Immunological markers and *Helicobacter pylori* in patients with stomach cancer: Expression and correlation

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Abstract. Programmed death-ligand 1 (PD-L1) and ICOS-L (also referred to as B7 homolog 1 and 2, respectively) modulate the immune inflammatory response. The aim of the present study was to examine the expression levels of these inflammatory mediators in two groups of patients with an *Helicobacter pylori* (*H. pylori*) infection; patients with and without gastric cancer. The association between bacterial virulence factors, CagA and VacA, was also examined, as well as their correlation with the inflammatory profile. Endoscopy analysis indicated that 18 patients suffered from cancer and 28 patients suffered from other gastric pathologies. PCR and reverse transcription-quantitative PCR were used to analyze gastric biopsies and determine the expression levels of the inflammatory modulators PD-L1 and ICOS-L, transcription factors, cytokines and other genes associated with inflammation and pathogenicity. All 46 patients were determined positive for markers of *H. pylori*. Patients with stomach cancer had lower levels of ICOS-L (P<0.05) and GATA3 (P<0.01), a negative correlation between CagA and IL-17 (P<0.05), a positive correlation between CagA and IL-10 (P<0.05), a negative correlation between *vacA-m1* and retinoid orphan receptor γt (RORγt) (P<0.001), and a positive correlation between RORγt and ICOS-L (P<0.001). The reduced levels of ICOS-L and GATA3 along with the negative correlation between CagA and IL-17, and between *vacA-m1* and RORγt were all associated with an increased risk of gastric cancer in the present cohort.

Introduction

Helicobacter pylori (*H. pylori*) is present in the stomach of ~50% of the population worldwide (1) and is considered to be the primary cause of chronic gastritis, gastric cancer and peptic ulcers (2). Around 89% of non-cardia gastric cancer cases, representing 78% of all gastric cancer case, are now estimated to be attributable to chronic *H. pylori* infection (3). Gastric cancer is among the five most common types of malignant tumors, and has the second highest cause of cancer-associated death worldwide (4). However, only 1-3% of the individuals with an *H. pylori* infection develop gastric cancer (5), as this pathology is dependent on the virulence of the bacteria, the environment and genetic factors of the host (6,7). For instance, common variable immunodeficiency (CVID) syndrome is associated with a 45-fold increase in the risk of gastric cancer and a 30-fold increase in the risk of gastric lymphoma (8). Although the etiology of CVID is not completely understood, in adults it is associated with deletion of a gene which encodes the inducible T-cell co-stimulator ICOS (9), which is expressed by T-cells when activated by their antigen. The only known ligand of ICOS (ICOS-L) is expressed constitutively by B lymphocytes (10). The interaction between ICOS:ICOS-L serves an important role in mediating the cooperation between T and B cells, as well as promoting the terminal differentiation of B cells to plasma B cells. ICOS activation induces the secretion of IL-4, IL-5, IL-6, IL-10, IL-21, tumor necrosis factor-a and interferon gamma (IFN-γ). In doing so, ICOS co-induces the secretion of interleukins and activates the function of Th1,

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Th2 and Th17 cells (11,12). Patients with deletion of ICOS have a reduced number of naïve B cells and memory cells, and low levels of serum antibodies, but do not exhibit a change in antibody isotype (9).

Another molecule which modulates the immune response is programmed death-ligand 1 (PD-L1; also known as B7 homolog 1). Encoded by the CD274 gene, PD-L1 activates a membrane receptor of programmed death 1 (PD-1). The PD-L1:PD-1 axis maintains the balance between tolerance and autoimmunity. A deficiency or excess in the function of PD-1 can result in various diseases, for example arthritis and lupus (13). One mechanism of regulating the expression of PD-L1, is binding of STAT3 to its promoter (14). Research has shown that in patients with tumors, upregulation of hypoxia-inducible factor 1α is associated with elevated levels of PD-L1 (15). PD-L1 functions primarily in a microenvironment enriched with lactate (16).

In relation to the virulence of *H. pylori*, the most studied molecule is the cytotoxin associated gene (CagA), which is translocated by the type IV secretion system of *H. pylori* into gastric cells, generating intracellular signals that facilitate malignancy (17). Individuals have an increased risk of developing gastric cancer if they express cagA+ instead of cagA-, and the strains of *H. pylori* that carry CagA are associated with an increased risk of developing chronic gastritis or peptic ulcers, as demonstrated in a meta-analysis (18). CagA is considered the primary virulence factor of *H. pylori*, and results in the downregulation of the inflammatory modulator ICOS-L, the attenuation of which occurs through the P70-S6 kinase signaling pathway in gastric epithelial cells (19).

Vacuolating cytotoxin A is another virulence mechanism of *H. pylori* correlated with gastric cancer (20,21). The DNA sequence analysis of the vacA gene shows a mosaic structure comprising allelic variations with different biological activity, resulting in the s1 and m1 regions as the two regions most frequently associated with peptic ulcers and an increased risk of gastric cancer (22). One of the mechanisms attributed to VacA is its interference with IL-2 production and IL-2 receptor (IL-2R) expression, which in turn reduces the proliferation of T lymphocytes (23). In mice, purified VacA results in the loss of gastric epithelial cells *in vivo* and *in vitro* by increasing apoptosis, which is initiated through the release of mitochondrial cytochrome C and the activation of Caspase 3 (24).

