Influence of Helicobacter pylori infection on hepcidin expression in the gastric mucosa

Running title: Helicobacter pylori and gastric hepcidin

Yuki Nishigaki, MD[1], Yuichi Sato, MD, PhD[1], Hiroki Sato, MD, PhD[1], Mitsuya Iwafuchi, MD, PhD[2], and Shuji Terai, MD, PhD[1]

[1] Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, 951-8510, Japan

[2] Division of Pathology, Niigata University School of Health Sciences, Niigata, 951-8518, Japan

Correspondence should be addressed to Yuichi Sato

Division of Gastroenterology, Graduate School of Medical and Dental Sciences, Niigata University
757-1, Asahimachidori, Chuo-ku, Niigata City, Niigata 951-8510, Japan

Tel: +81-25-223-2207; Fax: +81-25-223-0776; Email: yuichi@med.niigata-u.ac.jp

ABSTRACT

Background: Hepcidin is an antimicrobial peptide and a key hormone involved in iron homeostasis. Hepcidin level is elevated in the serum during the course of *Helicobacter pylori* infection and hepcidin is considered to contribute to iron deficiency anemia. However, it is unclear whether *H. pylori* infection influences hepcidin expression in the gastric mucosa.

Method: In this study, 15 patients with *H. pylori* infected nodular gastritis, 43 patients with *H. pylori* infected chronic gastritis, and 33 patients without *H. pylori* infection were enrolled. Endoscopic biopsy and immunohistochemical analysis were performed to evaluate the expression of hepcidin and its distribution in the gastric mucosa

Result: Hepcidin was strongly expressed in the lymph follicles of patients with nodular gastritis. The detection rates of gastric hepcidin-positive lymphocytes in patients with nodular gastritis and chronic gastritis were significantly higher than that without *H. pylori* infection. Moreover, regardless of the *H. pylori* infection status, hepcidin was expressed in the cytoplasm and intracellular canaliculi of gastric parietal cells.

Conclusion: Hepcidin is expressed in gastric parietal cells in a steady state, and *H. pylori* infection can induce hepcidin expression in lymphocytes present in the gastric mucosal lymphoid follicles. This phenomenon may be associated with systemic hepcidin overexpression and iron deficiency anemia in patients with *H.pylori*-infected nodular gastritis.

KEYWORDS

Hepcidin, nodular gastritis, chronic gastritis, *Helicobacter pylori, endoscopy*

INTRODUCTION

Hepcidin, a disulfide-rich peptide comprising 25-amino acids, was initially identified as an endogenous antimicrobial peptide belonging to the defensin family[1-3]. Recently, it was reported that hepcidin also acts as a regulatory hormone of iron homeostasis by binding to ferroportin, which decreases iron absorption in duodenal enterocytes and iron release from macrophages. Hepcidin is mainly expressed in the liver and is weakly expressed in other organs and cells, such as the kidney, stomach, small intestine, large intestine, muscles, heart, lungs, macrophages, monocytes, lymphocytes, and adipocytes[2,4-6]. However, the role of hepcidin in organs other than the liver is unclear.

Helicobacter pylori infection is associated with iron deficiency and iron deficiency anemia (IDA)[7-9], which also appears to be linked with elevated hepcidin production[10-14]. Our previous study showed that iron deficiency was accompanied by high levels of serum prohepcidin, a precursor of hepcidin, in patients with *H. pylori*-infected nodular gastritis (NG); this suggested that iron deficiency in the patients with NG is related to hepcidin[15]. NG is characterized by lymph follicles in the gastric mucosa with a gross appearance of goose flesh-like markings; thus, it is described as gastric lymphoid hyperplasia, follicular gastritis, or antral NG according to its endoscopic appearance (Figure 1) or histological findings[16]. A few other reports have also described the presence of IDA in patients with NG; however, the cause of iron deficiency or IDA in patients with NG is unknown. Furthermore, no studies have evaluated hepcidin expression in the gastric mucosa of patients with NG, and little is known about the relationship between gastric mucosal expression of hepcidin and *H. pylori* infection. Therefore, in this study, we investigated the expression of hepcidin in the gastric mucosa of patients with *H. pylori*-infected chronic gastritis (CG), patients with *H. pylori*-infected NG, and compared with uninfected patients using

immunohistochemistry to determine the effect of *H. pylori* infection on hepcidin expression in the gastric mucosa.

