Table 5 The ABC(D) stratification

Group	A	B	C	D
Anti-*H. pylori* antibody	Negative	Positive	Positive	Negative
Pepsinogen method	Negative	Negative	Positive	Positive
Risk of gastric cancer	Low	→	→	High
Odds rate	1.00	4.20	11.23	14.81
Interval of endoscopy	Every 5 yrs	Every 3 yrs	Every 2 yrs	Every 1 yrs

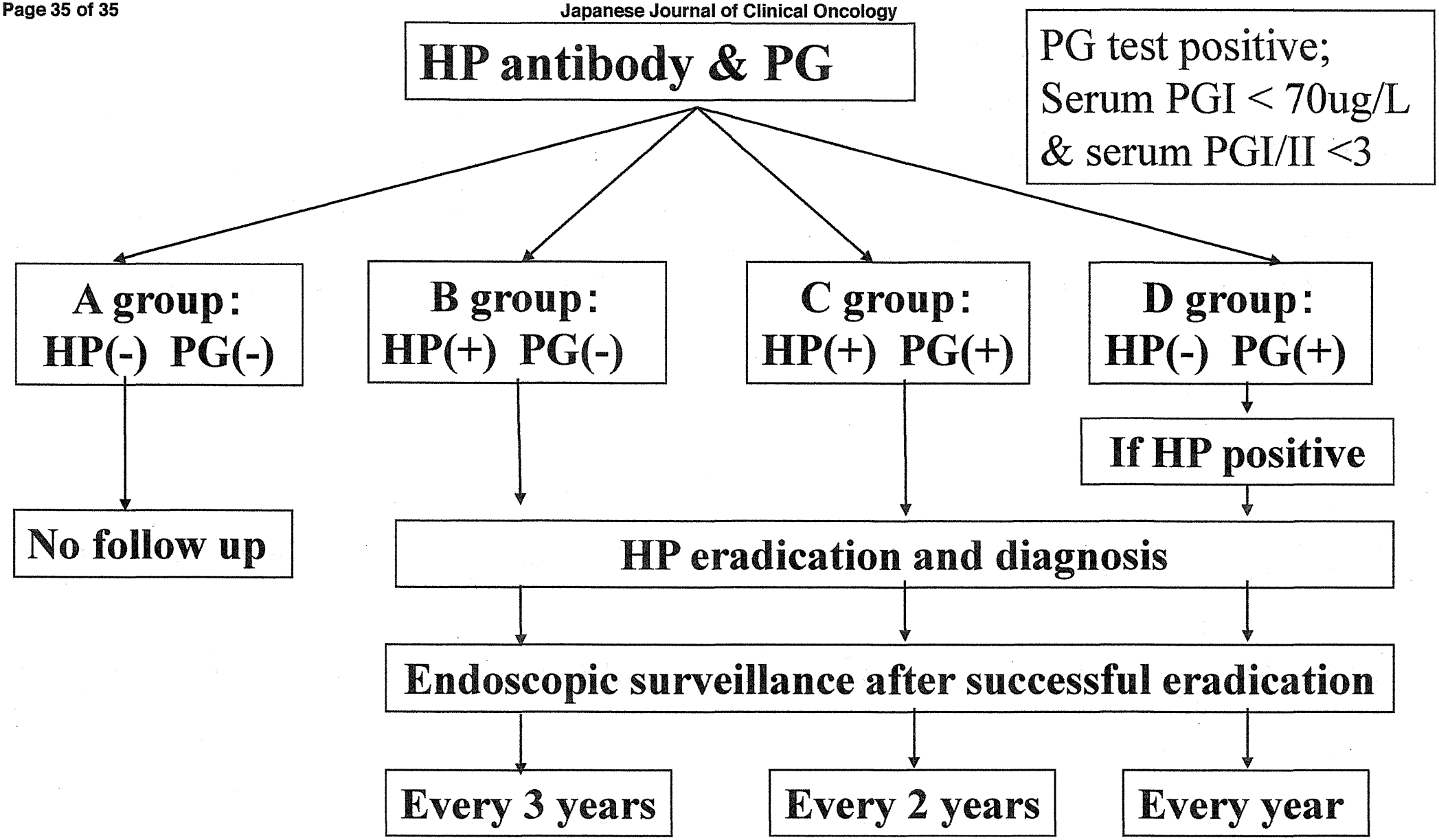

Figure 1 The strategy for elimination of gastric cancer

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Original Article

Changes in endoscopic findings of gastritis after cure of *H. pylori* infection: Multicenter prospective trial

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Background and Aim: Successful eradication of *H. pylori* changes pathological findings of gastritis dramatically. However, change of endoscopic mucosal findings is not fully understood. To clarify the short-term changes of endoscopic mucosal findings after cure of *H. pylori* infection, a multicenter prospective trial was conducted.

Methods: One hundred and forty-seven patients with *H. pylori* infection from 12 institutions were enrolled into this prospective cohort trial. Nineteen endoscopic findings using high-resolution white light electronic endoscopy were assessed before and 2–4 months after eradication treatment of *H. pylori*. *H. pylori* infection was diagnosed by pathology of three stomach sites using hematoxylin-eosin stain or *H. pylori*-specific immunostaining. Endoscopic features of the successful eradication group and the failed eradication group were compared. The change of severity of endoscopic features before and after *H. pylori* eradication were compared between successful eradication and failed eradication.

Results: One hundred and twenty-six patients were analyzed. Eradication rate was 81% (102/126). Non-transparency of gastric juice, diffuse redness of fundic mucosa, enlarged fold, spotty redness of fundic mucosa, flat erosion of stomach, and hemoglobin index of fundic mucosa were significantly different between the successful eradication group and the failed eradication group. Gastric flat erosion was of higher frequency in the successful eradication group. When eradication was successful, spotty redness of fundic gland improved significantly.

Conclusion: Assessment of endoscopic findings of spotty redness after eradication treatment is useful in the diagnosis of *H. pylori* eradication.

Key words: chronic gastritis, diffuse redness, endoscopic findings, erythema, *H. pylori* eradication

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INTRODUCTION

HELICOBACTER PYLORI INFECTS the human stomach for life and causes chronic inflammation of the gastric mucosa.[1] *H. pylori* infection induces infiltration of mononuclear cells and polynuclear cells into the gastric mucosa.[2] Atrophic change and intestinal metaplasia often occur during long-term persistent infection. *H. pylori* infection leads to a wide variety of upper gastrointestinal tract diseases, such as gastroduodenal ulcer, gastric adenocarcinoma, gastric mucosal-associated lymphoid tissue lymphoma, and gastric hyperplastic polyps.[3–7] Successful eradication of *H. pylori* improves histological gastritis and may prevent various diseases associated with *H. pylori* infection.[8]

It has long been believed that the features of conventional white light endoscopy correlate poorly with histopathological findings of *H. pylori*-induced gastritis.[9,10] Regular arrangement of collecting venules (RAC) was reported to be an endoscopic feature with high sensitivity and high specificity for the *H. pylori*-negative normal stomach.[11] Studies using magnifying endoscopy have shown that endoscopic features are associated with histopathological findings related to *H. pylori* infection.[12–14] Successful eradication of *H. pylori* dramatically changes the histopathological findings of gastritis. Recently, changes of magnifying endoscopic features with narrow band imaging (NBI) were investigated during *H. pylori* eradication.[15,16] However, change of conventional white light endoscopic features have not been clarified. A multicenter prospective trial was conducted to elucidate short-term changes of conventional white light endoscopic features after cure of *H. pylori* infection.

