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ORIGINAL ARTICLE

Retrospective Study

Association between *Helicobacter pylori* infection, mismatch repair, HER2 and tumor-infiltrating lymphocytes in gastric cancer

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Abstract

BACKGROUND

The influence of *Helicobacter-pylori* (*H. pylori*) infection and the characteristics of gastric cancer (GC) on tumor-infiltrating lymphocyte (TIL) levels has not been extensively studied. Analysis of infiltrating-immune-cell subtypes as well as survival is necessary to obtain comprehensive information.

AIM

To determine the rates of deficient mismatch-repair (dMMR), HER2-status and *H. pylori* infection and their association with TIL levels in GC.

METHODS

Samples from 503 resected GC tumors were included and TIL levels were evaluated following the international-TILs-working-group recommendations with assessment of the intratumoral (IT), stromal (ST) and invasive-border (IB) compartments. The density of CD3, CD8 and CD163 immune cells, and dMMR and HER2-status were determined by immunohistochemistry (IHC). *H. pylori* infection was evaluated by routine histology and quantitative PCR (qPCR) in a subset of samples.

RESULTS

dMMR was found in 34.4%, HER2+ in 5% and *H. pylori*-positive in 55.7% of samples. High IT-TIL was associated with grade-3 ($P = 0.038$), while ST-TIL with grade-1 ($P < 0.001$), intestinal-histology ($P < 0.001$) and no-recurrence ($P = 0.003$). dMMR was associated with high TIL levels in the ST ($P = 0.019$) and IB ($P = 0.01$) compartments, and ST-CD3 ($P = 0.049$) and ST-CD8 ($P = 0.05$) densities. HER2- was associated with high IT-CD8 ($P = 0.009$). *H. pylori*-negative was associated with high IT-TIL levels ($P = 0.009$) when assessed by routine-histology, and with high TIL levels in the 3 compartments ($P = 0.002$-0.047) and CD8 density in the IT and ST compartments ($P = 0.001$) when assessed by qPCR. A longer overall survival was associated with low IT-CD163 ($P = 0.003$) and CD8/CD3 ($P = 0.001$ in IT and $P = 0.002$ in ST) and high IT-CD3 ($P = 0.021$), ST-CD3 ($P = 0.003$) and CD3/CD163 ($P = 0.002$).

CONCLUSION

TIL levels were related to dMMR and *H. pylori*-negativity. Low CD8/CD3 and high CD163/CD3 were associated with lower recurrence and longer survival.

Key Words: Lymphocytes; Macrophages; Gastric cancer; *Helicobacter pylori*; HER2; Mismatch repair

Core Tip: Absence of *Helicobacter pylori* was associated with high tumor-infiltrating lymphocyte (TIL) levels and CD8 density. Deficient mismatch-repair was associated with high TIL levels, and CD3 and CD8 density. Longer overall survival was associated with a low CD8/CD3 ratio, and high CD3 and CD3/CD163 ratio.

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INTRODUCTION

Gastric cancer (GC) is one of the most common cancers throughout the world and the second most frequent in Peruvian

males, and carries a poor prognosis[1-3]. The pathological features of GC can define tumor behavior and prognosis[4]. *Helicobacter pylori* (*H. pylori*) infection, which is highly prevalent in Peru, is an accepted trigger of GC and has recently been suggested to predict a lower effect of immunotherapy with checkpoint immune inhibitors[5].

Microsatellite instability (MSI) defines one of the four molecular GC subtypes[6]. It is usually detected by deficient mismatch-repair (dMMR) and has been associated with high levels of neoantigens, and along with HER2 positive status are biomarkers of response to checkpoint immune inhibitors and trastuzumab treatment, respectively[7-11]. A recent study found that the addition of checkpoint inhibitors to antiHER2 therapy in HER2 positive cases increases clinical response[12].

High levels of tumor-infiltrating lymphocytes (TILs) have been associated with longer survival and greater response to checkpoint inhibitors in different malignancies[13,14]. Information on the type, density and location of TIL subpopulations has been associated with tumor features, such as Epstein-Barr-Virus infection[4] and survival in gastrointestinal malignancies[15,16], and predicts response to checkpoint inhibitors in advanced GC[11,17-19]. Despite the relevance of *H. pylori* infection, dMMR and HER2 status in GC, few studies have evaluated their association with TIL levels in resected non-metastatic tumors. The studies available differ in the methodologies used to evaluate TIL and very few have included South American populations.

In the present study, TIL levels were determined following the International Immuno-Oncology Biomarkers Working Group: Part 2 recommendations[20] as well as the density of CD3+ T lymphocytes, CD8+ cytotoxic T lymphocytes and CD163+ M2 macrophages. In addition, the relationship of TIL levels with the clinicopathological features, including *H. pylori* infection, dMMR, HER2 status and survival in resected GC, was evaluated.

MATERIALS AND METHODS

Study population

We included information from 503 GC patients who underwent surgery at the Instituto Nacional de Enfermedades Neoplasicas in Lima, Peru from January 2008 to December 2018 and in whom pathology material was available. Clinicopathological features were obtained from medical histories and pathology reports of the patients, and hematoxylin and eosin (HE)-stained slides were prospectively reviewed when no specific information was found[21,22].

This single-center retrospective cohort study was approved by the Research and Ethics Committee (Protocol Number 050-2015-CIE/INEN), and the patients provided signed informed consent.

Evaluation of TIL levels and the presence of H. pylori

Several original sections from each primary tumor were re-examined and the most representative tissue block, with a 5 μm thickness and stained in HE was selected. Original and new sections were examined by experienced histopathologists (Sanchez J & Taxa L) for review of the standard pathological features, including the presence of *H. pylori,* and contrasted with original reports. The level of TILs was estimated avoiding ulcerated or necrotic areas and classified by spatial location [intratumoral (IT), stromal (ST) and invasive-border (IB) compartments (Figures 1 and 2)]. TIL levels above the median (calculated for every compartment) were classified as high[20].

