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Follow-up analysis and histopathological study of gastric mucosa in patients with *Helicobacter pylori* infection

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Abstract

Objective: To investigate the histomorphological characteristics of the gastric mucosa and the prognosis in patients with *Helicobacter pylori* infection.

Methods: Progressive damage to the gastric mucosa was examined by immunohistochemistry in 2294 patients with *H. pylori* infection and follow-up information was analyzed.

Results: *H. pylori* initially colonized the mucus layer covered by the gastric mucosa epithelium, then selectively adhered to and destroyed the surface mucus cells causing intra-gastric and extra-gastric lesions. Gastric mucosal damage induced by *H. pylori* was divided into five stages according to the depth of *H. pylori* invasion and degree of lesion deterioration: mucilaginous, surface mucocellular, lamina propria lesion, mucosal atrophy, and intraepithelial neoplasia stages. Morphological follow-up analysis revealed no significant difference in 6-month curative effects between stage I and stage II, but significant differences were found between stages II and III, stages III and IV, and between stages IV and stage V, respectively.

Conclusions: This novel staging strategy may be a valuable tool for diagnosing and predicting the results of gastric mucosal damage induced by *H. pylori* infection.

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Introduction

Helicobacter pylori is a major cause of chronic gastritis, gastric ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[1,2] The methods of acquisition and transmission of *H. pylori* infection remain debatable, but direct person-to-person transmission (oral-oral, gastro-oral, fecal-oral, breastfeeding and iatrogenic pathways) appears to be the main route.[3] The prevalence of *H. pylori* varies geographically, with high prevalence rates in Russia, Jordan, Iran, China, and Latin American countries, as well as in Arctic populations in Canada.[4] Several studies in Asia have addressed annual reinfection rates of *H. pylori*, ranging from 1.5% in China to 3.1% in Korea.[5] *H. pylori* infection is the most important risk factor for gastric cancer, and its eradication is generally considered to reduce the risk of gastric cancer.[6,7] *H. pylori* infections are currently classified by clinical pathology as mild, moderate, or severe, and the corresponding pathology reports are described as +, ++, and +++. However, this classification does not reflect the histopathological features and does not provide any information on the relationship between clinical *H. pylori* infection and gastric cancer. In this study, we collected gastroscopic biopsy specimens from the gastric antrum and corpus and analyzed the morphological characteristics and evolution of *H. pylori* infection in the gastric mucosa, to further our understanding of the process of *H. pylori* infection and its optimal treatment.

Materials and methods

Sample collection

This study was performed in four hospitals in China: the Shenzhen Hospital of Southern Medical University, the 989th Hospital of the Joint Logistic Support Force of the PLA, the 3rd Affiliated Hospital of Zhengzhou University, and the 990th Hospital of the Joint Logistic Support Force of the PLA. Consecutive patients with laboratory-confirmed *H. pylori* infection admitted one of the hospitals were enrolled between November 2017 and November 2019. Biopsies were taken from the gastric antrum or gastric corpus, including at least two pieces of tissue per site, and sent to the pathology laboratory within 2 hours. We recorded the most severe stage among the different sites as the histopathological stage for follow-up analysis of each patient.

The reporting of this study conforms to the STROBE guidelines.[8] The study protocol (ID: 132102310008, approval date: 21 July 2016) was approved by the Medical Ethics Committee of the 989th Hospital of the Joint Logistic Support Force of PLA. Written informed consent was obtained from all patients before the study.

H. pylori *eradication protocol*

All patients were initially treated with a 14-day bismuth quadruple therapy program. The history of macrolide (clarithromycin, azithromycin, roxithromycin, erythromycin, spiramycin) use was

determined before enrollment. Patients without prior macrolide use received colloidal pectin bismuth capsules (Hunan Warrant Pharmaceutical Co., Ltd., Changsha, China) 140 mg (before meals), amoxicillin capsules (Hunan Kelun Pharmaceutical Co., Ltd., Yueyang, China) 1000 mg and clarithromycin tablets (Shandong Xinhua Pharmaceutical Co., Ltd., Zibo, China) 500 mg (after breakfast and dinner), plus rabeprazole sodium enteric-coated tablets 20 mg (before breakfast). Patients with prior macrolide use received colloidal pectin bismuth capsules 140 mg (before meals), amoxicillin capsules 1000 mg and metronidazole tablets (Sichuan Kelun Pharmaceutical Co., Ltd., Chengdu, China) 400 mg (after breakfast and dinner), plus rabeprazole sodium enteric-coated tablets 20 mg (before breakfast).