METHODS

Subjects

THIS MULTICENTER PROSPECTIVE trial comprised 12 institutions affiliated with the 'Study group for establishing endoscopic diagnosis of chronic gastritis' founded by the Japan Gastroenterological Endoscopy Society. This study group conducted other studies on the relationship between findings of white light endoscopy and histological findings. One hundred and forty-seven patients with *H. pylori* infection were initially enrolled from January 2009 to December 2009. Patients eligible for enrollment aged 20 years or older received eradication treatment of *H. pylori* infection. Exclusion criteria were histories of gastric surgery, gastrectomy, and eradication of *H. pylori*, treatment with non-steroidal anti-inflammatory drugs, antiplatelet agents, anticoagulants, steroids, antibiotics, and proton pump inhibitors within 4 weeks prior to entry, severe liver, renal, and cardiopulmonary dysfunctions, blood diseases including anemia, and a hemorrhagic tendency.

This study was approved by the Ethics Committee of each institution and carried out in conformity with the Declaration of Helsinki. All subjects gave written informed consent.

Procedures

Enrolled patients received high-resolution white light endoscopic examination to assess endoscopic findings before *H. pylori* eradication. *H. pylori* infection was diagnosed by rapid urease test upon initial endoscopic examination or by ^{13}C urea breath test prior to study. After the initial endoscopy, 10 mg rabeprazole, 200 mg clarithromycin, and 750 mg amoxicillin were given twice a day for 1 week, according to Japanese guidelines for management of *H. pylori* infection.[17] In patients with active gastric and duodenal ulcer disease, a proton pump inhibitor or a histamine receptor antagonist was given for 5 to 7 weeks after eradication therapy. A second endoscopic examination was carried out 2–4 months after eradication treatment and at least 4 weeks after completion of proton pump inhibitor treatment. Results of *H. pylori* eradication were diagnosed by pathological examination of three stomach sites during the second endoscopy. Diagnostic tools in which the result is known within a short time, such as the urea breath test, were excluded from this study in order to keep the endoscopist blinded to the eradication result. Biopsy samples were taken from one site each in the greater curvature of the antrum, the greater curvature of the upper body, and the lesser curvature of the angle. One specialized pathologist (H.W.) carried out blind assessment of *H. pylori* infection using hematoxylin-eosin (HE) staining or *H. pylori*-specific immunostaining. As immunostaining was added for distinguishing *H. pylori* from other microorganisms and also for detecting coccoid forms of *H. pylori,* the accuracy of histological diagnosis was expected to be the same as that of the urea breath test. Comparisons were made of the endoscopic features of successful and failed eradication groups and of the endoscopic features before and after successful eradication. End point was the diagnostic characteristics of endoscopic findings after successful eradication of *H. pylori*.

Endoscopic assessment

All endoscopists involved in the present study were accredited members of the Japan Gastroenterological Endoscopy Society. The high-resolution white light endoscope in this study was the GIF-240 series or the GIF-260 series (Olympus Medical Systems, Tokyo, Japan). Chromoendoscopy using 0.2% indigocarmine was carried out after the completion of conventional observation of the target region. Hemoglobin (Hb) index values of the fundic mucosa were carried out by institutions familiar with this method. Hb index

was measured using an image-processing system according to a previous report.[18] Two close-up pictures of the fundic mucosa without specific lesions, such as erosion and patchy redness, were obtained at the posterior wall of the upper gastric body. Characteristics of 10 endoscopic features were defined mainly based on endoscopic division of the Sydney System.[19] Another nine features, such as non-transparency of gastric juice, diffuse redness, RAC, adhesive mucus, xanthoma, fundic gland polyp, extent of atrophy, swelling of pyloric gland region with indigocarmine staining, and Hb index of fundic mucosa, were added to evaluate the endoscopic findings. The 19 features are described below.

1 Non-transparency of gastric juice: This is determined by visibility of gastric mucosa at the bottom of gastric juice. Severity increases as visibility decreases.
2 Diffuse redness of fundic mucosa: This refers to uniform redness involving the entire mucosa of the fundic gland. RAC is visible without diffuse redness.
3 Mucosal edema (fundic/pyloric mucosa): This is characterized by soft, thick, and swollen gastric mucosa.
4 Enlarged fold: This constitutes fold enlargement. Normal fold is straight, smooth, and approximately 5 mm in diameter.
5 Visibility of vascular pattern: Atrophy is diagnosed by the visibility of the vascular pattern and rugal atrophy.
6 RAC: Starfish-like red spots in a regular arrangement are visible through the mucosal surface in the fundic gland region. Visibility of RAC is affected by inflammation and atrophy[11]
7 Nodularity: Nodular protrusions measuring 2–3 mm are uniformly distributed in the antrum and angle.
Severities of the qualitative findings from categories 1 to 7 listed above were divided into four grades: none (–), intermediate (+/–), clear (+), and remarkable (2+) (Fig. 1).
8 Adhesive mucus: Grayish or yellowish mucus adheres to the mucosal surface prior to washing with water.
9 Spotty redness of fundic mucosa: Multiple tiny reddish spots are observed in the fundic gland region. This finding should be strictly differentiated from patchy redness in the point of location, size and number. Typical spotty redness is defined as tiny reddish lesions <1 mm in diameter that occur infinitely on the cardiac side of the fundic gland region.
10 Patchy redness (stomach/duodenum): It is defined as localized reddish macula of various sizes. It occurs once or frequently, but it is isolated.
11 Red streaking: It is defined as reddish longitudinal streaks in the antrum and corpus.
12 Flat erosion (stomach/duodenum): It is characterized by mucosal defects and whitish patches that vary in size.
13 Raised erosion: It is characterized as elevated mucosa with white excavation at the center.
14 Bleeding spot: It is defined as punctuated or ecchymotic reddish or brown- blackish flecks present in the gastric wall.
15 Xanthoma: It is characterized as yellow-white, well-demarcated, single or multiple nodules or plaques that vary in size.
16 Fundic gland polyp: It is characterized as tiny, numerous and sessile, usually scattered in the fundic gland region. They have the same color as the gastric mucosa.
Severities of the quantitative findings from categories 8 to 16 listed above were divided into four grades: 0 (–), 1 (+/–), 2–9 (+), and >10 (2+) (Fig. 2).
17 Extent of atrophy: The extent of atrophy was recorded according to the classification of Kimura and Takemoto.[20]
18 Swelling of areae gastricae in the pyloric gland region with indigocarmine staining: In the swollen areae gastricae, the inter-area groove is narrow. The classification was recorded according to Ida's paper.[21]
19 Hb index of fundic mucosa: Hb index is used as a parameter of the mucosal hemoglobin concentration and mucosal blood flow.[18] Calculated Hb index correlates value with the intensity of diffuse mucosal redness.

Statistical analysis

Statistical calculations were carried out with STATA ver. 11 software (StataCorp LP, College Station, TX, USA). The characteristics of eradicated subjects and failed subjects were compared by Wilcoxon signed-rank test, chi-squared test, or Student's *t*-test. Mann–Whitney rank-sum test or Student's *t*-test was used to assess the difference of endoscopic findings between the eradicated group and the failed group. Comparison of endoscopic findings before and after eradication in the two groups was analyzed using Wilcoxon signed-rank test. *P*-values <0.05 were considered to indicate statistical significance.