MATERIALS AND METHODS

Patients and sample collection

Patients diagnosed with *H.pylori* infected NG, *H.pylori* infected CG, and those without *H.pylori* infection were recruited. Diagnosis of *H.pylori* infection was based on the result of fecal antigen test and bacterial culture test of *H. pylori*. Esophagogastroduodenoscopy was performed, and biopsy specimens were obtained from the greater curvature of the gastric antrum and corpus. More than two biopsy specimens from the greater curvature of the gastric antrum and corpus were used for the bacterial culture test of *H. pylori*. In general, hepcidin are expressed in the fundic ground[17], therfore, the biopsy specimens from the corpus were used for histological analysis in this study. The specimen was fixed in 10% formalin, embedded in paraffin, and sectioned into 4-μm segments. Histological sections were stained with hematoxylin and eosin (H&E) and toluidine blue, and immunohistochemical staining was performed.

This study was approved by the ethics committee of our institution (2015-2192). Written informed consent was obtained from all patients.

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated in an ethanol series with phosphate-buffered saline. Antigen activation was performed in an autoclave at 121°C for 20 min with citrate buffer solution. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. To eliminate non-specific staining, the sections were incubated with 5% normal goat serum for 20 min. Sections were then incubated with anti-hepcidin antibody

(diluted 1:1600; Abnova, Taipei City, Taiwan), anti-H^+/K^+ ATPase antibody (diluted 1:6000; BMC, Inc., Tokyo, Japan), and anti-CD20 antibody (diluted 1:100; Abcam plc, Cambridge, UK) at 4°C overnight. After primary antibody treatment, the sections were incubated with Histofine Simple Stain MAX-PO MULTI (Nichirei Bioscience, Tokyo, Japan) for 40 min. After washing with phosphate-buffered saline, the sections were incubated with DAB (Nichirei Bioscience, Tokyo, Japan) and immediately washed with tap water after color development. Finally, the sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted for microscopic observation.

Statistical analyses

Continuous data were compared using an independent samples two-tailed *t*-test, whereas the categorical data were analyzed using the χ^2 test or Fisher's exact test. $P < 0.05$ was considered to represent a statistically significant difference for all tests. Analyses were performed using SPSS 20.0 for Windows (SPSS, Inc., Chicago, IL, USA).

RESULT

A total of 91 patients were enrolled in this study: 15 patients (2 men and 13 women; mean age, 43 years; age range, 21–63 years) with *H. pylori*-infected NG, 43 patients (16 men and 27 women; mean age, 55 years; age range, 34–78 years) with *H. pylori*-infected CG, and 33 patients (16 men and 17 women; mean age, 46 years; age range, 20–74 years) without *H. pylori* infection.

In patients with *H. pylori*-infected NG, hepcidin was strongly expressed in the lymph follicles of the gastric mucosa (Figure 2A, 2B). B cell marker CD20 was diffusely positive for the

lymphocytes of the lymph follicles (Figure 2C). However, in patients with NG, most lymphocytes infiltrating the mucosa did not show hepcidin expression, although a few hepcidin-positive lymphocytes were found around the muscularis mucosae (Figure 2D).

In patients with *H. pylori*-infected CG, hepcidin-positive lymphocytes were found around the muscularis mucosae, similar to those in patients with NG; however, hepcidin-positive lymphocytes were not detected in the mucosa despite moderate infiltration of lymphocytes. Hepcidin was expressed in both the cytoplasm and intracellular canaliculi of the parietal cells of the gastric mucosa (Figure 3C, 3D) but not in other cell types of the gastric epithelia. There were no differences in immunostaining patterns (distribution and staining intensity) of hepcidin in both the cytoplasm and intracellular canaliculi of the gastric parietal cells among the three patient groups. In addition, the intracellular canaliculi of the gastric parietal cells were positive for H^+/K^+-ATPase (Figure 3E, 3F), which is the proton pump for gastric acid secretion. Hepcidin expression disappeared after the absorption test using an serial section.

In contrast, patients without *H. pylori* infection exhibited much lower density of mucosal-lymphocyte infiltration and few hepcidin-positive lymphocytes in their mucosa and muscularis mucosae (Figure 4A, 4B).

