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False Sero-Negative Results for *Helicobacter pylori* Infection Indicate Increased Risk of Severe Atrophic Gastritis in Japanese Patients

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Abstract

Background/objective: *H. pylori* infection is commonly diagnosed using an anti-*H. pylori* IgG antibody test. However, a proportion of results are falsely sero-negative. We investigated characteristics of patients falsely sero-negative for *H. pylori* in relation to gastric atrophy.

Methods: *H. pylori* infection (Hp+ or Hp-) was determined based on culture test, rapid urease test (RUT), and polymerase chain reaction (PCR) test in 280 outpatients. Anti-*H. pylori* antibody titers ≥ 10 U/ml were diagnosed as sero-positive for *H. pylori* (IgG+), while those <10 U/ml were sero-negative (IgG-). Serum pepsinogen (PG) I/PG II ratios were calculated as a serological marker of gastric atrophy. Endoscopic gastric mucosal atrophy was also assessed according to the Kimura-Takemoto classification system.

Results: The mean PG I/PG II ratio in each group was as follows: Hp-/IgG- (4.99 ± 1.04, n=10), Hp+/IgG+ (2.59 ± 1.51, n=240), Hp-/IgG+ (5.65 ± 2.72, n=4) and Hp+/IgG- (3.02 ± 2.61, n=26). The mean serum PG I/PG II ratio in the Hp+/IgG- group was lower than those of Hp-/IgG- and Hp-/IgG+ groups (P=0.028 and 0.072). Incidence of severe gastric mucosal atrophy in the Hp+/IgG- group was highest of the four groups.

Conclusions: Individuals falsely sero-negative for *H. pylori* infection is at increased risk of severe atrophic gastritis, which is well known as precancerous lesion.

Keywords: Anti-*H. pylori* IgG antibody; Pepsinogen; Gastric atrophy

Abbreviations:

H. pylori: *Helicobacter pylori*; PG: Pepsinogen; IgG: Immunoglobulin G; RUT: Rapid Urease Test; UBT: Urea Breath test

Introduction

Chronic *Helicobacter pylori* (*H. pylori*) infection in gastric mucosa increases the risk of developing gastric cancer [1-5]. In particular, patients with *H. pylori*-associated severe atrophic gastric mucosa are at high risk of developing intestinal metaplasia, gastric adenoma, and gastric cancer [2]. Recently, the Japanese health insurance system permitted to check and eradicate *H. pylori* when the infection is present in patients with endoscopically proven gastritis [6]. Therefore, the accurate diagnosis of *H. pylori* infection is of clinical importance.

Several tests requiring endoscopy (i.e. culture, rapid urease test [RUT] and histology) and other tests without endoscopy (i.e. 13C-urea breath test [13C-UBT], stool antigen test and serological anti-*H. pylori* IgG antibody test) have been established for the detection of *H. pylori* infection in clinical practice [7]. The Maastricht IV/Florence Consensus Report recommends the 13C-UBT and stool antigen test to detect *H. pylori* infection, due to high sensitivity and specificity [7]. However, the anti-*H. pylori* IgG antibody test is typically used to check infection, particularly in health check-ups in Japan, because it is simple and inexpensive [8]. As sensitivity and specificity of the anti-*H.pylori* IgG antibody test is approximately 90%-95% [7,9], some 5%-10% of those assessed are falsely diagnosed by this test. If patients are misdiagnosed as uninfected with *H. pylori*, they will not undergo gastroscopy, which increases the risk of oversight of gastric cancer and other *H. pylori* related disorders. Therefore, elucidation of the clinical characteristics of individuals that are falsely negative for anti-*H. pylori* IgG antibody is of clinical importance.