RESULTS

H. pylori eradication

ONE HUNDRED AND forty-seven patients with *H. pylori* infection were enrolled in the present study (Fig. 3). Seventeen patients were lost at the second endoscopic examination. Four patients were excluded for lack of histological specimens. Of the 126 patients in the final analysis, there were 69 with chronic gastritis, 20 with gastric ulcer scar, 12 with duodenal ulcer scar, 11 with active gastric ulcer, one with active duodenal ulcer, four after endoscopic resection of early gastric cancer, three with hyperplastic polyp, and six with miscellaneous diseases. The male-to-female ratio and mean age were 1.3 and 61.7 years, respec-

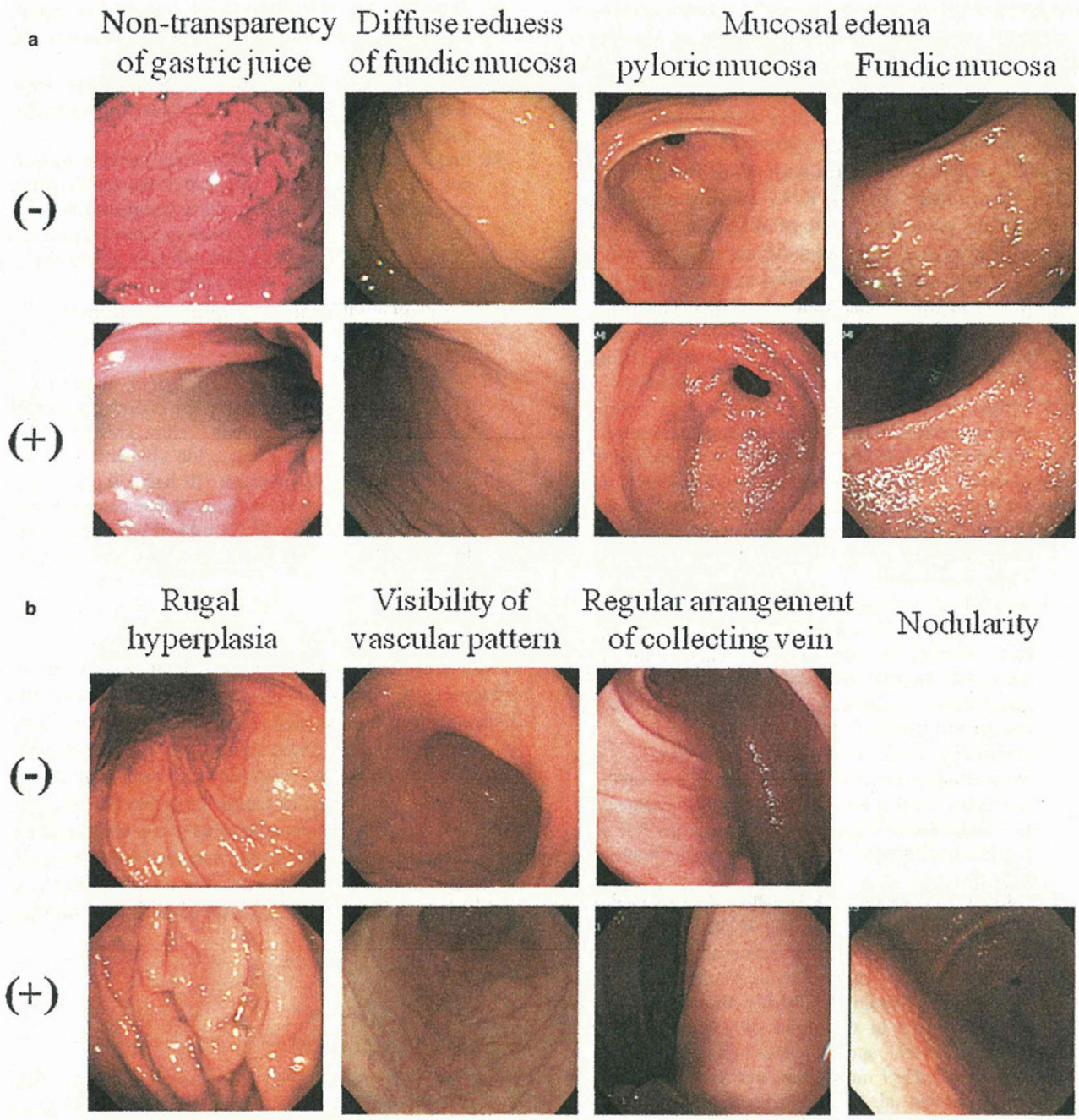

Figure 1 Grading of clear (+) and none (–) in the severity of seven qualitative findings.

tively. After eradication therapy of *H. pylori*, 102 patients were diagnosed with negative *H. pylori* infection using pathological examination and 24 patients were diagnosed with persistent *H. pylori* infection. Final eradication rate was 81% (102/126).

Comparison between successful and failed eradication group

Significant differences between the successful and failed eradication group were not seen in background characteris-

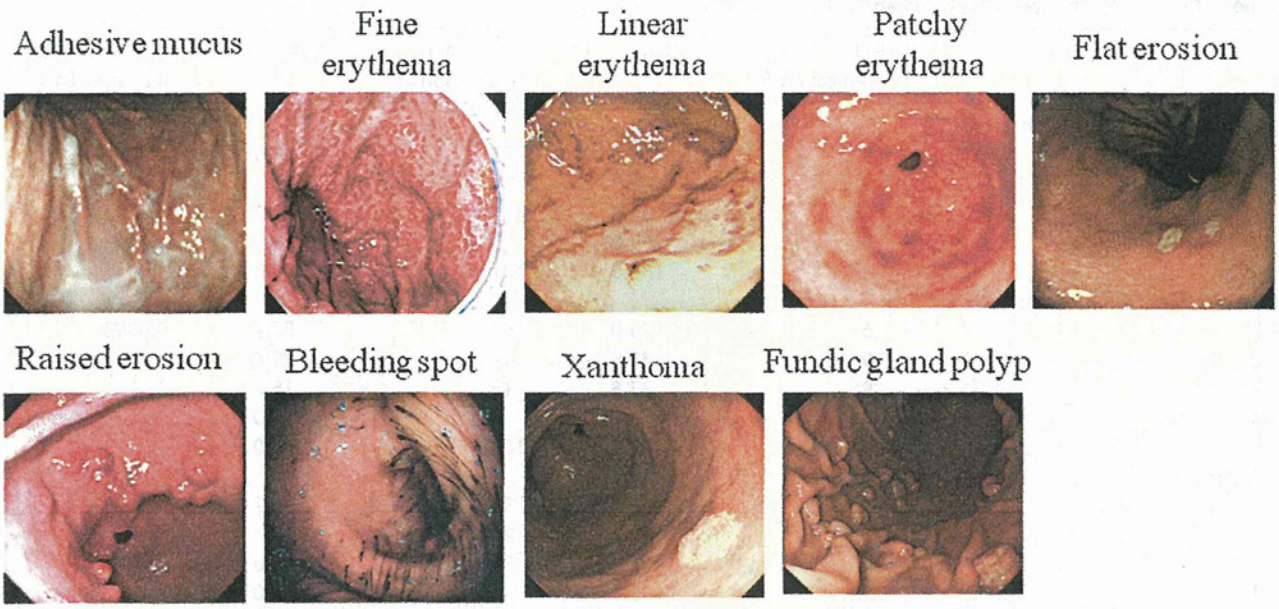

Figure 2 Nine quantitative findings.

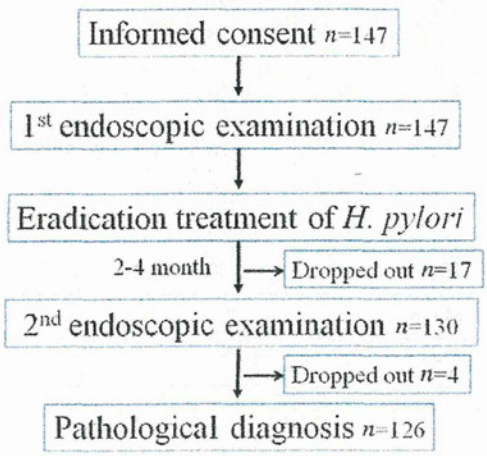

Figure 3 Protocol design.

tics such as age, sex, disease, and endoscopic findings, except diffuse redness of fundic mucosa. The successful eradication group had a lower grading in diffuse redness of the fundic mucosa than the failed group ($P = 0.0014$). Non-transparency of gastric juice, diffuse redness of fundic mucosa, enlarged fold, spotty redness of fundic mucosa, flat erosion of stomach, and Hb index of fundic mucosa after eradication were significantly different between the successful eradication group and the failed eradication group (Table 1). Other endoscopic findings had no significant differences (data not shown). Grading of endoscopic findings including diffuse redness, spotty redness, non-transparency of gastric juice, and enlarged fold were lower in the successful eradication group. Mean value of Hb index in the successful eradication group was lower than that in the failed eradication group. However, grading of gastric flat erosion was higher in frequency in the successful eradication group.

