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ORIGINAL ARTICLE

Esophageal *Helicobacter pylori* colonization aggravates esophageal injury caused by reflux

Yun-Xiang Chu, Wei-Hong Wang, Yun Dai, Gui-Gen Teng, Shu-Jun Wang

Yun-Xiang Chu, Wei-Hong Wang, Yun Dai, Gui-Gen Teng, Shu-Jun Wang, Department of Gastroenterology, Peking University First Hospital, Beijing 100034, China
Author contributions: Chu YX and Wang WH contributed equally to this work; Chu YX performed the majority of experiments and wrote the manuscript; Wang WH designed the study and was involved in writing of the manuscript; Dai Y, Teng GG, and Wang SJ provided technical assistance; all of the authors read and approved the final version of the manuscript.

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Correspondence to: Wei-Hong Wang, Professor, Department of Gastroenterology, Peking University First Hospital, 8 Xishiku Avenue, Xicheng District, Beijing 100034, China. wangweihong@medmail.com.cn
Telephone: +86-10-83572616 Fax: +86-10-66518105
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Abstract

AIM: To investigate esophageal *Helicobacter pylori* (*H. pylori*) colonization on esophageal injury caused by reflux and the related mechanisms.

METHODS: An esophagitis model, with acid and bile reflux, was surgically produced in male rats. The rats were randomly divided into either: (1) an esophagogastroduodenal anastomosis (EGDA) group; (2) an EGDA with *H. pylori* infection group; (3) a pseudo-operation with *H. pylori* infection group; or (4) a pseudo-operation group. All rats were kept for 36 wk. Based on the location of *H. pylori* colonization, the EGDA rats with *H. pylori* infection were subdivided into those with concomitant esophageal *H. pylori* colonization or those with only gastric *H. pylori* colonization. The esophageal injuries were evaluated grossly and microscopically. The expressions of CDX2 and MUC2 were determined by real-time polymerase chain reaction (RT-PCR) and immunohistochemistry. Ki-67 antigen expression was determined by immunohistochemistry. The mRNA levels of cyclin D1, c-Myc, Bax and Bcl-2 were determined by RT-PCR. Cell apoptosis was evaluated using the TdT-mediated dUTP nick-end labeling method.

RESULTS: Esophagitis, Barrett's esophagus (BE), and esophageal adenocarcinoma (EAC) developed in rats that underwent EGDA. When comparing rats with EGDA and concomitant esophageal *H. pylori* colonization to EGDA-only rats, the severity of injury (87.9 ± 5.2 *vs* 77.2 ± 8.6, macroscopically, 92.5 ± 8.0 *vs* 83.8 ± 5.5, microscopically, both $P < 0.05$) and the incidences of BE (80.0% *vs* 33.3%, $P = 0.055$) and EAC (60.0% *vs* 11.1%, $P < 0.05$) were increased. These increases were associated with upregulation of CDX2 and MUC2 mRNA (10.1 ± 5.4 *vs* 3.0 ± 2.9, 8.4 ± 4.6 *vs* 2.0 ± 3.2, respectively, *P*s < 0.01) and protein (8.1 ± 2.3 *vs* 3.3 ± 3.1, 7.3 ± 4.0 *vs* 1.8 ± 2.7, respectively, all $P < 0.05$). The expression of Ki-67 (8.9 ± 0.7 *vs* 6.0 ± 1.7, $P < 0.01$) and the presence of apoptotic cells (8.3 ± 1.1 *vs* 5.3 ± 1.7, $P < 0.01$) were also increased significantly in rats with EGDA and concomitant esophageal *H. pylori* colonization compared with rats with EGDA only. The mRNA levels of cyclin D1 (5.8 ± 1.9 *vs* 3.4 ± 1.3, $P < 0.01$), c-Myc (6.4 ± 1.7 *vs* 3.7 ± 1.2, $P < 0.01$), and Bax (8.6 ± 1.6 *vs* 5.1 ± 1.3, $P < 0.01$) were significantly increased, whereas the mRNA level of Bcl-2 (0.6 ± 0.3 *vs* 0.8 ± 0.3, $P < 0.01$) was significantly reduced in rats with EGDA and concomitant esophageal *H. pylori* colonization compared with rats with EGDA only.

CONCLUSION: Esophageal *H. pylori* colonization increases esophagitis severity, and facilitates the development of BE and EAC with the augmentation of cell proliferation and apoptosis in esophageal mucosa.

Key words: *Helicobacter pylori*; Esophagus; Metaplasia;

Adenocarcinoma; Animal model

Core tip: The relationship between gastroesophageal reflux disease and *Helicobacter pylori* (*H. pylori*) is controversial. This study demonstrates that esophageal *H. pylori* colonization can aggravate esophageal injury and promote the incidence of Barrett's esophagus and esophageal adenocarcinoma. Gastric *H. pylori* colonization did not aggravate esophageal mucosal lesions in rats with mixed reflux. However, esophageal *H. pylori* infection was associated with increased cell proliferation and apoptosis in the esophagi of rats with mixed reflux. Loss of balance between cell proliferation and apoptosis may be important in *H. pylori*-induced esophageal malignancy.

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INTRODUCTION

Esophageal adenocarcinoma (EAC) has received considerable attention in recent years due to the rapid increase in incidence rate and poor prognosis[1]. As a result, the number of studies attempting to elucidate the mechanisms of this increasingly prevalent cancer has increased. Barrett's esophagus (BE) is the result of intestinal metaplasia, where the normal esophageal squamous epithelium is replaced by simple columnar intestinal-type epithelium. BE is generally recognized as the major precursor for EAC. The estimated risk of BE patients developing EAC is 30-125 times higher than that of the general population[2]. Although various factors have been suggested to cause BE development, the pathogenesis of the disease remains unknown.

Chronic gastroesophageal reflux with bile plays a crucial role in the development of BE[3]. Acid and bile, the main components of gastroesophageal refluxate, act synergistically to induce mucosal injury[4]. Both components have been suggested to promote intestinal-type differentiation in esophageal keratinocytes by inducing the expression of the caudal type homeobox 2 (*cdx2*) gene, an intestine-specific transcription factor involved in intestinal differentiation. Aberrant expression of this gene is associated with intestinal metaplasia and tumorigenesis[5]. Homeodomain protein CDX2 regulates goblet-specific mucin 2 (*muc2*) gene expression resulting in the differentiation of intestinal epithelium[6]. Experimental models of gastroesophageal reflux in rodents provide a basis for understanding the molecular events, which characterize the metaplasia-dysplasia-adenocarcinoma process in the esophagus.

Helicobacter pylori (*H. pylori*) has been widely recognized as an important causative factor in the development of gastric adenocarcinoma. Studies indicate that *H. pylori* may colonize in the lower esophagus with gastric metaplasia. The prevalence of *H. pylori* infection in the lower esophagus varies from 20%-70%[7,8]. A preliminary study on the association between *H. pylori* infection and gastroesophageal reflux disease (GERD) suggested that the colonization of *H. pylori* occurs in the lower esophagus. Hence, the bacteria potentially aggravate esophageal mucosa injury and increase the incidence of BE and EAC with the upregulation of CDX2[9]. However, that particular study lacked an additional group of rats with both chronic reflux and esophageal *H. pylori* infection. Data analyses combined all the groups of rats with chronic reflux and esophageal *H. pylori* infection, as well as rats treated with celecoxib. The implicit mechanisms for the increase in esophageal injury and the subsequent progression from esophagitis to BE and EAC associated with *H. pylori* colonization are unknown.