Pepsinogen (PG) I and PG II, the two main precursors of pepsin, are both produced by chief cells and mucous neck cells of the stomach [10,11]. PG II is also produced by pyloric gland cells. Approximately 1% of PG I and PG II reach the systemic circulation, which can be detected and measured in the serum. Gastric inflammation increases the proportion of PG I and PG II that reach the blood stream. As gastric atrophy progresses, chief cells in the gastric corpus decrease and are replaced with pyloric glands, leading to a decrease in serum PG I levels. Therefore, both a low serum PG I and low PG I/PG II ratio are recognized as serological markers of gastric atrophy [12-14]. In Japan, serum PG I level ≤70 ng/ml and PG I/PG II ratio ≤3.0 indicate

severe gastric mucosal atrophy, which is called as "positive for the PG method" [14].

Recently, a combination of serum PG and anti-*H. pylori* IgG antibody tests, called the ABC classification, has been used to evaluate an individual risk for gastric cancer [2,15,16]. In the ABC classification, the risk of gastric cancer is classified into four groups as follows; Subjects classified into the group A are both negative for anti-*H. pylori* IgG antibody and the PG method. Those classified into the group B are positive for anti-*H. pylori* IgG antibody but negative for the PG method. Those classified into the group C are positive for both anti-*H. pylori* IgG antibody and the PG method. Those classified into group D are negative for anti-*H. pylori* IgG antibody but positive for PG method. Interestingly, individuals in Group D of this classification (serum PG I level ≤70 ng/ml, PG I/PG II ratio ≤3.0 and negative for anti-*H. pylori* IgG antibody) are known to be at the highest risk of developing gastric cancer. Although the sensitivity of this classification was reported as 70.5% and specificity as 97.0% [17], a considerable problem remains in that a certain proportion of patients in Group A (serum PG I level >70 ng/ml or PG I/PG II ratio >3.0, and negative for anti-*H. pylori* IgG antibody) and D are false negative in the IgG serology test.

Here, we investigated the characteristics of patients who were falsely sero-negative for *H. pylori* in relation to serum PG levels and endoscopic gastric mucosal atrophy.

Patients and Methods

Patients:

A total of 280 outpatients who underwent gastroscopy for screening of *H. pylori* infection at the University Hospital of Hamamatsu University School of Medicine were enrolled. Mean age was 58.0 ± 11.8 years. The cohort consisted of 170 males (60.7%) and 110 females (39.3%). They consisted of patients with hyperplastic polyp (n=8), gastric cancer (n=6), MALT lymphoma (n=4), gastritis alone (n=227), or either or both gastric or duodenal ulcer (n=32) and functional dyspepsia (n=3).

Serum levels of anti-*H. pylori* antibody titer and PGs:

We obtained sera from all subjects. Serum anti-*H. pylori* IgG antibody was determined using a commercial kit (E-plate Eiken®; Eiken Chemical Co., Ltd., Tochigi, Japan). Samples with an anti-*H. pylori* IgG titer <10 U/ml were diagnosed as sero-negative for *H. pylori*, defined as "IgG-". Samples with an anti-*H. pylori* IgG titer ≥ 10 U/ml were diagnosed as sero-positive for *H. pylori*, defined as "IgG +" in accordance with the manufacture's protocol [18]. Serum levels of PG I and II were measured using a commercial kit (Pepsinogen CLEIA®; Fuji Rebio., Ltd., Tokyo, Japan). PG I/PG II ratios were calculated as a serological marker of gastric atrophy. PG I/PG II ratio ≤ 3.0 indicated severe gastric atrophy and an increased risk of gastric cancer[14].

Gastroduodenoscopy and diagnosis of *H. pylori* infection:

Endoscopists were unaware of clinical information of each patient, such as serum PG I, PG II and anti-*H. pylori* antibody.

During gastroduodenoscopy, immediately after entering the stomach, 5 ml of gastric juice was aspirated through the suction channel of the endoscope and collected in a trap placed in the suction line [19]. Routine inspection of the upper gastrointestinal tract was performed, and several biopsy specimens from both the antrum and the corpus were then obtained for RUT and bacteriological culture as described below.

