182*		**0.026**		0.121		0.492*		0.325*	
<10%			12 (22.6)	1 (11.1)	4 (50.0)	12 (36.4)	5 (13.5)	9 (34.6)	8 (18.2)	15 (26.8)	2 (14.3)	0 (0.0)	17 (26.2)
≥10%			41 (77.4)	8 (88.9)	4 (50.0)	21 (63.6)	32 (86.5)	17 (65.4)	36 (81.8)	41 (73.2)	12 (85.7)	5 (100.0)	48 (73.8)
TCs-PD-1	0.182*					0.720*		0.288*		0.413*		0.088*	
Negative	12 (70.6)	41 (77.4)				26 (78.8)	27 (73.0)	17 (65.4)	36 (81.8)	41 (73.2)	12 (85.7)	2 (40.0)	51 (78.5)
1-50%	1 (5.9)	8 (15.1)				3 (9.1)	6 (16.2)	5 (19.2)	4 (9.1)	7 (12.5)	2 (14.3)	2 (40.0)	7 (10.8)
>50%	4 (23.5)	4 (7.5)				4 (12.1)	4 (10.8)	4 (15.4)	4 (9.1)	8 (14.3)	0 (0.0)	1 (20.0)	7 (10.8)
TILs-*CD45RO*	**0.026**			0.720*				0.174		0.338		0.056*	
≤135	12 (70.6)	21 (39.6)	26 (49.1)	3 (33.3)	4 (50.0)			15 (57.7)	18 (40.9)	28 (50.0)	5 (35.7)	0 (0.0)	33 (50.8)
>135	5 (29.4)	32 (60.4)	27 (50.9)	6 (66.7)	4 (50.0)			11 (42.3)	26 (59.1)	28 (50.0)	9 (64.3)	5 (100.0)	32 (49.2)
TICs-PD-L1	0.121			0.288*		0.174				**0.001**		1.000*	
<1%	9 (52.9)	17 (32.1)	17 (32.1)	5 (55.6)	4 (50.0)	15 (45.5)	11 (29.7)			26 (46.4)	0 (0.0)	2 (40.0)	24 (36.9)
≥1%	8 (47.1)	36 (67.9)	36 (67.9)	4 (44.4)	4 (50.0)	18 (54.5)	26 (70.3)			30 (53.6)	14 (100.0)	3 (60.0)	41 (63.1)

TCs-PD-L1	0.492*		0.413*		0.338		**0.001**				0.051*	
<1%	15 (88.2)	41 (77.4)	41 (77.4)	7 (77.8)	8 (100.0)	28 (84.8)	28 (75.7)	26 (100.0)	30 (68.2)		2 (40.0)	54 (83.1)
≥1%	2 (11.8)	12 (22.6)	12 (22.6)	2 (22.2)	0 (0.0)	5 (15.2)	9 (24.3)	0 (0.0)	14 (31.8)		3 (60.0)	11 (16.9)
MMR status	0.325*		0.088*		0.056*		1.000*		0.051*			
Proficient	17 (100.0)	48 (90.6)	51 (96.2)	7 (77.8)	7 (87.5)	33 (100.0)	32 (86.5)	24 (92.3)	41 (93.2)	54 (96.4)	11 (78.6)	
Deficient	0 (0.0)	5 (9.4)	2 (3.8)	2 (22.2)	1 (12.5)	0 (0.0)	5 (13.5)	2 (7.7)	3 (6.8)	2 (3.6)	3 (21.4)	

PD-1: programmed death-1, PD-L1: programmed death-ligand 1, TCs: tumor cells, TICs: tumor-infiltrating cells, MMR: mismatch repair. Values with $p<0.05$ are represented in bold, * the results of Fisher's exact test.