The imbalance between cell proliferation and apoptosis is common in neoplasia development. Ki-67 expression, a marker of cell proliferation, is increased in Barrett's epithelium and adenocarcinoma[10]. Cyclin D1, a regulating factor of the cell cycle, and c-Myc, a transcriptional enhancer, are overexpressed in gastric tumorigenesis induced by *H. pylori* infection[11,12]. Cell apoptosis is a complex process regulated by various apoptotic proteins, such Bcl-2 and Bcl2-associated X protein (Bax). While Bax promotes apoptosis, Bcl-2 inhibits the process. Both Bax and Bcl-2 have been shown to be associated with the development of gastric adenocarcinoma caused by *H. pylori* infection[13,14]. However, the alteration of cell proliferation and apoptosis in the esophagus in response to *H. pylori* infection has not been investigated.

The aims of the present study were to verify our previous findings by using a group of rats with chronic reflux and esophageal *H. pylori* colonization, and to investigate the possible mechanisms in the sequence of inflammation-metaplasia-adenocarcinoma in response to *H. pylori* colonization of the esophagus. A rat model of chronic acid and bile reflux with *H. pylori* infection was successfully established. As a result, the severity of esophagitis, the incidence of BE and EAC, the index of proliferation and apoptosis of esophageal mucosa, and the expression of possible regulators could be determined.

MATERIALS AND METHODS

Animals and surgery

Sixty-five specific-pathogen-free male Sprague-Dawley rats (8-wk-old) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were allowed to acclimate for one week with standard solid laboratory chow and distilled water, in an animal room with controlled temperature, and a 12 h light-dark cycle. The rats were randomly divided into four groups: (1) an esophagogastroduodenal anastomosis (EGDA) group (n = 15); (2) an EGDA-*H. pylori* infection group

(n = 34); (3) a pseudo-operation with *H. pylori* infection group (pseudo-operation with *H. pylori* gastric gavage; n = 8); and (4) a pseudo-operation group [pseudo-operation with brain heart infusion (BHI) gastric gavage; n = 8]. In our previous study, the mortality of rats that underwent EGDA was 40%-50%, and the prevalence of esophageal *H. pylori* colonization was 64.3%[9]. As a result, more rats were assigned to both the EGDA and EGDA-*H. pylori* infection groups. Based on the *H. pylori* colonization site, the rats in the EGDA-*H. pylori* infection group were further divided into two subgroups: EGDA with concomitant esophageal *H. pylori* colonization and EGDA with gastric *H. pylori* colonization. EGDA was performed as described previously to create a rat model with acid and bile reflux[15]. Briefly, a side-to-side anastomosis was made between the gastroesophageal junction and the middle duodenum on the anti-mesenteric border with an accurate mucosa-to-mucosa position. In the two pseudo-operation groups, the rats' abdomens were opened, the bowels were flipped, and the abdomens were sutured. *H. pylori* infection was established by inoculating the *H. pylori* strain SS1 intragastrically two weeks after EGDA. The study was approved by the Animal Care Committee of Peking University First Hospital (Beijing, China).

Rats were anesthetized 36 wk after surgery with chloral hydrate (0.3 mg/kg, intraperitoneal). The esophagus was removed 0.2 cm above the anastomotic stoma. The macroscopic score of esophageal mucosal injury was evaluated according to the presence of: (1) hyperemia; (2) edema; (3) erosion; (4) ulcer; (5) intramural; or (6) intraluminal hemorrhage[16]. Once removed, the esophagus was longitudinally cut into three segments. One segment was fixed in 40 g/L formaldehyde, and the other two were kept in liquid nitrogen for subsequent mRNA extraction. Gastric specimens were taken and opened along the greater curvature. The anterior and posterior wall of the stomach, including the body and the antrum, were cut roughly into two 3 mm × 10 mm segments along the greater curvature. One segment was used for a rapid urease test and the other was immediately immersed in 10% buffered formalin and embedded in paraffin. Blood samples were collected from the abdominal aorta and centrifuged for 20 min at 3500 rpm. The supernatants were aliquoted and stored at -70 ℃ until further use.

Histologic and immunohistochemical studies

Paraffin sections (4 μm thick) were stained with hematoxylin and eosin (HE) to examine inflammation of the esophagus and the presence of BE and EAC. BE was defined as the presence of columnar epithelium with intestinal-type goblet cells[17]. EAC was diagnosed when dysplastic columnar epithelial cells invaded the basement membrane[18]. The microscopic scores of the esophageal mucosal injury were evaluated as previously described[16]. High-iron diamine-Alcian blue-Periodic acid-Schiff (HID-AB-PAS) staining was performed according to the HID and AB-PAS kit protocols (Shijiheli Co. Ltd., Beijing, China). For immunohistochemical staining, paraffin sections were immersed in xylene and hydrated using a graded alcohol series. Antigen retrieval was performed by immersing the sections in 0.01 mol/L citrate buffer (pH 6.0) and heating in a microwave oven at 80 W for 20 min. After a 10 min peroxide-block treatment (BioGenex Laboratories, Fremont, CA, United States) to eliminate endogenous peroxidase activity, the sections were blocked with power block (BioGenex Laboratories) for 10 min in a humidified box at room temperature to eliminate non-specific binding. Sections were incubated overnight at 4 ℃ with primary antibodies: rabbit anti-rat Ki-67 monoclonal antibody (Abcam, Cambridge, United Kingdom), mouse anti-rat CDX2 monoclonal antibody (Cell Signaling Technology Inc., Beverly, MA, United States), or rabbit anti-rat MUC2 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, United States). The sections were rinsed in phosphate-buffered saline (PBS) and incubated with super enhancer (BioGenex Laboratories) for 20 min, followed by Polymer-HRP (BioGenex Laboratories) for 40 min. Staining was developed with diaminobenzidine (BioGenex Laboratories) under a light microscope. Sections were counterstained with hematoxylin. Full-tissue section slides were scored using an "*H*-score approach"[19]. The percentage of cells positively stained was scored as: a = 0% (score 0); 1%-20% (score 1); 21%-40% (score 2); 41%-60% (score 3); 61%-80% (score 4); or 81%-100% (score 5). The intensity of staining was categorized as i = absent (score 0); faint (score 1); moderate (score 2); or strong (score 3). High power magnification (400 ×) was used to count the number of positive cells. The final score for the counted cells was calculated by multiplying a by i with a minimum possible value of 0 and a maximum of 15.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from frozen esophageal tissues. The esophageal tissue was cleaved with 1 mL TRIzol® (Life Technologies, Carlsbad, CA, United States), followed by 200 μL of chloroform. The mixture was then shaken vigorously and centrifuged at 12000 rpm for 15 min at 4 ℃. The supernatant was mixed with 500 μL of isopropanol and centrifuged again at 12000 rpm for 15 min at 4 ℃. The RNA pellet was washed with 75% ethanol, dried, resuspended in sterile water, and quantified using a NanoDrop spectrophotometer (ND-1000V3.5.2 software, NanoDrop Technologies, Wilmington, DE, United States). Reverse transcription of mRNA to cDNA was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). The total reaction volumes were 20 μL, and consisted of 10 μL of 2 × master mix and 10 μL of RNA sample (2 μg total RNA). Reverse transcription was performed in a thermal cycler (Eppendorf, Hamburg, Germany) at 25 ℃ for 10 min, 37 ℃ for 120 min, and 85 ℃ for 5 min. The cDNAs were aliquoted and stored at -70 ℃. Real-time polymerase chain reaction (RT-PCR) was performed using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems of Thermo Fisher Scientific, Waltham, MA, United States). Primers were designed by Primer

