ORIGINAL ARTICLE

Mucosal barrier defects in gastric intestinal metaplasia: in vivo evaluation by confocal endomicroscopy

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Background: *Helicobacter pylori* infection and intestinal metaplasia (IM) are associated with gastric cancer. An impaired gastric mucosal barrier could be involved in this carcinogenesis.

Objective: To evaluate laser confocal laser endomicroscopy (CLE) for in vivo functional imaging of mucosal barrier defects in patients with IM.

Design: Prospective, controlled study.

Setting: A tertiary-care academic center.

Patients: This study involved patients with IM of the gastric mucosa who underwent CLE for surveillance.

Interventions: Specific IM mucosa and non-IM mucosa in patients were identified by CLE, and targeted biopsy samples were taken for histopathology and electron microscopy.

Main Outcome Measurements: Post-CLE assessment of paracellular fluorescein leakage was devised and validated by electron microscopy. We also evaluated the effect of *H pylori* eradication on the mucosal barrier.

Results: Forty-two patients were included. Of non-IM samples, the paracellular permeability was significantly increased in *H pylori*–positive samples compared with *H pylori*–negative controls (54 ± 31% vs 3 ± 6%, $P < .05$). Of IM samples, the permeability was significantly increased in both *H pylori*–negative and *H pylori*–positive samples (67 ± 34% and 72 ± 28% vs 3 ± 6%, both $P < .05$). The results of post-CLE assessment correlated well with the electron microscopy findings (R^2 0.834, $P < .0001$). After the eradication of *H pylori*, the paracellular barrier dysfunction of non-IM mucosa was significantly improved as shown by electron microscopy and CLE (both $P < .001$). However, there was no significant change in IM mucosa.

Limitations: Single-center study.

Conclusions: CLE allows functional imaging of mucosal barrier defects. Gastric IM is associated with an impaired paracellular barrier irrespective of *H pylori* eradication. (Gastrointest Endosc 2012;xx:xxx.)

Helicobacter pylori infection plays a crucial role in the multistep carcinogenic process of gastric cancer.[1] According to Correa's model of gastric carcinogenesis, the gastric mucosa evolves through the stages of chronic gastritis, glandular atrophy, intestinal metaplasia (IM), and dysplasia before developing gastric cancer.[2] On the basis of these observations, it is believed that eradication of *H pylori* may be an appropriate target for the prevention of gastric cancer. However, although *H pylori* eradication causes regression of inflammatory changes in the gastric mucosa, it remains unclear whether this prevents gastric cancer.[3-5] In particular, the effect of *H pylori* eradication in patients

Abbreviations: CLE, confocal laser endomicroscopy; IM, intestinal metaplasia.

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with IM is highly uncertain, which hinders clinical decision making in practice.[6-11] The discrepancies are partly due to the patchy distribution of IM, making IM difficult to assess by using limited random biopsy specimens and leading to imprecise results as a consequence of sampling errors.

The gastric mucosa is continuously exposed to potentially noxious factors in the lumen. Interconnected by junctional complexes, the surface epithelial cells form the main structural barrier preventing the access of acid, *H pylori*, antigens, and toxins. The junctional complexes also play critical roles in cell polarity, proliferation, and differentiation.[12,13] Dysfunction of the epithelial barrier is characteristic of many human diseases of the GI tract, including carcinogenesis.[14-17] Recent studies have shown that barrier function is compromised in *H pylori*–induced gastritis,[18-20] and precancerous gastric lesions in rats exhibit disappearance of apical tight junctions. However, although these lesions are frequently precancerous, the epithelial barrier function has rarely been investigated in IM patients because of the patchy nature.

The recent development of confocal laser endomicroscopy (CLE) provides an opportunity to allow in vivo microscopic examination during endoscopy. Endomicroscopy has been demonstrated to validate the results of tissue pathology in a multitude of diseases, and it also offers a unique possibility to study pathophysiologic changes in their natural environment.[21] Fluorescein sodium is the most widely used contrast agent during endomicroscopy. Furthermore, it is also a small molecule (molecular weight = 376) and is widely used as hydrophilic marker molecule for paracellular permeability studies.[22-25] Our previous study showed that CLE can identify gastric IM with high accuracy, and fluorescein leakage was an important marker in mucosal functional changes.[26,27]

The aims of the present study were to (1) evaluate CLE for in vivo functional imaging of mucosal barrier defects in IM patients and (2) compare the epithelial barrier integrity in *H pylori*–negative and *H pylori*–positive IM mucosa with that in *H pylori*–negative and *H pylori*–positive non-IM mucosa.