Survival Analysis

In univariate survival analysis, we evaluated the prognostic significance of clinicopathological markers (Table 5). Among the common clinicopathological factors, the following parameters were significantly associated with OS in the univariate Cox regression analysis: tumor location, gastrectomy type, LVI, extracellular mucin, T stage, N stage, and TNM stage. Among our IHC-evaluated cells, only PD-1^+ TCs were significantly associated with a lower OS (p=0.031). Patients with PD-1^- TCs and 1-50% expression of PD-1 in TCs had significantly longer OS than patients expressing PD-1 in more than 50% of TCs (p=0.012 and p=0.041, respectively). Nevertheless, there was no statistically significant difference between the patient's with 1-50% expression of PD-1 in TCs and the negative group (p=0.643).

Patients with $CD45RO^{high}$ TILs and PD-$L1^+$ TCs had longer OS, but they were not statistically significant (p=0.099 and p=0.141, respectively). Furthermore, survival analysis for MMR status indicated that d-MMR GC patients had longer OS than the proficient group, although it was not statistically significant (p=0.118) (Figure 5). According to the results of multivariate analysis, TNM stage, extracellular mucin, and tumor location but not PD-1 in TCs, are independent prognostic factors for intestinal-type gastric adenocarcinoma (p=0.003, p=0.011 and p<0.001, respectively) (Table 6).

Table 5. Univariate Cox proportional hazard analysis for overall survival of intestinal-type gastric adenocarcinoma

Variables		*HR*	*95% CI for HR*	*p value*
(Age <64)/(age≥64)		1.124	0.611-2.066	0.707
Sex (male/female)		1.810	0.836-3.919	0.132
Tumor location (cardia/non-cardia)		2.344	1.236-4.443	**0.009**
Gastrectomy (total/distal-subtotal)		3.631	1.524-8.652	**0.004**
Lymphovascular invasion (involve/free)		1.945	1.028-3.679	**0.041**
Neural invasion (positive/negative)		1.698	0.908-3.176	0.097
Margin involvement (involve/free)		1.260	0.614-2.583	0.528
Extracellular mucin (positive/negative)		2.901	1.458-5.770	**0.002**
Intracellular mucin (positive/negative)		1.984	0.773-5.094	0.154
T stage (T3+T4/T1+T2)		3.382	1.027-11.138	**0.045**
N stage (Involve/Free)		3.177	1.405-7.183	**0.005**
TNM stage (III/I+II)		3.730	1.855-7.502	**<0.001**
Histological grade (III/I+II)		1.271	0.533-3.035	0.589
TICs-PD-1 (positive/negative)		1.186	0.596-2.362	0.627
TCs-PD-1	Negative versus >50%	0.357	0.160-0.799	**0.012**
	1-50% versus >50%	0.279	0.082-0.949	**0.041**
TILs-CD45RO (low/high)		1.676	0.908-3.091	0.099
TICs-PD-L1 (positive/negative)		1.041	0.560-1.934	0.899
TCs-PD-L1 (positive/negative)		1.917	0.806-4.559	0.141
MMR status (deficient/proficient)		0.205	0.28-1.497	0.118

PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TCs, tumor cells; TIC, tumor-infiltrating cell; MMR, mismatch repair. Values with p<0.05 are represented in bold.