A meta-analysis recently suggested that the presence of the *vacA-s1* and *vacA-m1* genotype results in a greater risk of gastric cancer and account for a 33.4% and 2.08-fold increase, respectively, with an age-standardized rate (ASR) of 11-19 cases per 100,000 individual. A 40.2 and 66.6% increase, respectively, of gastric cancer was reported for a group with an ASR <10 per 100,000. Therefore, the *vacA-m1* genotype of *H. pylori* appears to be more potent than *vacA-s1* for inducing gastric cancer (25).

In the present study, the differences in the mRNA expression levels of cytokines, master transcription factors associated with the T-cell profile, and the co-immunomodulatory molecules PD-L1 and ICOS-L in two groups of patients infected with *H. pylori*, those with and without gastric cancer were examined. Additionally, the correlation between each group, the polymorphisms of *vacA* and the presence of *cagA* was assessed. The aim of the present study was to determine effect the co-modulating molecules, ICOS-L and PD-L1, conferred on the immune response of patients infected with *H. pylori* with or without gastric cancer.

Materials and methods

Patients. The present study was performed in the National Medical Center (Centro Médico Nacional 20 de November) of the ISSSTE Medical Service (a health plan for government workers) between June 2016 and August 2017. A total of 1,462 endoscopies were performed during this period on adults (>18 years of age), and 46 patients exhibited signs of a gastric pathology. These patients were referred to our group. The patients were given a detailed description of the study and all patients provided written informed consent, in accordance with the Helsinki Declaration and the Ethics Committee of the National Medical Center (Centro Médico Nacional 20 de November) of the ISSSTE Medical Service. The protocol used in the present study was approved by the Bioethics Committee of the Autonomous University of Aguascalientes (approval no. CIB-UAA-26).

The patients were divided into two groups; those with cancer (n=18), males (n=10, 55.56%), female (n=8, 44.44%), mean age 64.72±2.54 years (age range 48-82), and those without cancer (n=28) males (n=16, 21.42%), female (n=22, 78.58%), age mean 55.68±2.59 years (age range 33-77). Of the patients without gastric cancer, a duodenal ulcer was present in 10 patients, whereas the other 18 patients exhibited various other types of gastritis (epidemiological variables shown in Table I).

Extraction of gastric biopsies. The endoscopy procedure was handled by an experienced endoscopist on a Jaw Radial Endoscopy apparatus (160.24 cm in length with a 6 mm opening; Boston Scientific) and a 590 EG ZW (with a working channel 2.8 mm in diameter) (Fujinon; FUJIFILM, Inc.) with an EPX 4400 processor. By using the conventional brightfield endoscopy procedure, the mucus was carefully observed to detect visible alterations indicative of a gastropathy (for examples gastritis, gastric ulcers or duodenal ulcers) in patients with symptoms suggestive of these disorders. In individuals suspected of having gastric cancer, the neoplasm was examined to determine its characteristics and location. Gastric biopsies were obtained from the pyloric antrum and were placed in small vials containing TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and ethanol for extraction of RNA and DNA, respectively. The samples were stored at -40°C until required. Additional biopsies of the pyloric antrum were extracted and placed in 4% formaldehyde for histopathological confirmation using the Sydney protocol (26). In the event of a gastric neoplasm, a biopsy of the lesion was taken to perform histopathological analysis using hematoxylin and eosin staining (27).

DNA purification from gastric tissue. DNA was extracted using phenol-chloroform (28). Gastric tissue was homogenized with 500 µl lysis buffer (2 mM Tris-HCl, 10% SDS, 1 mM EDTA and 1.5 µg/µl proteinase K) and the sample was incubated at 65°C for 24 h. Subsequently, 500 µl phenol:isoamyl alcohol solution (24:1) was added and the

Table I. Clinicopathological characteristics of the patients recruited in the present study.

Clinicopathological characteristics	With cancer, n=18	Without cancer, n=28	P-value
Age, mean ± standard deviation (age range)	64.72±2.547 (48-82)	55.68±2.594 (33-77)	0.0227[a]
Males, n (%)	10 (55.56)	6 (21.42)	0.0172[a]
Female, n (%)	8 (44.44)	22 (78.58)	0.0271[a]
Smoker, n (%)	8 (44.45)	9 (32.14)	0.4102
Atrophy, n (%)	5 (27.78)	6 (21.42)	0.6314
Non-Atrophy, n (%)	13 (72.22)	22 (78.58)	0.6314
Type 2 diabetes, n (%)	3 (16.66)	7 (25)	0.7172
High blood pressure, n (%)	3 (16.66)	10 (35.71)	0.1968
Duodenal ulcer, n (%)	2 (11.11)	12 (42.85)	0.0465[a]

[a]P<0.05.

Table II. Primer sequences used to identify *Helicobacter pylori* positivity based on the presence of *cagA* and for analysis of the *vacA* polymorphisms.