Overall, hepcidin-positive lymphocytes around the muscularis mucosae were observed in 7 of the 15 (46.7%) patients with *H. pylori*-infected NG and in 18 of the 43 (41.9%) patients with *H. pylori*-infected CG, but in only 4 of the 33 (12.1%) patients without *H. pylori* infection. The detection rates of hepcidin-positive lymphocytes around the muscularis mucosae were significantly higher in patients with NG and CG than in those without *H. pylori* infection ($P < 0.01$ and $p < 0.05$, respectively; Table 1).

DISCUSSION

This study revealed strong staining for hepcidin in lymphoid follicles of *H. pylori* infected patients with NG. In addition, the proportion of hepcidin-positive lymphocytes in the gastric mucosa was significantly higher in *H. pylori*-infected patients than in uninfected patients.

H. pylori is known to cause NG and CG. Furthermore, it is also associated with iron deficiency and/or IDA[7-9]. Therefore, *H. pylori* eradication therapy is recommended in patients of unexplained IDA with *H. pylori* infection based on Maastricht V/Florence consensus as well as other guidelines[18-22]. The mechanisms by which *H. pylori* infection can cause iron deficiency and/or IDA remain unclear; however, several potential mechanisms have been suggested[23,24]: (1) *H. pylori* infection can cause hypochlorhydria or achlorhydria via atrophic gastritis leading to iron malabsorption; (2) *H. pylori* infection can reduce ascorbic acid (vitamin C) levels in gastric juice and thus inhibit non-heme iron absorption; (3) *H. pylori* infection can increase bacterial uptake of iron, an essential bacterial growth factor[24-26]; and (4) recent studies showed that hepcidin is a key regulator of systemic iron homeostasis that down-regulates duodenal iron absorption, and is related to IDA-associated *H. pylori* infection[10,11,14].

A recent meta-analysis showed that the relationship between *H. pylori* and IDA is stronger in children and adolescents than in adults[27]; however, the reason is unknown. In young *H. pylori*-infected patients, NG is a common type of gastritis that is also considered an early gastritis indicator in *H. pylori* infection[14]. In pediatric patients with NG, iron deficiency or IDA is often observed and such conditions are improved by *H. pylori* eradication[28,29]. Our previous study showed that patients with NG are younger than other *H. pylori*-infected patients and that these patients have elevated serum prohepcidin levels along with iron deficiency[15]. Other studies have also suggested that hepcidin is associated with iron

deficiency and *H. pylori* infection in children[10-15,30]. This study revealed that hepcidin was strongly stained in the lymph follicles of patients with NG. Taken together, pediatric and young patients with *H. pylori* infection may develop NG; hepcidin expression in lymph follicles of the gastric mucosa is upregulated in these patients, leading to an iron deficient state.

Additionally, in this study, hepcidin-positive lymphocytes in the mucosal layer were significantly higher in patients with *H. pylori* infection than in uninfected patients. This suggests that *H. pylori* infection affects hepcidin production in intramucosal lymphocytes. However, we found that only a small number of lymphocytes infiltrating the mucosa were hepcidin-positive, suggesting that *H. pylori* infection had a limited effect on hepcidin production in gastric mucosal lymphocytes, except for lymph follicles; therefore, the relationship between *H. pylori* and iron deficiency cannot always be explained by local hepcidin expression alone. Indeed, several reports[31-33] have revealed that hepcidin is not involved in the relationship between *H. pylori* and IDA.

We also demonstrated that hepcidin was localized in the intracellular canaliculi of gastric parietal cells in humans, in addition to the cytoplasm. Previous research[17] suggests that hepcidin is expressed in parietal cells of the stomach; however, the specific localization of hepcidin in these cells was not determined. This is the first study to determine the localization of hepcidin in parietal cells. Intracellular canaliculi, which were stained for H+/K+-ATPase in this study, are closely related to acid secretion of parietal cells. The hepcidin expression observed in the intracellular canaliculi suggests that hepcidin in the parietal cells is related to an acid secretion function. Indeed, a strong correlation between hepcidin and acid secretion has been previously reported[17].

In contrast, we observed no significant differences in the staining patterns of hepcidin in the intracellular canaliculi of the gastric parietal cells of *H. pylori*-positive and -negative patients. These results suggest that hepcidin is constantly produced in parietal cells, regardless of the *H. pylori* infection status.