Endoscopic gastric mucosal atrophy was assessed according to the Kimura-Takemoto classification system and classified into three groups as follows: none/mild, C-0 - C-II; moderate, C-III - O-I; and severe, O-II - O-III [20]. For the RUT, biopsy specimens were inoculated into a commercial kit (Helicocheck®; Otsuka Pharmaceutical Co, Ltd., Tokyo, Japan), and any color change was noted after 30 min and 2 h. A color change from yellow to pink within 2 h was judge as a positive result [21].

For the bacterial culture test, biopsy specimens were inoculated onto agar plates (E-MR82; Eiken Chemical Co, Ltd., Tochigi, Japan) and incubated at 37 °C under microaerophilic conditions for up to 7 days [19]. Colonies were identified as *H. pylori* based on morphology in Gram stains, oxidase test, and RUT [19].

For PCR analysis, DNA was extracted from gastric juice samples by the boiling method and subjected to PCR analysis of *H. pylori* infection using the automated gene analyzer, GENECUBE® (Toyobo Co., Ltd., Fukui, Japan) as previously described [22].

When any one of the three detection tests (PCR, culture or RUT) were positive, subjects were diagnosed with *H. pylori* infection and defined as "Hp+". When all tests were negative, subjects were diagnosed as uninfected with *H. pylori* and defined as "Hp-".

Subjects uninfected with *H. pylori* and negative for anti-*H. pylori* IgG antibody were classified as Hp-/IgG-, those infected with *H. pylori* and positive for anti-*H. pylori* IgG antibody were classified as Hp+/IgG+, those infected with *H. pylori* but negative for anti-*H. pylori* IgG antibody were classified as Hp+/IgG-, and those uninfected with *H. pylori* but positive for anti-*H. pylori* IgG antibody were classified as Hp-/IgG+.

The protocol was approved in advance by the Human Institutional Review Board of Hamamatsu University School of Medicine, and a written informed consent was obtained from each subject.

Statistics:

All numerical values are expressed as means ± standard deviations (SD). Statistical differences in serum PG values among subgroups were assessed via one-way ANOVA (analysis of variance) with Scheffe's multiple comparison test. Whether or not incidences of different grades of endoscopic gastric mucosal atrophy differed among subgroups was assessed via the chi-square test. All P-values were two-sided. $P<0.05$ was considered as statistically significant. Calculations were carried out using StatView 5.0 statistical software (SAS Institute, Cary, NC, USA).

Results

A total of 280 subjects were enrolled in the study. Mean serum level of PG I was 58.0 ± 36.9 ng/ml, and that of PG II was 24.0 ± 15.2 ng/ml. Mean PG I/PG II ratio was 3.0 ± 1.7, and mean anti-*H. pylori* IgG titer was 49.0 ± 39.8 U/ml. Two hundred and forty-four (87.1%) subjects tested positive in the anti-*H. pylori* IgG antibody test, 251 (89.3%) in the RUT, 258 (92.1%) in the PCR test, and 194 (69.3%) in the culture test (Table 1). A total of 266 subjects had positive result with any of the three tests (i.e., RUT, PCR and culture test) and were diagnosed to be

infected with *H. pylori* (Hp+). Remaining 14 were diagnosed to be uninfected with *H. pylori* (Hp-).