Express 3.0 software (Applied Biosystems) and were as follows: *Cdx2*, forward, 5'- CCATGAGGAGCACGGACACT-3' and reverse, 5'-TTCTGCCCTCTGT CCTCGAT-3'; *Muc2*, forward, 5'-CGTGCCACGGCAAGGT-3' and reverse, 5'-AGTGTCACAGG AGCAGGAGTC-3'; *C-Myc,* forward, 5'-GCGTTATTTGAAGCCTGAATTTCC-3' and reverse, 5'-CCTGTTAGCGAAGCTCACGTTG-3'; *Ccnd1* (cyclin D1), forward, 5'-CTA ATGTAAAGCCAGCCGCAATG-3' and reverse, 5'-TGGACACAGCAGCCCTCAAG -3'; *Bcl-2*, forward, 5'-GGGATGCCTTTGTGGAACTATATG-3' and reverse, 5'-TGAGCAGCGTCTTCAGAGACA-3'; *Bax,* forward, 5'-GCGATGAACTGGACAA CAACAT-3' and reverse, 5'-TAGCAAAGTAGAAAAGGGCAACC-3'; and *Actb* (β-actin; housekeeping gene), forward, 5'-GCCTCACTGTCCACCTTCCA-3' and reverse, 5'-GTCCGCCTAGAAGCATTTGC-3'. RT-PCR was performed in a final volume of 15 μL containing 1 μL of cDNA, 7.5 μL of SYBR Green RT-PCR Master Mix (Applied Biosystems), 1 μL of forward and reverse primers, and 5.5 μL of sterile water. The reactions were carried out according to the following parameters: denaturation at 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 15 s, 60 ℃ for 1 min, and a further melting curve step at 95 ℃ for 15 s, 60 ℃ for 30 s, and 95 ℃ for 15 s. All reactions were performed in triplicate. The PCR products were quantified using the $2^{-\Delta\Delta CT}$ comparative method.

H. pylori culture and inoculation

H. pylori strain SS1 obtained from the National Collection of Type Cultures (Colindale, London, United Kingdom) was cultured in a microaerobic humidified atmosphere on a Columbia agar (Oxoid, Hampshire, United Kingdom) plate with 80 mL/L defibrinogen goat blood (Baote Ltd., Beijing, China) at 37 ℃. After 48 h incubation, the bacteria were harvested in BHI broth (Oxoid, Hampshire, United Kingdom) and adjusted to 1×10^8 CFU/mL. EGDA rats were inoculated intragastrically for two weeks with 1 mL of *H. pylori* suspension every other day for a total of three times.

H. pylori detection

Warthin-Starry silver staining, a rapid urease test (RUT), and a serological test were used to confirm *H. pylori* infection. Paraffin-embedded esophageal and gastric sections were processed according to the Warthin-Starry silver staining protocol with *H. pylori* appearing dark-brown to black. For the RUT, samples from rat stomach and the lower esophagus were placed in a tube filled with RUT reagent and monitored for color change (from yellow to red) for up to 24 h. For the serological test, an antigen-specific antibody in serum was detected by an enzyme-linked immunosorbent assay (CagA-Hp-IgG ELISA kit, Jingying Co. Ltd., Shanghai, China)[9,20]. Samples with optical density (*A*) readings 2.1-fold greater than uninfected rats were considered positive. Rats with positive Warthin-Starry silver staining, or with positive RUT and anti-*H. pylori* serological tests were considered positive for *H. pylori* infection.

TdT-mediated dUTP nick-end labeling

Apoptosis was determined by the TdT-mediated dUTP nick-end labeling (TUNEL) method according to the protocol of the *In Situ* Cell Death Detection kit (Roche, Basel, Germany). Sections were de-waxed with xylene and hydrated, and then placed in 0.1 M citrate buffer (pH 6.0) in a microwave oven at 80 W for 20 min. After rinsing with PBS, sections were mixed with TUNEL mixture (enzyme solution and label solution) and incubated at 37 ℃ for 60 min in a dark humidified box. Following this incubation, the converter-peroxidase was added and sections were incubated at 37 ℃ for 30 min. Staining was developed with diaminobenzidine, and sections were counterstained with hematoxylin. TUNEL scoring was the same as that used for immunohistochemistry.

Statistical analysis

All quantitative values are expressed as mean ± SD. Statistically significant differences between groups were determined using χ^2, Fisher's exact, or nonparametric Wilcoxon and Mann-Whitney *U* tests. Data management and statistical analyses were performed using SPSS software 16.0 (SPSS Inc., Chicago, IL, United States). All analyses were considered significant when the *P* value was less than 0.05.

RESULTS

General observations

Within eight weeks of surgery, 21 rats died: six in the EGDA group, and 15 in the EGDA-*H. pylori* infection group. Of these, ten were due to anastomotic infarction, seven to malnutrition caused by vomiting, and four were due to undetermined reasons. Forty-four rats survived 36 wk after operation and completed the study: eight were in the pseudo-operation group, eight were in the pseudo-operation-*H. pylori* infection group, nine were in the EGDA group, and 19 were in the EGDA-*H. pylori* infection group. Gastric colonization of *H. pylori* was detected in all *H. pylori* inoculated rats. Of the 19 rats in the EGDA-*H. pylori* infection group, 10 were found to have concomitant *H. pylori* colonization in the esophagus (Figure 1).

Esophageal *H. pylori* colonization aggravates esophageal injury caused by chronic reflux

The esophagi in the two pseudo-operated groups appeared smooth and light pink (Figure 2A). The middle and lower esophagi of EGDA rats were markedly dilated and thickened with hyperkeratinization. Epithelial sloughing and ulcerations were found in large areas of the lower esophagi in EGDA rats. Esophagitis was identified as either a "white cobblestone" appearance or a "white tree bark" appearance (Figure 2A).

Under the microscope, normal rat esophagus displayed stratified keratinized squamous epithelium (Figure 2B). The esophagi of rats in the two pseudo-operation

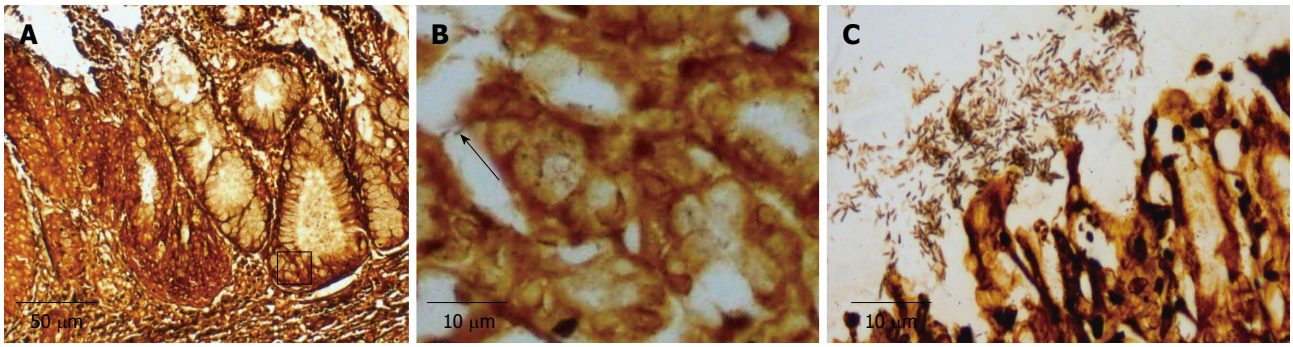


Figure 1 *Helicobacter pylori* infection detected with the Warthin-Starry silver staining method. Warthin-Starry silver staining revealed A: Intestinal metaplasia in the esophagus (200 × magnification); B: *Helicobacter pylori* (*H. pylori*) colonizing the esophagus (arrow) (1000 × magnification); C: *H. pylori* colonizing the stomach (1000 × magnification).

