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Helicobacter pylori infection and relationship with gastric epithelial cell proliferation and apoptosis

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Aim: To evaluate the effects of *Helicobacter pylori* on cell proliferation and apoptosis in gastric cancer (GC) both at tumoral and nontumoral sites and in gastritis.

Materials and methods: Biopsies from 96 patients (gastric cancer = 52, gastritis = 44) were examined immunohistochemically and histopathologically.

Results: In *H. pylori*-positive GC patients the average proliferative index (PI) in tumor and nontumor sites was 40.7% and 24.8%, while in *H. pylori*-negative patients it was 32.9% and 17.8%, respectively. The PI was significantly higher in the tumor than in the nontumor sites, regardless of *H. pylori* infection. Cell proliferation was also significantly associated with intestinal metaplasia in nontumor sections. The apoptotic index (AI) in *H. pylori*-positive GC tumor and nontumor sites showed an average of 61.8% and 50.0%, while in *H. pylori*-negative patients it was 38.1% and 25.0%, respectively. The AI was significantly higher in both groups in the presence of *H. pylori*. In gastritis, *H. pylori* infections lead to a significantly higher PI than AI. *H. pylori* infection increased the PI and the AI in cases of GC.

Conclusion: *H. pylori* induced higher proliferative and apoptotic activities in both tumor and nontumor sites of the GC sections, a fact that provides supportive evidence for its effect.

Key words: *Helicobacter pylori*, cell proliferation, apoptosis, gastric cancer, gastritis

1. Introduction

Infection of the human gastric mucosa with *Helicobacter pylori* generally causes gastritis. In addition, the organism has been implicated in the development of other gastrointestinal diseases, such as peptic ulceration, adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (1).

Although the mechanisms causing chronic active gastritis from other diseases are unclear, various bacterial factors, as well as the host's responses, are believed to contribute to the outcome of infection with *H. pylori* (2,3).

The development of gastric cancer (GC) is a multistep process that follows a path of progression from chronic gastritis, mucosal atrophy, intestinal metaplasia (IM), dysplasia, and finally cancer (4). *H. pylori*, which is considered an oncogenic agent, may influence telomerase activity, particularly after IM (5).This result supports the hypothesis that *H. pylori* may increase the risk of gastric cancer after IM (5).

H. pylori invades the gastric mucosa and starts alterations in the normal cell turnover in epithelial cells. Changes in the activity of apoptosis and/or cell proliferation ultimately may lead to ulceration or cancer (6). p53, CD95 (Fas), the Bcl-2 family, and caspases are the factors involved in the regulation of the balance between apoptosis and proliferation (7). Apoptosis has an essential function in maintaining the integrity of the gastrointestinal mucosa; however, deregulation of this process is associated with the occurrence of severe lesions (8).

Apoptosis is an important factor that results in cell death. Caspase 8 activation, oxidative stress, the Bcl-2 family, stress-induced protein genes, transcription factor p53, neutrophil activation, and TNF-α may be involved in leading up to apoptosis. Alhan et al. showed an increased apoptotic index (AI) in experimental acute pancreatitis in rats, probably due to oxidative stress-related activation of caspase pathways (9). They did not find any effect of p53 inactivation therapy during the course of pancreatitis, which suggested that p53 does not play a role in the apoptotic mechanism in acute experimental pancreatitis design (9).

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H. pylori strains bearing the cag pathogenicity island (PAI), including the cagA gene, have been shown to be associated with increased gastric inflammation, increased bacterial load, and both peptic ulcer disease and GC (2). During infection with cagA-positive–*H. pylori*, the bacterium injects its cagA protein into the gastric epithelial cells, leading to deregulation of the intracellular signaling pathways (1). According to some previous studies, the presence or absence of *H. pylori* infection does not have an effect on the AI or the degree of atrophy of the gastric mucosa (10,11). Another report showed that *H. pylori* infection may cause a decrease in apoptotic activity followed by an increase in cell proliferation (8). Eradication of *H. pylori* resulted in a significant decrease in gastric epithelial cell proliferation and apoptosis, indicating an important role for *H. pylori* in this process (8).

Ki-67 is a nuclear antigen that has been used as a proliferating marker that is expressed in all phases of the cell cycle except G0 (12,13). It is unlikely that apoptosis involves just a few cells at a specific site of the tissue examined, which is why it may be found at different steps of this process (14). It is also detected by immunohistochemical assays such as caspase and TUNEL (15,16).

