Actual status of involvement of *Helicobacter pylori* infection that developed gastric cancer from Group A of ABC (D) stratification —study of early gastric cancer cases that underwent endoscopic submucosal dissection—

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Running Title: *Helicobacter pylori* infection in patients at low risk of developing gastric cancer

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Abstract

Background/Aims: Patients who are *Helicobacter pylori* (*H. pylori*) antibody negative and have normal pepsinogen levels (group A of ABC (D) stratification) are considered unlikely to develop gastric cancer. This study aimed to clarify the involvement (uninfection, present infection, or previous infection) of *H. pylori* in group A patients with early gastric cancer who underwent endoscopic submucosal dissection (ESD) by examining their background gastric mucosa endoscopically and histologically.

Methods: This study included 166 patients with gastric cancer who were treated by ESD. Patients were classified according to pepsinogen levels and *H. pylori* antibody titers. Three biopsies (greater curvature of the antrum, lesser curvature of the middle corpus, and greater curvature of the middle corpus) from group A were histologically analyzed and compared with those of groups B, C, D, and after eradication).

Results: In group A (34 patients), 32 patients had endoscopic atrophy (group A′). Histological neutrophil activity, chronic inflammation, and atrophy scores were lower in group A′ than in other groups. Group A' scores were similar to those of the after eradication group.

Conclusion: Most group A patients with early gastric cancer were not uninfected with *H. pylori*, but had previous infections, thus carrying carcinogenic risk.

Introduction

Accumulating evidence suggests that *Helicobacter pylori* (*H. pylori*) infection plays a role in gastric carcinogenesis [1]. As a well-accepted model of the preneoplastic state, chronic gastritis resulting from *H. pylori* infection may evolve from inflammation to atrophy, intestinal metaplasia, and, ultimately, noninvasive neoplasia [2]. Retrospective studies conducted in Japan indicate that the incidence of *H. pylori*-negative patients among those with early gastric cancer is approximately 2%–10% [3-4]. However, taking mucosal atrophy into consideration, the incidence of gastric cancer in patients with no history of *H. pylori* infection is considerably lower. Matsuo et al. reported that the frequency of *H. pylori*-negative carcinoma among 3161 gastric cancer samples was 0.66% [5]. Likewise, Ono et al. reported that 0.42% of early gastric cancers developed without current or past *H. pylori* infection [6].

Serological parameters, such as pepsinogen (PG) levels, are routinely used to monitor atrophic or inflammatory conditions in the gastric mucosa. Ninety-nine percent of PG, the inactive precursor of pepsin specifically produced in the stomach, is secreted into the gastric lumen, while 1% is secreted into the blood stream [7]. PG is composed mainly of two biochemically and immunologically distinct isozymes (PG I and PG II). Both PG I and PG II are produced by chief and mucous neck cells in the stomach. In addition, PG II is produced by cardiac, pyloric, and Brunner's gland cells [8, 9].

Some studies have demonstrated that a low PG I concentration and PG I/II ratio are indicators of atrophic gastritis [10-14]. In addition, a high PG II level reflects chronic inflammation in *H. pylori*-related chronic gastritis [13, 14]. The ABC (D) method, which measures both *H. pylori* antibody titer and serum PG levels, provides an efficient evaluation of gastric cancer risk [15-18]. Group A patients are classified as having normal PG levels and are negative for *H. pylori* antibodies. Thus, these patients are assumed to have no *H. pylori* infection or atrophic gastritis, with little risk of developing gastric cancer. However, in clinical practice, gastric cancer is occasionally identified in group A patients [19]. Thus, group A is considered to include both patients truly negative for *H. pylori* and high-risk patients. These high-risk patients may include those with past *H. pylori* infection or false negative test results for PG and/or *H. pylori* antibodies despite ongoing *H. pylori* infection. Therefore, this study aims to clarify the involvement (uninfection, present infection, or previous infection) of *H. pylori* in group A patients with early gastric cancer who underwent ESD by examining their background gastric mucosa using histological (based on the updated Sydney System) and endoscopic evaluations.