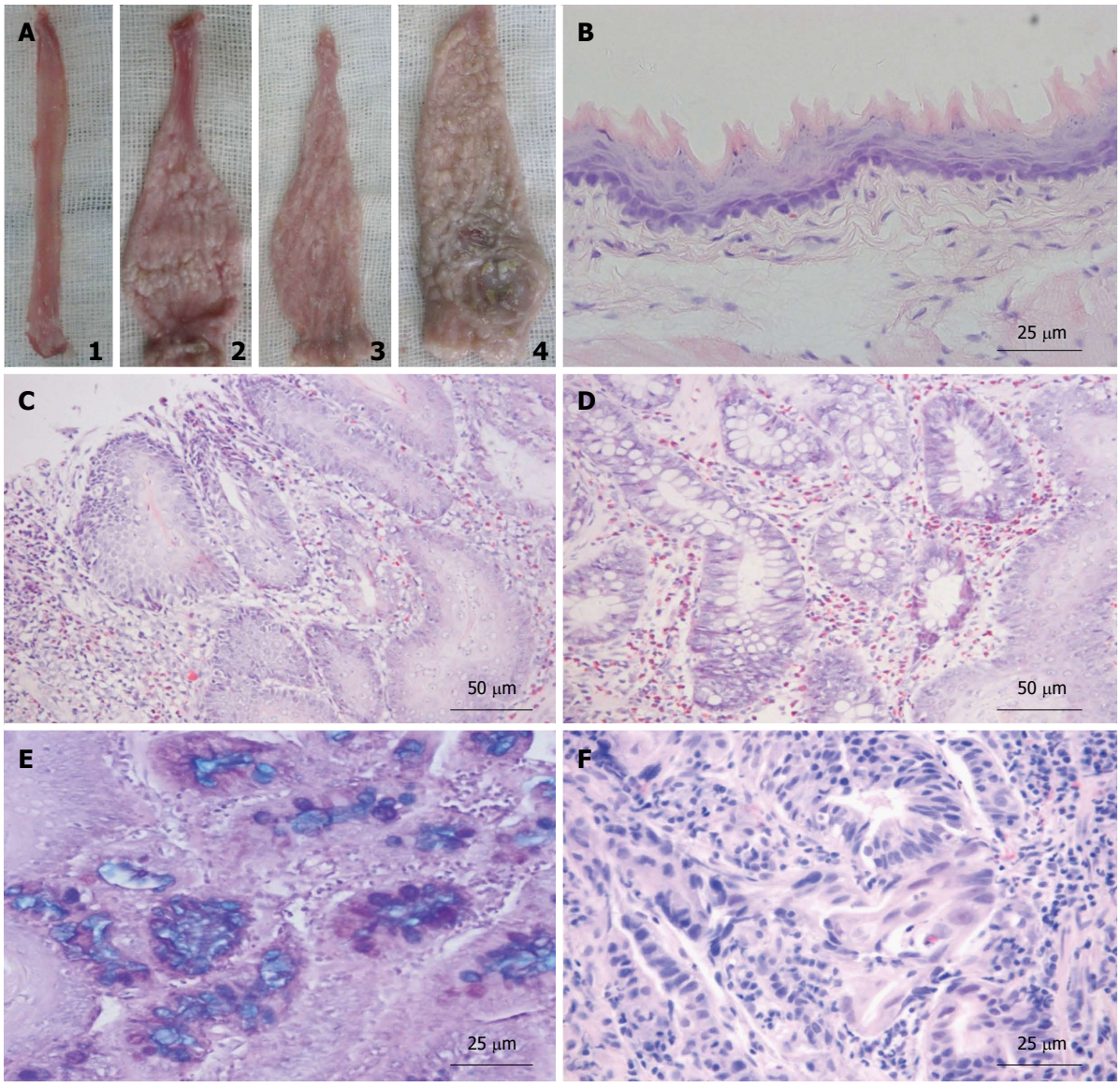


Figure 2 Macroscopic and microscopic findings in the rat esophagus. A: Macroscopic findings in the esophagus: 1, Normal esophagus; 2, Esophagitis with tree bark appearance; 3, Barrett's esophagus; and 4, esophageal adenocarcinoma. Microscopic findings; B: Normal squamous epithelium (400 × magnification); C: Reflux esophagitis (200 × magnification); D: Barrett's esophagus (200 × magnification); E: Intestinal metaplasia in the esophagus detected by High-iron diamine-Alcian Blue-Periodic acid-Schiff (400 × magnification); F: Esophageal adenocarcinoma (400 × magnification).

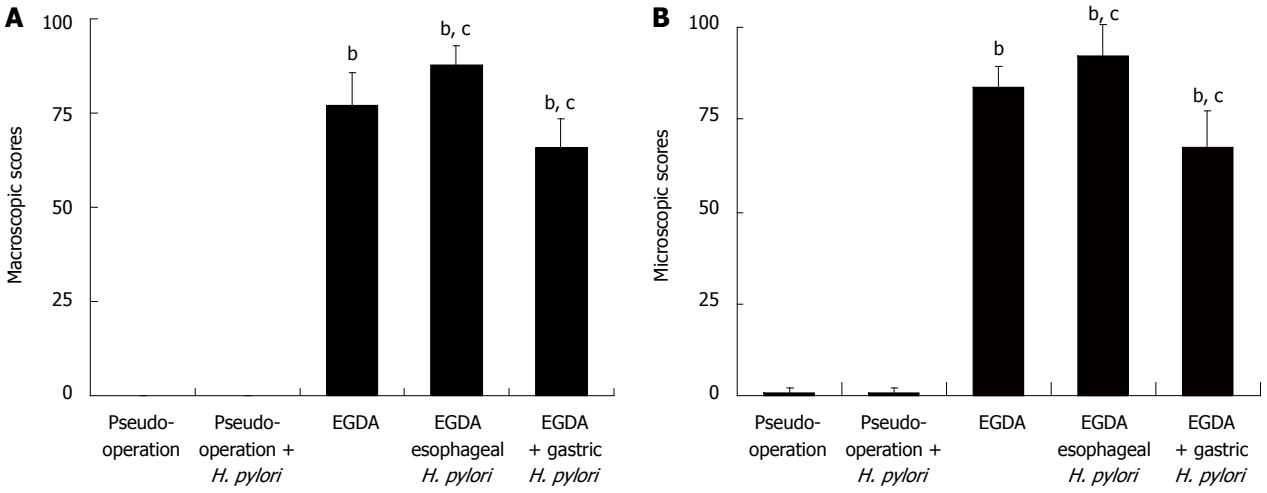


Figure 3 Findings of mucosal injury in the lower esophagus. A: Macroscopic scoring of esophageal mucosal injury; B: Microscopic scoring of esophageal mucosal injury. [b]$P < 0.01$ *vs* pseudo-operation and pseudo-operation with *Helicobacter pylori* (*H. pylori*) infection groups; [c]$P < 0.05$ *vs* esophagogastroduodenal anastomosis (EGDA) group.