Comparison of change before and after *H. pylori* eradication between successful eradication and failed eradication

To evaluate specific endoscopic findings related to successful eradication and not to failed eradication, change of severity of endoscopic findings before and after *H. pylori* eradication were compared between successful eradication and failed eradication. Spotty redness of fundic mucosa was improved significantly in successful eradication cases compared with a small change in failed eradication cases (Table 2). Other significant endoscopic findings between the successful and failed eradication groups, in particular non-transparency of gastric juice, diffuse redness of fundic mucosa, enlarged fold, flat erosion of the stomach, and Hb index of fundic mucosa, did not show a significant difference because of an improvement in failed eradication cases (Table 3).

DISCUSSION

HELICOBACTER PYLORI INFECTION leads to various upper gastrointestinal tract diseases and influences gastric function, including gastric acid secretion. Successful

Table 1 Comparison between the failed and successful eradication groups

	Failed eradication (*n*)	(%)	Successful eradication (*n*)	(%)	*P* (rank-sum test if unspecified)
Non-transparency of gastric juice					
1	3	13.0	37	36.6	0.026
2	9	39.2	31	30.7	
3	8	34.8	30	29.7	
4	3	13.0	3	3.0	
Total	23	100.0	101	100.0	
Diffuse redness of fundic mucosa					
1	6	25.0	54	52.9	0.0093
2	9	37.5	30	29.4	
3	9	37.5	16	15.7	
4	0	0.0	2	2.0	
Total	24	100.0	102	100.0	
Enlarged fold					
1	4	16.7	31	30.7	0.038
2	5	20.8	29	28.7	
3	13	54.2	39	38.6	
4	2	8.3	2	2.0	
Total	24	100.0	101	100.0	
Spotty redness of fundic mucosa					
1	10	41.7	59	57.8	0.020
2	1	4.2	22	21.6	
3	11	45.8	19	18.6	
4	2	8.3	2	2.0	
Total	24	100.0	102	100.0	
Flat erosion of stomach					
1	22	91.7	72	70.5	0.035
2	0	0.0	2	2.0	
3	2	8.3	27	26.5	
4	0	0.0	1	1.0	
Total	24	100.0	102	100.0	
	Mean	SD	Mean	SD	
Hb index of fundic mucosa	62.4	4.6	57.8	5.7	0.030

Hb, hemoglobin.

Table 2 Change of spotty redness of fundic mucosa before and after *H. pylori* eradication between failed and successful eradication

	Failed eradication		Successful eradication		*P* (chi-squared test)
	(*n*)	(%)	(*n*)	(%)	
Spotty redness of fundic mucosa					
Non-improvement	19	79.2	56	54.9	0.029
Improvement	5	20.8	46	45.1	
Total	24	100.0	102	100.0	

eradication of *H. pylori* improves histological gastritis and may prevent various diseases associated with *H. pylori* infection, such as gastric/duodenal ulcer and gastric cancer.[22,23] Moreover, *H. pylori* eradication therapy is necessary to prevent the spread of this infection. The detection of *H. pylori* infection after eradication treatment is carried out using invasive and non-invasive tests such as pathological examination, culture, ^{13}C-urea breath test, and stool antigen test. The aim of

Table 3 Comparison of change before and after *H. pylori* eradication between failed and successful eradication

	Failed eradication		Successful eradication		*P* (chi-squared test or *Fisher's exact test)
	(*n*)	(%)	(*n*)	(%)	
Non-transparency of gastric juice					
Non-improvement	12	54.5	48	49.5	0.67
Improvement	10	45.5	49	50.5	
Total	22	100	97	100	
Diffuse redness of fundic mucosa					
Non-improvement	8	33.3	37	36.3	0.79
Improvement	16	66.7	65	63.7	
Total	24	100	102	100	
Mucosa edema of fundic mucosa					
Non-improvement	9	45.0	40	45.5	0.97
Improvement	11	55.0	48	54.5	
Total	20	100	88	100	
Mucosa edema of pyloric mucosa					
Non-improvement	8	40.0	36	42.4	0.85
Improvement	12	60.0	49	57.3	
Total	20	100	85	100	
Enlarged fold					
Non-improvement	16	66.67	48	48.98	0.12
Improvement	8	33.33	50	51.02	
Total	24	100	98	100	
Visibility of vascular pattern					
Non-improvement	19	79.2	81	79.4	0.98
Improvement	5	20.8	21	20.6	
Total	24	100	102	100	
Regular arrangement of collecting venules					
Non-improvement	23	95.8	90	89.1	0.46*
Improvement	1	4.2	11	10.9	
Total	24	100	101	100	
Nodularity					
Non-improvement	21	87.5	99	97.1	0.083*
Improvement	3	12.5	3	2.9	
Total	24	100	102	100	
Adhesive mucus					
Non-improvement	14	58.3	43	42.2	0.15
Improvement	10	41.7	59	57.8	
Total	24	100	102	100	
Patchy redness of stomach					
Non-improvement	20	83.3	79	77.5	0.53
Improvement	4	16.7	23	22.5	
Total	24	100	102	100	
Patchy redness of duodenum					
Non-improvement	23	95.8	94	94.0	1.00*
Improvement	1	4.2	6	6.0	
Total	24	100	100	100	
Red streaking					
Non-improvement	22	91.7	100	98.0	0.16*
Improvement	2	8.3	2	2.0	
Total	24	100	102	100	

Table 3 Comparison of change before and after *H. pylori* eradication between failed and successful eradication (*contintued*)

	Failed eradication		Successful eradication		*P* (chi-squared test or *Fisher's exact test)
	(*n*)	(%)	(*n*)	(%)	
Flat erosion of stomach					
Non-aggravation	22	91.67	95	93.14	0.68*
Aggravation	2	8.33	7	6.86	
Total	24	100	102	100	
Raised erosion					
Non-improvement	23	95.8	99	97.1	0.58*
Improvement	1	4.2	3	2.9	
Total	24	100	102	100	
Bleeding spot					
Non-improvement	22	91.7	95	93.1	0.35*
Improvement	2	8.3	7	6.9	
Total	24	100	102	100	
Xanthoma					
Non-improvement	24	100.0	95	93.1	0.35*
Improvement	0	0.0	7	6.9	
Total	24	100	102	100	
Fundic gland polyp					
Non-improvement	23	95.8	102	100.0	0.19*
Improvement	1	4.2	0	0	
Total	24	100	102	100	
Extent of atrophy					
Non-improvement	22	91.7	84	82.4	0.36*
Improvement	2	8.3	18	17.6	
Total	24	100	102	100	
Swelling of areae gastricae					
Non-improvement	15	83.3	63	78.7	1.00*
Improvement	3	16.7	17	21.3	
Total	18	100	80	100	
Hb index of fundic mucosa					
Non-improvement	2	25	12	36.36	0.692
Improvement	6	75	21	63.64	
Total	8	100	33	100	

Hb, hemoglobin.

the present study was to evaluate endoscopic diagnosis for successful eradication of *H. pylori* infection. Endoscopy can improve the accuracy of diagnosis of *H. pylori* infection during examination without the need for biopsy. In this study, various kinds of white light endoscopic features were assessed before and after *H. pylori* eradication. Almost of these features were described in an endoscopic division of the Sydney System.[19] Other findings such as non-transparency of gastric juice, diffuse redness, RAC, adhesive mucus, xanthoma, fundic gland polyp, extent of atrophy, swelling of pyloric gland region with indigocarmine staining, and Hb index of fundic mucosa were reported to be associated with *H. pylori* infection.[11–14]

From our results, a decrease in spotty redness after eradication treatment was a significantly useful endoscopic finding for the diagnosis of successful eradication. Comparison between the successful and failed eradication groups showed a significant difference in six endoscopic findings. However, five endoscopic findings such as diffuse redness of fundic mucosa, non-transparency of gastric juice, enlarged fold, flat erosion of stomach, and Hb index of fundic mucosa were not specific changes in cases of successful eradication. These endoscopic findings could possibly be associated with temporary inhibition of gastric inflammation by suppression of *H. pylori*. Spotty redness of the fundic gland region is strictly influenced by curing *H. pylori* infection. As the

disappearance of polymorphonucleocytes is a histologically significant change shortly after *H. pylori* eradication, spotty redness of the fundic gland region is suggested to be related to histological activity.