Take-home Message

- Besides morphologic visualization, confocal endomicroscopy offers the possibility to study pathophysiologic events, providing dynamic microscopic imaging of perfusion and cellular function (shedding, apoptosis). In vivo functional imaging of mucosal barrier defects is possible in patients with gastric intestinal metaplasia (IM).
- Gastric IM is associated with an impaired paracellular barrier irrespective of *Helicobacter pylori* eradication. These findings may offer new insights into gastric carcinogenesis and IM surveillance.

METHODS

Patients

Consecutive outpatients with previous histologically confirmed IM of the gastric mucosa were recruited for endoscopic surveillance from January 2009 through November 2009. After being informed about the purpose, patients who were willing to choose CLE instead of conventional endoscopy were included in the study. The study protocol was approved by the ethics committee of Qilu Hospital, Shandong University, and informed consent was obtained from all participants.

Exclusion criteria were as follows: advanced adenocarcinoma of the GI tract; IM with known intraepithelial neoplasia; pregnancy or breastfeeding; allergic diseases; inability to give informed consent; and use of nonsteroidal anti-inflammatory drugs, proton pump inhibitors, or other injurious drugs (including immunosuppressants, antibiotics, and steroids) in the prior 4 weeks. A questionnaire concerning demographic data, the Hospital Anxiety and Depression Scale, and smoking and drinking habits was collected.

CLE procedure

Confocal laser endoscopy (Pentax EC3870CIK, Tokyo, Japan) was performed with the patients under conscious sedation by 3 experienced endoscopists (T.Y., X.M.G., and XLZ). Endomicroscopy was performed as described previously by using intravenous fluorescein as a contrast agent.[26] Ten standardized intragastric sites were observed separately on CLE: 4 sites from the antrum (2 from the lesser curvature, 2 from the greater curvature), 2 from the angulus, and 4 from the corpus (2 from the lesser curvature, 2 from the greater curvature). All of the sites showed endoscopic normal-looking or inconspicuous changes. If other suggestive lesions were seen, they were additionally scanned, and biopsy specimens were taken if necessary, but these additional specimens were not included in the analysis. Optical biopsies were performed by imaging from the mucosal epithelium to the lamina propria. Specific nonmetaplastic and IM mucosa were identified according to the confocal criteria.[26] Five to 10 CLE images of different mucosal depths were collected from each site, and the images were stored in a computer database for later assessment. During endoscopy, 5 biopsy specimens were taken. An antral biopsy specimen was taken for a rapid urease test, and 2 target biopsy specimens each were obtained from the metaplastic areas and nonmetaplastic areas (1 for histologic analysis and the other for electron microscopy). The IM samples were obtained from the sites that displayed the typical signs of diagnostic criteria and large extent so as to reduce sampling error. The targeted biopsy of the examined site was performed 5 mm immediately to the left of the "polyp" created by suction.

H pylori eradication and follow-up

H pylori infection was diagnosed by histology and rapid urease test. A positive result in either test was diagnosed as *H pylori* infection. Gastric biopsy specimens were divided into 4 groups as follows: *H pylori*–negative gastric mucosa, *H pylori*–negative IM mucosa, *H pylori*–positive gastric mucosa, and *H pylori*–positive IM mucosa.

Patients with *H pylori* infection received a 2-week triple therapy consisting of omeprazole (20 mg), clarithromycin (500 mg), and amoxicillin (1 g), all taken twice daily. Follow-up CLE was arranged 6 months after the eradication. For comparison, target biopsy specimens were taken from the same sites, guided by both CLE imaging and the recorded data. The ^{13}C urea breath test was performed 6 weeks after treatment to monitor *H pylori* status.

Histopathology

Mucosal biopsy specimens were placed in 10% formalin, embedded in paraffin, sectioned at 4-mm thickness, and stained with hematoxylin-eosin and modified Giemsa techniques. An experienced pathologist (C.J.Z.) who was blinded to the results of the endoscopic findings reviewed all the biopsy specimens. IM was recognized morphologically by the presence of goblet cells and absorptive cells.