Immunohistochemical and hematoxylin and eosin (HE) staining

Specimens were fixed with 10% neutral formalin for 24 hours and the gross examination findings were noted. Specimens were processed by standard paraffin embedding, and one part was prepared for HE staining and the other for En-Vision staining. Laboratory confirmation was carried out in accordance with the guidelines for the primary care of *H. pylori* infection (2019, Chinese Medical Association).[9] En-Vision staining was used to determine the presence of *H. pylori*. The specific antibody was used to label the antigen components of *H. pylori*, which was stained yellow. Pretreated sections were first rinsed with distilled water, followed by TBS for 10 minutes, and then incubated with primary antibody for 24 hours and rinsed in TBS for 10 minutes. The sections were then incubated with second antibody for 10 minutes, followed by the color-source substrate solution, and finally rinsed with distilled water for microscopic observation. All antibodies were obtained from Shenzhen Dartmon Biotechnology Co. Ltd. (Guangdong, China) and the procedures were performed in accordance with the kit instructions.

HE staining was applied to observe the histomorphology and evolution of gastric mucosa damage in patients with *H. pylori* infection. All the reagents were purchased from Fuzhou Maixin Biotech. Co., Ltd. (China) and prepared in the respective laboratories.

Follow-up analysis

All cases were followed-up by histopathological examination following endoscopic biopsy carried out after an interval of at least 6 months. Follow-up specimens were taken from the same sites as the previous histopathological examination. The deadline for follow-up analysis was 31 May 2020. 'Valid' was defined as the eradication of *H. pylori* infection or reduced histopathological stage of gastric mucosal damage induced by *H. pylori* infection; 'invalid' was defined as no significant change in histopathological stage of damage; and 'aggravating' was defined as increased histopathological stage of damage.

During the follow-up period, each patient was reminded of the patient compliance requirements by telephone every month. The main purpose was to remind them not to drink untreated water or eat cold dishes, to refrain from regular drinking and smoking and reduce their tobacco and alcohol consumptions to $<25\%$ of the levels before enrollment.

Statistical analysis

Differences in the curative effects between the stages were determined by nonparametric rank sum tests. All statistical analyses were carried out using IBM SPSS

Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA).

Results

Patients

A total of 2294 patients with laboratory-confirmed *H. pylori* infection were enrolled, including 1321 men. The patients were aged 19 to 82 years (mean 42.7 years). According to the gastroscopy records, 2107 biopsies were taken from the gastric antrum and 452 biopsies from the gastric corpus. Of these, 2067 patients (90.1%) without prior macrolides received treatment including clarithromycin and 227 (9.9%) with prior macrolides received treatment including metronidazole.

Damage stage of gastric mucosa

We divided the damage to the gastric mucosa into five stages according to the depth of *H. pylori* invasion and degree of lesion deterioration (Table 1, Figures 1, 2, and 3). There were 74 cases of stage I (mucilaginous stage), 619 cases of stage II (surface mucocellular stage), 979 cases of stage III (lamina propria lesion stage), 422 cases of stage IV (mucosal atrophy stage), and 200 cases of stage V (intraepithelial neoplasia stage).