Tissue array method and IHC staining

Core tissue biopsies (6 mm in diameter) were taken from tumoral areas with a high density of TIL in every individual paraffin-embedded tumor and 10-12 cores were re-arranged in a new recipient paraffin block (tissue array block) using the Quick-Ray Manual Tissue Microarrayer (Unitma Co., Ltd., Seoul, Korea). Sections (4 mm) were taken from each tissue array block, deparaffinized, and dehydrated[23].

Staining of GC tissue sections was performed using the EnVision FLEX Kit (K8000, Dako Glostrup, Denmark) in paraffin-embedded sections. Briefly, each paraffin section was deparaffinized, followed by antigen retrieval with Epitope Retrieval Solution, Tris/EDTA buffer pH 9 (DM830, Dako, Glostrup, Denmark) in a preheated water bath (95°C, 20 min). Endogenous peroxidase was blocked, and antihuman primary antibodies were applied for 25 to 45 min at 25ºC. Immune cells were evaluated using anti-mouse CD3 antibody (Is503, Dako), anti-human CD8 (IS623, Dako) and CD163 antibodies (clone EP324, Master Diagnostica, Granada, Spain).

The MMR proteins evaluated were mouse anti-human MutL protein homolog 1 (MLH1, ES01 Dako), mouse anti-human MutS protein homolog 2 (MSH2, FE11 Dako), rabbit anti-human MutS protein homolog 6 (MSH6, EP49 Dako) and rabbit anti-human postmeiotic segregation increased 2 (PMS2, EP51 Dako). HER2 was evaluated with the polyclonal rabbit anti-human c-erbB-2 oncoprotein (AO485 Dako).

Thereafter, the sections were incubated with secondary Abs as per the EnVision FLEX Kit, and counter-stained with hematoxylin. Positive staining controls were performed with paraffin sections of normal human tonsil.

Assessment of HER-2 status was performed following standard scoring criteria specific for GC[7,8,24], while dMMR was determined when the expression of at least one of the 4 MMR proteins evaluated was lost (Figure 3)[11,17-19].

Evaluation of H. pylori gene expression

H. pylori gene expression was determined by the constitutive *hspA* and *UreA* genes in DNA obtained from frozen gastric samples by quantitative PCR (qPCR) in the LightCycler 96 Instrument Thermal Cycler (Roche, Mannheim, Germany).

Values were considered positive when ≥ 10 copies/μL were detected. The virulence of the *cagA*, *vacAs*, and *vacAm* genes as well as *vacAs1* and *vacAm1* alleles was tested by an experienced cancer biologist (NS) in *H. pylori*-positive

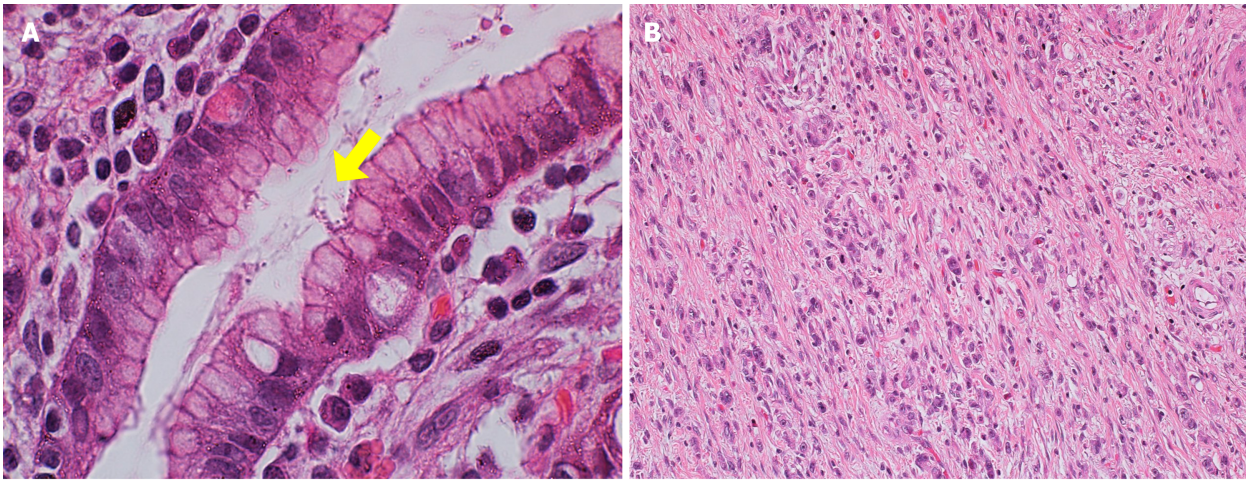

Figure 1 Pathological images of a case of gastric cancer with *Helicobacter pylori* infection with a low level of tumor-infiltrating lymphocytes. A: Hematoxylin and eosin (HE) staining showing the presence of *Helicobacter pylori* (yellow arrow) at 100 × magnification; B: HE staining of the intratumoral compartment with a low level of tumor-infiltrating lymphocytes at 20 ×.

patients, as described in a previous study by our group[3].

Quantitative analysis of TIL subpopulations

Immunostained slides were scanned with a digital virtual microscope BX63 Olympus (Tokyo, Japan), and the region with the highest immune cell density was selected. Five high power fields (HPF; or three when there was not enough stained tissue) within the IT and ST compartments were captured at 20 × magnification and analyzed using Visiopharm Tissuemorph Digital Pathology image analysis software (Visiopharm, Hoersholm, Denmark) under the supervision of a pathologist (Sanchez J). The density of the immune cells was calculated by the mean of positive cells in the captured HPF [25] (Figure 3). The optimal cutoff values for defining a higher density of immune cells (CD3, CD8 and CD163) were calculated using the maximally selected rank statistics according to Lausen [in relation to overall survival (OS)][26].