Table 1 Incidence of Barrett's esophagus and esophageal adenocarcinoma in rats *n* (%)

Groups (*n*)		BE	EAC
Ⅰ	Pseudo-operation (8)	0	0
Ⅱ	Pseudo-operation with *H. pylori* infection (8)	0	0
Ⅲ	EGDA (9)	3 (33.3)	1 (11.1)
Ⅳ	EGDA with *H. pylori* infection		
	Concomitant esophageal colonization (10)	8 (80.0)[a]	6 (60.0)[a,c]
	Only gastric colonization (9)	2 (22.2)	1 (11.1)

[a]$P < 0.05$ *vs* pseudo-operation and pseudo-operation with *Helicobacter pylori* (*H. pylori*) infection groups; [c]$P < 0.05$ *vs* esophagogastroduodenal anastomosis (EGDA) group. BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma.

groups did not reveal any abnormalities. However, mucosal injury and typical appearance of reflux esophagitis were found in the middle and lower esophagi of rats that underwent EGDA. An increase in the thickness of squamous epithelium, with basal cell hyperplasia and elongation of lamina propria papillae, was found in the EGDA rat esophagi (Figure 2C). Other pathologic findings included the infiltration of inflammatory cells, squamous epithelium deletion, columnar epithelium replacement, intestinal metaplasia, dysplasia, and even EAC (Figure 2D-F). The scores of esophageal mucosal injury were increased in EGDA rats compared to rats without EGDA ($P < 0.05$). There were significant reductions in the scores of esophageal mucosal injury in EGDA rats with only gastric *H. pylori* colonization compared to EGDA rats ($P < 0.05$). The EGDA rats with concomitant esophageal *H. pylori* colonization had higher mucosal injury scores in the lower esophagus than EGDA rats ($P < 0.05$) (Figure 3).

Esophageal H. pylori colonization promotes the development of BE and EAC in the presence of chronic reflux with CDX2 and MUC2 overexpression

None of the rats in the two pseudo-operation groups suffered from BE or EAC. There was no statistical difference in the incidence of BE and EAC in EGDA rats compared to rats in the two pseudo-operation groups (Table 1). Concomitant esophageal *H. pylori* colonization accompanied by chronic reflux increased the incidence of BE and EAC ($P < 0.05$). However, the frequency of BE and EAC remained unchanged between the EGDA rats with only gastric *H. pylori* colonization and the EGDA rats (Table 1).

In an effort to determine the role of CDX2 and its downstream molecules, the expression of the markers of intestinal metaplasia was evaluated. Chronic reflux caused by EGDA induced the expression of CDX2 and MUC2. Overexpression of CDX2 and MUC2 in the esophagus was found in EGDA rats with concomitant esophageal *H. pylori* colonization, as compared to EGDA rats ($P < 0.05$) (Figure 4).

Esophageal H. pylori colonization disrupts the balance between cell proliferation and apoptosis in esophageal mucosa

Loss of balance between cell proliferation and apoptosis is common in the development of neoplasia. Ki-67 expression was analyzed to evaluate the extent of cell proliferation, while TUNEL staining was used to indicate apoptosis in esophageal mucosa. In both normal esophageal mucosa and mucosa with esophagitis, basal layer cells in squamous epithelium expressed Ki-67. The TUNEL-positive cells were horny layer cells located in squamous epithelium. Ki-67 expression and TUNEL-positive cells were also detected in metaplastic glands and mesenchymal cells in Barrett's mucosa and EAC.

Sporadic expression of Ki-67 was found in esophageal squamous epithelial cells in the two pseudo-operation groups. Similarly, there were few TUNEL-positive cells in the esophagi of rats from the two pseudo-operation groups. In contrast, the expression of Ki-67 protein and the number of TUNEL-positive cells increased in

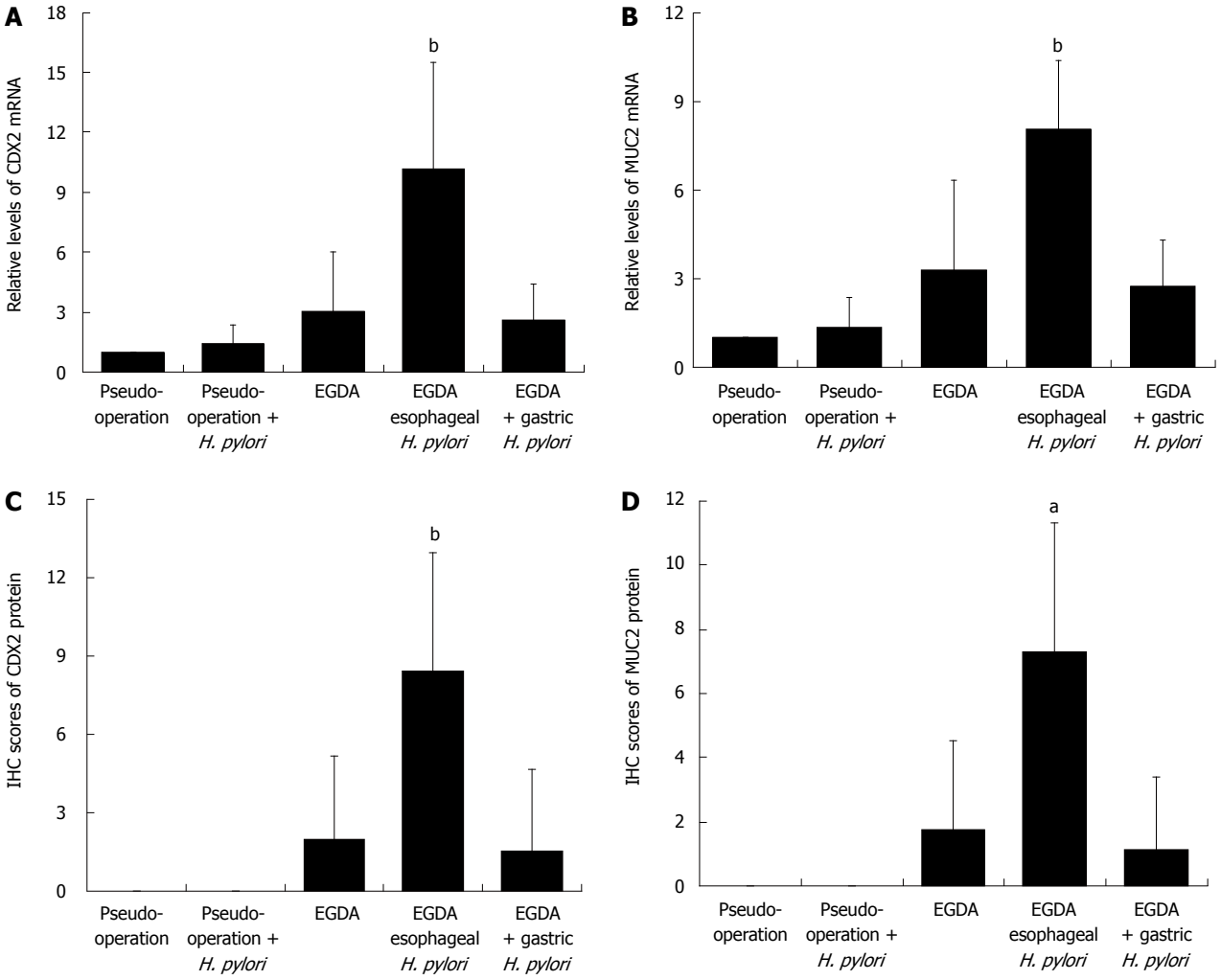


Figure 4 mRNA and protein expressions of CDX2 and MUC2 in the rat esophagus. The relative mRNA expression levels of CDX2 (A) and MUC2 (B) in the esophagus; Immunohistochemistry (IHC) scoring of CDX2 (C) and MUC2 (D) protein expression in the esophagus. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* esophagogastroduodenal anastomosis (EGDA), pseudo-operation, and pseudo-operation with *Helicobacter pylori* (*H. pylori*) infection groups.

the esophagi of rats that underwent EGDA ($P < 0.05$). In EGDA rats with concomitant esophageal *H. pylori* colonization, Ki-67 expression was elevated, and an increased number of esophageal apoptotic cells was detected ($P < 0.05$). The expression of Ki-67 in the esophagus was significantly decreased ($P < 0.05$), whereas the numbers of apoptotic cells remained unchanged in EGDA rats with gastric *H. pylori* colonization compared to EGDA rats (Figure 5).