Follow-up analysis

After treatment with the recommended regimen, follow-up analysis showed an overall valid rate of 44.4%, invalid rate of 44.8%, and aggravating rate of 10.8%. The highest valid rate of 59.5% was found in patients with stage I gastric mucosa damage and the lowest valid rate of 11.9% in patients with stage VA, while the lowest invalid and aggravating rates (35.1% and 5.4%, respectively) were also found in patients with stage I and the highest rates (68.9% and 19.2, respectively) in patients with stage V. There was no significant difference in 6-month curative effects, determined by changes in pathological stage before and 6 months after treatment, between the substages in each stage. There was also no significant difference in the 6-month curative effects between stages I and II, but the curative effect was significantly better in patients with stage II compared with stage III ($P = 0.018$), stage III compared with stage IV ($P < 0.001$), and stage IV compared with stage V ($P < 0.001$). All 49 cases with stage VB were treated with endoscopic submucosal dissection and no longer followed-up (Table 2).

Discussion

The stomach was long believed to provide a sterile environment because of its acid barrier; however, in 1983, Marshall and Warren[10] co-discovered the spiral Gram-negative bacterium, *H. pylori*, and subsequent studies have found that this pathogen has been co-evolving with humans for more than 60,000 years.[11]

H. pylori infection results in the destruction of the gastric mucosal barrier, which may in turn lead to the stepwise development of carcinogenesis. *H. pylori* is believed to be one of the main driving forces in the progression of non-cardia gastric cancers from acute gastritis to chronic gastritis, followed by atrophic gastritis and eventually progressing to malignancy.[12] *H. pylori* has the characteristics of cluster reproduction and tissue-specific adsorption. *H. pylori* initially colonizes the mucus layer covered by the epithelium of the gastric mucosa, and then penetrates to the mucus layer and specifically adheres to the surface mucus cytoplasm.[13] *H. pylori* toxin selectively destroys the surface mucus cell cytoplasm, causing significant swelling of the cytoplasm and vacuolar degeneration,[14] with complete destruction and loss of the cytoplasm in severe cases. *H. pylori* infection can induce

Table 1. Stages of gastric mucosa damage following *Helicobacter pylori* infection.

Stage	Depth of invasion	Status of lesion deterioration
I, ML stage	ML	
IIA, surface MC degeneration stage	Surface MC	Swelling and vacuolar degeneration in cytoplasm of surface MC
IIB, mucosal erosion stage	Surface MC	Degeneration and shedding of surface MCs; exuding of inflammatory cells; regional massive erosive lesions
IIIA, diffuse inflammation stage	Surface MC	Infiltration of many lymphocytes, plasma cells, and neutrophils, and vacuolar degeneration of glandular epithelial cells and inflammatory cells
IIIB, compensatory hyperplasia stage	Surface MC	Neck MCs and glands show compensatory hyperplasia, especially intestinal metaplasia
IIIC, mucosal ulcerative stage	Neck MC	Mucosal ulcers constituting necrotic cells, tissue fragments, fibroid material, small vessels, and inflammatory cells
IIID, diffuse lymphocyte proliferation stage	Neck MC	Diffuse small lymphocyte proliferation accompanied by small number of lymphoid follicles and glandular hyperplasia
IVA, lamina propria glandular atrophy stage	Neck MC	Intrinsic glands significantly smaller and decreased, accompanied by interstitial fibrous tissue and smooth muscle tissue hyperplasia, and lymphocytes and plasma cell infiltration
IVB, atrophic enterocytosis stage	Neck MC	Intestinal metaplasia glands proliferate and begin to replace lamina propria cells; atrophy of lamina propria gland coexists with intestinal metaplasia
VA, low-level intraepithelial neoplasia stage	Neck MC	Glands irregularly branched; cytoplasm basophilic; nucleus longer; obvious infiltration of lymphocytes and plasma cells
VB, high-level intraepithelial neoplasia stage	Neck MC	Irregular branching of glands; large nuclei; increased nucleoplasm ratio; obvious nucleoli; pathological mitosis

MC, mucinous cells; ML, mucus layer.

an immune response and promote lymphocytes, plasma cells, and macrophages to aggregate to the glands and basement membrane. The main feature of chronic active gastritis is neutrophil infiltration. In the absence of treatment, the inflammation and immune reaction caused by infection may lead to reduction or atrophy of the inherent glands and their replacement by fibrous or fibromuscular tissue or inflammatory cells.[15] The disease then progresses to atrophic gastritis. Continuous atrophy or reduction of the propria gland disrupts the normal repair process of the gastric mucosa. The propria gland is replaced by goblet cells and develops into small and large intestinal metaplastic foci, resulting in intestinal metaplasia.[11] Persistent *H. pylori* infection, inflammation, and an immune response can induce gastric mucosal damage, progressing to malignancy.[11]