To our knowledge, no previous study has looked in depth into the proliferative index (PI) and AI of tumor and nontumor sites of the same resected GC sections. The objectives of this study were to evaluate the effect of *H. pylori* on cell proliferative and apoptotic activities in tumor and nontumor sites of each resected GC section, to compare such activities with that of gastritis sections, and to detect the role of cagA gene and EPIYA motifs in these processes.

2. Material and methods

2.1. Biopsy samples

Included in this study were 96 patients who attended the İstanbul Teaching Hospital. There were 52 GC patients, from whom biopsy samples were obtained during the period of March 2007 to March 2010. The age range of these patients was 33–82 years (average: 61), and 12 were female. From each patient 2 biopsy samples from the resected GC tissues, 1 from a nontumor site and 1 from a tumor site (formalin-fixed paraffin-embedded tissues) were collected. The other 44 were patients with gastritis from whom antral biopsy samples were collected during the period of May 2010 to January 2011. The age range of these patients was 15–84 years (average: 46.4), and 21 were females. Sections were processed and examined by immunohistochemical analysis and histopathology. Written consent was obtained from each patient and the study was approved by the hospital's ethics committee.

2.2. DNA isolation and PCR

DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions, and stored at –20 °C until used. For the amplification of cagA gene the following primers were used: cagA1 (GATAACAGGCAACGTTTTCAGGGA) and cagA2 (CCGAACGGATCAAAAATTCATGG). The amplification conditions were 96 °C for 5 min, then 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. PCR products were separated by 2% agarose gel electrophoresis. The following primers were used to amplify the EPIYA motif encoding sequences A, B, C, and D, respectively: the forward primer cagA2, and the reverse primers cagA-P1C (GTCCTGCTTTCTTTTTATTAACTTKAGC), cagA-P2CG (TTTAGCAACTTGAGCGTAAATGGG), cagA-P2TA (TTTAGCAACTTGAGTATAAATGGG), and cagA-P3E (ATCAATTGTAGCGTAAATGGG) (17).

2.3. Immunohistochemical analysis

2.3.1. Cell proliferation assay (Ki-67 staining)

The Ki-67 staining for detecting cell proliferation in paraffin-embedded sections of biopsy samples from GC and gastritis patients was performed using the monoclonal antibody Clone SP6 (Thermo Scientific, USA). Sections of 5 µm were cut using a Tissue-Tek Cryo$_3$ Microtome (Sakura Finetek Inc., USA), then deparaffinized in a hot air oven at 70 °C. Immunostaining was performed using the fully automated Leica BOND-MAX instrument (Leica Microsystems, Germany). Sections were then examined under the light microscope and positive cells were detected by brown nuclear staining. The PI in *H. pylori*-positive and -negative sections was determined by calculating the percentage of positive cells in around 500 epithelial cells observed in 3 randomly selected gastric pit zones.

2.3.2 Apoptosis assays

2.3.2.1. Caspase-3 staining (CPP32 assay)

The detection of apoptotic cells in paraffin-embedded sections was performed using monoclonal antibody to CPP32 (ABC Kit, Novocastra, UK). Preparation of sections and immunostaining were performed as described above with the automated Leica BOND-MAX instrument (Leica Microsystems). Sections were then examined under the light microscope and positive cells were detected by brown cytoplasmic staining. The AI in *H. pylori*-positive and negative sections was calculated as the percentage of CPP32-positive cells in 500 epithelial cells examined for each section in 3 randomly selected zones under the light microscope.

2.3.2.2. TUNEL assay

Apoptotic cells were also detected by TUNEL using the TACS 2 TdT DAB In Situ Apoptosis Detection Kit (Trevigen, USA). Immunostaining and the calculation of AI were done similarly to that for CPP32 and positive cells (nuclear immunostaining) were recorded.

2.3.3. Histopathology

Tissue sections obtained from both nontumor and tumor sites of GC patients and antral biopsies from gastritis patients were stained with Giemsa and hematoxylin and eosin (HE) stains. *H. pylori* status and density, chronic gastritis (mononuclear cell activity), neutrophil infiltration, glandular atrophy, and IM were evaluated and graded from 0 to 3 according to the updated Sydney system (18). The type of GC (intestinal or diffuse) was classified according to the Lauren classification. The morphological evaluation of all sections was done by an experienced histopathologist who was not aware of the section's origin.