Electron microscopic evaluation of paracellular permeability

The lanthanum tracer method was used to evaluate epithelial paracellular permeability. Specimens were immediately fixed by a solution containing 2.5% glutaraldehyde and 4% lanthanum nitrate in 0.1 M cacodylate buffer, pH 7.4. After fixation, specimens were rinsed for 30 minutes in 0.1 M cacodylate buffer containing 4% nitrate lanthanum and then postfixed in 1% osmium tetroxide for 1 hour at 4°C. Subsequently, tissue samples were dehydrated through a graded alcohol series, embedded in Epon 812 resin. Ultrathin sections were prepared and examined by transmission electron microscopy (Hitachi H-600, Tokyo, Japan).

Normal gastric epithelium is intact and will not allow permeation of the lanthanum nitrate. However, during gastric damage, lanthanum can cross the tight junctions of gastric epithelium and appear in the paracellular space. In all samples, at least 50 junctions were examined. Results are expressed as percentage of leaky junctions with paracellular invasion of lanthanum nitrate.[28]

CLE evaluation of paracellular permeability

Fluorescein sodium predominantly stays in the vessels after intravenous injection, and the unbound fluorescein molecules (about 20%-30%) can leak from the capillaries into the tissue and label mucosal epithelial cells.[29] As a paracellular leakage marker in many permeability studies, the healthy epithelial cells allow less than 1% fluorescein permeation through the paracellular pathway, whereas in mucosa with an impaired epithelial barrier, more fluorescein within the lamina propria can pass the epithelial paracellular space, and it ultimately appears as a bright borderline in CLE images.

The distribution of fluorescein signals in gastric surface mucosa were further confirmed by immunohistochemistry as described previously.[30] Mouse monoclonal antibody to fluorescein (Abcam, Cambridge, UK) was used as the primary antibody at a 1:1000 dilution.

The CLE images within a specific area were stored as digital files separately. For each target biopsy site, 3 confocal images of sufficient quality from different z-stacks were selected by an experienced CLE investigator (Y.Q.L.), who was blinded to the histopathology results. Gastric epithelium with a reticular pattern of paracellular fluorescein leakage appeared with a bright borderline. The proportion of these leaky cells among surface epitheliums was graded semiquantitatively on a 0 to 4 scale: 0 = absent; 1 = 1% to 25%; 2 = 26% to 50%; 3 = 51% to 75%, and 4 = 76% to 100%. The semiquantitative results for each patient are expressed as the mean scores of the 3 confocal images. The evaluations were performed by another investigator (R.J.), who was also blinded to the histopathology results. The CLE images were enlarged electronically with ACDsee v7.0 (ACD Systems, Victoria, Canada) during leakage counting.

Statistical analysis

Statistical evaluation was performed by using SPSS v13.0 (SPSS Inc, Chicago, IL). The demographic data were compared by χ^2 test or Fisher's exact test where appropriate. The results of permeability are given as means ± SD. Comparisons between groups were made with the Kruskal-Wallis rank sum test. Correlations between CLE and electron microscopy findings were calculated by the Spearman correlation test. A P value of $<.05$ was considered statistically significant.

RESULTS

Patient characteristics

Forty-six patients with IM were screened for participation, of whom 4 were excluded after histopathology (3 patients because of detection of low-grade intraepithelial neoplasia during surveillance and 1 because of histopathologically reported focal IM in CLE nonmetaplastic mucosa). A total of 42 patients were enrolled in this study. The median follow-up time before CLE examination was 22.8 months (range 2.6-87.2). All of the metaplastic sites sampled for electron microscopy were diagnosed as IM by histopathology. *H pylori* infection was present in 20 patients (47.6%). In a per-biopsy analysis, *H pylori* infection was found in all the 20 non-IM biopsy specimens, 12 IM biopsy specimens, and 18 urease test biopsy specimens, respectively. There were no significant differences regarding demographic and clinical data between *H pylori*–positive and *H pylori*–negative patients (Table 1).

TABLE 1. Patients' clinical characteristics

	Helicobacter pylori–positive	*Helicobacter pylori*–negative
Patients (n)	20	22
Mean age, y (range)	48.6 (35-73)	52.2 (37-76)
Sex (M/F)	14/6	14/8
History of PPI use (n)	12	10
Smoking habits (n)	10	8
Drinking habits (n)	8	9
Anxiety (n)	2	2
Depression (n)	0	1

PPI, Proton pump inhibitors.