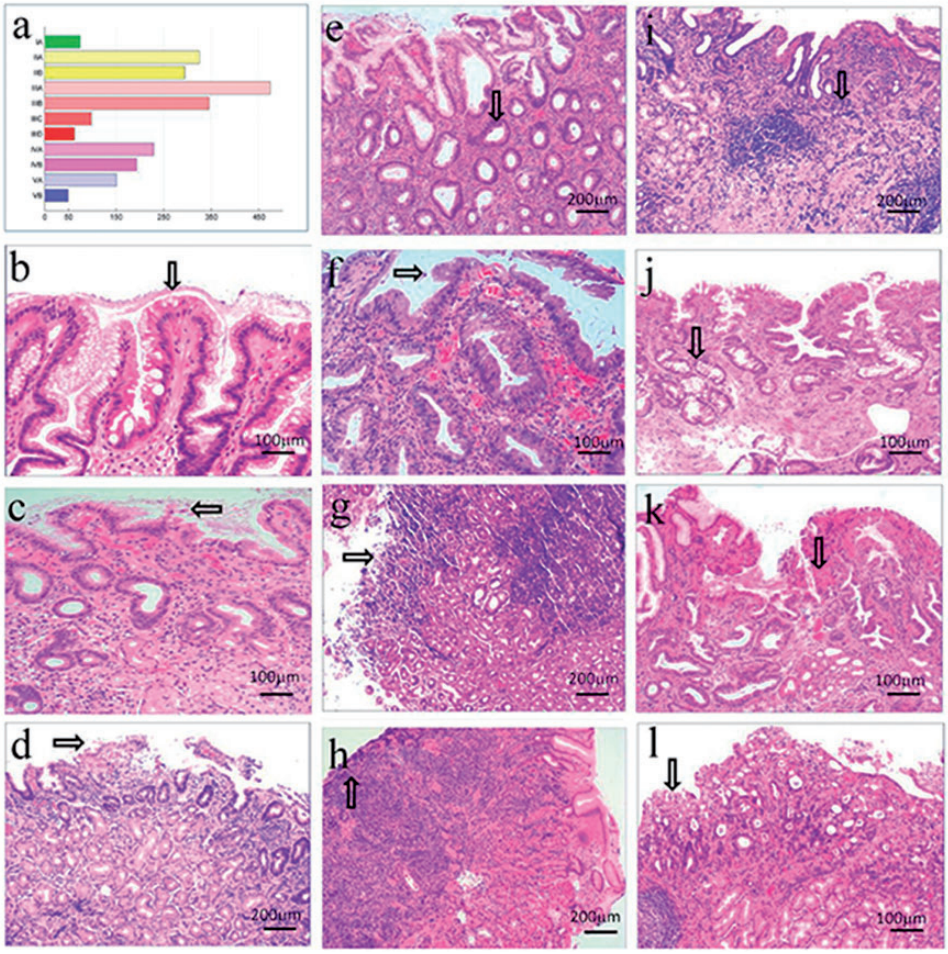

Figure 1. Case distribution (a) and common histomorphological characteristics (hematoxylin and eosin staining) of different stages of gastric mucosa damage causing by *Helicobacter pylori* infection (b–l). Black arrows show locations of common histomorphological characteristics in different stages. (b) Stage I, mucilaginous stage (200×); (c) stage IIA, surface mucocellular degeneration stage (200×); (d) stage IIB, mucosal erosion stage (100×); (e) stage IIIA, diffuse inflammation stage (100×); (f) stage IIIB, compensatory hyperplasia stage (200×); (g) stage IIIC, mucosal ulcerative stage (100×); (h) stage IIID, diffuse lymphocyte proliferation stage (100×); (i) stage IVA, lamina propria glandular atrophy stage (100×); (j) stage IVB, - atrophic enterocytosis stage (200×); (k) stage VA, low-level intraepithelial neoplasia stage (200×); and (l) stage VB, high-level intraepithelial neoplasia stage (200×).