Methods

Patients

Between January 2009 and September 2013, 507 consecutive patients with primary gastric cancer were treated by endoscopic submucosal dissection (ESD) at Okayama University Hospital. We analyzed 273 patients via histological examination as well as prior evaluation of serum *H. pylori* antibody titers and serum PG concentration. Patients were excluded if they had a history of upper gastrointestinal tract surgery (n = 36) or were taking proton pump inhibitors, as this could affect serum PG levels (n = 71). Therefore, 149 patients, none of whom received *H. pylori* eradication therapy, were enrolled in this study. Previous eradication history was decided based on the files of our hospital and the medical information of referral patients from other clinics. In addition, 17 patients with a history of *H. pylori* eradication therapy before ESD were included for comparison. Three biopsy samples (greater curvature of the antrum, lesser curvature of the middle corpus, and greater curvature of the middle corpus) were endoscopically obtained from each patient for histological analysis.

The study was approved by the Okayama University School of Medicine Clinical Ethics Committee on Human Experiments (approval number 2162; October 28, 2014), in accordance with the Declaration of Helsinki. Each patient provided informed consent.

Serum measurements

Fasting blood samples were collected from all patients immediately before endoscopy. Serum *H. pylori* IgG antibody titers were measured using a commercially available kit (E-plate; Eiken, Tokyo, Japan). Antibody titers were measured by optical density using standards and a cut-off value of 10 U/ml according to the manufacturer's protocol. Serum PG I and PG II levels were measured using a commercial chemiluminescent enzyme immunoassay kit (Lumipulse pepsinogen I & II; Fujirebio Inc., Tokyo, Japan). Positive cut-off values were set at PG I $\leq$ 70 ng/ml and PG I/II $\leq$ 3.0 ng/ml, as previously described [20-22].

Endoscopic examination

Endoscopic mucosal atrophy was evaluated at the atrophic border, as described by Kimura and Takemoto [23]. This boundary between the pyloric and fundic gland regions was endoscopically recognized by differences in color and the height of the gastric mucosa between the two sides of the border. Gastric mucosal atrophy was classified into 3 categories: no atrophy, closed-type, or open-type. And it is reported that there is a higher risk of developing gastric cancer in the case of open type atrophy than that of closed type atrophy [24]. As it is difficult

to distinguish cases of the mild atrophy (only on antrum) and no-atrophy cases endoscopically, we perform indigo carmine dye spraying in the routine esophagogastroduodenoscopy and to diagnose no-atrophy cases for those which have smooth mucosa.

Histological examination

Serial sections of the three biopsy specimens (greater curvature of the antrum, lesser curvature of the middle corpus, and greater curvature of the middle corpus) were stained with hematoxylin and eosin and Giemsa. The status of the gastric mucosa was evaluated by expert pathologists. The degree of polymorphonuclear neutrophil activity and chronic inflammatory cells (mononuclear cells) indicate chronic gastritis, atrophy, intestinal metaplasia (IM), and *H. pylori* density. Biopsies were classified into four grades according to the updated Sydney system [25]: 0, normal; 1, mild; 2, moderate; and 3, severe.

ABC (D) stratification

Group A consisted of patients negative for *H. pylori* antibody and with normal PG levels; Group B patients had normal PG levels and were positive for *H. pylori* antibody; Group C patients had atrophic PG levels and were positive for *H. pylori* antibody; and Group D patients had atrophic PG levels and were negative for *H. pylori* antibody. Clinical practice has shown that group A frequently contains high-risk patients with atrophic gastritis [19]. Therefore, group A patients were separated into those endoscopically and/or histologically diagnosed with atrophic gastritis (group A′) as well as those with no atrophic gastritis who were truly negative for *H. pylori* infection. To clarify *H. pylori* involvement in group A′, we compared the gastric histological features of group A′ with those of groups B, C, and D, as well as patients who had a history of *H. pylori* eradication therapy before ESD.

Statistical analysis

Statistical analyses for comparing categorical data were performed using Pearson's chi-square tests. A Student's *t*-test or Wilcoxon rank sum test was used for numerical data and scored categorical data, as appropriate. A p value < 0.05 was considered statistically significant. JMP statistical software (SAS Institute Inc., Cary, NC, USA) was used for all calculations. The statistical analyses performed were verified by a qualified biostatistician.