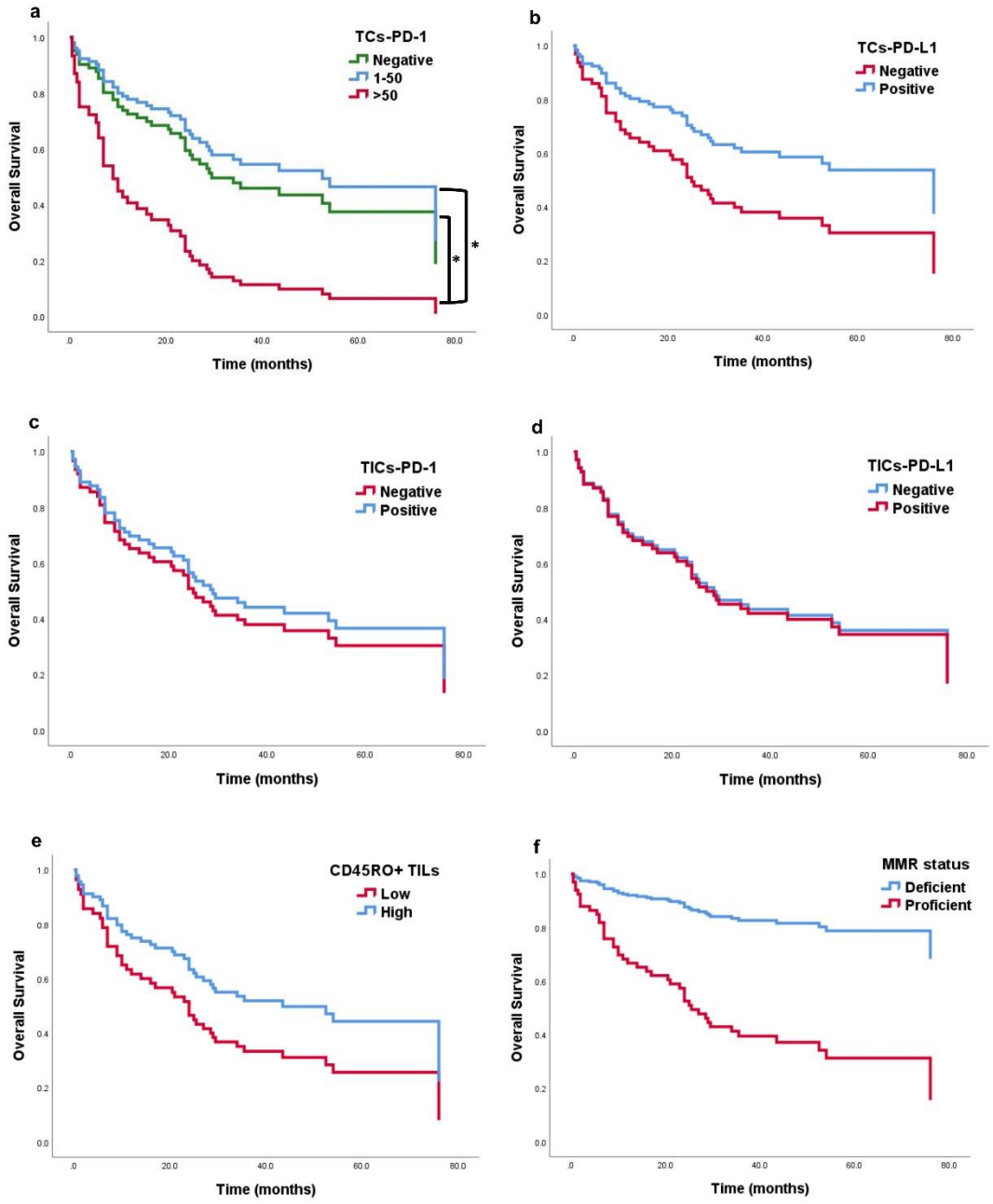

Figure 5. Kaplan-Meier estimates of overall survival in non-metastatic intestinal-type gastric adenocarcinoma according to the expression of (a) programmed death 1 (PD-1) and (b) programmed death-ligand 1 (PD-L1) on tumor cells, (c) PD-1 and (d) PD-L1 on tumor infiltrating cells, (e) CD45RO on tumor infiltrating lymphocytes and (f) mismatch repair status; *p<0.05.

Table 6. Multivariate Cox proportional hazard analysis for overall survival of intestinal-type gastric adenocarcinoma

Variables	HR	95% CI for HR	*P* value
Tumor location	0.362	0.184-0.710	**0.003**
Extracellular mucin	2.895	1.271-6.590	**0.011**
TNM stage	4.1194	2.017-8.712	**<0.001**

T: Depth of invasion; N: Lymph Nodes; M: Metastases, Values with $p<0.05$ are represented in bold.