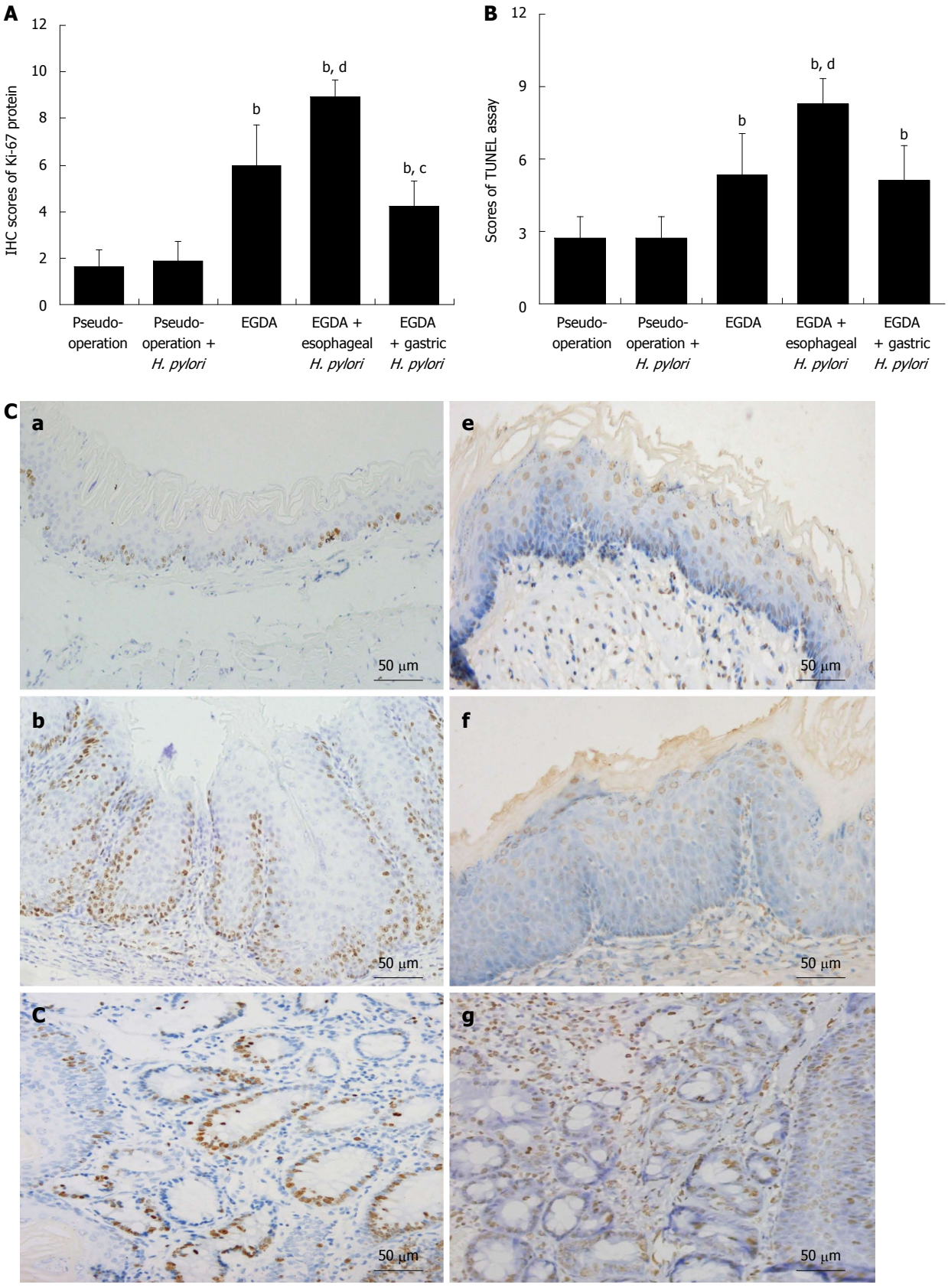
Esophageal H. pylori colonization increases the expression of genes involved in the regulation of cell proliferation and apoptosis

To elucidate the regulatory mechanisms for the imbalance between cell proliferation and apoptosis, the mRNA expression of associated genes was investigated. There was no statistical difference in the mRNA expression of cyclin D1 and c-Myc in the esophagi of the two pseudo-operation groups, however, both were significantly induced in EGDA rats (both $P < 0.05$). Further increases in the expression of cyclin D1 and c-Myc mRNAs were evident in EGDA rats with concomitant esophageal *H. pylori* colonization ($P < 0.01$). The mRNA expression of cyclin D1 and c-Myc was reduced in EGDA rats with only gastric *H. pylori* colonization compared to EGDA rats ($P < 0.05$).

There were no significant differences in the mRNA expression of Bax or Bcl-2 in the esophagi of rats in the pseudo-operation and pseudo-operation-*H. pylori* infection groups. However, Bax mRNA in the esophagus increased significantly, whereas Bcl-2 mRNA was reduced in EGDA rats compared to the rats of the two pseudo-operation groups (*P*s < 0.05). Bax mRNA was further induced, and Bcl-2 reduced, in the esophagi of EGDA rats with concomitant esophageal *H. pylori* colonization compared to EGDA rats (both $P < 0.01$). There was no significant difference in the expression of Bax or Bcl-2 mRNA between EGDA rats and EGDA rats with only gastric *H. pylori* colonization (Figure 6).

DISCUSSION

H. pylori has been shown to increase the risk of precancerous lesions in the stomach; however, the role *H. pylori*


A
IHC scores of Ki-67 protein
12
10
8
6
4
2
0
b
b, d
b, c
Pseudo-operation
Pseudo-operation + H. pylori
EGDA
EGDA + esophageal H. pylori
EGDA + gastric H. pylori
B
Scores of TUNEL assay
12
9
6
3
0
b
b, d
b
Pseudo-operation
Pseudo-operation + H. pylori
EGDA
EGDA + esophageal H. pylori
EGDA + gastric H. pylori
C
a
e
b
f
C
g
50 μm