Results

Patient characteristics

This study included 166 patients, 149 of whom had not received *H. pylori* eradication therapy and 17 of whom had a history of *H. pylori* eradication therapy before ESD. The 149 patients (99 men and 50 women) who had not received *H. pylori* eradication therapy had a mean age of 72 years (range, 35–91 years; Table 1). Based on endoscopy results, 123 (83%) patients had open-type atrophy, 21 (14%) had closed-type atrophy, and 5 did not have apparent mucosal atrophy. Ninety-seven patients were positive for *H. pylori* IgG antibodies, and 63 had normal PG levels. ABC (D) stratification identified 34 patients (23%) in group A, 29 (19%) in group B, 68 (46%) in group C, and 18 (12%) in group D (Fig. 1).

Characteristics of groups A and A′

In group A, only 2 patients (1.3%) did not have endoscopic atrophic gastritis. Histologically, these patients had no inflammation, activity, atrophy, or IM at all three sites. Therefore, they were considered truly uninfected with *H. pylori.* The remaining 32 patients had endoscopic and/or histological atrophy (group A′; Table 2). This group included 24 men and 8 women with an average age of 73 years. Serum anti-*H. pylori* antibody titer was < 5U/ml in 26 out of 32 cases while it was <3U/ml in 20 cases in them. On the other hand, all 32 patients had endoscopic atrophy, with 3 (9%) cases of closed-type atrophy and 29 (91%) cases of open-type atrophy. In group A′, the median PG I level was 40.4 ng/ml and the average PG II level was 7.8 ng/ml. The PG I levels of 27 patients (84%) were < 70 ng/ml, and the PG II levels of 25 patients (78%) were < 10 ng/ml. A urea breath test was performed for nine group A′patients; all nine patients tested negative (data not shown).

The histological characteristics of group A′ are shown in Table 3. Ten patients were normal for inflammation at all three sites, and 27 patients were normal for activity at all three sites. By histological analysis, each patient in group A′ had atrophy, intestinal metaplasia, or inflammation. There was no *H. pylori* colonization in any group A patient.

Histological distinctions between group A′and groups B, C, and D

The histological characteristics of group A′ were compared with those of groups B, C, and D, as well as those of patients who previously received eradication therapy (after eradication group). The activity and inflammation scores of group A′ were lower than those of groups B and C at all three sites, and were lower than those of group D (Fig. 2a, b). Activity scores in group A′ and the after eradication group were almost zero (Fig. 2a). Regarding atrophy scores,

no significant differences were detected between groups in the greater curvature of the antrum. However, in the corpus, atrophy scores of group A′ were lower than those of groups C and D, and were similar to those of the after eradication group (Fig. 2c). Regarding IM, no significant difference was detected between groups except that scores of group A′ were lower than those of group D in the greater curvature of the corpus (Fig. 2d).

Discussion

This retrospective study evaluated 149 patients without an apparent history of *H. pylori* eradication therapy and 17 patients who received prior eradication therapy, all of whom were treated for primary gastric cancer with ESD. Group A (i.e. both normal PG test and negative for *H. pylori* antibody) patients accounted for 23% (34 of 149 patients) of the patients without a history of eradication therapy. While there have been several reports that showed patients who developed gastric cancer due to the involvement of *H. pylori* infection in Group A, we have not yet seen detailed study about them. We examined serum antibody titer for group A' patients, and then compared their status of histological gastritis with those of group B, group C, group D and the group who had eradication history. As a result, we found that group A' patients' serum antibody was very low and that there was a similarity of status of histological gastritis in group A' patients and patients with eradication history. Based on this procedure, we think our study firstly proved that the most of group A' patients were the cases of former infection with unexpected successful eradication, not the cases of false negative of present infection.

In group A, only two patients (1.3%) were truly uninfected with *H. pylori*; similar rates have been reported previously. Matsuo et al. reported a frequency of *H. pylori*-negative gastric cancer of 0.66% (21 of 3161 patients) [5]. Similarly, Ono et al. reported that only 1 of 240 cases of early gastric cancer (0.42%) occurred in patients without current or past *H. pylori* infection [6]. Boda et al. reported that 3 of 271 patients (1.1%) with gastric epithelial neoplasms were truly uninfected with *H. pylori* [19]. Thus, gastric cancer is rare in patients uninfected with *H. pylori*.