Moreover, our results demonstrated that lymphocytes in the lymph follicles were CD20-positive B cells, which is consistent with the results of previous studies[34,35]. Although hepcidin mRNA is known to be expressed in both T cells and B cells, our results suggest that B cells infiltrating the mucosa play a major role in the production of hepcidin in the *H. pylori*-infected gastric mucosa.

This study has the following limitations: Firstly, we did not measure the hepcidin or gastric acid concentrations in the gastric mucosa; secondly, the small number of cases restricted the power of analysis; lastly, although we demonstrated that *H. pylori* infection can induce hepcidin expression in gastric mucosal lymphocytes, with particularly strong expression in the lymphoid follicles, hepcidin was expressed in the intracellular canaliculi in the gastric parietal cells irrespective of the *H. pylori* infection status. Further investigation is required in order to elucidate the relationship between *H.pylori* infection, gastric hepcidin expression, and IDA.

In conclusion, we revealed that *H. pylori* infection can induce hepcidin expression in the lymphocytes of lymph follicles in patients with NG. In contrast, the role of *H. pylori* infection on hepcidin expression in gastric mucosal lymphocytes was found to be limited. Hepcidin expression in gastric parietal cells was independent of *H. pylori* infection.

Conflicts of interest and source of funding: none declared

1

References

1. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001; 276 (11):7806-7810.
2. Atanasiu V, Manolescu B, Stoian I. Hepcidin--central regulator of iron metabolism. Eur J Haematol 2007; 78 (1):1-10.
3. Ganz T. Hepcidin and iron regulation, 10 years later. Blood 2011; 117 (17):4425-4433.
4. Dudkowiak R, Neubauer K, Poniewierka E. Hepcidin and its role in inflammatory bowel disease. Adv Clin Exp Med 2013; 22 (4):585-591.
5. Zhang X, Rovin BH. Hepcidin expression by human monocytes in response to adhesion and pro-inflammatory cytokines. Biochim Biophys Acta 2010; 1800 (12):1262-1267.
6. Pinto JP, Dias V, Zoller H, Porto G, Carmo H, Carvalho F, et al. Hepcidin messenger RNA expression in human lymphocytes. Immunology 2010; 130 (2):217-230.
7. Muhsen K, Cohen D. Helicobacter pylori infection and iron stores: a systematic review and meta-analysis. Helicobacter 2008; 13 (5):323-340.
8. Qu XH, Huang XL, Xiong P, Zhu CY, Huang YL, Lu LG, et al. Does Helicobacter pylori infection play a role in iron deficiency anemia? A meta-analysis. World J Gastroenterol 2010; 16 (7):886-896.
9. Yuan W, Li Y, Yang K, Ma B, Guan Q, Wang D, et al. Iron deficiency anemia in Helicobacter pylori infection: meta-analysis of randomized controlled trials. Scand J Gastroenterol 2010; 45 (6):665-676.
10. Azab SF, Esh AM. Serum hepcidin levels in Helicobacter pylori-infected children with iron-deficiency anemia: a case-control study. Ann Hematol 2013; 92 (11):1477-1483.
11. Ozkasap S, Yarali N, Isik P, Bay A, Kara A, Tunc B. The role of prohepcidin in anemia due to Helicobacter pylori infection. Pediatr Hematol Oncol 2013; 30 (5):425-431.
12. Emiralioglu N, Yenicesu I, Sari S, Egritas O, Poyraz A, Pasaoglu OT, et al. An insight into the relationships between prohepcidin, iron deficiency anemia, and interleukin-6 values in pediatric Helicobacter pylori gastritis. Eur J Pediatr 2015; 174 (7):903-910.
13. Baingana RK, Enyaru JK, Tjalsma H, Swinkels DW, Davidsson L. The aetiology of anaemia during pregnancy: a study to evaluate the contribution of iron deficiency and common infections in pregnant Ugandan women. Public Health Nutr 2015; 18 (8):1423-1435.
14. Lee SY, Song EY, Yun YM, Yoon SY, Cho YH, Kim SY, et al. Serum prohepcidin levels in Helicobacter pylori infected patients with iron deficiency anemia. Korean J Intern Med 2010; 25 (2):195-200.
15. Sato Y, Yoneyama O, Azumaya M, Takeuchi M, Sasaki SY, Yokoyama J, et al. The relationship between iron deficiency in patients with Helicobacter pylori-infected nodular gastritis and the serum prohepcidin level. Helicobacter 2015; 20 (1):11-18.
16. Yang HR. Updates on the Diagnosis of Helicobacter pylori Infection in Children: What Are the Differences between Adults and Children? Pediatr Gastroenterol Hepatol Nutr 2016; 19 (2):96-103.
17. Schwarz P, Kubler JA, Strnad P, Muller K, Barth TF, Gerloff A, et al. Hepcidin is localised in gastric parietal cells, regulates acid secretion and is induced by Helicobacter pylori infection. Gut 2012; 61 (2):193-201.
18. Malfertheiner P, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, et al. Management of Helicobacter pylori infection-the Maastricht V/Florence Consensus Report. Gut 2017; 66 (1):6-30.
19. Fock KM, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, et al. Second Asia-Pacific Consensus Guidelines for Helicobacter pylori infection. J Gastroenterol Hepatol 2009; 24 (10):1587-1600.