DISCUSSION

Among the rare studies looking for the expression of PD-1 and PD-L1 on both tumor cells and tumor-infiltrating cells, ours was one of the first to examine their expression in non-metastatic intestinal-type gastric adenocarcinoma. We also investigated the association between CD45RO$^+$ memory TILs, PD-1, PD-L1, MMR status, and clinicopathological factors in non-metastatic intestinal-type gastric adenocarcinoma, as well as the role of these markers and clinicopathological factors in patients' OS.

PD-1 expression has been frequently observed in tumor-infiltrating immune cells but not in TCs. Besides reports of PD-1 expression on immune cells,[22-24] studies have indicated that non-small-cell lung cancer, ovarian high-grade serous carcinoma, Ewing's sarcoma, liver, and melanoma cancer cell lines express PD-1 protein.[22,25-28] A recent study demonstrated that hepatocellular carcinoma cell lines and clinical tissues probably contain subpopulations expressing PD-1 that lead to tumor growth even in the absence of immune stimulation.[26] The search results in The Cancer Genome Atlas (TCGA) database for human PDCD1 gene coding PD-1 protein in TME and PDCD1 transcriptomes from Cancer Cell Line Encyclopedia (CCLE) indicated that many tumor cells, including GC, express PD-1.[29]

In line with our study, a cutoff of 50% was considered by Van Erp et al, for analysing tumor cells expressing PD-1.[18] We found that cytoplasmic expression of PD-1 in more than 50% TCs was associated with shorter OS. In contrast, Machado et al, revealed that cytoplasmic and membranous expression of PD-1 in Ewing's sarcoma was related to increased survival.[28] However, due to a lack of consensus for a positive expression threshold of PD-1 in TCs the results vary between studies. This is at least in part due to different antibodies with different sensitivities used to detect these markers. Our study demonstrated that most of the PD-1$^+$ TCs cases were females. This result may be related to studies demonstrating gender-related responses to anti-PD-1/PD-L1 inhibitors in various cancers.[30,31] Therefore, we suggest a gender-based analysis of this molecule in future studies. Multivariate survival analysis excluded PD-1 from the model, indicating PD-1 expression may not be an independent prognostic factor, whereas extracellular mucin, tumor location, and TNM stage were significant independent prognostic factors. However, PD-1 needs to be evaluated in a larger population.

Furthermore, in line with a study by Angell et al,[32] we observed an association between PD-L1 expression on TCs and longer OS. In our study, it did not reach statistical significance. The inverse relationship between TCs-PD-L1 and neural invasion as well as TCs-PD-L1 and TNM stage supports this conclusion. According to Svensson et al, TICs expressing PD-1 and PD-L1 are associated with longer survival,[33] but in our study, they did not affect survival.

Infiltrating CD45RO$^+$ TILs can promise a potent prognostic tool for many solid tumors.[34-36] However, its role as a prognostic factor in GC remains unclear. Wakatsuki et al, studied the prognostic power of CD45RO$^+$ TILs in 101 patients with primary GC. They observed no prognostic significance for CD45RO$^+$ TILs in early-stage GC. By contrast, there was a significant positive association between CD45RO$^+$ TILs and better OS in advanced GC.[19] Lee et al, indicated that the CD45RO$^+$ TILs, CD3$^+$, and CD8$^+$ TILs levels are independent prognostic factors in GC.[37] Although statistically insignificant, we found that patients with higher CD45RO$^+$ TILs had a better OS. In our study, high infiltration of CD45RO$^+$ TILs was significantly associated with PD-1$^+$ TICs (p=0.026). This result indicates that most of the PD-1-expressing TICs might be memory TILs. In line with this result, Pauken et al, demonstrated that the PD-1 pathway regulates memory cell formation.[38] Furthermore, the memory cell effector

function may be reduced by PD-1 expression due to exhaustion.[39] These results may provide new insights regarding targeted immunotherapy for GC. However, more research is needed for confirmation.