Statistical analysis

The non-paired Student's *t*-test was used to examine differences between groups. Correlations between values were evaluated using the non-parametric Spearman rank correlation. The intraclass correlation test was used to compare TIL levels and the density of immune cells in different compartments. OS was calculated from the date of surgery until death or until the date patients were last known to be alive (obtained from National Registry of Identification and Marital Status). Disease-free survival (DFS) was calculated from the date of surgery until relapse (patient records) or last known alive status. The last review of state of life was carried out in May 2022. In the univariate analysis, the survival curves were compared according to clinical characteristics using the log-rank or Breslow test, and in multivariate analysis using the Cox regression model with a stepwise selection method. A P value < 0.05 was considered significant. Analyses were performed using the SPSS statistical software (IBM SPSS Statistical 19) and R program. The statistical methods of this study were reviewed by Flores CJ from Oncosalud-AUNA.

RESULTS

General features

The clinicopathological features of the patients included are described in Table 1. Most cases underwent subtotal gastrectomy (62.4%). Adjuvant chemotherapy was administered in 45.1% and radiation in 13.9%.

H. pylori (+) was found in 231 of 415 (55.7%) cases when evaluated in HE staining and in 149 of 234 (63.7%) cases when evaluated with qPCR. The *cagA* gene was detected in 87.2% (130/149) of *H. pylori* (+) patients identified by qPCR, while the v*acAs* gene was detected in 79.1% (117/148) and *vacAm* in 75.2% (112/149) of *H. pylori* (+) patients. *VacAs1* and *vacAm1* alleles were detected in 47.9% (56/117) and 70.5% (79/112) of *H. pylori* (+) patients, respectively, and concurrent presence was found in 40.2% (39/97).

The presence of dMMR was found in 141 (34.4%; negative in 269 cases and not conclusive staining in 69), HER2-positive status in 23 (5%; negative in 434 and equivocal in 11 cases) and both features were found in 8 (2.2%) cases (Table 1). dMMR was associated with grade 1 (P = 0.028). HER2 overexpression was associated with an intestinal subtype (P < 0.001), grade 3 (P < 0.001) and low stage (P = 0.009).

Regarding survival analysis, the median follow-up was 6.4 years (95%CI 5.9-6.8 years), and recurrence and death were found in 181 cases and 270 cases, respectively. The clinicopathological features related to survival are described in Table 1.

Table 1 Clinicopathological features and prognostic value

Feature	Total (*n* = 503)	%	Median	5 yr DFS	*P* value	Median	5 yr OS	*P* value
Age, yr (19-95 yr)								
< 60	231	45.9	4.6	49.1	0.043	7.0	52.5	0.009
≥ 60	272	54.1	3.0	39.6		3.7	41.6	
Sex								
Female	252	50.1	3.3	43.7	0.582	3.8	45.3	0.319
Male	251	49.9	4.0	44.6		4.5	48.2	
Bormann								
I-II	90	18.1	NA	65.4	< 0.001	NA	66.0	0.002
III	311	62.7	2.9	40.6		4.0	44.4	
IV-V	95	19.2	2.6	36.8		2.8	37.6	
Lauren (*n* = 496)								
Intestinal	222	44.8	4.3	39.9	0.716	4.8	49.3	0.598
Diffuse	181	36.5	3.0	46.2		4.1	43.9	
Mixed	93	18.8	2.3	42.9		3.2	43.2	
Grade								
1	46	9.1	NA	62.2	0.025	NA	67.8	0.044
2	160	31.8	4.4	46.9		4.5	49.0	
3	297	59.1	2.5	39.7		3.6	42.3	
ILV								
No	149	29.6	6.9	62.4	< 0.001	8.1	66.4	< 0.001
Yes	354	70.4	2.4	36.5		3.0	38.5	
Antrum								
Yes	323	64.2	2.7	47.9	0.064	3.5	51.0	0.104
No	180	35.8	4.5	38.8		5.1	40.8	
Clinical stage								
I	64	12.7	NA	80.7	< 0.001	NA	83.0	< 0.001
II	138	27.4	7.7	59.6		7.7	59.5	
III	301	59.8	1.9	29.3		2.3	33.2	
Node involvement								
No	139	27.6	10.5	67.0	< 0.001	NA	68.2	< 0.001
Yes	364	72.4	2.3	35.4		3.0	38.4	
Recurrence								
No	322	64.0	-	-	-	NA	68.5	< 0.001
Yes	181	36.0	-	-		1.7	12.3	
H. pylori HE (*n* = 415)								
Absent	184	44.3	3.8	42.9	0.252	4.5	46.0	0.637
Present	231	55.7	7.3	48.3		5.3	51.2	
H. pylori qPCR (*n* = 234)								
Negative	85	36.3	2.5	36.8	0.229	3.1	39.1	0.219
Positive	149	63.7	3.8	38.3		4.0	39.7	
dMMR (*n* = 479)								