20. Goddard AF, James MW, McIntyre AS, Scott BB, British Society of G. Guidelines for the management of iron deficiency anaemia. Gut 2011; 60 (10):1309-1316.
21. Asaka M, Kato M, Takahashi S, Fukuda Y, Sugiyama T, Ota H, et al. Guidelines for the management of Helicobacter pylori infection in Japan: 2009 revised edition. Helicobacter 2010; 15 (1):1-20.
22. Kato M, Ota H, Okuda M, Kikuchi S, Satoh K, Shimoyama T, et al. Guidelines for the management of Helicobacter pylori infection in Japan: 2016 Revised Edition. Helicobacter 2019; 24 (4):e12597.
23. Annibale B, Capurso G, Delle Fave G. The stomach and iron deficiency anaemia: a forgotten link. Dig Liver Dis 2003; 35 (4):288-295.
24. Barabino A. Helicobacter pylori-related iron deficiency anemia: a review. Helicobacter 2002; 7 (2):71-75.
25. Yokota S, Konno M, Mino E, Sato K, Takahashi M, Fujii N. Enhanced Fe ion-uptake activity in Helicobacter pylori strains isolated from patients with iron-deficiency anemia. Clin Infect Dis 2008; 46 (4):e31-33.
26. Lee JH, Choe YH, Choi YO. The expression of iron-repressible outer membrane proteins in Helicobacter pylori and its association with iron deficiency anemia. Helicobacter 2009; 14 (1):36-39.
27. Hudak L, Jaraisy A, Haj S, Muhsen K. An updated systematic review and meta-analysis on the association between Helicobacter pylori infection and iron deficiency anemia. Helicobacter 2017; 22 (1).
28. Cardamone M, Alex G, Harari MD, Moss WP, Oliver MR. Severe iron-deficiency anaemia in adolescents: consider Helicobacter pylori infection. J Paediatr Child Health 2008; 44 (11):647-650.
29. Gulen H, Kasirga E, Yildirim SA, Kader S, Sahin G, Ayhan S. Diagnostic yield of upper gastrointestinal endoscopy in the evaluation of iron deficiency anemia in older children and adolescents. Pediatr Hematol Oncol 2011; 28 (8):694-701.
30. Mendoza E, Duque X, Hernandez Franco JI, Reyes Maldonado E, Moran S, Martinez G, et al. Association between Active H. pylori Infection and Iron Deficiency Assessed by Serum Hepcidin Levels in School-Age Children. Nutrients 2019; 11 (9).
31. Kim HK, Jang EC, Yeom JO, Kim SY, Cho H, Kim SS, et al. Serum prohepcidin levels are lower in patients with atrophic gastritis. Gastroenterol Res Pract 2013; 2013:201810.
32. Cherian S, Forbes DA, Cook AG, Sanfilippo FM, Kemna EH, Swinkels DW, et al. An insight into the relationships between hepcidin, anemia, infections and inflammatory cytokines in pediatric refugees: a cross-sectional study. PLoS One 2008; 3 (12):e4030.
33. Chen ST, Ni YH, Li CC, Liu SH. Hepcidin correlates with interleukin-1beta and interleukin-6 but not iron deficiency in children with Helicobacter pylori infection. Pediatr Neonatol 2018; 59 (6):611-617.
34. Elitsur Y, Jackman S, Keerthy S, Lawrence Z, Maynard VL, Triest WE. T and B cell repertoire in gastric lymph follicles in children with Helicobacter pylori infection. Pediatr Pathol Mol Med 2002; 21 (1):31-39.
35. Terres AM, Pajares JM. An increased number of follicles containing activated CD69+ helper T cells and proliferating CD71+ B cells are found in H. pylori-infected gastric mucosa. Am J Gastroenterol 1998; 93 (4):579-583.