2.4. Statistical analysis

The correlation between the dependent variables (cell proliferation, apoptosis) and the independent variables (*H. pylori*, sex, age, IM, atrophy, tumor type) was analyzed using regression analysis for both GC and gastritis patients. Differences between cell proliferation and AIs in the presence or absence of *H. pylori* were analyzed using the independent sample t-test. Comparison between GC nontumor and tumor sites was made by the paired sample t-test. $P < 0.05$ was considered as statistically significant.

3. Results

In paraffin-embedded tissue sections, *H. pylori* was detected by either staining with Giemsa stain or by PCR. *H. pylori* was positive in 35 (67.3%) of 52 samples from GC patients. The presence of *H. pylori* in the gastric antral biopsies was detected either by rapid urease test or PCR. *H. pylori* was positive in 31 (70.4%) of 44 gastric biopsies from gastritis patients. The cagA gene was detected in 15 (48.3%) of 31 *H. pylori*-positive samples in gastric biopsies. The EPIYA motifs were of the ABC type in 14 (93.3%) and ABCC type in 1 (6.7%) of the isolates from gastritis patients.

Immunohistochemical analysis of cell proliferation detected by Ki-67 staining (Figure 1) in *H. pylori*-positive GC sections showed that the average PI in tumor and nontumor sites was 40.7% and 24.8%, respectively, while in both sites of *H. pylori*-negative sections it was 32.9% and 17.8%, respectively (Table 1). Correlations between cell proliferation and the independent variables (*H. pylori*, sex, age, IM, atrophy, and tumor type) are shown in Table 2.

The AI in GC sections detected by caspase-3 staining in *H. pylori*-positive sections showed that the average results in tumor and nontumor sites were 61.8% and 50.0%, respectively, while in *H. pylori*-negative sections they were 38.1% and 25.0%, respectively (Table 1). Statistical analysis showed that the AI in GC nontumor and tumor sections was significantly higher in the presence of *H. pylori* than that in the absence of *H. pylori* (Figure 2). Correlation between the AI in GC nontumor sites was not significantly associated with any of the independent variables (*H. pylori*, sex, age, IM, atrophy, and tumor type). However, in tumor sites, a significant association with the independent variables *H. pylori* ($P = 0.011$) and sex ($P = 0.047$) was observed (data not shown).

On the other hand, immunohistochemical analysis of gastric biopsy sections from gastritis patients showed that the average results of the PI in *H. pylori*-positive sections were 54.8% while in *H. pylori*-negative they were 30.0% (Table 1). The PI in these sections was significantly higher in the presence of *H. pylori* than in the absence of *H. pylori*. In these sections cell proliferation was significantly associated with the independent variables of chronic inflammation ($P = 0.011$), neutrophil infiltration ($P = 0.000$), and increase in *H. pylori* density (data not shown). We also found that the PI was significantly higher than the AI in the presence of *H. pylori*. The average results of the AI in *H. pylori*-positive sections were 36.1% and in *H. pylori*-negative were 42.4% (Table 1), and the AI was

Table 1. Average percentages of cell proliferative index (PI) and apoptotic index (AI) detected in paraffin-embedded sections of *H. pylori*-negative and -positive biopsy samples from gastric cancer and gastritis patients.

		Gastric cancer section						Gastritis section			
		PI			AI				PI	AI	
Hp	**No.**	**NT**	**T**	**t-test**	**NT**	**T**	**t-test**	**No.**			**t-test**
Hp–	**17**	17.8%	32.9%	t = 3.66* SE = 4.14	25.0%	38.1%	t = 2.78* SE = 4.72	**13**	30.0%	42.4%	t = 1.53 SE = 7.96
Hp+	**35**	24.8%	40.7%	t = 3.23* SE = 4.92	50.0%	61.8%	t = 1.98 SE = 6.01	**31**	54.8%	36.1%	t = 3.72* SE = 5.03
t-test		t = 1.02 SE = 6.9	t = 1.27 SE = 6.14		t = 2.73* SE = 8.4	t = 3.75* SE = 7.11			t = 4.12* SE = 5.96	t = 1.0 SE = 6.24	

Hp: *H. pylori*, NT: nontumor site, T: tumor site.

*: $P < 0.05$, SE: standard error.

Table 2. The results of regression analysis showing the relation between Ki-67 staining of nontumor sites of gastric cancer sections and factors analyzed for the study's dependent variable: Ki-67 nontumor sections.