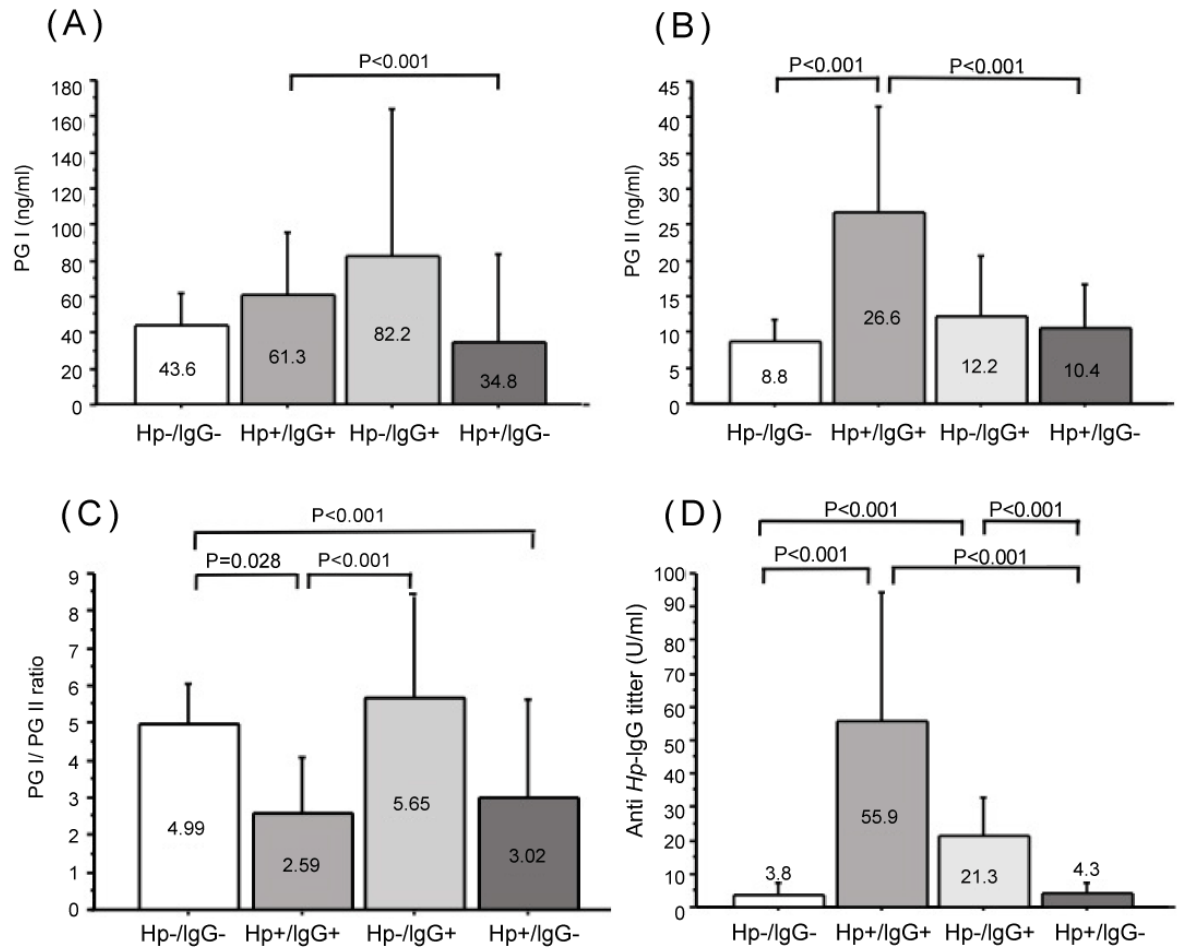

Figure 1: Mean serum PG I (A), PG II (B), PG I/PG II ratios (C) and anti- *H. pylori* IgG antibody titer (D) in Hp-/IgG-, HP+/IgG+, Hp-/IgG+ and Hp+/IgG- groups. Bars indicate standard deviations. Abbreviations are: Hp-/IgG-, uninfected with *H. pylori* and negative for anti-*H. pylori* IgG antibody; Hp+/IgG+, infected with *H. pylori* and positive for anti-*H. pylori* IgG antibody; Hp-/IgG+, uninfected with *H. pylori* but positive for anti-*H. pylori* IgG antibody; Hp+/IgG-, infected with *H. pylori* but negative for anti-*H. pylori* IgG antibody.