MMR deficiency or proficiency of TCs can indicate its MSI status, representing the tumor's genetic instability and, in turn, its behavior (based on evidence that MMR status detected by IHC is a surrogate for the MSI genotype).[40,41] Tumors with d-MMR/MSI-H have an increased TMB, which can lead to an augmentation of neoantigens. As a result, immune cells tend to infiltrate into tumors, leading to a hot TME, and contributing to a prolonged survival. In contrast, p-MMR/MSI-L have a lower TMB and neoantigen load and, thus, a less effective antitumor immune response.[42,43] Also, our results demonstrated that d-MMR/MSI-H patients had longer OS than p-MMR/MSI-L patients, although the difference was not statistically significant.

Our data demonstrate that GC patients with d-MMR/MSI-H had a high count of $CD45RO^+$ TILs and more $PD\text{-}1^+$ TICs and $PD\text{-}L1^+$ TCs, representing a hot TME. This observation suggests that the immune response varied from microsatellite status in GC. In contrast to our results, Jin et al, did not observe any significant correlation between $PD\text{-}1^+$ TICs and $PD\text{-}L1^+$ TCs with d-MMR/MSI-H.[44] However, in line with our study, Sun et al, and Wang et al, observed that $PD\text{-}L1^+$ TCs were significantly associated with d-MMR/MSI-H.[45,46] Shin et al, and Wang et al, investigated the difference in the accumulation of $CD8^+$ cells in p-MMR and d-MMR GC and observed that $CD8^+$ cells increased in d-MMR GC patients.[16,47] We investigated $CD45RO^+$ TILs and observed that the proportion of $CD45RO^+$ TILs increased in compared to p-MMR patients. However, further research on $CD45RO^+$ TILs in GC is needed to confirm this result. Some studies have indicated that d-MMR/MSI-H tumors are chemoresistant.[48] As an alternative to chemotherapy, due to the highly infiltrated immune cells, d-MMR/MSI-H tumors are the best candidates for immune checkpoint inhibitor immunotherapy.

A phase II clinical trial (NCT02335411), assessing the safety and efficacy of pembrolizumab monotherapy in patients with advanced GC, demonstrated that MSI-H patients had a higher objective response rate (ORR). However, the data was not statistically significant due to the small group of patients with known MSI status.[49] In a phase III randomized clinical trial (NCT02494583), advanced GC patients with MSI-H experienced improved outcomes with pembrolizumab therapy.[50] A phase I/II trial examined nivolumab and nivolumab plus ipilimumab in patients with advanced esophagogastric cancer. The ORR was higher in patients with MSI-H advanced esophagogastric cancer.[51] Additionally, a meta-analysis of randomized clinical trials exploring CheckMate-649, JAVELIN Gastric 100, and KEYNOTE-061 trials demonstrated that remedial effects of anti-PD-1 agents were significantly higher in MSI-H patients compared to microsatellite stable.[52] Also, in a recent clinical trial PD-1 blockade resulted in a complete response, with dostarlimab in all d-MMR locally advanced rectal cancer patients.[53]

Gastric tumors have the potential for PD-1 expression, which may have clinical significance. At the univariate survival analysis level, PD-1 expression in more than 50% of TCs was significantly associated with shorter OS. Patients with d-MMR status had a higher proportion of $CD45RO^+$ TILs and $PD\text{-}L1^+$ TCs related to more prolonged survival; however, these data did not reach statistical significance. Finally, such studies on GC are limited, so we had limitations regarding the lack of standard references for the cutoff points. We highly recommend conducting several studies with larger sample sizes to find the most reliable cutoff points for the markers evaluated in this study.

STATEMENT OF ETHICS

All procedures performed in this study were performed in accordance with the Declaration of Helsinki. This study was approved by the Ethics Committee of the Shiraz University of Medical Sciences (Ethics code: IR.SUMS.REC.1400.280).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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