Baseline lanthanum permeability

In normal gastric epithelium, lanthanum accumulation was stopped at the apical site of the paracellular space (Fig. 1A), suggesting that normal tight junctions prevent apical diffusion of lanthanum, whereas in patients with impaired epithelial barriers, lanthanum penetrated into the lateral intercellular space of the epithelium (Fig. 1B). For non-IM samples, quantitative analysis showed that the percentage of leaky junctions was significantly increased in *H pylori*–positive samples compared with *H pylori*–negative controls (54 ± 31% vs 3 ± 6%, $P < .05$). For IM samples, paracellular permeability was significantly increased in both *H pylori*–negative and *H pylori*–positive samples compared with controls (67 ± 34% vs 3 ± 6% and 72 ± 28% vs 3 ± 6%, both $P < .05$). However, there were no significant differences among the 3 higher groups (Fig. 2). Furthermore, in a separate analysis of the 19 patients who neither smoked nor used alcohol, similar results could still be observed. Permeability was significantly increased in *H pylori*–negative IM samples, *H pylori*–positive gastric samples, and *H pylori*–positive IM samples (62 ± 31%, 45 ± 34% and 72 ± 30%) compared with controls (2 ± 4%, all $P < .05$).

Baseline CLE permeability and immunohistochemistry

Confocal imaging permitted in vivo visualization of epithelial layers at a high resolution with clear visible borders between individual cells. In *H pylori*–negative patients, normal gastric epithelium showed polygonal cells with a cobblestone appearance without paracellular fluorescein leakage (Fig. 3A). However, during epithelial barrier damage, fluorescein sodium can cross the tight junctions of gastric epithelium, and the fluorescence signals showed a uniform and linear distribution localized at the apical portion of cell-to-cell contact of the epitheliums (Fig. 3B-D).

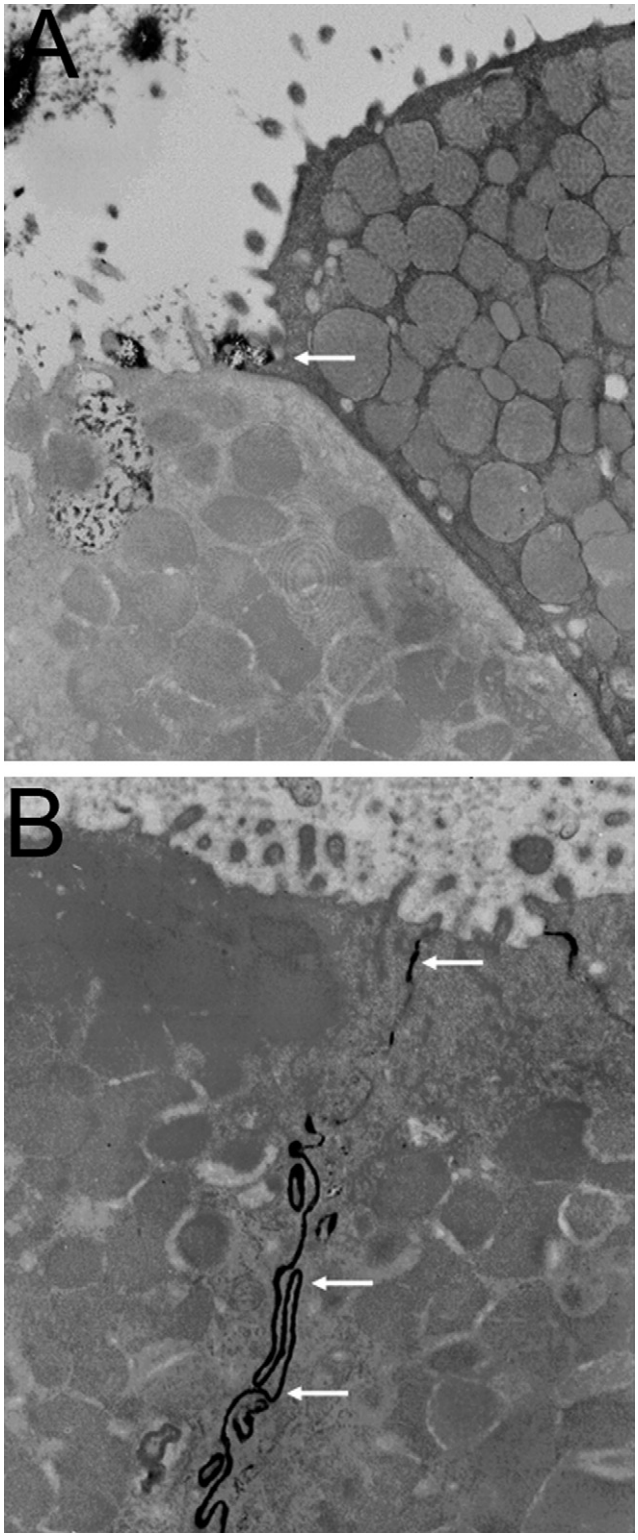

Figure 1. Electron microscopic view after perfusion with lanthanum nitrate. **A,** The invasion of lanthanum nitrate was stopped at the apical site of the paracellular space in normal gastric epithelium (*arrow*). **B,** *Arrows* indicate penetration of lanthanum within the junctional complex in patients with intestinal metaplasia.