Author, year	Gene	Forward	Reverse	Product length	(Refs.)
Abdi *et al*, 2016	cag2-4	GGAACCCTAGTCGGTAATG	ATCTTTGAGCTTGTCTATCG	450/558	(25)
Sipponen *et al*, 2011	cagA1	TGGCAGTGGGTTAGTCATAGCAG	AGGACTCTTGCAGGCGTTGGTG	481	(26)
	cagA2	ATAATGCTAAATTAGACAACTTGAGCGA	TTAGAATAATCAACAAACATCACGCCAT	298	
Luna, 1968	G-Hp1/2	AAGCTTTTAGGGGTGTTAGGGGTTT	AAGCTTACTTTCTAACACTAAACGC	294	(27)
	G-H3/4	CTTTCTTCTCAAGCGGTTGTC	CAAGCCATCGCCGGTTTTAGC	252	
Sambrook and Green, 2012	*s1 vac1*	GAAATACAACAAACACACCGC	GGCTTGTTTGAGCCCCCAG	201	(28)
	s2 vac1	GAAATACAACAAACACACCGC	GGCTTGTTTGAGCCCCCAG	228	
	m1 Vac3	GGTCAAAATGCGGTCATGG	CATCAGTATTTCGCACCACA	388	
	m2 Vac4	CCAGGAAACATTGCCGGCAAA	CATAACTAGCGCCTTGCAC	346	

mixture was centrifuged at 15,800 x g for 15 min at 25°C. The aqueous phase was separated, and a second extraction was performed using the supernatant with 500 μl chloroform:isoamyl alcohol solution (24:1). The sample was centrifuged at 15,800 x g for 15 min at 25°C and the aqueous phase was collected. After precipitating the DNA present in the aqueous phase with 500 μl isopropanol and centrifuging at 15,800 x g for 5 min at 25°C, the pellet was washed with 70% ethanol at -20°C and resuspended in nuclease-free water free of chelating agents. DNA was quantified by spectrophotometry at 260 nm and its integrity was evaluated by electrophoresis on a 1.25% agarose gel.

Sequence typing of vacA and determination of cagA positivity. The genotypes of *H. pylori* with the presence/absence of *cagA* and distinct polymorphic regions for *vacA* (*s1s2* or *m1m2*) were analyzed by endpoint PCR from DNA samples extracted from the gastric tissue. PCR was performed using a Platinum® PCR SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) on a Techne 3 Prime Base/02 thermocycler with the following thermocycling conditions: Denaturation at 95°C for 1 min, annealing at 58°C for 30 sec and extension at 72°C for 30 sec for 40 cycles. The sequences the primers used are presented in Table II (29-32).

RNA extraction and reverse transcription (RT). Total RNA was extracted using the organic protocol with TRIzol (33). To eliminate genomic DNA contamination, digestion of DNA was performed using 2 U DNase I (DNase amplification grade) (Invitrogen; Thermo Fisher Scientific, Inc.) per μg RNA. The sample was incubated at room temperature for 15 min. Subsequently, the enzyme was inactivated with 1 μl

Table III. Primer sequences used to analyze the expression of the genes encoding transcription factors, cytokines and immunomodulatory molecules.

Author, year	Gene	Forward	Reverse	Product length	(Refs.)
Wagih et al, 2015	GATA3	GGAGGTGGGATGTGCTTTTTAACA	ACCTGGCTCCCGTGGTG	116	(30)
	RORγt	AGGAAGTGACTGGCTACCAGAGG	GAACTCCACCACGTACTGAATG	94	
	IL10	TTCCCTGTGAAAACAAGAGC	TCACTCATGGCTTTGTAGATGC	90	
	IL17A	CTCATTGGTGTCACTGCTACTG	CCTGGATTTCGTGGGATTGTG	77	
Osman et al, 2015	IFN-γ	GTGTGGAGACCATCAAGGAAGACA	TTGGACATTCAAGTCAGTTACC	112	(31)
	TGF-β1	GGTGGAAACCCACAACGAAAT	TCTCGGAGCTCTGATGTGTTGA	86	
	GAPDH	TGGTCTCCTCTGACTTCAACA	AGCCAAATTCGTTGTCATACC	116	
	Foxp3	GAGAAGGGCAGGGCACAAT	GTGGGCCTGCATGGCAC	101	
Rhead et al, 2007	IL-4	CACAGGCACAAGCAGCTGAT	CCTTCACAGGACAGGAATTCAAG	87	(32)
	IL-12p40	CATCTTCTTGGTTTTCCCTGGTT	CATAAACATCTTTCTTCAGTTCCCATAT	73	
Chomczynski and Sacchi, 1987	PD-L1	ACAGAGGGCCCGGCTGTTGA	AGCGGTACACCCCTGCATCCT	94	(33)
Yilmaz et al, 2015	ICOS-L	GCAAACCAGTGAGTCGAAAACC	GGTGACATCAGGGCTCGGT	101	(34)

PD-L1, programmed death ligand 1; RORγt, retinoid orphan receptor γt.

EDTA (25 mM) and 10 min of heating at 65˚C. Total RNA was quantified by spectrophotometry at 260 nm and the integrity of the RNA was assessed using electrophoresis on a 1.2% agarose gel.