Predictors	Coefficient	Std. error	t	Sig.
(Constant)	45.666	20.009	2.282	0.027
Sex	–0.571	7.586	–0.075	0.94
Age	–0.529	0.29	–1.826	0.074
Hp	6.874	9.233	0.745	0.46
IM	21.963	8.598	2.554	0.014
Atr	–17.488	8.821	–1.983	0.054
Tt	–0.745	10.401	–0.072	0.943

Independent variables: (constant), sex, age, Hp: H. pylori, IM: intestinal metaplasia, Atr: atrophy, Tt: tumor type.
$R^2 = 0.207$, Durbin–Watson = 1.821, F = 1.962, P = 0.091.

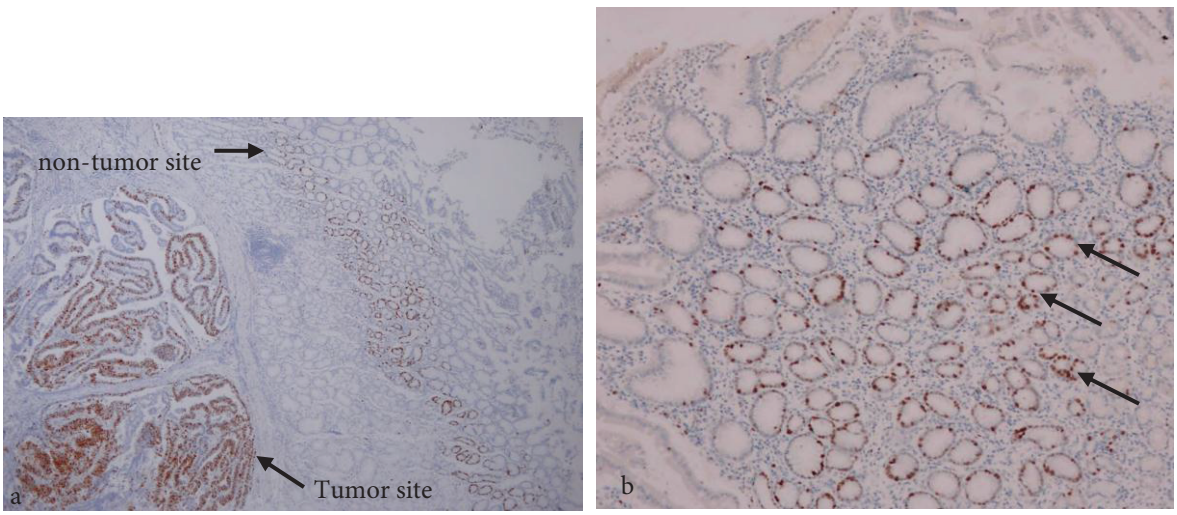

Figure 1. a) Immunohistochemical staining for Ki-67 of tumor and nontumor sites of intestinal type gastric cancer section (*H. pylori*-positive; magnification of 40×). b) Positive cells were commonly found in the neck region of the gastric pits (arrows; magnification of 100×).

significantly associated with IM (P = 0.033). The TUNEL assay was also used to detect the apoptotic activities in 22 gastric biopsy sections from gastritis patients and to compare the results with those of the caspase-3 staining. We found that the average results in 17 *H. pylori*-positive and 5 *H. pylori*-negative sections were 23.3% and 19.0%, respectively. The PI and AI detected by this assay were lower than those detected by the caspase-3 assay.

The results of a paired sample t-test revealed statistically significant differences for PI, which was lower than the AI in GC tumor and nontumor sites (P = 0.000). We also found that in the presence of *H. pylori*, the PI in gastritis

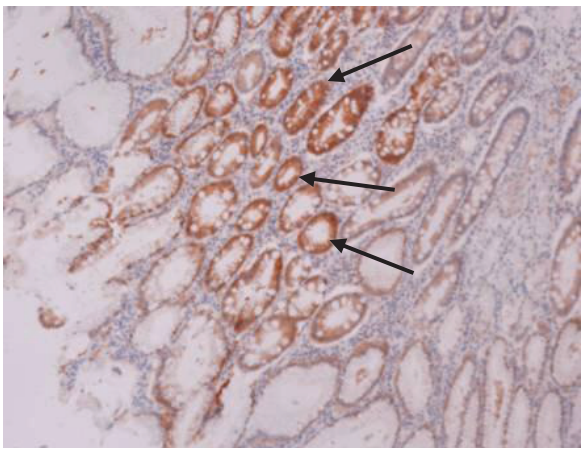

Figure 2. Immunohistochemical staining for caspase-3 of nontumor site of *H. pylori*-positive gastric cancer section. The cytoplasm of positive cells was stained with brown color (arrows) (magnification of 100×).

sections was significantly higher than that of GC tumor and nontumor sites (P = 0.001).