Flat erosion of stomach and duodenum is related to recovery of acid output after successful eradication.[24] Basal gastric acidity increases after successful *H. pylori* eradication, but does not change for subjects with persistent infection.[25] Specifically, Japanese patients have a high likelihood of acid recovery after successful eradication because approximately 80% of *H. pylori*-infected patients end up with corpus-predominant gastritis. Although an increase in duodenal erosion is due to acid recovery, it occurs less frequently and only for a short duration after successful eradication.

The change in conventional endoscopic features with white light imaging has not been clarified. However, the change in magnifying endoscopic features with narrow band imaging during *H. pylori* eradication has been reported. Yagi *et al.* evaluated magnifying endoscopic change focusing on mucosal and microvascular patterns 1 year after successful eradication.[15] Changes in magnified findings after successful eradication included disappearance of erythema and swelling of areas between gastric pits, pinhole-like changing of white pits, and recovery of RAC. Okubo *et al.* also reported changes in gastric mucosal patterns observable by magnifying NBI.[16] The patterns of enlarged or elongated pits improved to small oval or pinhole-like round pits, and the density of fine irregular vessels decreased. However, a 5-year follow-up study using conventional endoscopy by Oda *et al.* reported that although histological atrophy improved, endoscopic examination revealed no consistent alteration in atrophic border.[26] Antral erosion became more conspicuous 5 years after successful eradication. Spotty redness in the corpus disappeared after 5 years.

The present study has limitations. Assessment of endoscopic findings depended on the endoscopist; however, a meeting was held to agree upon standards for endoscopic assessment. Because endoscopic change varies with the interval after successful eradication, short-term change is never relevant.

In conclusion, assessment of spotty redness after eradication treatment is useful in the diagnosis of *H. pylori* eradication.

CONFLICT OF INTERESTS

AUTHORS DECLARE NO conflict of interests for this article.

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Scientific Research

Coccoid *Helicobacter pylori* Can Directly Adhere and Invade in Agminated Formation to Human Gastric Epithelial Cells

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ABSTRACT

Helicobacter pylori (*H. pylori*) can infect into the epithelial cell to cause benign or malignant disorders. Under stressful environment, a spiral form of *H. pylori* is transformed into a coccoid form. The infectivity of the coccoid form is still controversial. Since spiral forms are transformed into two types of coccoid forms *via* different mechanisms, the infectivity of the two types of coccoid forms into human gastric epithelial cell was examined. A laboratory and a clinical strain of *H. pylori* were cultured in liquid medium under different conditions to produce the two types of coccoid forms. These coccoid *H. pyloris* were then co-cultured with human derived gastric epithelial cell, MKN-28. Adhesion and penetration of bacteria into MKN-28 cells were monitored by scanning-, standard transmission- and immunotransmission-electron microscopy (SEM, TEM and ITEM). We observed that both coccoid forms were able to adhere onto the surface of MKN-28 cells in agminated formation and also penetrated into the gastric epithelial cells besides the spiral form of *H. pylori*. Coccoid *H. pylori* is not a passive entity but can actively infect the human gastric epithelial cell.

Keywords: *H.pylori*; Electron Microscopy; Coccoid Form; Spiral Form; Bacterial Infection; *Cag A*

1. Introduction

Helicobacter pylori (*H. pylori*) is a microaerophilic bacterium that produces many benign or malignant disorders [1-3]. Eradication of *H. pylori* in patients after endoscopic resection during the early stage of gastric cancer has been shown to greatly reduce the development of metachronous gastric carcinoma [4]. Under stressful conditions, *H. pylori* changes from a spiral form to a coccoid one [5].

We reported that two types of coccoid forms could be produced in culture using different glucose-concentrations via different mechanisms [6-8]. One form (Type A) has irregular surface with few flagella and an indistinct cytoplasmic membrane cultured in Brucella-specific broth (Difco; USA) culture medium with 10% heat-activated horse serum (designated as CLM), and the other form (Type B) has smooth surface with tightly encircled flagella and comparatively clear membrane cultured in 300 mM glucose added CLM (designated as 300 mM-LM).

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Though the viability and characteristics of the coccoid forms have been a subject of controversy [9,10], there are many reports that non-spiral *H. pylori* is viable and there is a morphological manifestation for cell adaptation to severely non-optimal environment [11].

H. pylori can be seen adhering onto the surface of not only the biopsied gastric epithelial cell [12,13] but also cultured cells [14,15]. However, there had been several studies on adhesion and invasion of coccoid forms into human epithelial cells. One stated that coccoid forms could adhere and invade into human gastric epithelial cells [16]. Others suggested that coccoid forms could adhere only poorly onto such human cultured cells [17] or that the non-spiral bacteria produced as a result of antibiotic treatment would be passively adhered to such human cultured cells to be eventually destroyed [18].

Therefore, in this study, for the direct observation of the adhesion and invasion of the coccoid *H. pylori* into the epithelial cell, the infectivity of two different types of coccoid forms to human cultured epithelial cells was examined by SEM, TEM and ITEM comparing with that

of the spiral form as a control. A standard bacterial stock and a clinical strain of *H. pylori* were co-cultured with cells from a human gastric epithelial cell line and then observed by electron microscopy. Some biopsied specimens containing the bacteria and the gastric epithelial cells extracted from the patients with abdominal discomforts were also used as control.

2. Materials and Methods

2.1. Bacterial Culture

Both the ATCC 43504 strain and a clinical strain established from an *H. pylori*-positive patient were cultured in CLM for 24 hours and then were co-cultured under 8% CO_2 for 1 hour with MKN-28 cells raised in RPMI-1640 medium using a 12 wells plate (Transwell-COL, Corning Costar Co., USA) for 2 days. On the 1st day, approximately 95% of the bacteria showed spiral forms [6-8].

Both the ATCC 43504 strain and a clinical strain established from an *H. pylori*-positive patient were also cultured in CLM and in 300 mM-LM for 4 days. Organisms on the 4th day culture were used for co-culture with MKN-28 cell to examine the adhesion between bacteria and gastric epithelial cell. On the 4th day, approximately 99% of the bacteria in CLM was Type A coccoid form and more than 90% of those in 300 mM-LM was Type B one [7,8].

2.2. Morphological Preparation

After discarding the suspended *H. pylori* in the well, the bacteria that adhered to the MKN-23 cell were fixed in a solution containing 0.1% glutaraldehyde plus 2% paraformaldehyde. The membrane at the bottom of the well, onto which the cells were attached, was cut into three pieces, each for SEM, TEM and ITEM, respectively, as previously reported. Briefly, for SEM [6] or TEM [7,8], the specimens were fixed in 2% glutaraldehyde, for observing with an electron microscope. For ITEM [19,20], the fixed specimens were immediately treated with Lowicryl K4M (Polysciences; Tokyo, Japan) followed by ultra-sectioning. Ultrathin sections mounted on nickel grids were reacted with a rabbit IgG fraction specific for *H. pylori* (DAKO Japan) followed by reacting with gold particle-labeled goat antiserum specific for rabbit IgG. Biopsy materials from the gastric antrum of 24 patients examined by endoscopy for dyspepsia symptoms were also processed by the standard procedure for SEM, TEM and ITEM. No malignant lesion was diagnosed in all biopsy materials by standard light micros-copy.

3. Results

By ITEM, the gold particles indicating positive reaction for the presence of *H. pylori* antigen were detected on the flagella, on the bacterial surface and in the cytoplasm of the bacteria. Our electron microscopic study showed that *H. pylori* bacteria first aligned in agminated pattern to adhere onto the surface of epithelial cell to be incorporated into the cytoplasm.