In this study, the remaining 32 patients (group A′) were also negative for *H. pylori* antibodies and had normal serum PG levels. However, they each exhibited gastric atrophy via endoscopic and/or histological analyses. Ono et al. reported that 14% of patients with early gastric cancer treated by ESD were negative for five *H. pylori* tests and had not received eradication therapy [6]; these patients exhibited histological or endoscopic atrophy. Boda et al. also reported that

10% of patients treated for gastric epithelial neoplasms by ESD were classified into group A′, and 94% of these patients had open-type atrophy diagnosed via endoscopy [19]. Thus, present or prior infection of *H. pylori* can only be confirmed by observing histological or endoscopic atrophy in approximately 10% to 25% of patients with early gastric cancer. Therefore, the development of gastric cancer in group A′ patients was considered to be related to *H. pylori* infection (present or previous infection).

As more detailed information is unclear from previous reports, the present study clarified the actual status of *H. pylori* infection by comparing histological data from several sites in the stomach between group A′ and groups B, C, and D as well as the group of patients with apparent prior eradication. First, in this study the *H. pylori* antibody titers of 20 patients (63%) in group A′ were < 3U/ml (the cut-off value was 10 U/ml; data not shown). Their very low *H. pylori* antibody titers demonstrate that titers may not indicate false negative results. Histological analysis according to the updated Sydney system revealed similar characteristics for group A′ patients and those with a history of *H. pylori* eradication therapy. These two groups had lower polymorphonuclear neutrophil activity as well as chronic inflammatory and atrophy scores than groups B, C, and D at all three sites surveyed. Kodama M et al. conducted a 10-year follow-up study monitoring histological changes at five points in the gastric mucosa after *H. pylori* eradication [26]. Activity scores were markedly reduced 6 months after successful eradication, nearly reaching zero. Moreover, Kodama M et al. also reported a gradual decrease in atrophy scores after *H. pylori* eradication. All sites, except the antral sites, reached a level similar to the *H. pylori*-negative group. In contrast, with the exception of scores for the lesser curvature of the corpus, IM scores fluctuated considerably during the entire observation period. In our study, patients in group A′ and patients with a history of *H. pylori* eradication therapy showed activity scores near zero. In addition, we observed that group A′ had lower atrophy scores than groups C and D in the lesser and greater curvatures of the corpus.

Antral atrophy was more dominant than corpus atrophy in group A′ patients. This finding is not compatible with autoimmune gastritis, which is another cause of atrophic gastritis. When mucosal atrophy is caused by autoimmune gastritis, corpus atrophy is more dominant [27]. Thus, we hypothesize that the activity and atrophy scores of group A′ decreased after unexpected eradication of *H. pylori* infection (ex. antibiotic administration for any bacterial infection). Accordingly, many patients in group A′ were regarded as having a history of unexpected successful eradication.

Our study had certain limitations. This was a retrospective, single-center study with a limited

number of cases. Next, though biopsy regions that got consensus by the updated Sydney system were five, we utilized lesser samples(one sample for three regions) to evaluate the histologic gastritis on the background mucosa for the purpose of less invasive procedure, which can be considered another limitation. Furthermore, subjects were limited to patients with early gastric cancer who were treated with ESD. In the general population of Japan, the prevalence of *H. pylori*-infected patients with a history of unexpected eradication is unknown. In this study, the median patient age was 72 years. The difference in age between our patient population and that of the general population may have influenced *H. pylori* antibody titers and PG levels. Thus, large-scale, multicenter studies are warranted.

Though it is useful to perform endoscopic examination for atrophic gastritis as is shown in this study to clarify whether or not patients in group A are uninfected with *H. pylori*, it will be too difficult to put it into practice considering a problem of cost and man power. On the other hand, the ABC (D) stratification is considered to be a reliable mass-screening method to examine a risk of gastric cancer in that it is simple despite several exceptional cases. It is expected to be carried out in the future. Considering that as we have extended coverage of national health insurance in Japan and there will be an increasing number of patients who undergo eradication therapy, group A patients are expected to increase more and more, we need to develop a criterion value system to examine whether or not patients in group A are uninfected with *H. pylori.*

In conclusion, our histological data suggest that approximately 20% of gastric cancer patients experience unexpected eradication for *H. pylori*, and would therefore be considered high-risk patients despite having normal PG levels and testing negative for *H. pylori* antibodies. It is important to consider that group A includes not only low-risk patients truly negative for *H. pylori,* but also patients at high risk for developing gastric cancer.