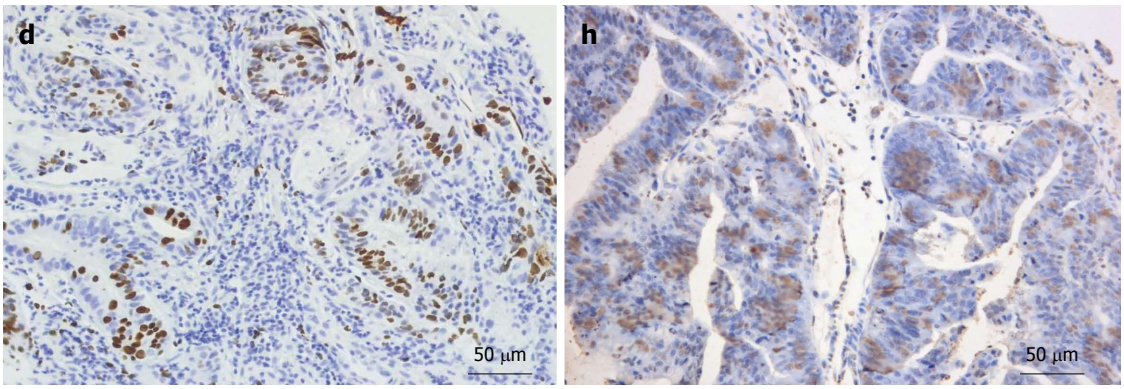


Figure 5 Ki-67 expression and TdT-mediated dUTP nick-end labeling assay in rat esophagus. A: Immunohistochemistry (IHC) scoring of Ki-67 expression in the esophagus; B: The scoring of apoptotic cells in the esophagus as determined by TdT-mediated dUTP nick-end labeling (TUNEL) assay; C: IHC staining of Ki-67-positive cells and TUNEL-positive apoptotic cells. a, e: Normal esophagus mucosa; b, f: Esophagitis; c, g: Barrett's esophagus; d, h: Esophageal adenocarcinoma; a-d: IHC staining of Ki-67; e-h: apoptotic cells stained by TUNEL. All of the samples were viewed under 200 × magnification. $^{b}P < 0.01$ *vs* pseudo-operation and pseudo-operation with *Helicobacter pylori* (*H. pylori*) infection groups; $^{c}P < 0.05$, $^{d}P < 0.01$ vs esophagogastroduodenal anastomosis (EGDA) group.

infection plays in the esophagus remains controversial. Epidemiologic studies have shown lower prevalence of *H. pylori* infection in patients with GERD, BE, and EAC[21]. Fallone *et al*[22] reported a correlation between *H. pylori* infection and a reduction in the severity of reflux esophagitis. These findings suggest an inverse association between *H. pylori* infection and the risk of esophagitis, as well as BE and EAC. However, Henihan *et al*[7] found that severe esophageal mucosal injury can result from *H. pylori* colonization of the esophagus.

Until recently, no research differentiated the importance of different *H. pylori* colonization sites on the development of GERD. We established a rat model with chronic acid and bile reflux using EGDA. To elucidate the effects of *H. pylori* infection on reflux esophagitis and development of BE and EAC, EGDA rats were infected with *H. pylori*. Gastric colonization of *H. pylori* was detected in all rats inoculated with *H. pylori*. In *H. pylori*-inoculated rats without reflux, no *H. pylori* colonization or related lesions were found in the esophagus. These findings suggest that *H. pylori* colonization of the stomach has no influence on the esophageal mucosa of rats in the absence of reflux. Acid and bile reflux, due to EGDA, led to injury of the lower esophagus, and initiated the replacement of squamous epithelium by columnar epithelium in the esophagus. *H. pylori* colonization was found in Barrett epithelium of the lower esophagus of some rats that underwent EGDA. The inflammation scores of esophageal injury were not aggravated in EGDA rats with gastric *H. pylori* colonization compared to EGDA rats. However, the severity of esophagitis was exacerbated and the incidence of BE and EAC increased in EGDA rats with concomitant esophageal *H. pylori* colonization. The ability of *H. pylori* infection to affect the esophagus may depend on the type of infection site. Esophageal *H. pylori* infection may play an important role as an aggressive factor and initiate a pathogenic process.

Previous studies have indicated that *H. pylori* can colonize the gastric-type epithelium of the lower esophagus[7]. In the present study, esophageal squamous epithelium damaged by chronic acid and bile reflux might be replaced by Barrett's epithelium, providing the prerequisite for *H. pylori* colonization. Long-term exposure of squamous epithelium to acid and bile produced by EGDA caused the development of columnar epithelium metaplasia through *de novo* metaplasia or proximal migration of duodenal epithelium[23]. The high incidence of columnar epithelium in the lower esophagus provided the high possibility of *H. pylori* colonization observed in the present study. Up to 52.6% (10/19) of rats inoculated with *H. pylori* strain SS1 after EGDA, were found to have *H. pylori* colonization in the lower esophageal mucosa with gastric metaplasia. The high *H. pylori* colonization rate of the esophagus was associated with the strong ability of SS1 strain to colonize[24], the chronic reflux of gastric contents that contained a large amount of the bacteria, and the high incidence of columnar epithelium replacement in the lower esophagus caused by EGDA. The results of the present study are consistent with our previous findings which showed that esophageal *H. pylori* colonization increased the severity of reflux esophagitis and augmented the risk for the development of BE and EAC.

CDX2 is expressed in the epithelium of the small intestine and colon[25] and is generally accepted as one of the key factors in the induction of intestinal metaplasia and the formation of intestinal-type carcinoma[5]. In rat models, acid and bile can induce CDX2 expression, an early event in lesions caused by reflux, and considered to be a key step in EAC morphogenesis[4,26]. *H. pylori* induces cag pathogenicity island-dependent mRNA expression of CDX2 and MUC2[27]. In our study, *H. pylori* further increased the expression of CDX2, while its downstream target, MUC2, was induced by mixed reflux leading to the development of BE and EAC. The findings of this study suggest that the combined presence of injurious refluxate and *H. pylori* infection synergistically activate ectopic expression of CDX2, which in turn initiates the development of the intestinal phenotype.