In the SEM study using spiral bacteria, the gastric epithelial cell was adhered onto the surface by the bacteria in agminated pattern (**Figure 1(a)**). By TEM and

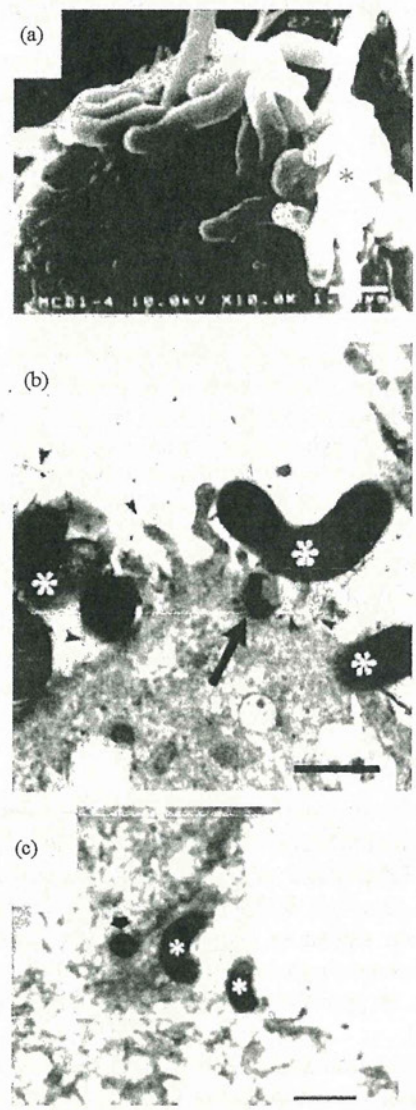

Figure 1. Spiral *H. pylori* as controls. (a) The bacterium (asterisk) agminates onto a certain spot of the surface of the epithelial cell (SEM: A clinical strain from the 1st day of culture. Bar indicates 1 μm); (b) The bacterial body (asterisks) directly attaches or is taken (black and white arrows) into the epithelial cell. Flagella (arrowheads) also adhere on the epithelial cell (TEM: ATCC43504 strain from the 1st day of culture, Bar indicates 0.5 μm); (c) The bacteria (asterisks) directly agminate to contact on the surface of the epithelial cell. A bacterial body (arrow) is observed in the cytoplasm (ITEM: A biopsy specimen, Bar indicates 1 μm).

ITEM, spiral bacteria were observed to be directly in close contact with the surface or microvilli of the epithelial cells (**Figures 1(b)** and **(c)**, asterisks) with flagella touching the microvilli (**Figure 1(b)**, arrowheads) and also their body being already in the cytoplasm of the epithelial cell (**Figures 1(b)** and **(c)**, arrow). We could not observe any agminated pattern of *H. pylori* on any of the biopsied specimens by SEM. Type A (**Figures 2(a)-(c)**) and Type B (**Figures 2(d)** and **(e)**) coccoid bacteria from the standard bacterial stock and a clinical strain of *H. pylori* adhered in similar manner to that of the spiral form. In Type A coccoid forms, the spherical bacteria were seen adhering in agminated pattern on the surface of the epithelial cells (**Figure 2(a)**). Remnants of the flagella and the bacterial body were seen to be directly in contact with the surface of the epithelial cells (**Figure 2(b)**, arrowheads). A vacuole as indicated by gold particles was considered to be the existence of the invaded bacteria in the cytoplasm (**Figure 2(c)**, arrow). Type B coccoid forms also adhered in agminated pattern on the surface of the epithelial cells (**Figure 2(d)**). Flagella (**Figure 2(e)**, arrowheads) and the bacterial body (**Figures 2(b)** and **(c)**, asterisks) were observed to be directly in contact with the surface of the epithelial cells. Amorphous structure with gold particles showing the presence of *H. pylori* antigen in the cytoplasm of the infected epithelial cells (**Figures 2(c)** and **(e)**, arrow) demonstrated that the adhered bacterium had been taken into the epithelial cell.

4. Discussion

In this study, we observed that not only the spiral *H. pylori* but also the coccoid ones assembled at a certain area on the surface of the epithelial cell before being taken into the cytoplasm. In general, a bacterium is thought to adhere to and invade into the epithelial cell only after the colony formation by its own cell-fission [21]. However, our study showed that many coccoid bacteria adhered simultaneously in a convergent fashion onto a narrow area on the surface of the epithelial cell and then invaded into the cytoplasm. In several biopsy specimens, we also observed the internalization of the agminated organisms by TEM and ITEM. In studies using cultured HEp-2 [14, 22] or AGS [23] cells, only spiral *H. pylori* has been reported to be taken into those cells. However, our study

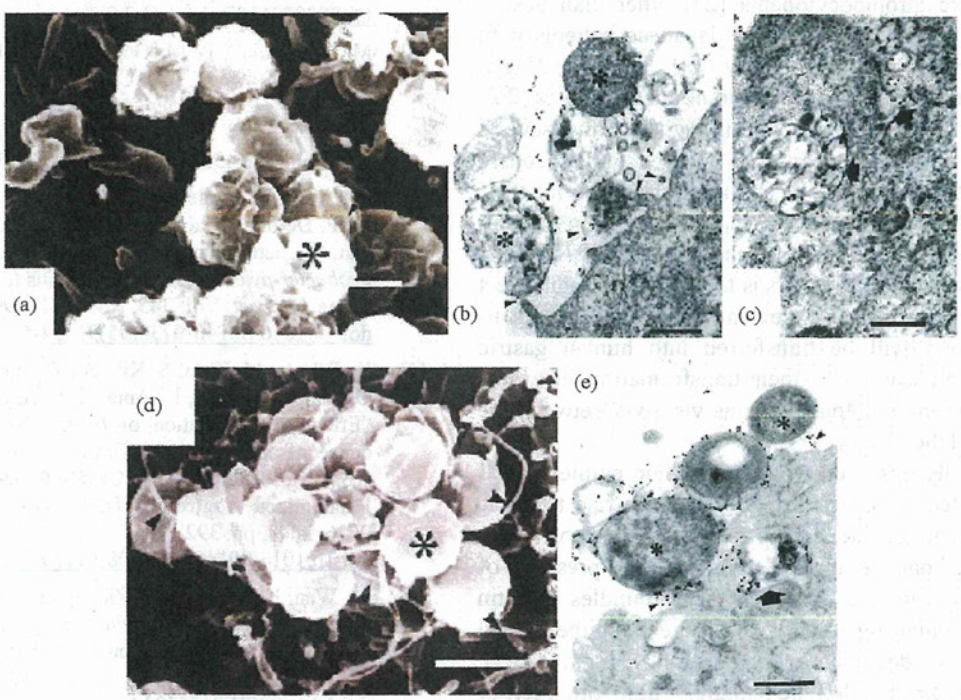

Figure 2. Coccoid *H. pylori* invasion into gastric epithelial cells (Bar indicates 0.5 μm). (a)-(c) show ATCC43504 strain from the 4th day in CLM. (a) Type A coccoid forms (asterisk) also agminates onto a certain area in similar way as shown by the spiral ones serving as control (SEM); (b) Type A coccoid forms (asterisks) are directly in contact with the surface of the epithelial cells using fragella and the bodies (arrowheads) (ITEM); (c) The anti-*H. pylori* antibody positive structures (arrows) are observed in the cytoplasm of the epithelial cells indicating successful penetration (ITEM); (d) and (e) show ATCC43504 strain from the 4th day in 300 mM-LM; (d) Type B coccoid forms (asterisks) also agminates onto a certain area as the spiral and coccoid A bacteria. (SEM); (e) Type B coccoid forms (asterisks) directly in contact with the epithelial cells in the same way as the type A coccoid ones (ITEM) Anti-*H. pylori* antibody positive flagella (arrowheads) are seen. Anti-*H. pylori* antibody positive structure (arrow) is seen in the cytoplasm of epithelial cell.

using Type A- and Type B-coccoid *H. pylori in vitro* [6-8,19] showed colony-like formation with attaching by themselves and invading into the epithelial cells, like that seen for the spiral form.