RT of total RNA was performed using a Superscript VILO kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The resulting cDNA was quantified by spectrophotometry at 260 nm and the samples were diluted to a concentration of 400 ng/μl. The samples were stored at -20˚C until further use.

Analysis of RNA expression in gastric biopsies. The RNA obtained from gastric tissue was evaluated by RT-quantitative (q)PCR using Express SYBR qPCR SuperMix Universal (Invitrogen; Thermo Fisher Scientific, Inc.). The expression levels of cytokines (IL-4, IL-10, IL-12, IL-17, IFN-γ and TGF-β), the co-modulating molecules of the immune system (PD-L1 and ICOS-L), and the master transcription factors, including forkhead box P3 (Foxp3), GATA3, and the retinoid orphan receptor γt (RORγt). Analysis was performed on a StepOne™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Initial denaturation, 50˚C for 2 min and 95˚C for 10 min; followed by 50 cycles of denaturation at 95˚C for 15 sec, and annealing and an extension at 60˚C for 60 sec. All primers were acquired from T4 Oligo. The sequences of the primers used are presented in Table III (34-38). GAPDH was used as the normalization control. The differential expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method (39).

Statistical analysis. The results from qPCR were examined on DataAssist™ Software version 3.01 (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the Cq values for relative expression. The relative expression of mRNA was compared between patients with and without cancer to determine the significance of differences using a Mann-Whitney U test and SPSS version 20 (IBM Corp.) and multiple comparisons were evaluated using a Kruskal-Wallis test with a Dunn's post-hoc. This test is suitable for comparison of two unpaired groups of continuous variables that are not normally distributed. $P<0.05$ was considered to indicate a statistically significant difference. To analyze if there was a relationship between the mRNA expression levels of the various genes within each group, a Spearman's Rho (ρ) value was calculated (40). The correlation was considered either positive/negative and either high/medium/low (high, ρ value of 0.7-0.9; medium, ρ value of 0.5-0.7; low ρ value <0.5) according to the scale described by Mukaka (41).

Results

Prevalence of the cagA and vacA genotype in gastric biopsies. All 46 biopsies were positive for *ureC* (a marker for *H. pylori*) and *vacA*. Only 18 biopsies were positive for *cagA* (39.1%). Regarding *vacA*, the frequency of polymorphisms in the subregions *s1* and *m1* in the group of patients with gastric cancer was higher compared with the rest of the subregions (Table IV). It has been reported that polymorphisms in these subregions increase the risk of gastric cancer (25). The possible association between these polymorphisms with the

Table IV. Frequency of two *vacA* polymorphisms and of positivity for *cagA* and *ureC* in patients with and without gastric cancer.

Groups	ureC	cagA	s regions of vacA		m regions of vacA	
			s1	s2	m1	m2
Cancer	18	10	14	1	9	4
Without Cancer	28	8	16	2	14	12
Total	46	18	30	3	23	16

expression of the mRNA of cytokines, nuclear transcription factors and co-modulating molecules related to the gastric pathology caused by *H. pylori* was further analyzed.

Analysis of mRNA expression in gastric biopsies. The expression of cytokines, transcription factors and co-modulating molecules of the immune system was determined using RT-qPCR. The expression levels of ICOS-L were significantly reduced in patients with cancer (Fig. 1A). Additionally, there was a lower level of ICOS-L relative to PD-L1, represented by the ratio PD-L1/ICOS-L, although this decrease was not significant (Fig. 1B).

The relative expression of the master transcription factors was higher with the phenotype of regulatory T cell (Treg), Th17 and GATA3 in the patients without cancer. The difference in the expression of Foxp3 (P=0.063) and RORγt (P=0.060) between patients with and without gastric cancer was not significant; however, the difference in the expression of GATA3 was significant between the two groups. (P<0.01; Fig. 1C).

The expression levels of IL-4, IL-10, IL-12 and IL-17, IFN-γ and TGF-β were similar between the two groups. The expression levels of IL-4 in the Th2 profile were decreased in patients with gastric cancer, but the difference was not significant (P=0.07; Fig. 1D).

Spearman's rank correlation coefficient. Spearman's bivariate correlation analysis of immune- and virulence-related variables in patients with and without cancer are presented in Table V. Patients without cancer showed a high positive correlation between ICOS-L and PD-L1 (ρ=0.707), IL-4 (ρ=0.719) and IL-12A (ρ=0.712); a moderate positive correlation with RORγt (ρ=0.687), GATA-3 (ρ=0.561) and Foxp3 (ρ=0.530); and low positive correlation with TGF-β (ρ=0.481) and IL-17 (ρ=0.411). Patients with cancer exhibited a high positive correlation between ICOS-L and IL-12 (ρ=0.772), RORγt (ρ=0.760) and IL-10 (ρ=0.708); a moderate positive correlation with GATA3 (ρ=0.689), IFN-γ (ρ=0.637), PD-L1 (ρ=0.610), *vacA-m2* (ρ=0.567) and IL-4 (ρ=0.566); and a low positive correlation factor with Foxp3 (ρ=0.490).