Histopathology evaluation of the tumor type in biopsy sections from 52 patients with GC showed that of 17 *H. pylori*-negative cases, 5 (29.4%) were of the intestinal type and 12 (70.6%) of the diffuse type. All sections showed chronic gastritis and, of these, 9 (52.9%) had IM and glandular atrophy. Biopsy sections from the other 35 *H. pylori*-positive patients with GC revealed that 34 (97.2%) were of the intestinal type and 1 (2.8%) was of the diffuse type. 3 shows tumor tissue of the intestinal type stained with HE stain. Chronic gastritis was detected in all sections, and of these 24 (68.5%) had IM and glandular atrophy. The type of IM detected in these sections was of the complete type in 81% of the GC sections. On the other hand, biopsy sections from 44 patients with gastritis revealed that of 13 patients with no *H. pylori*, 84.6% had chronic inflammation, 38.4% had neutrophil infiltration, and 3 (23.0%) had IM. Biopsy sections from the other 31 *H. pylori*-positive patients with gastritis showed 100% with chronic inflammation, 93.5% with neutrophil infiltration, and 10 (32.2%) with IM and glandular atrophy.

4. Discussion

The gastric mucosa continuously renews its cells and is capable of repair after mucosal injury. This is achieved by sloughing off the damaged cells and rapid migration of proliferated cells to replace the dead cells. This balance is maintained by cell proliferation and apoptotic mechanisms. An imbalance between these 2 mechanisms might lead to increased cell proliferation and the development of GC (7,19,20).

Subjects infected with *H. pylori*, a major etiological factor of GC reported to be the initial triggering mechanism, develop chronic active gastritis and subsequently progress to gastric atrophy and IM, and ultimately to cancer (21). Previous studies revealed significant difference between cell proliferation and apoptosis in patients with and without *H. pylori* infection. Some studies showed that *H. pylori* infection has a relationship with an increased apoptosis rate in gastric lesions such as chronic gastritis, GU, IM, and GC (14,22–26). However, some studies showed that the presence or absence of *H. pylori* did not influence the AI (10,11).

Several studies showed contradicting results in regards to *H. pylori* infection. No significant difference was found between the AI of *H. pylori*-negative normal and *H. pylori*-positive groups with gastritis and GC (27,28). Leite et al. (29) also reported no significant difference in AI between *H. pylori*-positive and -negative groups with gastritis, while Tiwari et al. (22) and Shiotani et al. (16) found a higher AI in *H. pylori*-positive GC and gastritis groups. These contradictory results might be due to the presence or absence of *H. pylori* virulence factors such as cagPAI, cagA gene, and EPIYA motifs, which may be responsible for the differences in apoptotic ratios reported in different groups of gastritis and GC patients infected with *H. pylori*. The CPP32 antibody detects cells undergoing apoptosis at an early stage, before DNA fragmentation, a late event in the apoptotic process measured by the TUNEL assay. This might also be attributed, as suggested earlier, to the fact that some cells may undergo apoptosis without the activation of caspase-3 (23,30,31).

In this study, the presence of *H. pylori* provoked higher proliferative activities in our GC tumor and nontumor sections than in the absence of *H. pylori*. Such activities were significantly higher in the tumor sections regardless of the presence or absence of *H. pylori*. A high PI detected in the tumor sections in the absence of *H. pylori* indicates a biological requirement for the tumor tissue to increase its proliferative activities. On the other hand, the presence of *H. pylori* also induced significantly higher apoptotic activities in the tumor and nontumor sections in the absence of *H. pylori*. Similarly, Shiotani et al. (16) found that *H. pylori* infection was associated with increased cell proliferation and apoptosis in GC patients but not the controls. However, van der Woude et al. (27), Yoo et al. (28), and Leite et al. (29) found no significant differences between the AIs of *H. pylori*-positive subjects with GC and *H. pylori*-negative normal subjects. These findings do not seem to be clear-cut for the effect of *H. pylori* on these activities since many published reports were in disagreement. Unlike previous reports, we have shown such an effect in both tumor and nontumor sites of the