No	269	65.6	4.3	45.8	0.396	4.7	47.9	0.158
Yes	141	34.4	3.1	42.6	-	3.6	44.7	-
HER-2 status (n = 468)								
Negative	434	95.0	3.5	43.4	0.868	4.3	46.2	0.892
Positive	23	5	3.1	39.8		3.3	39.3	
IT TIL (n = 462)								
< 10	186	40.3	2.7	41.8	0.763	3.7	43.6	0.395
≥ 10	276	59.7	3.7	44.0		4.5	47.1	
ST TIL (n = 461)								
< 30	258	55.9	2.6	41.5	0.458	3.8	45.0	0.853
≥ 30	203	44.0	3.8	44.8		4.4	46.3	
IB TIL (n = 332)								
< 70	178	53.6	3.3	46.0	0.991	4.4	47.8	0.660
≥ 70	154	46.4	4.4	46.4		4.9	49.4	
IT CD3/HPF (n = 453)								
< 58	126	27.8	2.4	34.3	0.028	2.9	36.5	0.021
≥ 58	327	72.2	4.4	47.0		5.4	50.5	
IT CD8/ HPF (n = 443)								
< 70	231	52.1	4.4	47.4	0.060	4.8	49.9	0.142
≥ 70	212	47.9	2.6	40.4		4.0	43.8	
IT CD163/HPF (n = 205)								
< 240	183	89.3	2.8	42.7	0.002	3.8	45.3	0.003
≥ 240	22	10.7	1.4	0.0		1.5	0.0	
IT CD8/CD3 ratio (n = 432)								
< 0.63	210	48.6	4.5	49.1	<0.001	6.0	52.9	0.001
≥ 0.63	222	51.4	2.3	36.0		2.8	38.5	
IT CD3/CD163 (n = 179)								
< 8	137	76.5	22	33.9	0.004	2.6	36.6	0.002
≥ 8	42	23.5	NA	59.5		NA	64.3	
ST CD3/HPF (n = 363)								
< 95	180	49.6	2.6	36.6	0.014	2.9	38.7	0.003
≥ 95	183	50.4	5.2	50.7		7.0	54.6	
ST CD8/HPF (n = 341)								
< 68	216	63.3	3.8	43.5	0.578	4.0	45.0	0.382
≥ 68	125	36.7	3.6	43.0		4.5	47.5	
ST CD8/CD3 ratio (n = 328)								
< 1.2	277	84.5	4.3	45.9	0.005	4.6	49.1	0.002
≥ 1.2	51	15.5	1.9	26.1		2.0	26.6	

NA: Not available; HE: Hematoxylin and eosin; IT: Intratumoral; ST: Stromal; IB: Invasive border; TIL: Tumor-infiltrating lymphocytes; dMMR: Deficient mismatch-repair; *H. pylori*: *Helicobacter pylori*; HPF: High power field; DFS: Disease-free survival; OS: Overall survival; qPCR: Quantitative PCR.

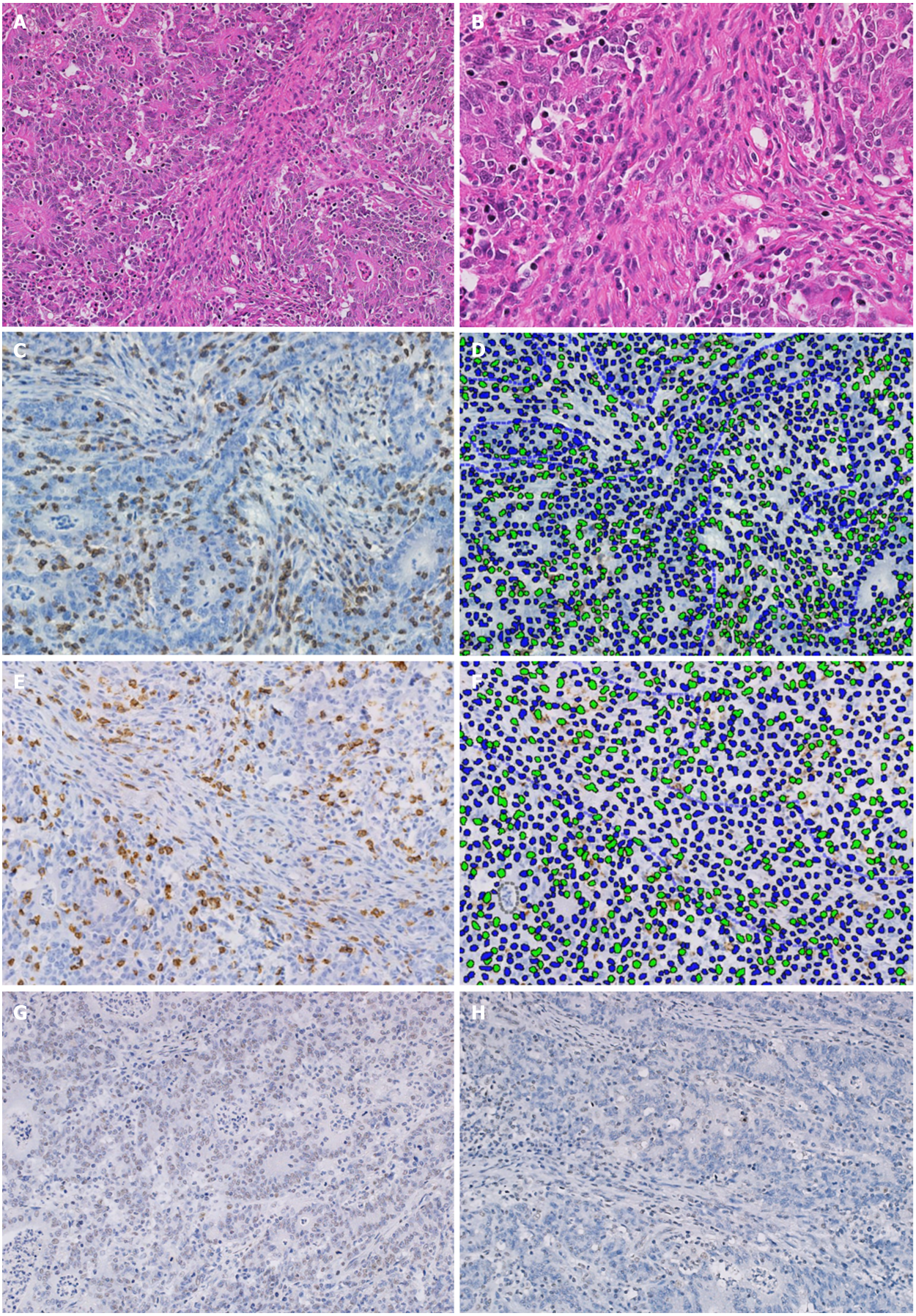

Figure 2 Pathological images of a case of gastric cancer with deficient mismatch-repair and a high level of tumor-infiltrating lymphocytes. A: Hematoxylin and eosin (HE) staining of stromal compartment with high level of tumor-infiltrating lymphocytes (TILs) at 20 × magnification; B: Field

magnification of HE with high TIL stain at 40 ×; C: CD3 immunohistochemistry (IHC) staining at 40 ×; D: Identification of CD3 density by machine learning-based image processing showing positive (green) and negative (blue) cells; E: CD8 IHC staining; F: Digital identification of CD8 density; G: IHC staining showing absence of MSH6 expression and; H: Absence of PMS2 expression.