Conflicts of Interest:

All authors declare that there are no conflicts of interest. The sponsor had no role in the design of the study, data collection, analysis, and interpretation, writing of the manuscript, or decision to submit for publication. The sponsor had no access to raw data.

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1 29. Table 1. Characteristics of analyzed patients

Patients, n	
Total	149
Median (range) age, years	72 (35-91)
Gender, n	
Male	99 (66%)
Female	50 (34%)
Extension of gastric atrophy, n (%)	
None	5 (3%)
Closed-type	21 (14%)
Open-type	123 (83%)
Histological atrophy, n (%)	
None	5 (3%)
Mild	28 (19%)
Moderate or severe	116 (78%)
Histological intestinal metaplasia, n	
(%) None	18 (12%)
Mild	31 (21%)
Moderate or severe	100 (67%)
***H. pylori* IgG**	
Median (range), U/ml	19 (0-177)
Negative, n (%)	52 (35%)
Positive, n (%)	97 (65%)
PG	
PG I median (range), ng/ml	32(3-456)
PG II median (range), ng/ml	12.5(3-121)
PG I/II (range)	2.4(0.3-12)
Negative, n (%)	63 (42%)
Positive, n (%)	86 (58%)
ABC (D) stratification, n (%)	
Group A	34 (23%)
Group B	29 (19%)
Group C	68 (46%)
Group D	18 (12%)

30. H. pylori, Helicobacter pylori

31. PG, pepsinogen.

33. Table 2. Comparison of characteristics between group A′ and group non-A

	Group A′ n = 32	**Group non-A n = 115**	**p**
Age, years			
Median (range)	73 (57–91)	71 (35–91)	0.31 [a]
Gender, n (%)			
Male	24 (75%)	75 (65%)	0.39 [b]
Female	8 (25%)	40 (35%)	
Endoscopic atrophy, n (%) None	0 (0%)	3 (3%)	0.41 [b]
Closed-type	3 (9%)	18 (15%)	
Open-type	29 (91%)	94 (82%)	
Median PG			
PG I (ng/ml)	40.4	69.7	0.02 [a]
PG II (ng/ml)	7.8	18.9	< 0.001 [a]

34. PG, pepsinogen.

35. [a]Wilcoxon rank sum test.

36. [b]Pearson's chi-square tests.

Table 3. Histological characteristics of group A′ (n = 32)

	Inflammation	Activity	Atrophy	Intestinal metaplasia	Bacterial density
Negative	10	27	3	3	32
Positive	22	5	29	29	0

37. Data are presented as the number of patients.

38. Figure legends

39. Fig. 1. Flow diagram of *H. pylori* infection and serological status in the present ESD series.

40. Group A consisted of patients negative for *H. pylori* antibody and with normal PG levels; Group B patients had normal PG levels and were positive for *H. pylori* antibody; Group C patients had atrophic PG levels and were positive for *H. pylori* antibody; and Group D patients had atrophic PG levels and were negative for *H.*