Patients without cancer exhibited a high positive correlation between PD-L1 and ICOS-L (ρ=0.707); a moderate positive correlation with IL-4 (ρ=0.668), IL-17A (ρ=0.570), Foxp3 (ρ=0.551) and TGF-β (ρ=0.525); and a low positive correlation with IFN-γ (ρ=0.454) and IL-10 (ρ=0.428). For the patients with cancer, there was a high positive correlation between PD-L1 and IL-10 (ρ=0.789), IL-12A (ρ=0.789) and GATA3 (ρ=0.757); and a low positive correlation with IFN-γ (ρ=0.485) and Foxp3 (ρ=0.483).

Patients without cancer demonstrated a high positive correlation between RORγt and IL-12A (ρ=0.772), IL-4 (ρ=0.737) and Foxp3 (ρ=0.708); a moderate positive correlation with IL-17A (ρ=0.603) and PD-L1 (ρ=0.549); and a low positive correlation with TGF-β (ρ=0.408). Patients with cancer exhibited a high positive relation of RORγt; a moderate positive correlation with IL-12A (ρ=0.623), *vacA-m2* (ρ=0.598) and GATA3 (ρ=0.522); and a low positive correlation with IL-10 (ρ=0.488). A high negative correlation was observed between RORγt and *vacA-m1* (ρ=-0.756).

In the patients without cancer, there was a high positive correlation between Foxp3 and IL-12A (ρ=0.784) and IL-4 (ρ=0.715); a moderate positive correlation with IL-17A (ρ=609), IFN-γ (ρ=0.585) and IL-10 (ρ=0.579); and a low positive correlation with TGF-β (ρ=0.457). In patients with cancer, there was a moderate correlation between Foxp3 and IL-12 (ρ=0.659) and IL-10 (ρ=0.569); and a low positive correlation with GATA3 (ρ=0.493).

Patients without cancer had a moderate positive correlation between IL-17A and IL-10 (ρ=0.690), TGF-β (ρ=0.637), IFN-γ (ρ=0.572), IL-4 (ρ=0.565) and IL-12A (ρ=0.530). In patients with cancer, there was only a low positive correlation between IL-17A and IL-10 (ρ=0.493) and a moderate negative correlation with CagA (ρ=-0.537).

Patients without cancer exhibited a moderate positive correlation between TGF-β with IFN-γ (ρ=0.676) and IL-10 (ρ=0.621); and a low positive correlation with IL-4 (ρ=0.413). Patients with cancer only showed a moderate positive correlation between TGF-β and IFN-γ (ρ=0.581).

Patients without cancer exhibited a high positive correlation between IL-10 with IFN-γ (ρ=0.775); a moderate positive correlation with TGF-β (ρ=0.621), Foxp3 (ρ=0.579). Patients with cancer demonstrated a high positive correlation between IL-10 a moderate positive correlation with IL-12A (ρ=0.696), GATA3 (ρ=0.672) and *vacA-m2* (ρ=0.630).

Discussion

In the present study, the presence of *H. pylori* in gastric biopsies from patients with and without gastric cancer was examined, as well as the correlation between the development of this disorder and the presence of virulence factors, CagA and VacA (42), including the polymorphisms of *vacA* (*s1, s2, m1* and *m2*) (22). The *s1m1* polymorphism of *vacA* was found to be the most frequently observed polymorphism in the participants, consistent with a previous study on an adult population in Mexico (43).

The host factors which may affect the elimination or proliferation of *H. pylori* (44) were also analyzed in the present study. Gastric biopsies were obtained to determine the mRNA expression levels of interleukins, master transcription factors and co-modulators of the immune response. RT-qPCR analysis showed significantly lower relative mRNA expression levels of the co-activating molecule ICOS-L in patients with cancer compared with patients without cancer. ICOS-L is the only known ligand for ICOS, a receptor which activates

Table V. ρ values from correlation analysis between immune- and virulence-related variables in patients with and without gastric cancer.

A, Without cancer

Factor	IL-4	IL-10	IL-12	IL-17	IFN-γ	TGF-β	ICOS-L	PD-L1	RORγt	GATA3	FoxP3	vacA-m1	vacA-m2	CagA
ICOS-L	0.719	<0.4	0.712	0.411	<0.4	0.481	-	0.707	0.687	0.561	0.53	-	<0.4	-
PD-L1	0.668	0.428	0.675	0.570	0.454	0.525	0.707	-	-	<0.4	0.551	-	-	-
IL-12	0.785	0.509	-	0.530	0.437	-	0.712	0.675	0.772	0.509	0.784	-	<0.4	-
IL-17	-	0.690	0.530	-	-	-	0.411	0.570	0.603	-	-	-	-	<0.4
RORγt	0.737	-	0.772	-	-	-	0.687	-	-	-	0.708	<0.4	<0.4	-
vacA-m2	<0.4	<0.4	<0.4	-	-	-	<0.4	-	<0.4	-	-	-	-	-