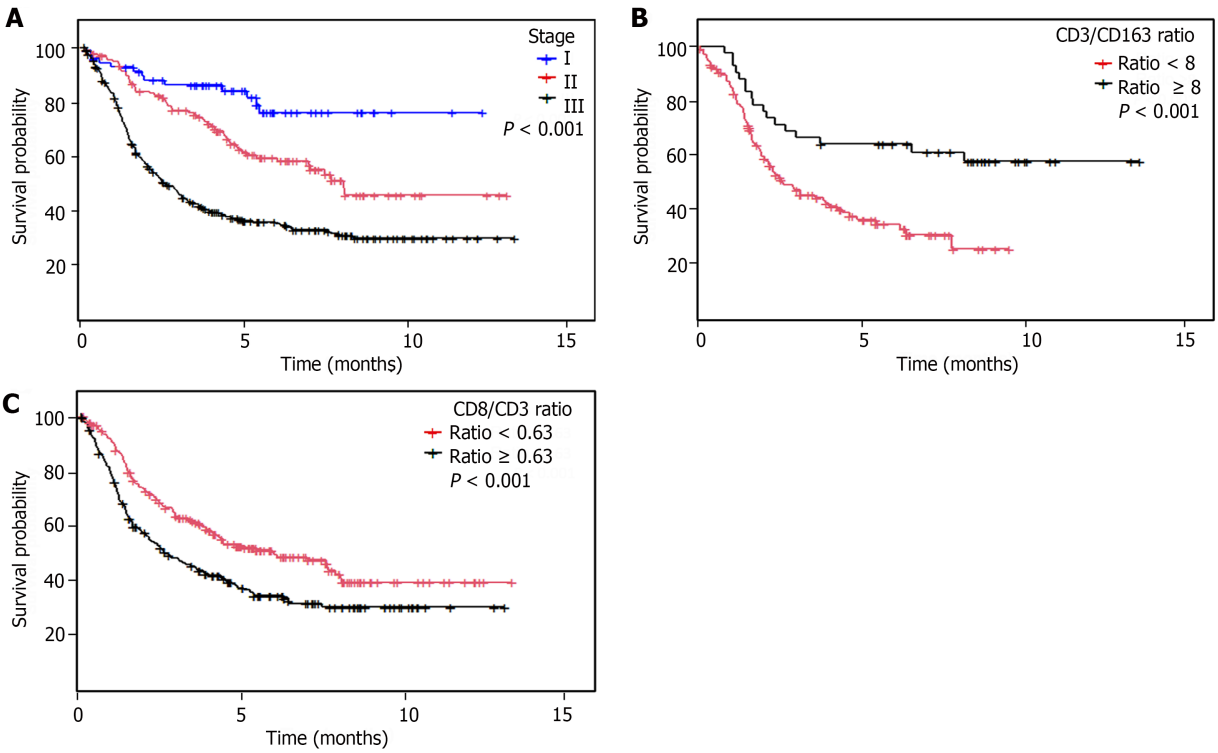

Figure 3 Overall survival analyses. A: Kaplan-Meier overall survival curve according to clinical stage; B: Intratumoral CD3/163 ratio; C: Intratumoral CD3/CD8 ratio.

Correlation between TILs and clinicopathological features

The median TIL level in the IT compartment was 10% (1%-80%) (n = 462), being 30% in the ST (1%-95%; n = 461) and 70% (1%-95%; n = 332) in the IB compartments, with a significant correlation among the three compartments [IT *vs* ST, intraclass correlation coefficient (ICC) = 0.613; IT *vs* IB, ICC = 0.340; IB *vs* ST, ICC = 0.726]. A high IT TIL level was associated with grade 3 (P = 0.038), lymphovascular invasion+ (P = 0.028) and stage 2 (P = 0.016). High ST TIL levels were associated with age ≥ 60 years (P < 0.001), non-antrum location (P = 0.049), intestinal histology (P < 0.001), grade 1 (P < 0.001), stage 2 (P = 0.001) and no recurrence (P = 0.003). Lastly, a high IB TIL level was associated with intestinal histology (P = 0.002), lymph node negative (P = 0.029) and earlier stages (P = 0.001; Table 2).

There was poor correlation between IT TIL levels and the densities of CD3+ (ICC = 0.106), CD8+ (ICC = 0.151) and CD163+ (ICC = 0.065) cells.

The mean densities of IT CD3+ (n = 453) and CD8+ (n = 443) cells/HPF were 108.6 (0.2-925.2) and 66.6 (1-777.4), respectively, while the mean density of IT CD163+ was 43 (0-519)/HPF (n = 205), and those of ST CD3+ (n = 363) and CD8+ (n = 341) cells/HPF were 95.2 (0.2-858.6) and 45.8 (0.5-581.8), respectively (Table 1). There was almost perfect agreement between the densities of CD3+ and CD8+ cells in the IT (ICC = 0.692) as well as in the ST compartments (ICC = 0.606). There was moderate agreement between the density of IT CD163+ and both CD3+ (ICC = 0.327) and CD8+ cells (ICC = 0.259) in the IT compartment.

A high density of IT CD3+ cells were associated with diffuse histology (P = 0.03), grade 3 (P < 0.001), absence of recurrence (P = 0.02) and longer DFS and OS (P = 0.028 and 0.021, respectively). A high density of IT CD8+ cells were associated with grade 3 (P < 0.001; Table 3) and a high density of IT CD163+ cells were associated with a non-antrum location (P = 0.031), grade 3 (P = 0.02) and shorter DFS and OS (P = 0.002 and P = 0.003, respectively). Cases with a high IT CD3/CD163 ratio presented a longer DFS (P = 0.004) and OS (P = 0.002; Table 1 and Figure 3).