H. pylori colonizes the stomach in more than half of the global population, but its prevalence shows geographical variations.[16] *H. pylori* infection almost always progresses to chronic gastritis when left untreated. *H. pylori* colonization leads to infiltration of the gastric mucosa by neutrophilic cells, which induce acute and chronic gastritis.[13] This chronic active gastritis is the primary state of *H. pylori* infection, and leads to other *H. pylori*-associated disorders.[17] Approximately 20% of *H. pylori*-positive patients are at risk of developing ulcers,[18] and some cohort studies suggested that the

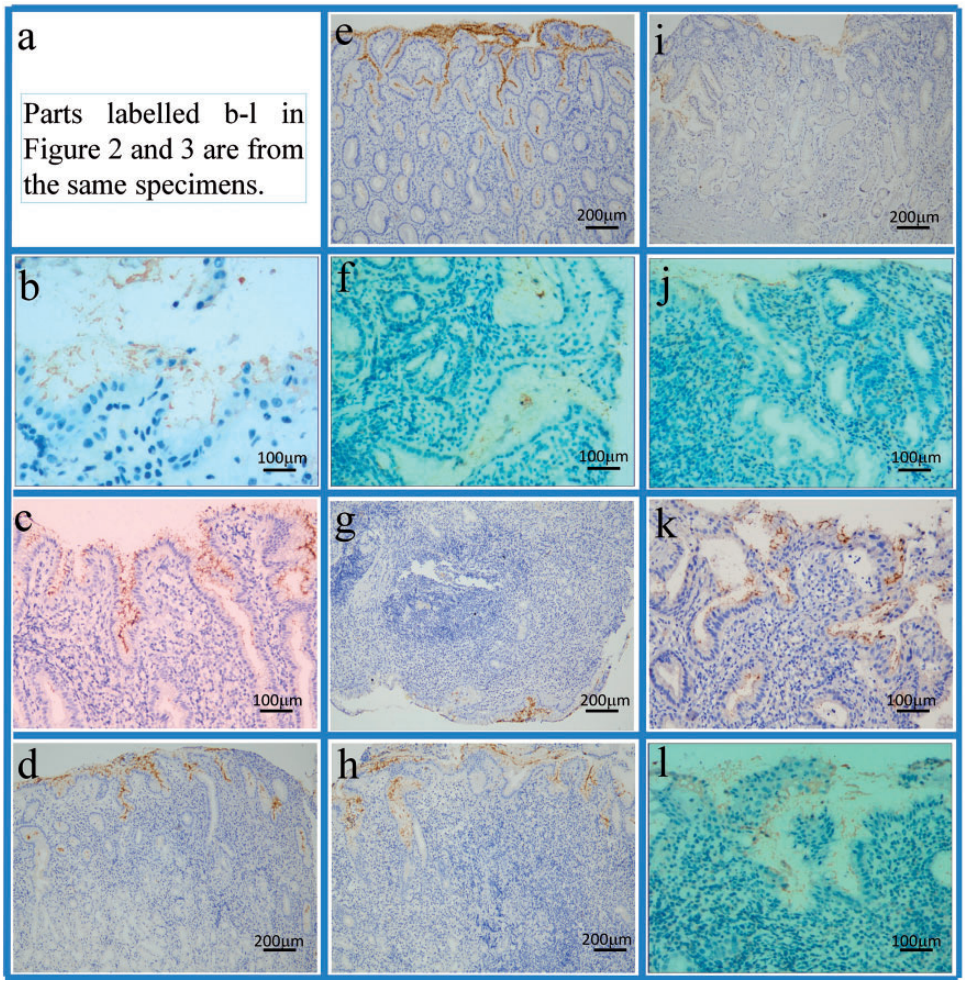

Figure 2. Common *Helicobacter pylori* colonizing status (En-Vision staining) in different stages of gastric mucosa damage. (a) Specimen sources. *H. pylori* colonized (b) the mucus layer, (c–f) mucinous cells, and (g–l) mucinous cells and neck mucinous cells.