B, Cancer

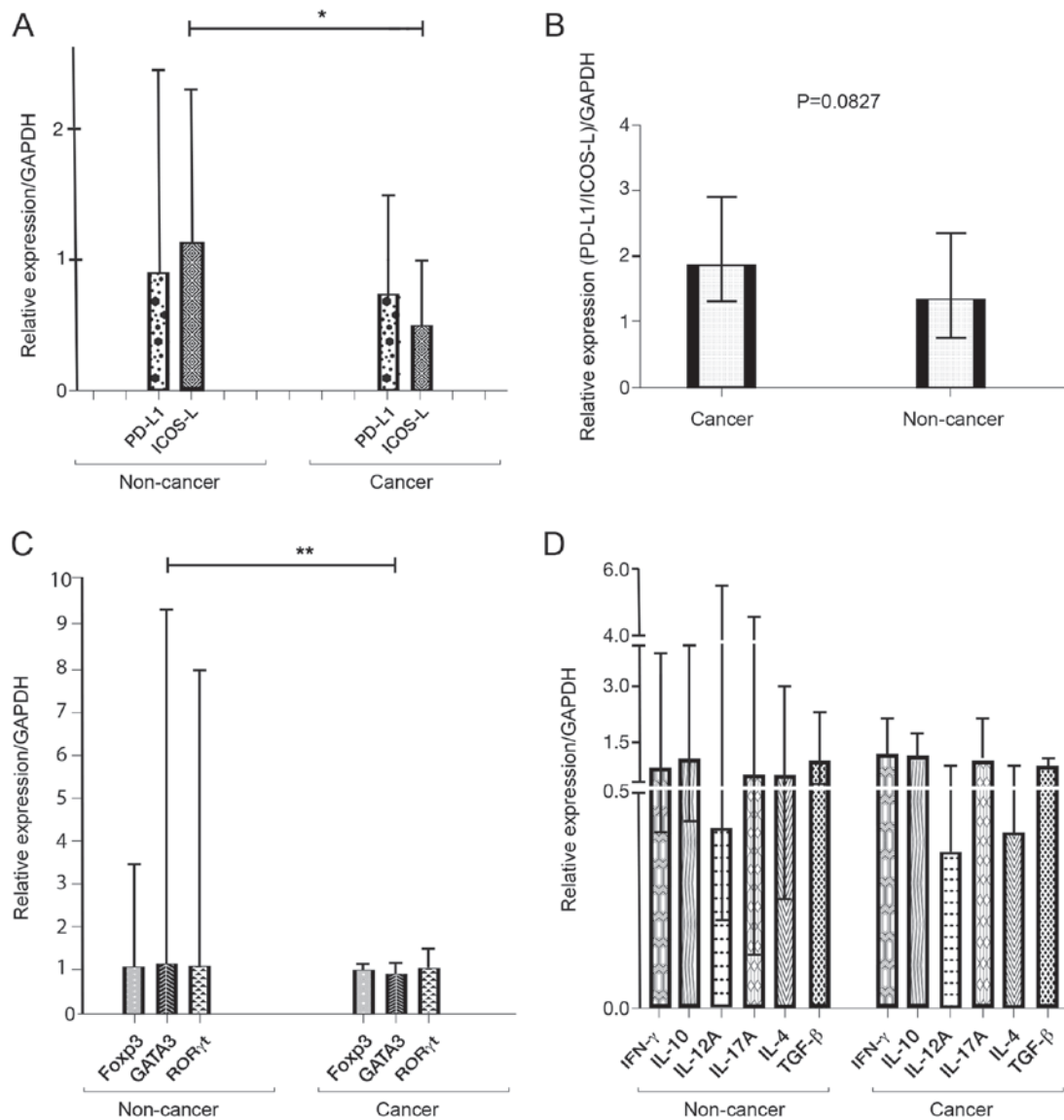| Factor	IL-4	IL-10	IL-12	IL-17	IFN-γ	TGF-β	ICOS-L	PD-L1	RORγt	GATA3	FoxP3	vacA-m1	vacA-m2	CagA
ICOS-L	0.566	0.708	0.772	<0.4	0.637	<0.4	-	0.610	0.760	0.689	0.490	-	0.567	-
PD-L1	<0.4	0.789	0.789	<0.4	0.485	<0.4	0.610	-	-	0.757	0.483	-	-	-
IL-12	0.593	0.696	-	<0.4	0.547	-	-	0.789	0.623	0.762	0.659	-	0.567	-
IL-17	-	0.493	-	-	-	-	-	-	<0.4	-	-	-	-	-0.537
RORγt	0.426	-	0.623	<0.4	-	-	0.760	-	-	-	<0.4	-0.756	-	-
vacA-m2	0.567	0.630	0.567	-	-	-	0.567	-	0.598	-	-	-	-	-

Figure 1. Analysis of cytokine, transcription factors and immunomodulatory molecules expression levels in gastric biopsies. (A) Relative mRNA expression levels of PD-L1 and ICOS-L in patients with and without cancer. *$P<0.05$. (B) Ratio of PD-L1/ICOS-L expression in patients with and without gastric cancer. (C) Relative expression of Foxp-3, GATA3 and RORγt in patients with and without gastric cancer. **$P<0.01$. (D) Relative expression of IL-4, IL-10, IL-12, IL-17, IFN-γ and TGF-β in patients with and without gastric cancer. Data are expressed as the median ± the interquartile range. PD-L1, programmed death ligand 1; RORγt, retinoid orphan receptor γt.

various inflammatory pathways implicated in the elimination of *H. pylori* (11).