Chronic acid and bile reflux produces severe esopha-

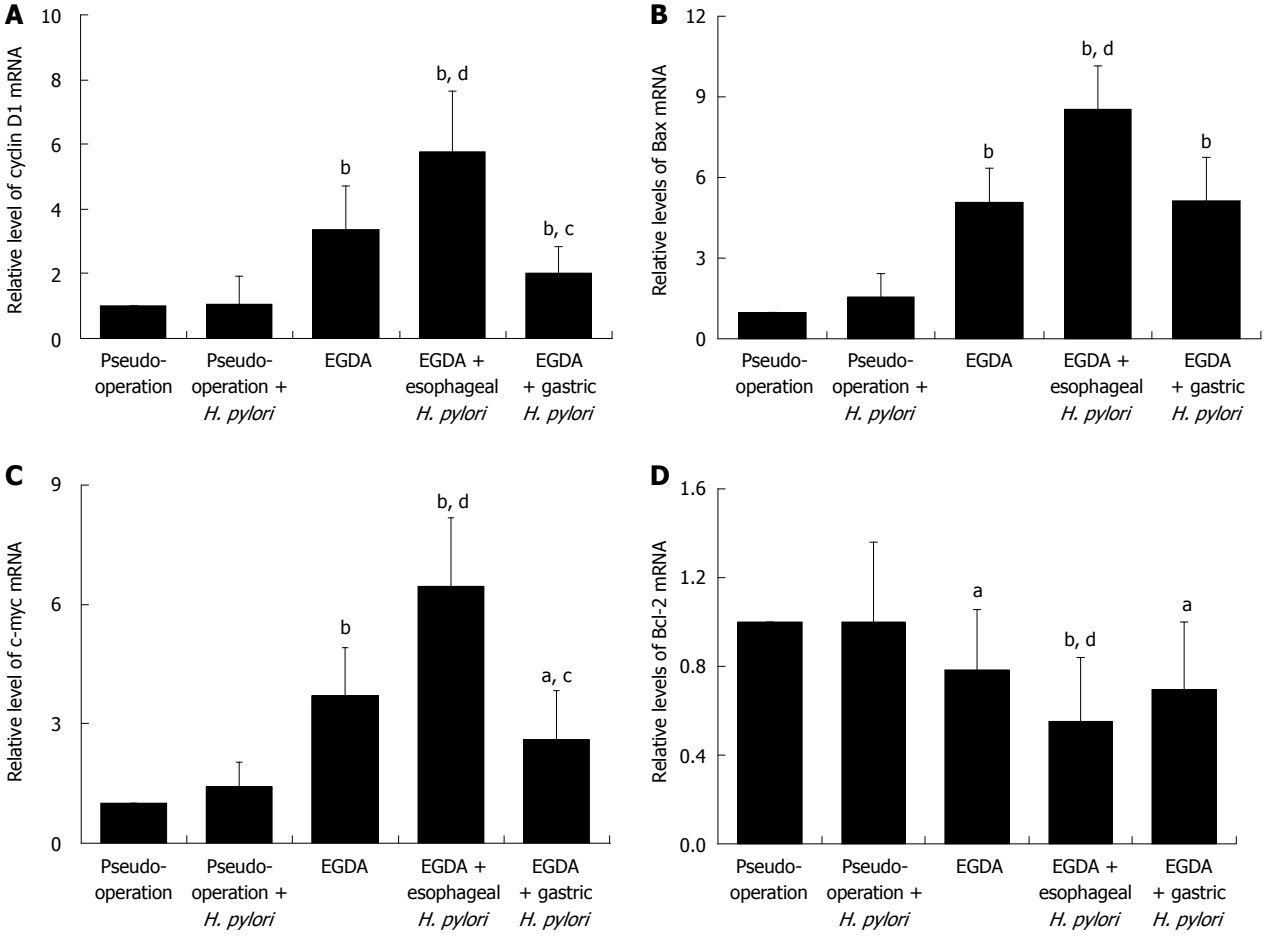


Figure 6 mRNA levels of cyclin D1, c-Myc, Bax, and Bcl-2 in the esophagus. Quantitative real-time polymerase chain reaction determination of cyclin D1 (A), Bax (B), c-Myc (C) and Bcl-2 mRNA levels (D). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* pseudo-operation and pseudo-operation with *Helicobacter pylori* (*H. pylori*) infection groups; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* esophagogastroduodenal anastomosis (EGDA) group.

geal injury and initiates metaplasia, dysplasia, and even EAC[4]. In the present study, esophageal *H. pylori* colonization further promoted the process. It is known that both *H. pylori* infection and bile exposure induce intestinal metaplasia of gastric mucosa and the development of gastric adenocarcinoma[28-30]. Therefore, we speculated that infection of *H. pylori* in the lower esophagus might initiate a pathologic process, as in the stomach, and facilitate the development of BE, and even EAC. The high incidence rates of BE and EAC found in this study were associated with the synergistic effect of mixed reflux caused by EGDA and *H. pylori* infection.

Disruption of the balance between cell proliferation and apoptosis can occur in chronic inflammation and tumorigenesis. In most neoplasms, proliferation is induced while apoptosis is inhibited. Previous animal studies have also reported promotion of cell proliferation and apoptosis in mixed reflux esophagitis[31], which is consistent with the results from this study. It is well known that *H. pylori* CagA- or VacA-positive strains can directly induce proliferation and apoptosis in human gastric cells[32-34]. CagA pathogenicity island-positive *H. pylori* induces apoptosis more rapidly[33]. In previous studies, VacA-positive *H. pylori* was able to induce apoptosis by a mitochondrial membrane permeability change[35]. The presence of CagA and VacA proteins is important for inducing apoptosis. *H. pylori* SS1, a virulent strain positive for both VacA and CagA, may induce the alteration of cell proliferation and apoptosis in the esophagus and initiate the development of EAC.

Cell cycle regulatory genes are known to be involved in the loss of control of cell proliferation and apoptosis and the development of EAC. Studies have revealed increased levels of Ki-67, cyclin D1, and c-Myc in BE and EAC[10,36,37]. In the present study, an increase in BE and EAC, in response to esophageal *H. pylori* colonization, further increased the expression of these genes compared to EGDA rats. Bax and Bcl-2 are required for the regulation of cell apoptosis[38,39]. Previous studies indicated that *H. pylori* induced apoptosis of gastric epithelium *via* a mitochondrial pathway. *H. pylori* VacA modulates the permeability of mitochondrial membrane in a transmembrane-potential dependent manner[40]. The imbalance of Bax/Bcl-2 expression, as indicated in this study, increased the apoptosis rate when *H. pylori* colonized the esophagus. In the current rat model with acid and bile reflux, the increased apoptotic rate might counteract the significant cell proliferation increase in the esophageal mucosa.

Over-proliferation enhances the possibility of aberrant gene expression; therefore, the compensated cell death may be a protective mechanism against this over-proliferation. The alteration in the expression of genes involved in the regulation of cell proliferation and apoptosis and the subsequent imbalance between the two may play an important role in the development of BE and EAC.

In conclusion, *H. pylori* strains may colonize the esophageal mucosa, aggravate the inflammation of the lower esophagus, and induce intestinal metaplasia or even adenocarcinoma. Loss of balance between proliferation and apoptosis may be important in *H. pylori*-induced esophageal diseases.

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COMMENTS

Background

The relationship between gastroesophageal reflux disease and *Helicobacter pylori* (*H. pylori*) is controversial. Previous studies have shown that *H. pylori* colonization in the esophagus increased the incidence of Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC). However, the relevant mechanism is not clear. The authors established a rat model of chronic acid and bile reflux with *H. pylori* infection and investigated the possible mechanisms in the process from inflammation to malignancy in response to *H. pylori* colonization in the esophagus.

Research frontiers

Chronic gastric *H. pylori* infection in an animal model induces gastric adenocarcinoma. Studies have shown *H. pylori* colonizing the BE columnar epithelium. However, the outcomes of esophageal *H. pylori* colonization and the relevant mechanisms have not been determined.

Innovations and breakthroughs

The authors found that *H. pylori* colonization of the esophagus might play an aggressive role and initiate a pathogenic process from esophagitis to BE and EAC. This is the first study to investigate the effect esophageal *H. pylori* colonization has on cell proliferation and apoptosis in the esophagus.

Applications

Eradication of *H. pylori* infection in patients with gastroesophageal reflux disease (GERD) is a hot topic. This study indicates that esophageal *H. pylori* colonization aggravates esophagitis, and as a result, promotes the occurrence of BE and EAC *via* loss of balance between cell proliferation and apoptosis. Consequently, in order to prevent esophageal malignancy in patients with GERD, *H. pylori* should be eradicated in patients with concomitant esophageal colonization. These findings further our knowledge on potential ways of eradicating *H. pylori* infection.

Terminology

The macroscopic and microscopic scoring systems of esophageal injury are valid scores for quantifying the severity of esophageal injury based on the epithelial damage, vascular damage, and inflammatory extension.

Peer review

In this study, the authors indicate that esophageal *H. pylori* colonization aggravates reflux esophagitis and increases the incidences of BE and EAC. Loss of balance between cell proliferation and apoptosis in the esophagus contributes to tumorigenesis caused by *H. pylori* infection. However, more studies are needed to further elucidate the mechanisms involved in the esophageal injury associated with *H. pylori* colonization.

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