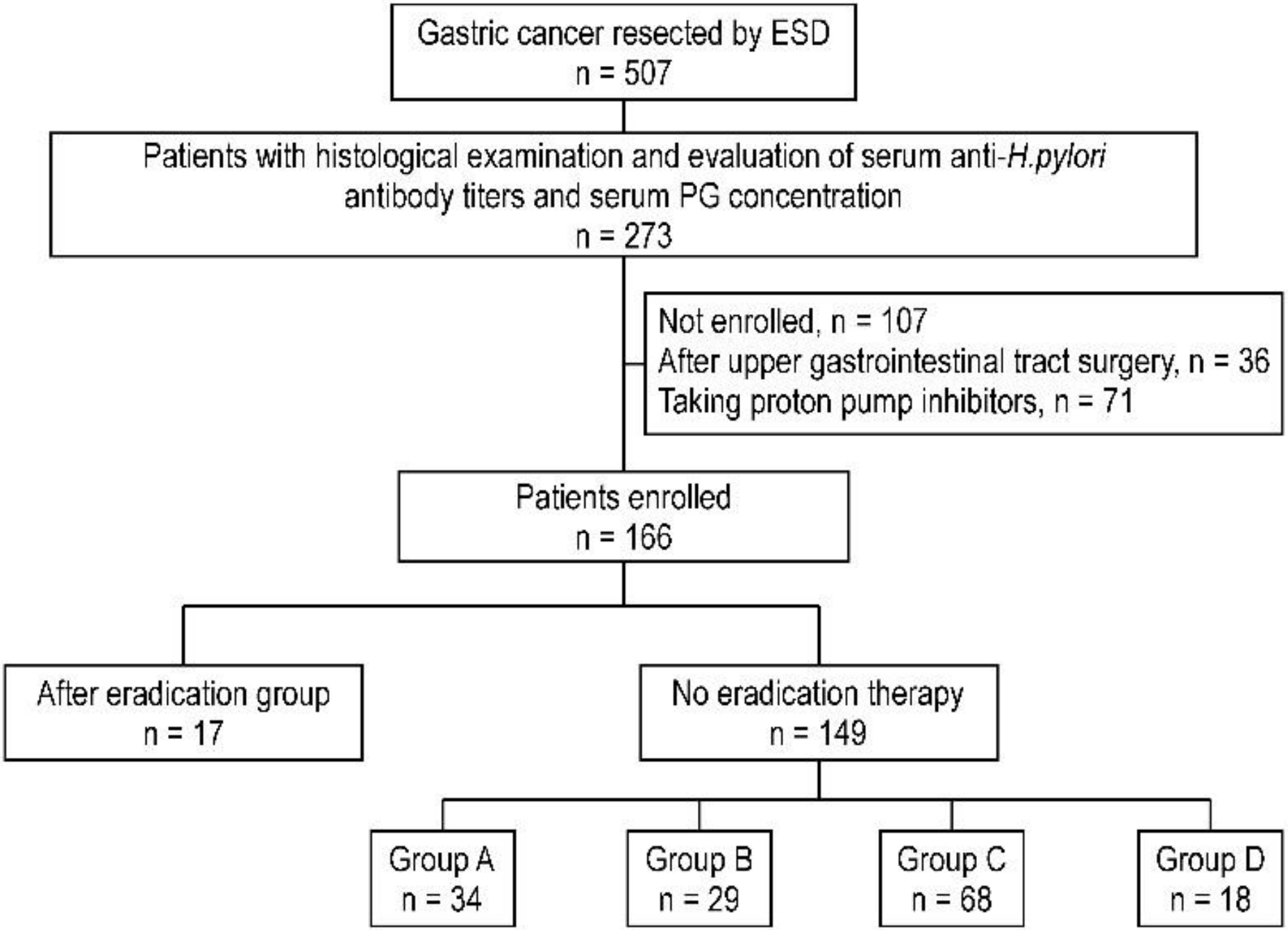
41. *pylori* antibody.