In the patients with cancer, the mRNA expression levels of the master transcription factor GATA3 was significantly reduced. GATA3 promotes the differentiation of T-cells into Th2 cells (45). The reported negative association between allergies and *H. pylori* infection can be explained, at least in part, by hygiene theory, which is based on the fact that microbial infections protect against allergic processes by suppressing T-helper 2 immune responses (46). The inflammation caused by *H. pylori* can induce an imbalance in T helper cells between the Th1- and Th2-types in the gastric mucosa (47). When stimulated *ex vivo* with *H. pylori*, dendritic cells of peripheral blood (derived from mononuclear cells) exhibit increased production of IL-12 (48).

The differentiation to the Th1 phenotype was determined based on the expression of IL-12A and IFN-γ which constitute the most important cytokines produced by Th1 cells and underlie the suppression of GATA-3 in T-cells (49); a result observed in the present study as well in the patients with cancer. The suppression of GATA-3 may be explained by the fact that the *Helicobacter pylori* neutrophil-activating protein HP-NAP is an antagonist of TLR2 and stimulates neutrophils and monocytes to produce inflammatory cytokines associated with Th1 lymphocytes (50). Bagheri *et al* (51-53) showed that in the evolution of gastric pathologies caused by *H. pylori* infection, there is a dynamic change in the phenotypes of inflammatory cells of the innate and adaptive immune system, as well as in the underlying mechanisms of regulation and damage repair, performed by cells of the immune system and their mediators, with significant differences in the levels of expression of inflammatory and anti-inflammatory cytokines between healthy patients (no *H. pylori* infection) compared with patients infected with *H. pylori*, and at different stages of gastric infection.

To improve our understanding of the microenvironment in the gastric tissues, Spearman's rank correlation coefficient analysis was performed between various factors of the immune response: Co-modulating molecules, cytokines, transcription factors and variants of the virulent factors of *H. pylori*. In the participants without cancer, there was a positive correlation between ICOS-L and IL-17A. The latter cytokine represents the Th17 group, which is associated with the elimination of *H. pylori* (54). This correlation did not exist in the patients with cancer, perhaps due to the phenomenon described by Downs (55); the microenvironment of the tumor has a mechanism for evading the immune response through the transdifferentiation of the lymphocytes from a Th17⁺/Foxp3⁻ phenotype to a Th17⁺/Foxp3⁺ phenotype, resulting in an increase in the population of Treg cells as well as anti-inflammatory cytokines.

Unlike the patients without cancer, those with cancer showed a positive correlation between ICOS-L and IL-10 (Treg) and IFN-γ (Th1). It has been reported that signaling through ICOS-L can have a direct effect on dendritic cells via phosphorylation of p38-MAPK and decrease the expression of IL-10/FoxP3 in cell lines (56). In the results of the present study, the high correlation between IL-10 and ICOS-L observed in patients with cancer suggests a possible alteration of the p38-MAPK pathway.

The expression of the transcription factor GATA3 was significantly different between the two groups. The most notable positive correlation of GATA 3 was with IL-4 (the primary cytokine of the Th2 inflammatory phenotype) in the patients without cancer. For the other transcription factors analyzed by RT-qPCR (RORγt and Foxp3), the relative expression levels of their mRNA was lower in the patients with cancer, but the difference was not significant. A key correlation, reported for the first time in the present study, to our knowledge, was the high negative correlation between the ROR transcription factor and the m1 vacA polymorphism, additionally a negative correlation between IL-17A and CagA was identified. Establishing that both proteins interfere with the Th17 inflammatory profile activation axis. The previous result indicates a possible synergy of both proteins to interfere with the axis of activation of the Th17 inflammatory profile. The synergy between CagA-VacA has only been documented to generate damage to gastric cells and facilitate iron acquisition (57).

Additionally, there was a moderate negative correlation between IL-17A and the oncoprotein CagA in the patients with (but not without) cancer. The same inverse association has been explained as a reduction in ICOS-L expression in gastric epithelial cells through CagA-induced activation of the mTOR kinase p70 S6 signaling pathway, associated with a reduction in Th17 cells (19). The mechanism of evasion used by *H. pylori* may be coordinated by the polymorphism of the middle region of *vacA* (*m1*) and the presence of the CagA protein to decrease the Th17 phenotype, and thus allow the bacteria to survive and proliferate in the host. The genotypes of *H. pylori* with these characteristics thus constitute an increased risk of gastric cancer. A meta-analysis revealed that *m1* of *vacA* is the greatest risk factor for gastric cancer (25).

In the participants without cancer, IL-17A exhibited a high correlation with IL-10, TGF-β, Foxp3 and RORγt.