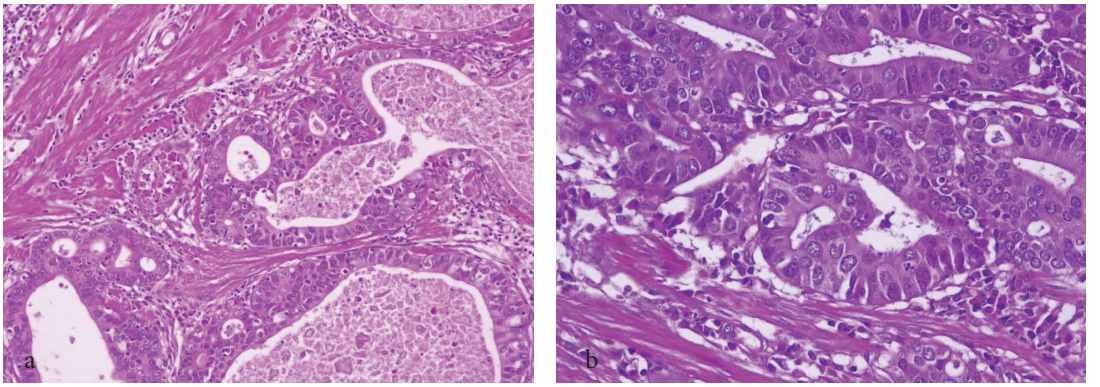

Figure 3. Intestinal-type gastric cancer section (*H. pylori*-positive) stained with HE showing invasive tumor cells: a) magnification of 200×, b) magnification of 400×.

same resected sections, a fact that substantiates the effect of *H. pylori* on these activities. The proliferative activities detected in our gastritis sections were also significantly higher in the presence of *H. pylori* while no such effect was noticed on the apoptotic activities. Thus, the comparison of these activities reveals that *H. pylori* infection increased both the PI and AI in our GC sections, while it only increased the PI in gastritis sections.

Several investigators have reported enhancement of cell proliferation and apoptosis in the gastric epithelium during *H. pylori* infection. Others failed, however, to establish such a straightforward correlation, emphasizing the importance of diversities of the strains.

In this study, the presence of cagA and the ABC-type EPIYA motifs of *H. pylori* detected in gastric biopsies from gastritis patients did not seem to affect the PI/AI ratio as compared to *H. pylori*-negative biopsies. Further samples from peptic ulcer and GC patients infected with *H. pylori* that are cagA-positive with an increased number of the EPIYA-C motifs are required to elaborate on the role of these factors in these processes. Tiwari et al. (22) also found no difference in AI in relation to the presence of cagA.

We have found that the PI and the AI detected by the TUNEL assay were lower than those detected by the CPP32 assay, regardless of the presence or absence of *H. pylori*. This might be explained, as suggested earlier, by the fact that immunohistochemical assay using CPP32 antibody detects cells undergoing apoptosis at an early stage, before DNA fragmentation, a late event in the apoptotic process measured by the TUNEL assay. Thus, the CPP32 assay is considered more sensitive and specific, and less dependent on the observer's subjective assessment.

Patients with IM are more likely to be at risk of developing GC since it is implicated in the imbalance of cell proliferation and apoptosis in the precancerous lesions. Shinotami et al. (16) reported that the PI in the antrum was significantly higher in GC patients with IM than in the control group. Wambura et al. (32) compared the rates of apoptosis and proliferation in mucosa with and without IM and demonstrated that, while antral apoptosis was significantly lower in IM, proliferation was significantly higher. We have also found that cell proliferation was significantly associated with the presence of IM in nontumor sites. This shows an agreement on the effect of *H. pylori* on cell proliferation during the precancerous stages, leading to an imbalance in cell kinetics. In addition, the majority of IM cases detected in our sections were of the complete type. In gastritis sections, infection with *H. pylori* provoked more severe histopathological changes and appeared to induce IM more so than in those without the bacterium.

We have found that the majority of the GC types detected in *H. pylori*-positive sections were of the intestinal type, while in *H. pylori*-negative they were of the diffuse type, emphasizing the important role of *H. pylori* in this process. We detected no significant differences in the staining of GC sections (intestinal type or diffuse type) or gastritis sections for cell proliferation and apoptosis in this study. Other investigators also reported no such differences (27,28).

In conclusion, *H. pylori* infection increased the proliferative and apoptotic activities in tumor and nontumor sites of GC sections. The PI was significantly higher in the tumor sites than in the nontumor sites, regardless of *H. pylori* infection, while the AI was significantly higher in both sites in the presence of *H. pylori*. Cell proliferation, but not apoptosis, was found to be significantly associated with IM in nontumor sites. In gastritis sections a significantly higher PI than AI was detected in the presence of *H. pylori*. Overall, the detection of cell proliferation and apoptotic activities in tumor and nontumor sites of each resected GC section provided additional supportive evidence for the effect of *H. pylori* on these activities.

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