lifetime risk of ulcers in *H. pylori*-positive patients was three to ten times higher than in *H. pylori*-negative patients.[19] *H. pylori* infection also carries an approximately 2% risk of developing gastric cancer,[18] and a meta-analysis demonstrated that eradication of *H. pylori* significantly reduced the future incidence of gastric cancer in both healthy individuals and patients with gastric neoplasia.[20] The virulence and pathogenicity of *H. pylori* have been ascribed to its ability to adapt and evade innate and adaptive immunologic defenses.[21] The intercellular apical junctions of epithelial cells are critical for maintaining the integrity of the gastric epithelial barrier and essential cellular functions.[22] *H. pylori* disrupts epithelial tight junctions by binding to specific cellular receptors and stimulating signaling pathways.[23] *H. pylori* was recently shown to reduce the acid-induced tightening of cell junctions in an

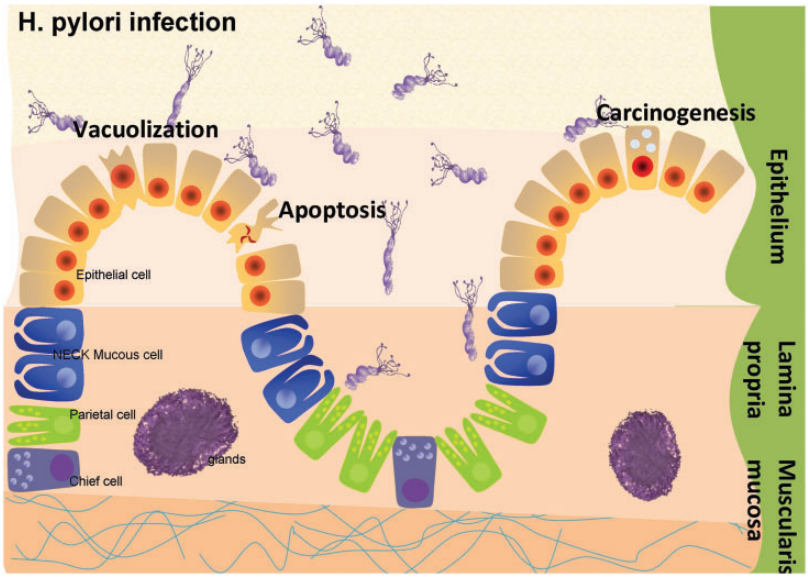

Figure 3. Diagram showing evolution of gastric mucosa damage following *Helicobacter pylori* invasion.

aurease-dependent manner, promoting barrier compromise and progression to an inflammatory response.[24] *H. pylori* also caused defects in epithelial cell polarity by targeting epithelial adhesion receptors such as E-cadherin and β1-integrin, to modulate formation of the cytoskeleton.[25] *H. pylori* not only colonizes the mucus layer covering the gastric mucosa, but also invades gastric epithelial cells and immunocytes, and recent studies demonstrated that *H. pylori* induced autophagy of epithelial cells and phagocytes.[26] The rapid turnover of epithelial cells helps to protect the epithelium from infection, but *H. pylori* disrupts the balance between the proliferation and turnover of the gastric epithelium to facilitate its survival.[27]

The World Health Organization has classified *H. pylori* infection as a gastric carcinogen, and there is currently a trend to prevent gastric cancer by eliminating *H. pylori* via its detection and treatment.[28] Numerous studies have shown that *H. pylori* causes structural and morphological changes in the gastric mucosa prior to gastric carcinogenesis, including changes in the morphology and signaling of gastric epithelial cells and in the expression of certain proteins.[29,30] However, the clinicopathological classification of *H. pylori* infection as mild, moderate, or severe is misleading; the number of *H. pylori* bacteria does not reflect the extent of gastric mucosal damage and is not conducive to its accurate treatment by clinicians and to the follow-up of malignant cell transformation.