We have already reported that coccoid *H. pylori* is divided into four categories, namely, the dying one, the living one with culturability, the viable *but* non-culturable one and a special form prepared for species-preservation [19]. In this study, we could observe that the adhered coccoid forms showed an agminated pattern converging at a certain site and then penetrated into the gastric epithelial cells. From this observation, we propose the existence of a fifth category of coccoid *H. pylori*, one which can aggressively adhere in agminated manner and infuse their own genes into the cytoplasm of epithelial cells just like those of the spiral forms. Finally, the genes of the coccoid *H. pylori* could be involved in the production of human gastric ulcers, cancers and other diseases after the invasion into the epithelial cell.

From our observation, whole genes of coccoid *H. pylori* appeared to be finally incorporated into the epithelial cells. It is well-known that this bacterium also elicits many benign disorders such as coronary heart disease [2] or idiopathic thrombocytopenia [24] other than gastric carcinoma. Moreover, almost all Japanese patients with peptic ulcer, who are infected by *Cag A*-positive *H. pylori* [25], do not develop further to duodenal cancer [26]. From this clinical observation, it is suggested that several different genes other than *Cag A* are simultaneously taken into the epithelial cell and are transported together with *Cag A* to play their own respective roles in the mutation of the human cell. Since the infection of *H. pylori* into the gastric epithelial cells is facilitated through direct contact between the cells, several genes from the agminated bacteria will be transferred into human gastric epithelial cell leading to their transformation in accordance to the immunological status vis-a-vis between the bacteria and the host cell.

We actually observed that a transgenic epithelial cell being inserted with just *Cag A* gene gave rise to ultrastructural changes, but did not ultimately led to the direct onset of oncogenesis. We observed the presence of glycogen clusters and increased in the bundles of actin filaments, in the *Cag A* inserted transgenic epithelial cell (not shown in detail here. In preparation for a paper). Thus, it is suggested that the malignant transformation of gastric epithelial cell would need the interaction of several other genes, rather than just *Cag A*. This study supports the clinical phenomena that all other genes attached together with *Cag A* are simultaneously taken into epithelial cells resulting in several disorders. Furthermore, coccoid *H. pylori*, while trying to adapt to the harsh environment, would probably also elicit several *H. pylori*-related disorders.

Since several biopsied specimens showed agminated and invading bacteria, we raised an alarm that these patients should be given due attention for the treatment of sporadic *H. pylori*-related disorders because the bacterial genes might have been inserted into their gastric epithetlial cells. Thus, our study demonstrated that coccoid *H. pylori* is not a passive entity but can actively infect into the human gastric epithelial cell.

In conclusion, from this study, we proposed that coccoid *H. pylori* possessed a fifth characteristic; the ability to directly infect into gastric epithelial cells. This will be added to the already known 4 categories, viz, the dying one, the living one with culturability, the viable *but* non-culturable one and the active form for species-preservation.

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Frequency of *Helicobacter pylori*-Negative Gastric Cancer and Gastric Mucosal Atrophy in a Japanese Endoscopic Submucosal Dissection Series Including Histological, Endoscopic and Serological Atrophy

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Key Words
Helicobacter pylori · Gastric cancer · Endoscopic submucosal dissection

Abstract
Background: The definition of *Helicobacter pylori*-negative gastric cancer depends on the accuracy of diagnosis of *H. pylori* infection. The aim of this study was to determine the frequency of *H. pylori*-negative gastric cancer and to clarify relationships with histological atrophy, endoscopic atrophy, and serological atrophy. ***Methods:*** A total of 240 early gastric cancers were included in this study. The status of *H. pylori* infection was determined from the rapid urease test, ^{13}C-urea breath test, *H. pylori* culture, histopathological examination and examination of IgG antibodies. In *H. pylori*-negative gastric cancer, histological atrophy and intestinal metaplasia, endoscopic atrophy and serological atrophy were assessed by pepsinogen. ***Results:*** The rate of *H. pylori* infection was 77.9% and 19 patients (7.9%) had a history of eradication. 34 patients (14.2%) were diagnosed with *H. pylori*-negative gastric cancer using diagnostic tools of *H. pylori*. However, most of the patients with *H. pylori*-negative gastric cancer had histological atrophy and intestinal metaplasia. Only 1 gastric cancer (0.42%) occurred in the mucosa without histological atrophy, endoscopic atrophy or serological atrophy. ***Conclusion:*** Early gastric cancers in the Japanese endoscopic submucosal dissection series were strongly related to current or past infection with *H. pylori* and to gastric mucosal atrophy.

Introduction

The technique of endoscopic surgery has undergone various developments, particularly in Japan, because of the high frequency of early gastric cancer [1]. The advantage of endoscopic submucosal dissection (ESD), which is a current standard method of endoscopic resection, is curative treatment and accurate pathological diagnosis based on en-bloc resection [2, 3]. On the other hand, the high frequency of metachronous gastric cancer is problematic [4, 5]. Fukase et al. [6] showed in a prospective study that eradication of *Helicobacter pylori (H. pylori)* reduced the incidence of metachronous gastric cancer in one-third of the patients. Maehata et al. [7] disagreed with this opinion and they showed that eradication of *H. pylori*

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did not reduce metachronous gastric cancer from a retrospective follow-up of more than 5 years. Since 2010, the Japanese insurance system has allowed patients who have undergone endoscopic resection to receive eradication of *H. pylori* [8]. However, no evidence of *H. pylori* infection was found in some patients with gastric cancer, and there have been few reports on *H. pylori* infection status of early gastric cancer resected by endoscopic surgery [9, 10].

Determination of *H. pylori* infection status for gastric cancer is problematic because of false negatives for *H. pylori* tests and because of spontaneous disappearance in severely atrophic mucosa [11, 12]. Some studies on *H. pylori*-negative gastric cancer have focused on serological atrophy, histological atrophy and intestinal metaplasia [13–15]. However, there have been few studies on *H. pylori*-negative gastric cancer that have focused on endoscopic atrophy. In this study, we determined the ratio of *H. pylori*-negative early gastric cancer resected by ESD according to diagnostic tools of *H. pylori* and estimated relations with histological atrophy, endoscopic atrophy, and serological atrophy.

Materials and Methods

Patients

A total of 294 gastric epithelial neoplasms that were consecutively treated by ESD between January 2004 and December 2010 were retrospectively investigated. Patients who met the following criteria were excluded from this study: patients who were using an immunosuppressant, patients who had a history of gastric surgery and radiation therapy, and patients in whom *H. pylori* infection status was not determined.

Determination of H. pylori *Infection Status*

H. pylori-Positive

The status of *H. pylori* was determined to be positive when the results of at least one of the following were positive before ESD: rapid urease test (RUT), ^{13}C-urea breath test (^{13}C-UBT), *H. pylori* culture, and histopathologic examination.

In the RUT, by using a Helicocheck kit (Otsuka Pharmaceutical Co., Tokyo, Japan), the presence of *H. pylori* was determined by color changes after 20 min in specimens of normal mucosa biopsy obtained from the gastric antrum and body [16].

H. pylori was cultured by using modified Skirrow agar in a certified central laboratory. Biopsy specimens were obtained from the antrum and the body of the stomach. Static culturing was performed with the use of sheep blood agar M58 (Eiken, Tokyo, Japan) and *H. pylori* isolation medium (Eiken) as culture media with the addition of 10% carbon dioxide (CO_2) at 35°C for 7 days.

For the ^{13}C-UBT, breath samples were obtained before and at 20 min after administration of 100 mg of ^{13}C-urea. $^{13}CO_2$ excretion was measured in a UBiT-IR200 (Photal Otsuka Electronics, Tokyo, Japan), and a level >2.5‰ indicated *H. pylori* infection [17].