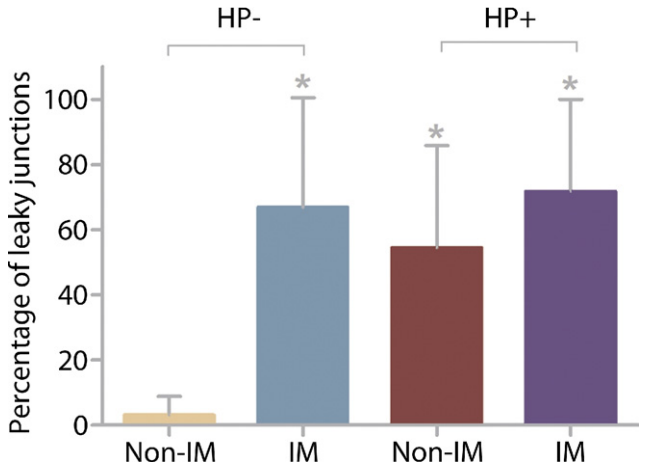

Figure 2. Percentage of leaky junctions of the gastric epithelium in *Helicobacter pylori*–negative gastric mucosa, *H pylori*–negative IM mucosa, *H pylori*–positive gastric mucosa, and *H pylori*–positive IM mucosa. *$P < .05$ vs *H pylori*–negative gastric mucosa. There were no significant differences among the 3 higher groups. HP, *Helicobacter pylori*.

The results of immunohistochemistry were consistent with the distribution of fluorescein under CLE. Fluorescein signals showed diffuse cytoplasmic staining with higher intensity at the surface layer, and goblet cells could not absorb fluorescein (Fig. 4).

In the post-CLE assessment, the mean confocal scores of *H pylori*–negative gastric epithelium (control) were 0.21 ± 0.24. This score was significantly increased in *H pylori*–positive gastric epithelium (2.60 ± 1.03, $P < .05$). For IM epithelium, the paracellular fluorescein leakage was significantly increased in both *H pylori*–negative and *H pylori*–positive epithelium compared with controls (2.82 ± 1.00 vs 0.21 ± 0.24, 2.83 ± 0.81 vs 0.21 ± 0.24, both $P < .05$). The results of semiquantitative analysis of CLE images correlated well and linearly with the electron microscopy findings ($R^2 = 0.834$, $P < .0001$, Fig. 5).

Effect of eradicating *H pylori*

Six months after the first endoscopy, 18 patients with eradication (90.0%) returned for follow-up endoscopy. *H pylori* infection was successfully eradicated in 14 patients (77.8%). The permeability results in paired samples of the 14 patients before and after treatment are shown in Table 2. After cure of infection, the permeability scores of non-IM samples significantly decreased as shown by both transmission electron microscopy and CLE ($P < .001$). However, no significant changes were observed in IM mucosa; the leaky cells remained present in IM mucosa (Fig. 6). For the 4 patients in whom eradication was unsuccessful, the barrier dysfunction of non-IM mucosa and IM mucosa had no significant improvement either.

DISCUSSION

The present study demonstrated that disruption of the gastric paracellular barrier was observed not only within *H pylori*–infected mucosa but also in IM mucosa. Furthermore, this dysfunction of IM mucosa may be a long-standing change irrespective of *H pylori* infection. It is not surprising to find that *H pylori* infection induces disruption of the gastric paracellular barrier, and cure of infection in our patients was associated with a significant fall in paracellular permeability. Specific *H pylori* constituents have been reported to be involved in the disruption of tight junctions, such as VacA cytotoxin, CagA, and urease activity.[18,31,32]