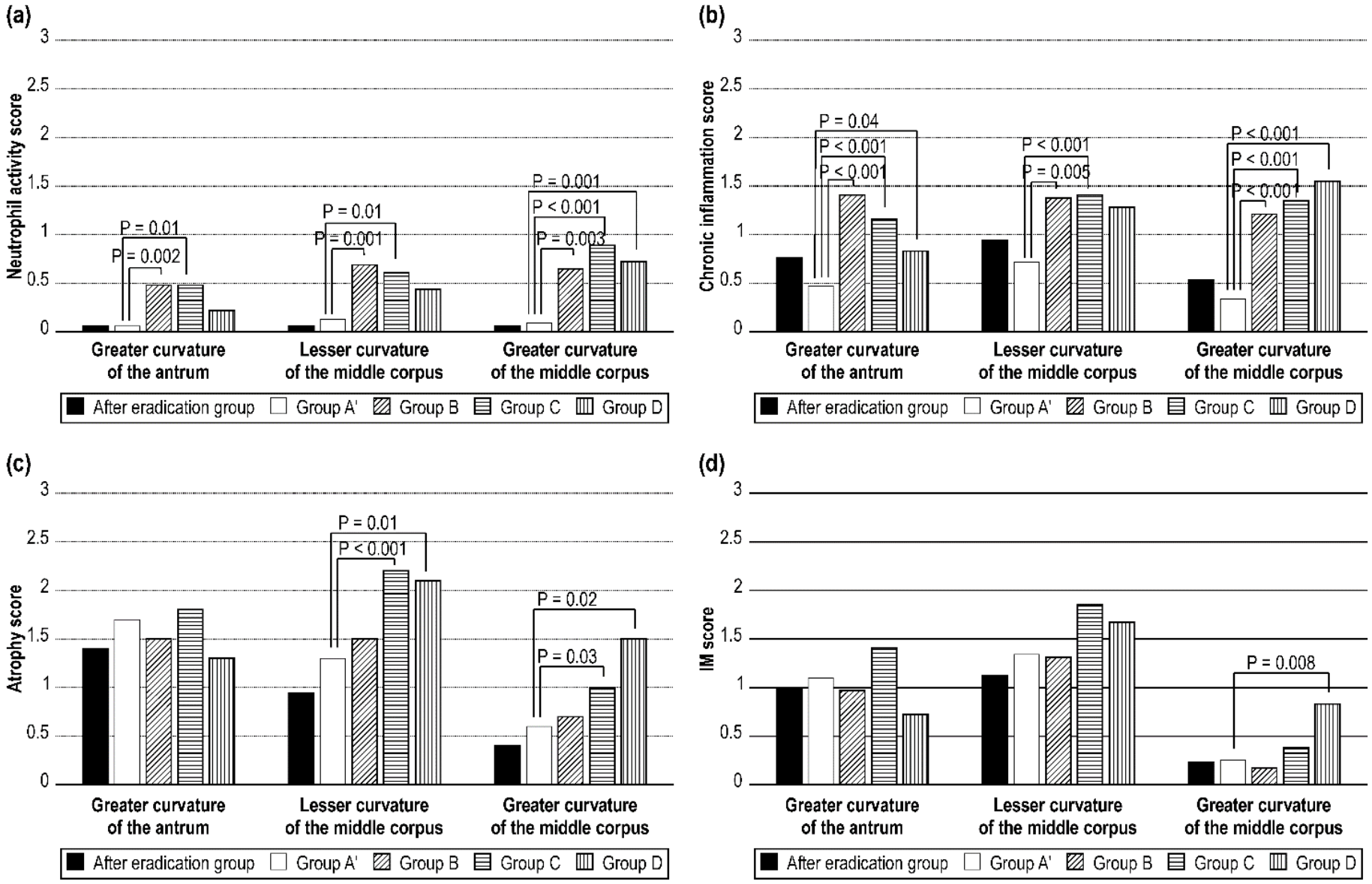
42. Fig. 2. Histological characteristics of group A′ and other groups.

43. The activity and inflammation scores of group A′ were lower than those of groups B and C at all three sites, and lower than those of group D in the greater curvature of the corpus (a, b). Activity scores in group A′ and the after eradication group were nearly zero (a). Regarding atrophy scores, no significant differences were detected between groups in the greater curvature of the antrum. However, in the corpus, atrophic scores of group A′ were lower than those of groups C and D and were similar to those of the after eradication group (c). Regarding intestinal metaplasia, no significant differences were detected between groups, with the exception of the greater curvature of the corpus (d). A Student's *t*-test was used to

44. compare data.

~~28.~~45.


Gastric cancer resected by ESD
n = 507
Patients with histological examination and evaluation of serum anti-H.pylori antibody titers and serum PG concentration
n = 273
Not enrolled, n = 107
After upper gastrointestinal tract surgery, n = 36
Taking proton pump inhibitors, n = 71
Patients enrolled
n = 166
After eradication group
n = 17
No eradication therapy
n = 149
Group A
n = 34
Group B
n = 29
Group C
n = 68
Group D
n = 18


(a)
Neutrophil activity score
3
2.5
2
1.5
1
0.5
0
P = 0.01
P = 0.002
P = 0.01
P = 0.001
P = 0.001
P < 0.001
P = 0.003
Greater curvature of the antrum
Lesser curvature of the middle corpus
Greater curvature of the middle corpus
After eradication group
Group A'
Group B
Group C
Group D
(b)
Chronic inflammation score
P = 0.04
P < 0.001
P < 0.001
P < 0.001
P = 0.005
P < 0.001
P < 0.001
P < 0.001
(c)
Atrophy score
P = 0.01
P < 0.001
P = 0.02
P = 0.03
(d)
IM score
P = 0.008