Table 1: Clinical characteristics of patients and hepcidin expression in histology

	H. pylori–infected nodular gastritis (n=15)	*H. pylori*–infected chronic gastritis (n=43)	*H. pylori*–uninfected (n=33)
Age (mean± SEM)	43±10.5	55±10.9	46±14.6
Male: Female	2:13	16:27	16:17
Hepcidin expression in the parietal cells	15 / 15 (100%)	43 / 43 (100%)	33 / 33 (100%)
Hepcidin expression in the lymphocytes	7 / 15 (46.7%)	18 / 43 (41.9%)	4 / 33 (12.1%)*
Hepcidin expression in the lymph follicles	15 / 15 (100%)	No lymph follicles	No lymph follicles

* $P < 0.05$, vs *H. pylori*–infected nodular gastritis and chronic gastritis group

SEM, Standard error of the mean.

Figure 1:

Endoscopic findings of *H. pylori* infected nodular gastritis (A, B). Nodularity is visible as uniform, small granular elevation, shaped like goose flesh in the antrum (yellow triangle). *H. pylori* infected chronic gastritis (C, D). Atrophy is recognized as a region discolored to a diffuse white tone (red triangle). Endoscopic images of gastric antrum and body without *H. pylori* infection (E, F).

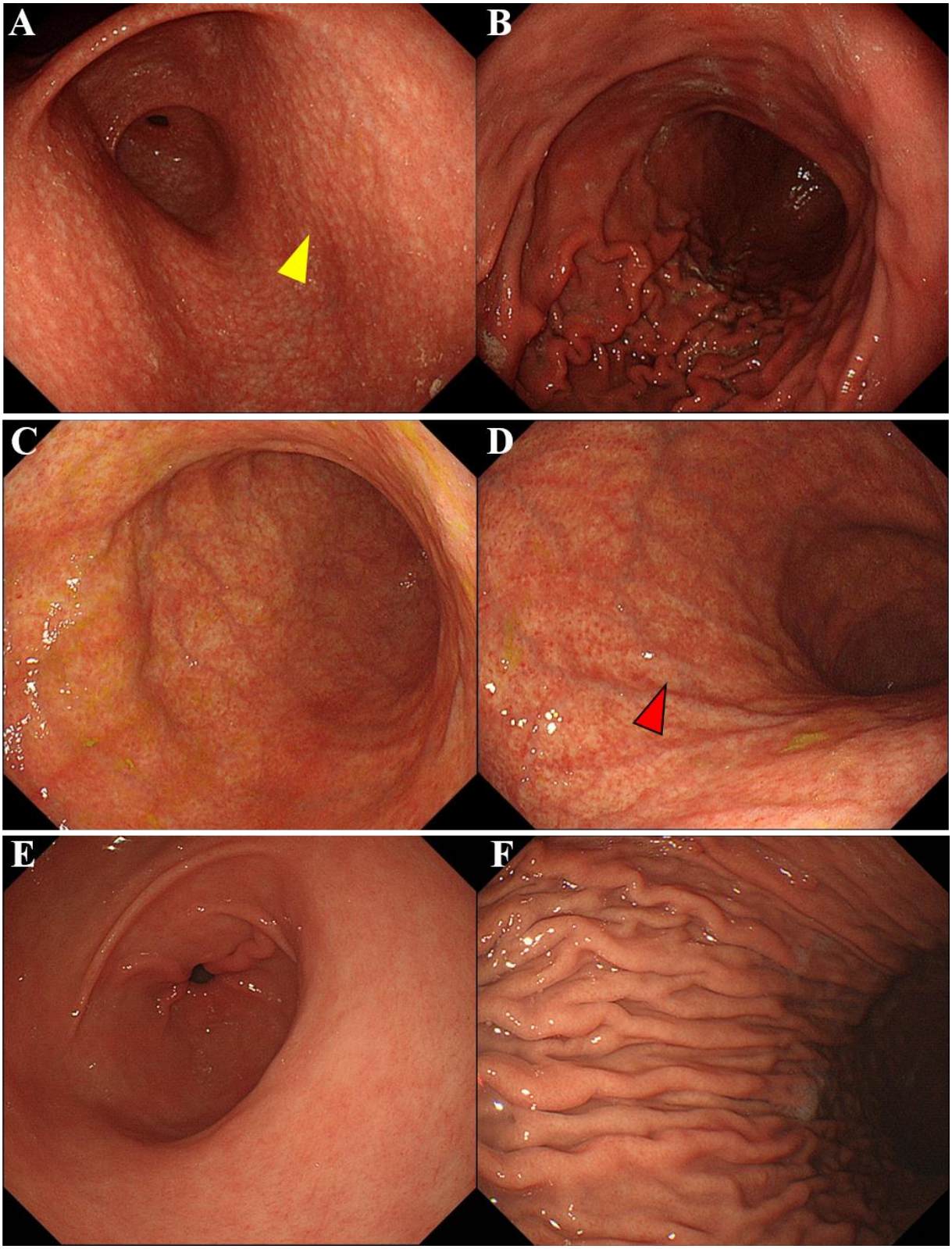
A
B
C
D
E
F

Figure 2: (A) Hematoxylin and eosin (H&E) staining (×100) in biopsy specimens obtained from patients with *H. pylori* infected nodular gastritis. (B) Immunostaining for hepcidin (×200). Hepcidin was expressed in the gastric parietal cells and mucosal lymphocytes (white arrow), particularly in the germinal center of the lymph follicles (black arrow). (C) CD20 was expressed only in the germinal center of lymph follicles. (D) Immunostaining for hepcidin (×400) showing Hepcidin-positive lymphocytes (black arrow) in the deep layer of the lamina propria mucosa.