Downs-Canner *et al* (55) showed that Th17 lymphocytes are dependent on TGF-β for their differentiation. Furthermore, plasticity has been described for the transdifferentiation of Th17 cells to Th17-IL-17neg-Foxp3⁺ cells, which is induced by the microenvironment of tumoral tissue (55). The significant correlation shown between IL-17A and Foxp3 in healthy tissue establishes a balance in the inflammatory/anti-inflammatory response, whereas in patients with cancer, the expression of Foxp3 with anti-inflammatory function, predominates in the tumor microenvironment.

Similarly, in the patients without (but not with) cancer, there a high positive correlation between RORγt and Foxp3. There is an antagonism between tumor tissue and Foxp3 T-cells, which has been reported for RORγt in mice (58). Recently it was documented that in the microenvironment of tumor tissue, a reduced number of Foxp3⁺ T-cells resulted in a change in the phenotype of Foxp3⁺ T-cells by the gene of the master transcription factor RORγt (55).

There was a very high correlation between TGF-β and IFN-γ and a high correlation between TGF-β and IL-17A in the patients without stomach cancer. The co-cultivation of CD4⁺ T-cells and macrophages was previously demonstrated to increase the secretion of IFN-γ and IL-17A. When infected with *H. pylori*, these cells exhibited upregulated expression of RORγt and an increase in the number of Th17 cells (59). The correlation between TGF-β and RORγt, IL-17A, Foxp3 and IL-10 was only observed in the patients without cancer. Additionally in the patients without cancer, a correlation was also observed between TGF-β and the two co-modulating molecules of the inflammatory response (ICOS-L and PD-L1).

RORγt is the master transcription factor for the differentiation of T-cells to Th17. Similarly, IL-6 and TGF-β concomitantly serve an important roles in initiating differentiation (58). It is thus hypothesized that if a chronic infection results in the transformation of tumor cells, the correlation between TGF-β and cytokines, including transcription factors, may be lost. In the patients with cancer in the present study, TGF-β was only correlated with cytokines, and moderately with IFN-γ.

Of the polymorphisms of *vacA*, only the *m2* polymorphism showed a high positive correlation with RORγt, IL-12A, IL-4 and the co-stimulatory molecule ICOS-L, in patients with cancer. Notably, a meta-analysis showed an inverse correlation between the *vacA-m2* polymorphism and the risk of gastric cancer (25). In the present study, cytokines and transcription factors were positively correlated with the *vacA-m2* polymorphism, suggesting that the cytokines were associated with elimination of *H. pylori* and protection against gastric cancer.

In patients with gastric cancer, IL-10 was the anti-inflammatory cytokine which exhibited the highest correlation with the co-inhibitory molecule PD-L1 and with IL-12A; whereas in the patients without cancer, IFN-γ and IL-17A exhibited the highest correlations with IL-10.

According to a previous study, *H. pylori* infection deregulates the expression of PD-L1, and the primary cytokine secreted during infection (principally by dendritic cells) is IL-10. The levels of IL-12A and IFN-γ are increased only if the dendritic cells are treated with CD40L (60). *H. pylori*

is capable of stimulating IL-23 secretion, which belongs to the family of IL-12A, and stimulates the production of IL-17 from TH-17 cells. However, following prolonged stimulation, the capacity of dendritic cells to produce IL-12A is reduced (61).

The results of the present study suggest that the evasion of the immune system by *H. pylori* occurs predominantly through the downregulation of the expression of ICOS-L in individuals with gastric cancer. These results suggest a coordination of the virulence factors *cagA* and *vacA* of *H. pylori* (particularly the *m1* polymorphism of *vacA*) to inhibit the differentiation of cells to the inflammatory Th17 phenotype.

In conclusion, the relative mRNA expression levels of the co-activating molecules ICOS-L and GATA3 (the master transcription factor for Th2 phenotype) were significantly lower in patients with cancer compared with patients without cancer. To the best of our knowledge, the present study is the first to show a high inverse correlation between the *m1* polymorphism of *vacA* and the master transcription factor RORγt. Additionally, a previously described negative correlation between *cagA* and IL-17A was confirmed (55). Based on these results, it is hypothesized that *vacA-m1* and *cagA* coordinate the inhibition of the inflammatory response of Th17 cells. There was no correlation between TGF-β and other cytokines or transcription factors in the patients with cancer. TGF-β and IL-6 are essential for the differentiation of the Th17 phenotype. The present study focused on correlations between the factors involved in the immune system, *H. pylori* and gastric cancer, showing novel associations of bacterial virulence factors and possible points of modulation of the inflammatory response. These results may highlight potential avenues for the design, diagnosis and therapeutics in oncological pathologies

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

The present study was performed by RGS and JGEC. Patient management was performed by EVMC, JTL, MITR, LAWG and RGJJ. Samples analysis was performed by MECV and JVJ. PCR analysis was performed by JGEC, MHMO and MEVC. Statistics analysis was performed by JELR and JGEC. The literature review, manuscript and references was performed by JGEC and RGS. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients provided written informed consent, in accordance with the Helsinki Declaration and the Ethics Committee of the National Medical Center (Centro Médico Nacional 20 de November) of the ISSSTE Medical Service. The protocol used in the present study was approved by the Bioethics Committee of the Autonomous University of Aguascalientes (approval no. CIB-UAA-26)

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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