In the histopathologic examination, all biopsy specimens from the antrum and the body were stained with Giemsa stain and classified by pathologists according to the updated Sydney System [18].

When results of the above four tests and examinations were all negative, serum *H. pylori* antibody was examined by using the E plate test (Eiken Kagaku, Tokyo, Japan) [19]. The cut-off value was 10 U/ml.

Post-Eradication of *H. pylori*

If the patient had a history of *H. pylori* eradication and no bacteria were found by the RUT, ^{13}C-UBT, culture and histopathology, the patient was diagnosed with post-*H. pylori* eradication without current infection.

H. pylori-Negative

Status of *H. pylori*-negative was determined when results of all *H. pylori* tests (RUT, ^{13}C-UBT, culture, histopathology, and IgG antibody) were negative without a history of eradication.

Definitions of Endoscopic Atrophy and Serological Atrophy

Endoscopic atrophy was defined according to the Kimura-Takemoto classification system before ESD. Kimura and Takemoto [20] divided gastric mucosal atrophy into six groups (closed type: C1, C2, C3, open type: O1, O2, and O3) according to endoscopically recognized differences in the color and height of the gastric mucosa. This classification correlated well with the histological features and showed progress from C1 to O3.

In addition, fasting pepsinogen (PG) I and II levels were assayed using the chemiluminescence enzyme immunoassay for determination of serum atrophy in patients who did not have a history of use of antacids within the previous 2 weeks [21]. Serum samples were collected before ESD. Serological mucosal atrophy was evaluated from these PG levels according to previous reports, and no severe atrophy was defined as PG I level >70 ng/ml and PG I/PG II (PG I/II) ratio >3.0 [22]. PG I and PGI/PG II ratio were compared according to histological atrophy and endoscopically defined atrophy using Student's t test.

Histological Score of Background Mucosa

Biopsy specimens were taken from five sites of the stomach, as recommended by the updated Sydney System [18]: the greater curvature of the antrum, the lesser curvature of the antrum, the lesser curvature of the angulus, the lesser curvature of the corpus, and the greater curvature of the corpus. The state of the gastric mucosa was evaluated according to the updated Sydney System by pathologists. The degrees of atrophy and intestinal metaplasia (IM) were classified into four grades: 0, 'normal'; 1, 'mild'; 2, 'moderate', and 3, 'marked'. Histological non-atrophy was defined as score 0 in both atrophy and IM. Mild atrophy was defined as score 1 of atrophy and/or IM in one site or more. The remainder was defined as severe atrophy. The scores of histological atrophy and IM were compared according to endoscopic atrophy using the Mann-Whitney U test.

Ethics

This study was carried out in accordance with the principles embodied in the Declaration of Helsinki 1975 and was approved by our institutional review board. Informed consent was obtained from all subjects.

Results

Frequency of H. pylori*-Negative Gastric Cancer Determined by Using Diagnostic Tools*

H. pylori infection status in our 294 consecutive patients with gastric cancer resected by ESD is shown in figure 1. 51 patients were excluded according to our exclusion criteria and 3 patients who had a history eradication of *H. pylori* after a finding of gastric lesions were excluded. The *H. pylori*-positive rate in our ESD series was 77.9% (187/240), and 19 patients (7.9%) had a history of eradication. Finally, 34 patients (14.2%) who were negative for all *H. pylori* tests and had not received eradication were diagnosed with *H. pylori*-negative gastric cancer using diagnostic tools.

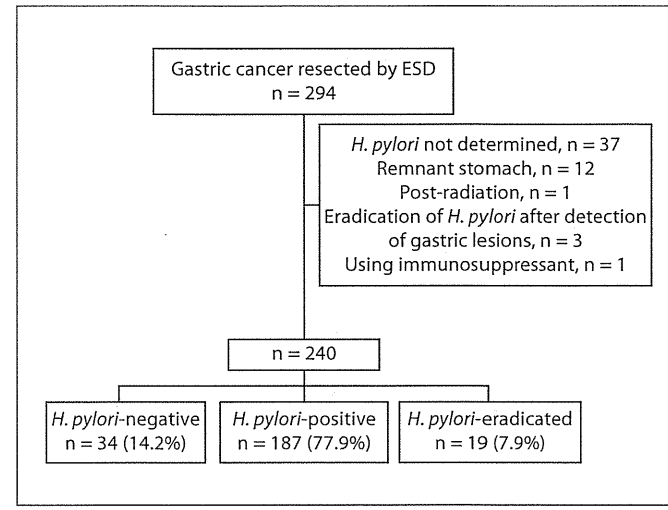

Fig. 1. Flow diagram of *H. pylori* infection status in the present ESD series. *H. pylori* infection was defined as positive from a RUT, histology, culture, UBT and IgG antibody.

Histological Atrophy and IM of H. pylori*-Negative Gastric Cancer*

Most of the *H. pylori*-negative gastric cancers occurred in histologically mild or severe atrophic mucosa, and only 3 patients had neither histological atrophy nor IM in background mucosa (fig. 2).

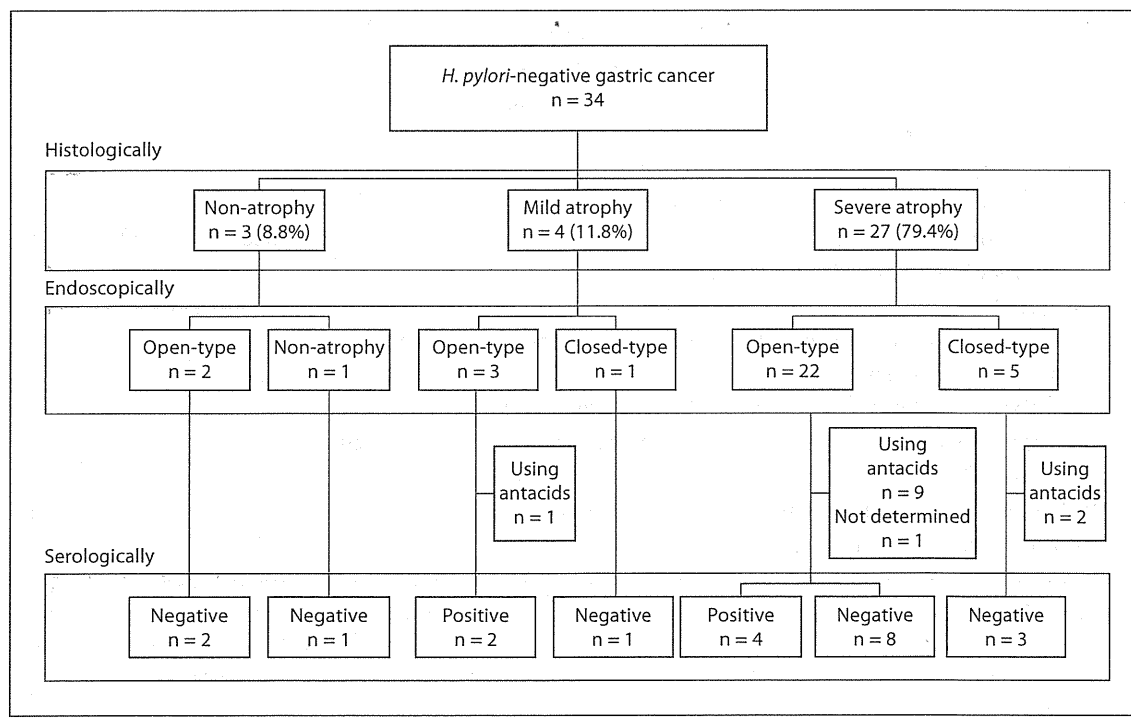

Fig. 2. Flow diagram of histological atrophy, endoscopic atrophy and serological atrophy of *H. pylori*-negative gastric cancer. Histological atrophy was classified into three groups according to updated Sydney System. Endoscopic atrophy was defined according to the Kimura-Takemoto classification system. Serological atrophy was determined when the PG I level was <70 ng/ml and/or the PG I/PG II ratio was <3.0.