In this study, we proposed a novel staging strategy based on the extent and depth of gastric mucosal damage caused by *H. pylori* infection, as well as the pattern and development of lesion occurrence. Early gastric mucosal biopsy of *H. pylori* infection can detect high levels of *H. pylori*, while *H. pylori* numbers are decreased in late-biopsy samples, mainly due to pyloric gland/basophilic gland degeneration, as well as the infiltration of numerous inflammatory cells and the

Table 2. Curative effects in patients with different stages of gastric mucosal damage.

Half-year curative effect	Stage I			Stage IIA			Stage IIB			Stage IIIA			Stage IIIB		
	V	I	A	V	I	A	V	I	A	V	I	A	V	I	A
	44	26	4	180	118	27	157	112	25	225	206	43	165	147	33
Priority		☆					☆					☆☆			
$P^{\$}$				0.882						0.998					
$P^{\#}$					0.574										
								0.018							

Half-year curative effect	Stage IIIC			Stage IIID			Stage IVA			Stage IVB			Stage VA		
	V	I	A	V	I	A	V	I	A	V	I	A	V	I	A
	46	42	10	31	26	5	83	110	36	53	109	31	18	104	29
Priority			☆☆						☆☆☆					☆☆☆☆	
$P^{\$}$			0.998					0.139							
$P^{\#}$						<0.001									
									<0.001						

V, valid; I, invalid; A, aggravating. $^{\$}$sub-stage versus sub-stage; $^{\#}$stage versus stage. *Better curative effect led to lower treatment priority.

detection of vacuole-like degeneration and other indirect morphological changes. It is important to understand the main pathological characteristics of the *H. pylori*-infection stages. Stage I refers to the presence of *H. pylori* bacteria in the mucus layer, with a normal surface-covered mucus layer thickness of 0.25 to 0.5 mm. The clinical symptoms at this stage are not obvious and the incidence of diagnosis based on histological analysis of endoscopic biopsy samples is low, with *H. pylori* infection usually being diagnosed based on health examinations. When the population of *H. pylori* increases, the bacteria are propelled towards the mucus layer in a corrosive manner, resulting in deeper destruction. When the mucus layer is completely disrupted, the infection enters stage II, the surface mucus cell stage. Stage IIA is characterized by *H. pylori*-specific adhesion to and selective destruction of the surface mucus cell cytoplasm, with significant swelling and vacuolation-like degeneration of the cytoplasm. The degeneration and shedding of surface mucus cells causes the mucus membranes to enter stage IIB of *H. pylori* infection, the vesicular phase. This degeneration and shedding of surface mucus cells, together with the exudation of inflammatory cells, result in the formation of regional flaky, crusty lesions, and a lack of timely treatment allows the infection to enter stage III, the laminar lesion stage. In stage IIIA, diffuse acute and chronic inflammation occurs throughout the lamina propria, together with enhanced basophilicity of the interstitial stroma and the infiltration of numerous lymphocytes, plasma cells, neutrophils, and eosinophils with varying degrees of vacuolar-like degeneration. This stage is characterized by inflammatory cells with unclear cytoplasmic boundaries, the breakdown of neutrophils and eosinophils, and the scattering of eosinophils in the matrix of the mesenchyme. Small interstitial vessels appear dilated, congested, and hemorrhagic, with the formation of common mucosal erosions and small abscesses with mild hyperplasia of surface mucus cells and lamina propria glands, and enhanced cytoplasmic eosinophilia. As the disease progresses, *H. pylori* infection enters stage III, the compensatory hyperplasia stage, with the main features of mucus neck cell surrogate hyperplasia, pyloric/gastric floor glands with varying degrees of