Clinical demographic characteristics of four groups, Hp-/IgG-, Hp +/IgG+, Hp-/IgG+ and Hp+/IgG-, were summarized in Table 2. Ten (3.6%) subjects were classified as Hp-/IgG-, 240 (85.7%) as Hp+/IgG+, 4 (1.4%) as Hp-/IgG+, and 26 (9.3%) as Hp+/IgG-. The mean age of the Hp+/IgG- group was 66.1 ± 13.8, which was slightly higher in comparison with other three groups. There were no significant differences in male/female ratios among the four groups (Table 2).

Characteristic	Value
Male/Female (% ratio)	170/110 (60.7%/39.3%)
Age (years)	58.0 ± 11.7
PG I (ng/ml)	58.0 ± 36.9
PG II (ng/ml)	24.0 ± 15.2
PG I/ PG II ratio	3.0 ± 1.7
Anti *H. pylori*-IgG titer (U/ml)	49.0 ± 39.8
Anti *H. pylori*-IgG (positive)	244 (87.1%)
Eradication history (none)	190 (67.8%)
RUT (positive)	250 (89.3%)
PCR (positive)	254 (90.7%)
Culture (positive)	195 (69.6%)

Table 1: Patient characteristics. Values are presented as mean ± standard deviation PG I, pepsinogen I; PG II, pepsinogen II; RUT, rapid urease test

Mean serum PG I level of each group was demonstrated in Figure 1A. Mean serum PG I level of the Hp+/IgG- group was significantly lower than that of the Hp+/IgG+ group (P<0.001), but not that of the Hp-/IgG- or Hp+/IgG- group. Mean serum PG II level of each group was demonstrated in Figure 1B. Mean serum PG II level of the Hp+/IgG- group was significantly lower than that of the Hp+/IgG+ group (P<0.001). Mean of serum PG I/PG II ratio of each group was demonstrated in Figure 1C. Mean of serum PG I/PG II ratio in the Hp+/IgG- group was lower than that in the Hp-/IgG- or Hp-/IgG+ group (P=0.028 and 0.072). Mean anti-*H. pylori* IgG titer of each group was demonstrated in Figure 1D. In the IgG- groups, the mean titer of anti-*H. pylori* IgG antibody in Hp+/IgG- or Hp-/IgG- groups were no 0, indicating that patients with low titers for anti-*H. pylori* IgG antibody were included in the Hp+/IgG- or Hp-/IgG- groups.

	HP-/IgG- N=10 (3.6%)	HP+/IgG+ N=240 (85.7%)	HP-/IgG+ N=4 (1.4%)	HP+/IgG- N=26 (9.3%)	P-value
Male/Female	8/2 (80.0%/20.0%)	141/99 (58.8%/41.2%)	3/1 (75.0%/25.0%)	18/8 (69.2%/30.8%)	0.379
Age (years)	63.4 ± 8.6	56.5 ± 11.0	63.3 ± 16.6	66.1 ± 13.8	<0.001
Eradication history (none)	5 (50.0%)	178 (74.2%)	1 (25.0%)	6 (23.1%)	0.383
RUT (positive)	0	234 (97.5%)	0	16 (64.0%)	<0.001
PCR (positive)	0	230 (95.8%)	0	24 (92.3%)	<0.001
Culture (positive)	0	193 (80.4%)	0	2 (7.7%)	<0.001
ABC classification A/B/C/D (n/n/n/n)	10/0/0/0	0/113/127/0	0/3/1/0	11/0/0/15	<0.001

Table 2: Demographic clinical characteristics of each group. Values are presented as mean ± standard deviation Hp-/IgG-, uninfected with *H. pylori* and negative for anti-*H. pylori* IgG antibody; Hp+/IgG+, infected with *H. pylori* and positive for anti-*H. pylori* IgG antibody; Hp-/IgG+, uninfected with *H. pylori* but positive for anti-*H. pylori* IgG antibody; Hp+/IgG-, infected with *H. pylori* but negative for anti-*H. pylori* IgG antibody.