A high density of ST CD3+ cells was associated with an intestinal subtype (P = 0.003), and longer DFS and OS (P = 0.014 and P = 0.003, respectively), while a high density of ST CD8+ cells was associated with Bormann III GC (P = 0.006) and an intestinal subtype (P = 0.003; Table 3).

Patients with a low CD8/CD3 ratio in the IT and ST compartments had a longer DFS (P < 0.001 and P = 0.005, respectively) and OS (P = 0.001 and P = 0.002, respectively; Table 1 and Figure 3).

Multivariate analysis showed that age, tumor grade, stage as well as a high density of IT CD3+ cells and a low IT CD8/CD3 ratio were associated with a longer DFS and OS (Table 4).

Table 2 Relationship between level of infiltrating lymphocytes and clinicopathological features

Features	IT TILs	*P* value	ST TILs	*P* value	IB TILs	*P* value
Median	> 10%		> 30%		> 70%	
Age		0.762		< 0.001		0.279
< 60	59.1		33.3		43	
≥ 60	60.4		54.8		48.9	
Sex		0.382		0.074		0.678
Female	57.7		39.8		45.1	
Male	61.7		48.1		47.4	
Gastric region location		0.450		0.049		0.160
No antrum	62		50		51.1	
Antrum	58.4		40.5		43.3	
Bormann		0.221		0.379		0.094
1	54.5		50		44.4	
2	47.3		36.4		38.8	
3	63.3		47.6		52.2	
4	61		38.3		33.3	
5	50		50		28.6	
Lauren histology		0.891		< 0.001		0.002
Intestinal	58.7		58.74		53.1	
Diffuse	58.9		20.4		28.6	
Mixed	61.6		50		42.6	
Grade		0.038		< 0.001		0.716
1	50		58.3		52.8	
2	53.5		55.5		45.6	
3	64.6		35.6		45.8	
Lymphovascular invasion		0.028		0.520		0.968
No	51.9		41.7		46.2	
Yes	62.9		44.9		46.5	
Lymph node involvement		0.403		0.714		0.029
Yes	60.9		43.5		42.5	
No	56.6		45.5		55.6	
Pathology stage		0.016		0.001		0.001
I	42		28		41.5	
II	65.4		55.8		60.4	
III	60.3		41.5		38.9	
Recurrence		0.230		0.003		0.369
No	61.9		49.5		48	
Yes	56.3		35.2		42.7	
H. pylori HE		0.009		0.445		0.411
Absent	65.9		47.6		47.5	
Present	52.4		43.6		42.5	
H. pylori qPCR		0.002		0.047		0.010

Absent	67.1		48.6		50.9	
Present	43.7		34.1		29.9	
CagA/H. pylori+		0.761		0.275		0.002
CagA-	40		46.7		64.3	
CagA+	44.1		32.4		24.1	
VacAs/H. pylori+		0.332		0.719		0.045
VacAs-	51.9		37		47.6	
VacAs+	41.4		33.3		25	
VacAs1/H. pylori+		0.530		0.887		0.894
VacAs1-	38.5		32.7		24.4	
VacAs1+	44.7		34		25.7	
VacAm/H. pylori+		0.824		0.535		0.265
VacAm-	41.9		38.7		38.5	
VacAm+	44.2		32.6		26.8	
VacAm1/H. pylori+		0.154		0.093		0.508
VacAm1-	55.2		44.8		21.7	
VacAm1+	39.4		27.3		29.2	
VacAs1+m1+/H. pylori+		0.937		0.953		0.527
No *VacAs1+m1+*	44.2		32.7		24.4	
VacAs1+m1+	43.3		66.7		31.8	
dMMR		0.875		0.019		0.010
No	58.7		38.9		38.6	
Yes	59.6		51.5		54.2	
HER2 positive		0.498		0.420		0.55
No	60.1		44.1		47.2	
Yes	52.4		47.6		35.3	

HE: Hematoxylin and eosin; IT: Intratumoral, ST: Stromal; TIL: Tumor-infiltrating lymphocytes; dMMR: Deficient mismatch-repair; *H. pylori*: *Helicobacter pylori*; HPF: High power field.

Correlation between TIL levels and H. pylori infection

A high IT TIL level was associated with absence of *H. pylori* (P = 0.009) when evaluated by HE in the whole series. When evaluated by qPCR (n = 234), *H. pylori* (-) was associated with high IT (P = 0.002), ST (P = 0.047) and IB (P = 0.01) TIL levels. High IB TIL levels were associated with infection by *H. pylori*+ *cagA*- (P = 0.002) and v*acA*- (P = 0.045; Table 2).

High densities of IT CD8+ (P = 0.001) and ST CD8+ (P = 0.001) cells were associated with *H. pylori* (-) and a high density of IT CD8+ cells were associated with *H. pylori*+ *cagA*- (P = 0.023; Table 3).

Correlation between TIL and both dMMR and HER2 expression

High ST (P = 0.019) and IB (P = 0.01) TIL levels were associated with dMMR. High densities of ST CD3+ (P = 0.049) and CD8+ (P = 0.05) cells were associated with dMMR (Table 3) and a high density of IT CD8+ cells were associated with HER2-negative (P = 0.009; Tables 2 and 3).

DISCUSSION

Our series shows that the association between TIL levels and clinical-pathological features varies according to the tumor compartments in which TILs are determined. This is the first study to describe that high TIL levels in the IT compartment are associated with the absence of *H. pylori* infection, while high TIL levels in both the IB and ST compartments are associated with dMMR, and ST TIL levels are also associated with low disease recurrence. High densities of CD3+ and CD8+ T lymphocytes were associated with dMMR in the ST compartment, and CD8+ T lymphocytes with HER2 negative in the IT compartment.