Our data showed that barrier function was altered in both *H pylori*–positive and *H pylori*–negative IM mucosa. Although IM is considered a precancerous lesion of the stomach, the causes underlying carcinogenesis in IM are still largely unknown. IM is an acquired replacement of gastric mucosa by intestinalized mucosa, but this replacement may be a faulty regeneration.[33] For example, IM in Barrett's esophagus exhibited a paracellular transepithelial leak to sucrose.[34] The frequency of gap junctions between the absorptive cells in the area of IM was low, and gap junctions cannot be observed in the lateral membranes of goblet cells.[35] The expression of tight-junction protein (such as claudin-7 and claudin-4) was also altered in IM mucosa.[36,37] There is increasing evidence that disruption of cell junctions has an effect on the processes of cell polarity, differentiation, and proliferation.[12] The gastric mucosal barrier dysfunction may lead to consistent transepithelial penetration of *H pylori*, bile, and antigenic and carcinogenic substances from the lumen, and thereby cause immune-inflammatory responses, dyspeptic symptoms, and neoplasia development. Previous ultrastructural findings have shown that *H pylori* bacteria can be detected in the intercellular spaces of metaplastic mucosa, even up to the underlying lamina propria.[38] This interaction between *H pylori* or its virulence factors and immune cells can trigger an immune-inflammatory response and the release of proinflammatory cytokines and free radicals, which in turn increase the paracellular permeability of the GI epithelium. This vicious cycle may contribute to DNA damage and mutation.[39] Taken together, these data suggest that the disruption of the paracellular barrier may be a potential mechanism contributing to gastric carcinogenesis in patients with IM.

In this study, no improvement in the paracellular barrier was observed in IM mucosa 6 months after *H pylori* eradication. This is consistent with many previous studies reporting the effect of treatment. In a large randomized controlled trial, Wong et al[9] suggested that *H pylori* eradication does not prevent gastric cancer once premalignant conditions have developed. A meta-analysis found that *H pylori* eradication was of long-term benefit only to patients with gastric atrophy not in those with IM.[40] It may be that in certain individuals, IM is a point of no return wherein many molecular changes can be detected and the progression will not be reversed.