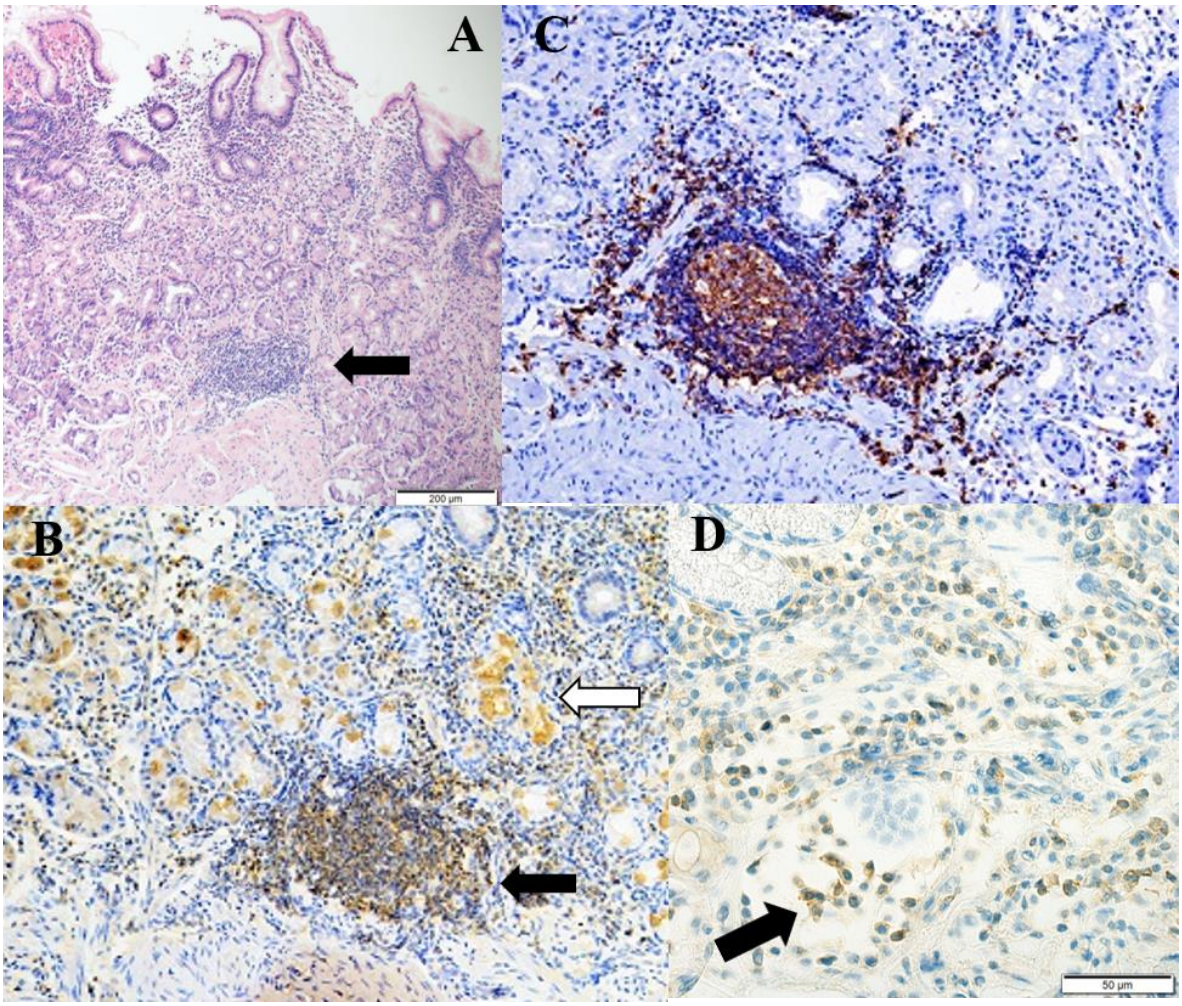

Figure 3: Hematoxylin and eosin (H&E) staining (A: ×100, B: ×200), and immunostaining for hepcidin (C: ×100, D: ×200) and H+/K+-ATPase (E: ×100, F: ×200) in biopsy specimens obtained from the fundic gland of patients with *H. pylori* infected chronic gastritis. Hepcidin was expressed in the cytoplasm (white arrow) and intracellular