Table 3 Relationship between density of infiltrating immune cells and clinico-pathological features

Features	IT CD3	*P* value	ST CD3	*P* value	IT CD8	*P* value	ST CD8	*P* value	IT CD163	*P* value
Median	> 58		> 95		> 70		> 68		> 240	
Age		0.714		0.275		0.026		0.224		0.162
< 60	73		47		42.1		32.9		7.4	
≥ 60	71.5		52.8		52.7		39.3		13.5	
Sex		0.039		0.557		0.814		0.699		0.354
Female	76.5		52		48.4		37.7		8.7	
Male	67.8		49		47.3		35.7		12.7	
Gastric region location		0.503		0.585		0.795		0.901		0.031
No antrum	74.1		52.4		48.7		36.2		16.7	
Antrum	71.1		49.4		47.4		36.9		7.1	
Bormann		0.648		0.441		0.338		0.006		0.462
I-II	70.4		47.4		41.8		22.6		5.3	
III	73.6		55.6		50.7		43		11.7	
IV-V	68.9		52.9		46.1		30		13	
Lauren histology		0.03		0.003		0.308		0.003		0.038
Intestinal	66.2		57.4		43.7		40.3		8.2	
Diffuse	78		36.6		50		22.2		17.8	
Mixed	75.9		50.8		52.7		46.3		2.9	
Grade		<0.001		0.311		<0.001		0.083		0.02
1	59		55.3		26.8		20.5		3.8	
2	62.9		54.2		40.7		38.2		4.3	
3	79.5		46.4		55.3		39.2		16.4	
Lymphovascular invasion		0.602		0.241		0.092		0.117		0.865
No	73.9		55.2		41.8		30.4		11.3	
Yes	71.5		48.4		50.5		39.3		10.5	
Lymph node involvement		0.723		0.983		0.487		0.141		0.252
Yes	72.6		50.4		48.9		39.1		12.1	
No	71		50.5		45.2		30.6		6.3	
Pathology stage		0.942		0.101		0.674		0.058		0.197
I	70.9		50		42.9		20		9.5	
II	73.2		59		47.2		41		4.1	
III	72		46.3		49.2		37.8		13.3	
Recurrence		0.02		0.079		0.448		0.475		0.389
No	76		53.9		46.6		35.2		9.3	
Yes	65.9		44.4		50		39.1		13.2	
H. pylori HE		0.547		0.817		0.356		0.97		0.637
Absent	71.1		51.4		49.1		36		13.4	
Present	73.9		52.8		44.2		36.2		11.1	
H. pylori qPCR		0.143		0.335		0.001		0.001		0.105
Absent	83.1		55.9		62.7		49.1		26.1	
Present	74		48.1		37		22.8		14.1	

CagA/H. pylori+		0.254		0.603		0.023		0.54		0.729
CagA-	82.9		42.9		57.5		21.2		14.3	
CagA+	74.2		48		37.2		26.6		17.2	
VacAs/H. pylori+		0.992		0.843		0.071		0.855		0.823
VacAs-	76.8		45.8		51.7		24.4		15.2	
VacAs+	76.9		47.6		37.3		25.9		16.9	
VacAs1/H. pylori+		0.397		0.432		0.942		0.234		0.543
VacAs1-	79.2		53.5		36.4		18.4		14.7	
VacAs1+	72.2		44.7		37		30		20.8	
VacAm/H. pylori+		0.1		0.248		0.255		0.884		0.865
VacAm-	69.4		40.4		47.6		24.5		17.1	
VacAm+	80.6		50.6		38.7		25.6		15.8	
VacAm1/H. pylori+		0.145		0.098		0.242		0.822		0.877
VacAm1-	89.7		65.2		48.1		23.8		16.7	
VacAm1+	77		44.8		35.4		26.3		15.2	
VacAs1+m1+/H. pylori+		0.113		0.289		0.913		0.253		0.345
No *VacAs1+m1+*	86		57.5		37.7		21.6		12.9	
VacAs1+m1+	72.2		44		38.9		34.6		23.5	
dMMR		0.393		0.049		0.095		0.05		0.169
No	75.4		47.6		45.5		32.3		11.5	
Yes	71.3		59.5		54.7		43.9		5.1	
HER2 status		0.06		0.204		0.009		0.404		0.835
Negative	74.4		52.4		49.9		37.7		11.1	
Positive	56.5		38.1		21.7		28.6		9.1	

HE: Hematoxylin and eosin; IT: Intratumoral; ST: Stromal; HPF: High power field; *H. pylori*: *Helicobacter pylori*; dMMR: Deficient mismatch-repair; qPCR: Quantitative PCR.

We found that patients with tumors with a high density of CD3+ T lymphocytes in the IT and ST compartments, a low CD8/CD3 ratio in the IT and ST compartments, a low density of CD163+ macrophages, and a high CD3/CD163 ratio had greater survival than the remaining patients.

H. pylori infection detected by 2 methodologies was consistently associated with low TIL levels in the IT compartment. In addition, a low TIL level in the IB compartment was associated with *H. pylori* detected by qPCR, as well as with strains without strong virulence factors (*CagA*- and *VacA*-). Furthermore, the high density of CD8+ T lymphocytes in the IT and ST compartments was also associated with the absence of *H. pylori* (determined by qPCR). Different studies find that the intestinal microbiota modulates the activity of the immune system against cancer and even the activity of checkpoint inhibitors[27,28]. However, to our knowledge, this is the first time that a strong association between *H. pylori* and immune activity against cancer in GC has been described. Our results need further validation and suggest that *H. pylori* status evaluated by qPCR should be analyzed in clinical trials evaluating checkpoint inhibitors in GC.