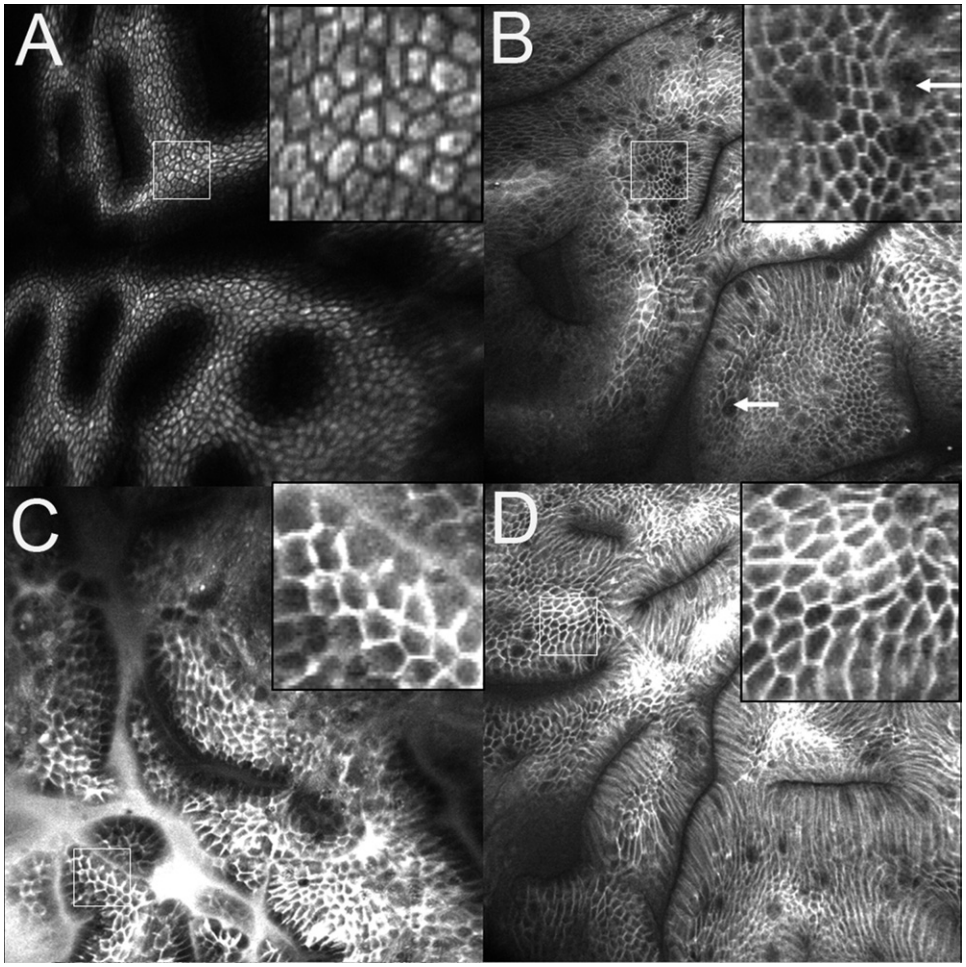

Figure 3. Confocal imaging of gastric epithelium. The boxed area of the images is shown magnified in the upper right corner. **A,** Normal gastric epithelium in *Helicobacter pylori*–negative patients. **B,** Intestinal metaplasia (IM) epithelium in *H pylori*–negative patients. The bright borderline of epithelial cells is clearly shown. *Arrows* show goblet cells. **C,** Gastric epithelium in *H pylori*–positive patients. **D,** IM epithelium in *H pylori*–positive patients.

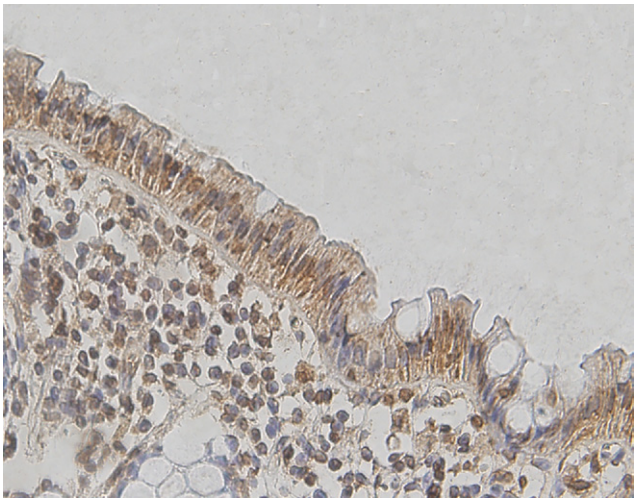

Figure 4. Fluorescein sodium distribution in intestinal metaplasia mucosa by immunohistochemistry. Fluorescein staining was present in the cytoplasm of the gastric epithelium, but mucin-containing goblet cells could not absorb fluorescein.

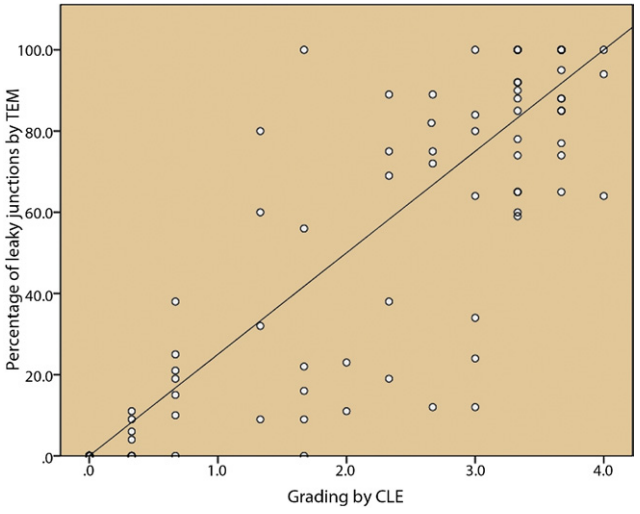

Figure 5. Correlation between confocal laser endomicroscopy (CLE) and transmission electron microscopy (TEM) findings. Permeability score of CLE image positively correlated with TEM findings.

TABLE 2. Permeability scores at baseline and 6 months after *Helicobacter pylori* eradication

	TEM			CLE		
	Baseline	Follow-up	*P*	Baseline	Follow-up	*P*
Non-IM (n = 14)	54 ± 31	9 ± 7	<.001	2.60 ± 1.03	0.31 ± 0.28	<.001
IM (n = 14)	72 ± 28	67 ± 32	.65	2.83 ± 0.81	2.92 ± 0.94	.78

TEM, Transmission electron microscopy; *CLE*, confocal endomicrocopy; *IM*, intestinal metaplasia.