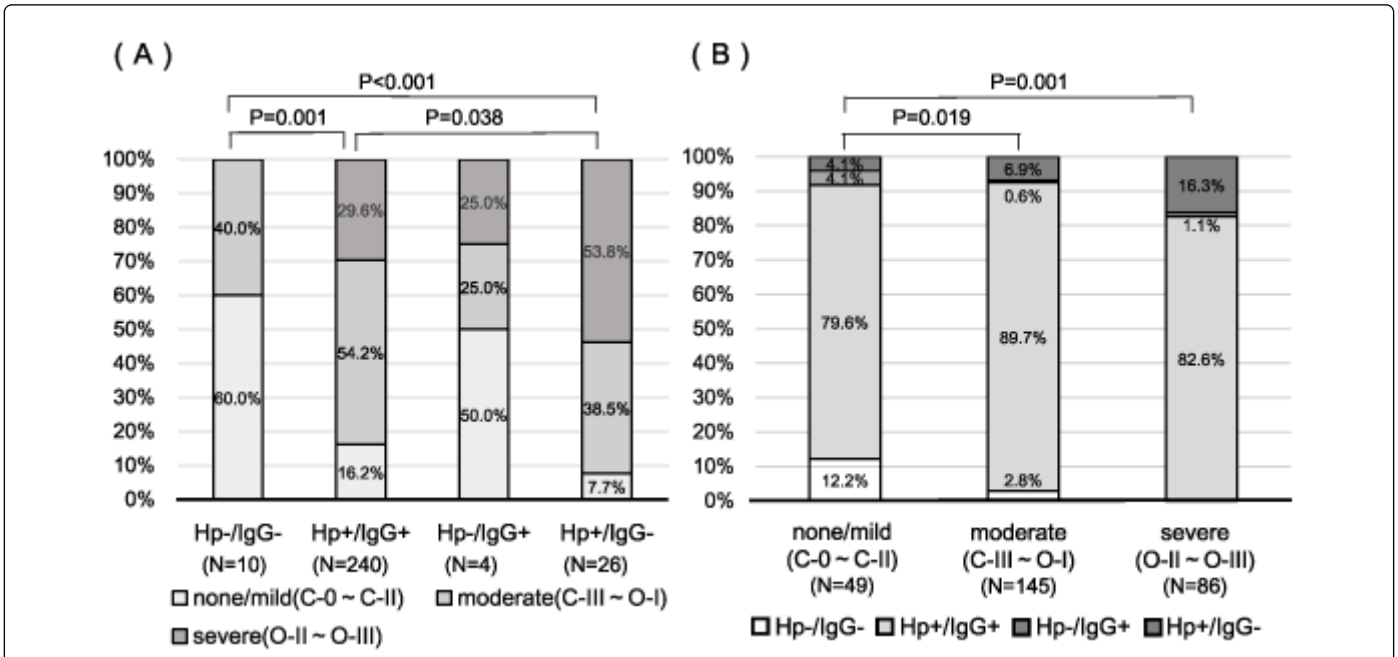

Figure 2: Incidence of endoscopic gastric mucosal atrophy in Hp-/IgG-, HP+/IgG+, Hp-/IgG+ and Hp+/IgG- groups (A) and the percentage of four groups (Hp-/IgG-, HP+/IgG+, Hp-/IgG+ and Hp+/IgG-) in different gastric mucosal atrophy grade (B). Abbreviations same as Figure 1.

Incidences of different grades of endoscopic gastric mucosal atrophy in the four groups are summarized in Figure 2. Incidence of moderate (C-III - O-I) and severe gastric mucosal atrophy (O-II - O-III), particularly severe atrophy, was highest in the Hp+/IgG- group (Figure 2A). The incidence of falsely sero-negative subjects (Hp+/IgG-) became higher as the grades of atrophic gastritis progressed

from non/mild (C-0 - C-II) to moderate (C-II - O-I) and severe (O-II - O-III) (Figure 2B).