Different series have evaluated the association between MSI and TIL, and Angell *et al*[29] found that 18.9% of GC cases were MSI- high and were associated with a high density of CD3+ and CD8+ T lymphocytes, as well as a better OS in a series including 380 cases of GC[30-33]. We found that the association between high levels of TIL and dMMR depends on the compartment evaluated, since the association was found in the ST and IB compartments but not in the IT compartment. Similarly, high densities of CD3+ and CD8+ T lymphocytes were associated with dMMR in the ST compartment but not in the IT compartment.

We found that a low density of CD8+ T lymphocytes in the IT compartment was associated with HER2 positivity. Similarly, Lv *et al*[34] reported a HER2 positive rate of 14% and an inverse relationship with the density of CD8+ T lymphocytes in a series of 120 patients with GC. However, other studies have described different findings[29].

The TIL levels were highest in the IB and ST compartments and were associated with less aggressive clinicopathological features (grade 1 and intestinal subtype for IB and ST, and negative lymph nodes for IB). The association between increased survival and a strong lymphocyte infiltrate has been described by different studies including up to 400 GC cases; however, these studies used different and non-standard methodologies (reporting rates of strong lymphocyte infiltration of 14% to 47%)[35-37].

Table 4 Multivariate analysis of factors associated with disease-free survival and overall survival

Features	DFS		OS	
	HR	*P* value	HR	*P* value
Age				
< 60	Reference	-	Reference	-
> 60	1.8 (1.2, 2.5)	0.002	1.9 (1.3, 2.8)	< 0.001
Histological grade				
Differentiated	Reference		Reference	
Undifferentiated	1.7 (1.01, 2.7)	0.043	2.0 (1.2, 3.3)	0.007
WHO classification				
1-2	Reference		Reference	
3-4	2.5 (1.6, 3.8)	< 0.001	2.4 (1.5, 3.7)	< 0.001
Clinical stage				
I-II	Reference		Reference	
III	2.1 (1.4, 3-2)	< 0.001	1.9 (1.2, 2.9)	0.004
IT CD3/HPF				
High (> 58)	Reference		Reference	
Low (< 58)	1.5 (1.02, 2.2)	0.037	1.6 (1.1, 2.4)	0.018
IT CD8/CD3 ratio				
< 0.63	Reference		Reference	
> 0.63	1.7 (1.2, 2.5)	0.002	1.6 (1.1, 2.3)	0.008
Non-significant variables				
Sex	-	0.939	-	0.51
Cardia-fundus	-	0.398	-	0.212
Bormann	-	0.328	-	0.315
Lauren	-	0.311	-	0.291
Lymph nodes	-	0.836	-	0.663
lymphovascular invasion	-	0.972	-	0.277
H. pylori	-	0.123	-	0.140
Deficient mismatch-repair	-	0.973	-	0.365
HER2	-	0.222	-	0.114
IT TIL	-	0.940	-	0.547
Stromal TIL	-	0.818	-	0.806
IT CD8/HPF	-	0.077	-	0.340

DFS: Disease-free survival; OS: Overall survival; HR: Hazard ratio; WHO: World Health Organization; IT: Intratumoral; TIL: tumor-infiltrating lymphocytes; HPF: High power field; *H. pylori*: *Helicobacter pylori*.

On the other hand, aggressive features such as grade 3 were associated with high TIL levels in the IT compartment but low TIL levels in the ST compartment. Similarly, grade 3 was associated with high density of CD3 in the IT compartment but not in the ST compartment. This different activity of TILs depending on their spatial location in relation to malignant cells could be explained by a higher percentage of anergic immune cells in the IT compartment[38].

We found that a high density of CD3+ T lymphocytes in the IT and ST compartments, as well as tumors with a low CD8/CD3 ratio in the IT and ST compartments, were associated with increased survival in univariate and multivariate analyses. In addition, as densities were calculated in small tumor cores (tissue microarray-stained samples), we expect that the prognostic value of the density of CD3+ T lymphocytes and the CD8/CD3 ratio would be maintained when evaluated in samples obtained from gastroscopies or conservative surgery. Therefore, we recommend that these densities

be evaluated in prospective GC trials.

The poor prognosis associated with the CD8/CD3 ratio has also been described by other groups and could be related to an anergic status of tumor-infiltrating CD8+ T lymphocytes[16,39]. Recent studies suggest that 30% to 38% of cases with a high CD8 T- cell density also have high levels of positive PD-L1[40], and Wang *et al*[41] found that the presence of both stains was associated with a shorter survival in a series of 147 GC cases.

In addition, our finding of a poor prognosis associated with high levels of CD163+ M2 macrophages has also been described by other groups and confirms the protumoral activity of these immune cells. The high ratio of CD3/CD163 was also associated with a favorable prognosis even in the smaller population size in which it was tested. Nonetheless, further studies on the ratio of immune cells in GC are necessary to confirm these findings[42,43].

Finally, this is the largest South American series evaluating molecular markers in GC, reporting dMMR and HER2 overexpression rates of 29% and 4.7% in early GC, respectively. The prevalence of both biomarkers is similar to what has previously been described in Caucasian series (5%-33% for dMMR and less than 18% for HER2)[10,44-46].

CONCLUSION

The levels of TILs are significantly related to dMMR and a *H. pylori*-negative status. However, the association of TIL levels with tumor features depends on the tumor compartment evaluated. Low CD8/CD3 and high CD163/CD3 values were strongly associated with a lower rate of recurrence and longer survival.

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FOOTNOTES

Author contributions: Castaneda CA and Castillo M contributed to the conception and design of the study; Castaneda CA, Castillo M and Flores CJ performed data analysis and interpretation; Bernabe LA, Sanchez J, Tello K, Fassan M, Bazan Y, Alatrista R, Poquioma E and Taxa L performed data acquisition, as well as providing technical support; Chavez Passiuri I, Barreda F, Ruiz E, Wistuba II, Abad-Licham M, Mengoa C, Fuentes H, Montenegro P and Valdivia D provided administrative and material support; all authors drafted the article, made critical revisions and approved the final version of the manuscript.

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