canaliculi (black arrow) of gastric parietal cells (C, D). H+/K+-ATPase was expressed in the intracellular canaliculi of gastric parietal cells (E, F).

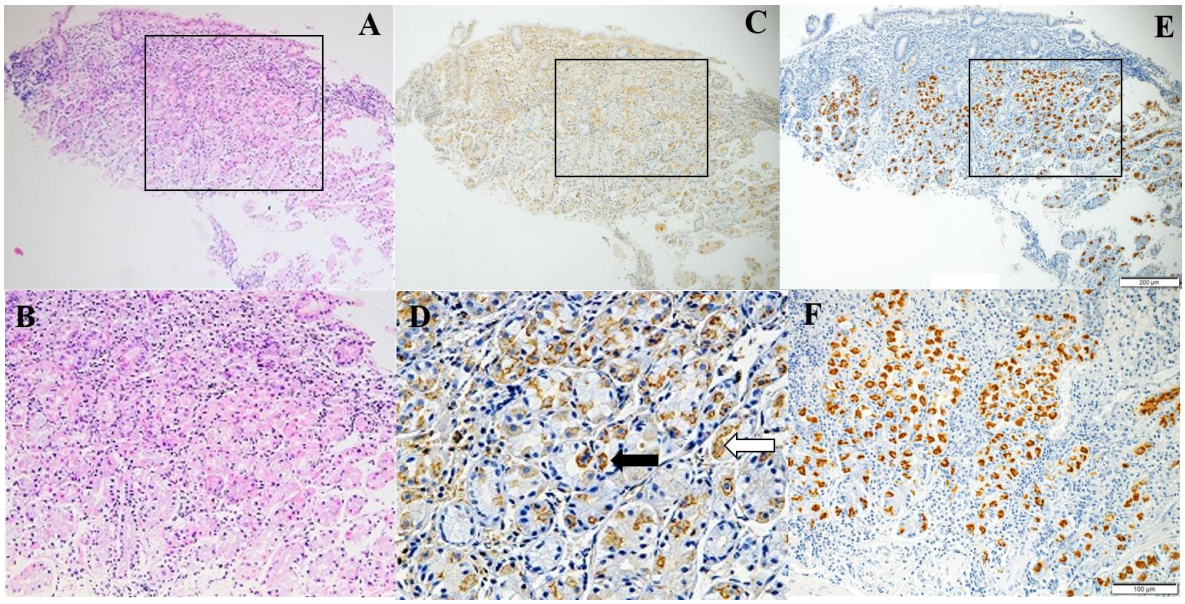

Figure 4: Hematoxylin and eosin (H&E) staining (A: ×100) and immunostaining for hepcidin (B: ×100) in biopsy specimens obtained from patients without *H. pylori* infection.