Sex	Age (years)	Anti-*H. pylori* IgG titer (U/ml)	ABC method	Endoscopic gastric mucosal atrophy	RUT	Culture	PCR
M	75	<3	D	O-II	P	N	P
F	64	<3	D	C-II	ND	N	P
F	66	<3	D	O-III	P	N	P
F	74	<3	D	O-III	P	N	P
M	72	3	D	O-III	P	N	P
M	74	4	D	O-III	P	N	P
M	75	4	D	O-III	N	N	P
M	81	4	D	O-III	N	N	P
M	81	4	D	O-III	N	N	P
M	74	5	D	O-III	P	N	P
F	67	5	D	O-III	N	N	P
M	70	6	D	O-III	P	P	P
M	67	8	D	O-I	P	N	P
M	62	9	D	O-I	N	N	P
M	74	9	D	O-III	N	N	P
M	41	<3	A	C-III	P	N	P
M	54	<3	A	O-I	N	N	P
M	70	<3	A	O-III	P	N	P
M	87	3	A	O-I	P	N	N
M	76	4	A	O-I	P	N	P
F	41	5	A	O-I	N	N	P
F	62	7	A	C-III	P	N	P
F	66	7	A	O-I	P	N	P
M	48	8	A	C-III	P	P	P
M	71	8	A	O-II	P	N	N
F	27	9	A	C-II	N	N	P

Table 3: Clinical characteristics of the Hp+/IgG- group. Hp+/IgG-, infected with *H. pylori* but negative for anti-*H. pylori* IgG antibody; RUT, rapid urease test; M, male; F, female; P, positive; N, negative; ND, no data.

Although only 2 of the 26 subjects of the Hp+/IgG- group were positive for the culture test, 16 (64.0%) were positive for RUT, and 24 (92.3%) were positive for PCR (Table 3). Based on the ABC classification, 15 of the Hp+/IgG- group belonged to Group D, while the remaining 11 patients belonged to Group A. Moreover, the anti-*H.pylori* IgG antibody titer<3 were observed in only 7 of 26 (26.9%) patients. Remaining 19 patients (73.1%) had the low titer (3 – 9 U/ml) of the anti-*H. pylori* IgG antibody.

Discussion

In the present study, we found that 9.3% of patients enrolled in this study were falsely sero-negative for *H. pylori* infection. This result was consistent with a previous report [23]. We also observed that mean age of Hp+/IgG- group was slightly higher than that of other groups, that the mean of serum PG I/PG II ratio in the Hp+/IgG- group was lower than that of in the *H. pylori*-negative groups and that endoscopic gastric mucosal atrophy of the Hp+/IgG- group was the most advanced of the four groups. Patients who were judged falsely sero-

negative (Hp+/IgG- group) were therefore considered to be at a high risk of developing gastric cancer.

The problems of falsely sero-negative for *H. pylori* have been reported previously. Ootani, et al. [24] reported that 9.5% of *H. pylori* sero-negative subjects had endoscopically atrophic gastritis. Yamaji et al. [25] reported that weakly positive for anti-*H. pylori* IgG antibody is a major risk factor for gastric cancer, particularly in the elderly. Given that a proportion of patients in the falsely sero-negative group had a low titer of anti-*H. pylori* antibody (e.g. 3-9 U/ml), they were considered the same as the weakly positive group. Some reports have noted that patients with gastric cancer had low titers of anti-*H. pylori* IgG antibody [26-29] and suggested that the weakly positive group might have diminished immune function and be unable to adequately respond to *H. pylori* antigen. Chen et al. [30] reported that degradation of the immune response to *H. pylori* might increase the risk of gastric cancer. Together, these findings suggest that changes in systemic and local immune response to *H. pylori* might be associated with sero-negativity and carcinogenesis in the stomach [31,32]. Further studies are needed to clarify the causative relationship between development of gastric cancer and low titers of anti-*H. pylori* IgG antibody.