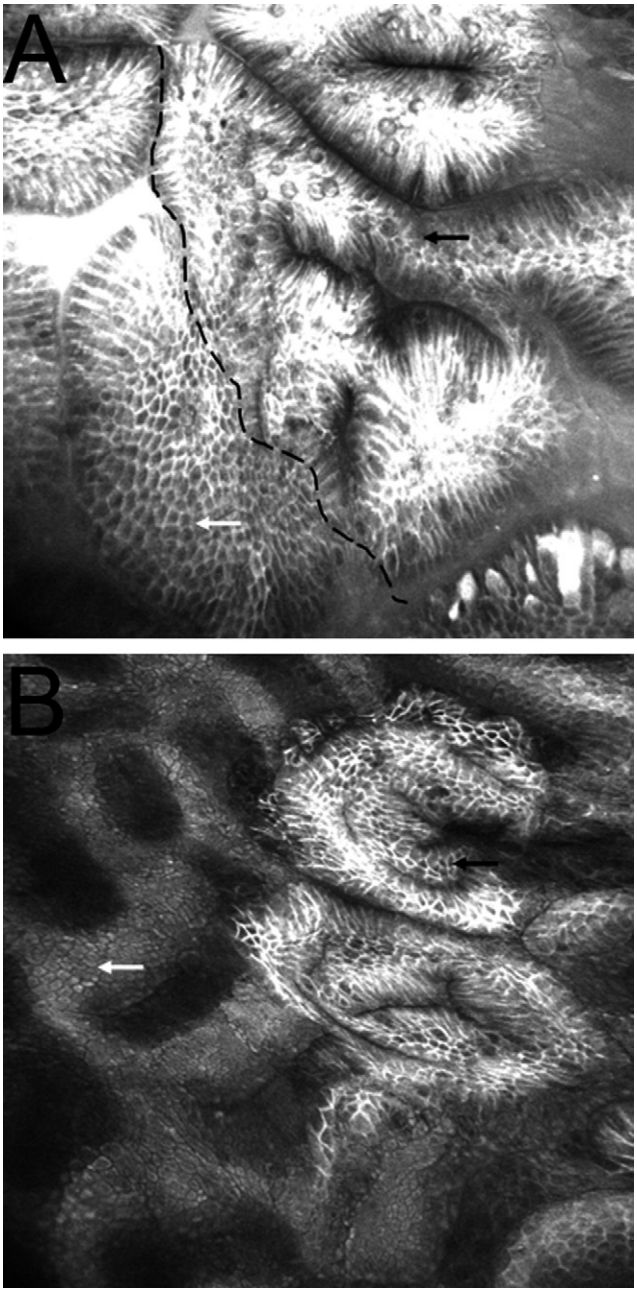

Figure 6. Representative images showing epithelial changes before (**A**) and after (**B**) the eradication of *H pylori*. *Black arrows* show IM mucosa, and *white arrows* show non-IM gastric mucosa.

The CLE findings correlated well with the electron microscopy results, and the paracellular leakage of fluorescein may be a functional feature of impaired paracellular barrier, which is a valuable complement to CLE histologic characterization. In vivo endomicroscopy is less prone to tissue artifacts than is routine histopathology.[41] CLE not only allows accurate resolution of tissue morphology but also enables functional imaging in the natural environment of tissue. Fluorescein sodium, the most widely used contrast agent, has been safely used for decades in ophthalmology in human beings. The observations of this study suggest that the use of fluorescein tracer is a noninvasive, simple, and endoscope-compatible method. Moreover, fluorescein tracer by CLE can also indicate the location of a lesion with abnormal permeability. The cancer risk of these lesions with paracellular fluorescein leakage should be further evaluated in IM patients, and this may provide new insights into IM surveillance.

Several potential limitations warrant consideration in this study. First, it did not include healthy control individuals; only IM patients were enrolled for surveillance. However, all target biopsy samples were taken far enough apart to avoid the potential influence of IM. This design benefitted the comparison of nonmetaplastic and metaplastic mucosa under the same baseline conditions. Second, IM mucosa showed an increase of gastric permeability to small molecules, like fluorescein sodium or lanthanum nitrate. The effect of macromolecules was not evaluated in this study. Finally, the number of patients included in each group was relatively small, the exact duration of IM was difficult to obtain, and the follow-up period was too short to allow confirmation of any long-term benefit of the treatment. Future studies are planned to address these issues.

In conclusion, we show that CLE allows functional imaging of mucosal barrier defects, and gastric IM is associated with an impaired paracellular barrier regarding small molecules. Moreover, this dysfunction of IM seems irrespective of *H pylori* eradication. CLE and fluorescein tracer may provide potentially important insights into our understanding of the pathophysiology of GI diseases. Future studies will further investigate both the biologic mechanisms and potential clinical applications.

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