hyperplasia and heteroplasia, enlarged glands with branching, and serrated/micropapillary structures. Glandular epithelial cells show enhanced cytoplasmic basophilia, enlarged nuclei, and gray staining of the nucleus. In stage IIIC, mucosal ulcers develop constituting necrotic cells and tissue fragments, as well as fibrous material, small blood vessels, and inflammatory cells, and *H. pylori* can be detected in the surface of mucus cells and neck mucus cells in the peripheral area. Glandular epithelial cells show different degrees of hyperplasia, repair hyperplasia, and heterotypic hyperplasia. Khatoon et al. showed the relationship between confirmed heterogeneity of the *cag* pathogenicity island gene and peptic ulcer disease in *H. pylori* strains and the effective prevention of peptic ulcer disease by the identification of *H. pylori*.[31,32] Stage IIID is characterized by histologically diffuse lymphocyte proliferation and a number of lymphatic follicles of varying sizes. *H. pylori* can also be detected in surface mucus cells surrounding lymphocyte proliferation and in areas of proliferating neck mucus cells. *H. pylori* infection has been reported to manifest not only as atrophic gastritis, but to also develop into mucin-associated lymphoma in the marginal zone, consistent with our proposed pathological stage of *H. pylori* infection.[33] The present study found many small lymphocytes surrounding the proliferative lymphatic follicles in this stage, as an early alteration of MALT lymphoma, forming follicular sets in the form of marginal zones. The lesion progresses further and the tumorigenic lymphocytes show erosive growth and infiltrate into and destroy the follicle. Cytologically, the tumor cells are of medium size, with white cytoplasm and irregular nuclei, with a centrocyte-like or monocyte-like appearance, and positive expression of CD20, CD79a, and BCL2.[34] *H. pylori* infection stage IV is considered as the mucosal atrophy stage. Stage IVA is characterized by atrophy of the lamina propria gland and the proliferation of interstitial fibers and smooth muscle tissue, and their intermingling with infiltrating lymphocytes and proliferating lymphatic follicles.[35] A thorough understanding of the characteristics of *H. pylori* infection leading to gastric mucosal atrophy and controlling the development of mucosal atrophy in a timely manner have been reported to reduce the incidence of early gastric cancer.[36] In stage IVB, the atrophic enterocytosis stage, enterocytic glands with varying degrees of proliferation and expansion and mild atypia are noted, mucus secretion in the enterocytic region is enhanced, and basophilic granules are formed in the cytoplasm of enterocytic cells. Enterocytes with and without *H. pylori* both proliferate in a compensatory manner, representing an important precancerous lesion. Stage V represents the intraepithelial neoplasia stage. The main features of stage VA, the low-level intraepithelial neoplasia stage, are irregular branching of the glands, enhanced cytoplasmic eosinophilia, elongated nuclei located at the glandular epithelial base, and 30% of the nuclei occupied by small kernels. Interstitial fibrous tissue hyperplasia and the infiltration of lymphocytes and plasma cells are noted. Low-grade intraepithelial neoplasia is generally closely followed-up, and we recommend that gastroscopic biopsies should be reviewed every 6 months to reveal exacerbated morphological changes, with endoscopic submucosal dissection employed as necessary.

We tried to minimize the confounding factors affecting the results and conclusions; however, this study still had some limitations. The absence of carbon-13 urea breath test results meant that we were unable to obtain information on the *H. pylori* eradication rate after 14 days of bismuth quadruple therapy. We also know that *H. pylori* was not eradicated in some patients, leading to the progression of

gastric mucosal injury. The eradication rate is affected by many factors, including genetic susceptibility to drugs, recrudescence, and reinfection, and the determination of these causes requires the use of polymerase chain reaction or sequencing methods. However, these techniques are not currently available in our laboratory.

In summary, *H. pylori* infection has been recognized as a major cause of chronic gastritis, gastric ulcers, MALT lymphoma, and gastric adenocarcinoma. To improve our understanding of the evolution of damage to the gastric mucosa, we conducted a histopathological study and follow-up analysis in 2294 patients with confirmed *H. pylori* infection. Morphological observations revealed that *H. pylori* initially colonized the mucus layer covered by the epithelium of the gastric mucosa, and then selectively adhered to and destroyed the surface mucus cells, causing intra-gastric and extra-gastric lesions. Furthermore, we classified the gastric mucosal damage caused by *H. pylori* into five stages, according to the depth of invasion and the degree of lesion deterioration. Follow-up analysis revealed significant differences in 6-month curative effects between patients with stage II and stage III, stage III and stage IV, and stage IV and stage V disease.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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