The characteristics of Group D under ABC classification appear similar to those of the Hp+/IgG- group in the present study. Individuals classified into Group D are considered to have cleared infection naturally by severe gastric mucosal atrophy [33]. However, the results of our present study demonstrated that more than half of the patients in the Hp+/IgG- group were classified into Group D. Therefore, the considerable proportion of individuals in Group D may actually be infected with *H. pylori*. In fact, all patients classified as Group D in the present study were deemed to be infected with *H. pylori* based on either culture, RUT or PCR or both findings. Diminished immune response to *H. pylori* is also suggested to occur in Group D [31,32].

The remaining 11 subjects in the Hp+/IgG- group were classified into Group A under the ABC classification system. Members of Group A are considered to have no *H. pylori* infection and be at low risk of gastric cancer and healthy gastric mucosa [1,34,35]; however, our study results demonstrated that some members of Group A may indeed be *H. pylori*-positive. All of the 11 individuals had atrophic gastritis from Grade C-II to O-III (Table 3). Therefore, diagnosis of *H. pylori* infection based on serum anti-*H. pylori* IgG antibody alone appears to be insufficient and risks oversight of severe atrophic gastritis. Endoscopy and other diagnostic tests for *H. pylori* infection are therefore necessary for more accurate diagnosis of *H. pylori* infection, even in Group A. Further, reassessment of the cut off level of the antibody titer is also required. In patients falsely sero-negative, the PCR method was able to detect *H. pylori* infection at 92.3% (n=24) and RUT at 64.0% (n=16). Combined detection might therefore diagnose *H. pylori* infection more accurately than using single method.

In this study, four subjects in the Hp-/IgG+ group were considered falsely sero-positive. Three of the four subjects had a history of eradication of *H. pylori* infection, suggesting the process that antibody titer was decreasing in the subjects after eradication who were misdiagnosed as having unsuccessful eradication. We therefore consider an interview concerning eradication history to be important when diagnosing *H. pylori*.

Several limitations to the present study warrant mention. First, this study had a small sample size and was a single center study. Second, *H. pylori* infection was detected via a culture test, RUT, and PCR test. For more accurate diagnosis, we should have conducted tests with higher sensitivity and specificity, such as the 13C-UBT or stool antigen test [7]. Third, as the population in this study included many *H. pylori*-infected subjects, there was a degree of bias in the results. We consider a similar study in the general population to be necessary. Therefore, the results of the present study should be considered with care and a multicenter study with large sample size performed to clarify the characteristics of falsely sero-negative subjects.

In conclusion, the results of the present study suggest that individuals who are falsely sero-negative for *H. pylori* infection have a high risk of severe atrophic gastritis, which is known as a precancerous lesion. Although the serological test for *H. pylori* is a simple and easy assay for screening *H. pylori* infection, it carries a risk of false-negative results. Combined detection, such as 13C-UBT, RUT, stool antigen test, culture test and/or histology, might therefore provide a more accurate diagnosis of *H. pylori* infection when serological test is negative. Then, we would like to offer the following recommendation; Patients with the anti-*H. pylori* IgG antibody ≥10 U/ml can be considered as *H. pylori* positive. Patients whose anti- *H. pylori* IgG antibody are 3–9 U/ml are suspicious for *H. pylori* infection and should undergo the alternative examination for *H. pylori* infection. Patients whose anti- *H. pylori* IgG antibody are <3 U/ml could be considered as *H. pylori*-negative, but should be recommend to have another examination if they are elderly. However, validity of our recommendation must be verified in the future prospective study.

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Conflict of interest:

This study was partly supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, and Sports of Japan (23590913). The Center for Clinical Research and the First Department of Medicine at Hamamatsu University School of Medicine have received grants from Takeda Pharmaceutical Co., Ltd.; AstraZeneca KK, Eisai Co., Ltd.; Daiichi-Sankyo Co. Ltd.; and Drs. Furuta and Sugimoto have received lecture fees from those companies. The authors have no other conflicts of interest that are directly relevant to the content of this article.

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