Characteristics of gastric cancer in negative test of serum anti-*Helicobacter pylori* antibody and pepsinogen test: a multicenter study

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Abstract

Background and aim The serological risk prediction system combines the pepsinogen test and anti-*Helicobacter pylori* (*H. pylori*) antibody determination. In this system, chronic atrophic gastritis (CAG) is diagnosed using the pepsinogen test. Patients who are *H. pylori* negative and pepsinogen negative are classified into group A, are assumed to be *H. pylori* uninfected, and are at an extremely low risk for gastric cancer. However, gastric cancers are

detected in this group. The aim of this study is to clarify the clinicopathological status of group A patients with gastric cancer.

Methods A total of 109 gastric cancer patients classified as group A were enrolled in a multicenter study. Group A patients were divided into two subgroups: group AN (*H. pylori* uninfected) and group AP (*H. pylori* infected). They were compared to 183 *H. pylori*-infected gastric cancer patients who were not in group A.

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Results Of the 109 patients, only 7 were classified as group AN; the other 102 were classified as group AP. The clinicopathological features of group AP included older age, predominantly differentiated type cancer, endoscopically visualized CAG, and pepsinogen (PG) I/II ratio lower than that of group AN. In group AN, the depressed type was dominant, and the PG I/II ratio was higher than in those gastric cancer patients who were infected with *H. pylori*.

Conclusion Patients in group AP had CAG, and their gastric cancers were similar to those of *H. pylori*-eradicated patients. Concerning the recent ABC classification system, advanced decision criteria should be proposed to decrease the false-negative evaluation of gastric cancer risk.

Keywords *Helicobacter pylori* · Gastric cancer · Serological risk prediction system · Pepsinogen · Gastritis

Introduction

In Japan, gastric cancer is the second most common cause of cancer-related death [1]. It is well known that *Helicobacter pylori* (*H. pylori*) infection is a major risk factor for gastric cancer. In addition, the gastric mucosal inflammation and atrophy induced by *H. pylori* are important risk factors for gastric cancer [2–4].

As biomarkers for gastritis and corpus atrophy, serum pepsinogen (PG) levels are the most reliable and established markers for gastritis including corpus atrophy [5, 6]. PG concentrations have been used with a set of recommended cutoff values to diagnose chronic atrophic gastritis (CAG) (PG I $\leq$ 70 ng/ml and PG I/II $\leq$ 3.0 ng/ml) [7]. Therefore, gastric cancer risk can be evaluated by measuring the serum anti-*H. pylori* antibody titer in combination with PG levels [8–10], and this system of risk prediction using the anti-*H. pylori* antibody serological test and the PG test is known as the ABC classification system in Japan [8–10]. Subjects with negative test results for either the anti-*H. pylori* antibody serology test or the PG test were classified in group A, anti-*H. pylori* antibody-positive/PG-negative subjects were in group B, anti-*H.*

pylori antibody-positive/PG-positive subjects in group C, and anti-*H. pylori* antibody-negative/PG-positive subjects were in group D. Specifically, multivariate meta-analyses suggested that group A had a lower risk than groups B/C/D and that group B had a lower risk compared with groups C/D [10].

Indeed, the ABC classification system is now one of the efficient risk prediction tools utilized for gastric cancer screening. Because the development of gastric cancer in those patients not infected with *H. pylori* is extremely rare in Japan [11], it may be expected that the *H. pylori*-uninfected healthy population could be excluded from the mass screening system for gastric cancer. Group A includes subjects with neither *H. pylori* infection nor CAG by testing; however, group A contains *H. pylori*-infected subjects. Boda et al. reported that approximately 10% of patients with gastric tumors (cancer or adenoma) belong to group A [12]. The cancer risk of these patients may be underappreciated using the current criteria of the ABC classification system. The presence of *H. pylori*-infected subjects in group A is a crucial problem because the subjects in group A are judged as having an extremely low risk for gastric cancer, similar to healthy, *H. pylori*-uninfected individuals.

In the present study, we constructed a multicenter study to clarify the clinicopathological features of the gastric cancer patients in group A (having test results negative for serum anti-*H. pylori* antibody and PG), and we discuss and advocate for the advancement of methods for evaluating gastric cancer risk using serum markers.

Methods

Patients

A total of 109 patients [82 men and 27 women of mean age 68.8 years (range, 40–87 years)] with 114 lesions were registered in this study from seven collaborating research units within hospitals (Hiroshima University Hospital, Kawasaki Medical School, Center of Cancer Screening Miyagi Cancer Society, Hokkaido University, Social Insurance Shiga Hospital, Kyoto Second Red Cross Hospital, Kohnodai Hospital). These subjects were patients with gastric cancer who underwent gastrointestinal endoscopic examinations, measurement of anti-*H. pylori* antibody titers (Eiken E-plate test), and serum PG I and II levels (CLIA Abbott; E-plate Eiken or LZ test Eiken) between January 2003 and October 2014. The diagnosis of gastric cancer was based on the following: ABC methods (1), X-ray examination (14), gastrointestinal endoscopy (11), visiting clinics with symptoms (4), and unknown (79). Five patients had received *H. pylori* eradication therapy,

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and proton pump inhibitors (PPI) were administered to 12 patients.

For the analysis, we classified group A patients into two subgroups: *H. pylori* uninfected and *H. pylori* infected. We defined the *H. pylori*-uninfected group A patients as those showing negative results in other *H. pylori* examinations [histological examination, urea breath test (Otsuka, Tokushima, Japan), rapid urease test, anti-*H. pylori* antibody in the urine, stool antigen test, or microbial culture test] and having no atrophic gastritis. The *H. pylori*-infected group A patients were defined as either those showing positive results in the *H. pylori* test or those having CAG. Moreover, it is speculated that the *H. pylori*-infected group A consists of *H. pylori*-infected and formerly infected subjects. In this study, the *H. pylori*-uninfected group A gastric cancer patients are shown as “group AN,” meaning group A with negative results for *H. pylori* infection tests. The *H. pylori*-infected group A is shown as “group AP,” meaning group A patients who are positive for *H. pylori* infection.

Group BCD gastric cancer patients

To clarify the clinicopathological characteristics of gastric cancer in group A, we enrolled gastric cancer patients who were classified into group B, C, or D between June 2003 and October 2014 in Hiroshima University Hospital ($n = 167$), Shiga Hospital ($n = 6$), or Kawasaki Medical hospital ($n = 10$). Regarding the patients in Hiroshima University Hospital, we chose the patients from the database used in the previous study by Boda et al. [12]. These 183 gastric cancer patients (136 men; mean age, 66.4 years) with *H. pylori*-positive results or with CAG were used as controls. We excluded those patients with gastric adenoma, those who were post gastrectomy, and those who had severe renal dysfunction. The use of PPI was checked during a medical interview. Fasting sera were similarly collected for the measurement of anti-*H. pylori* antibody titers (Eiken E-plate test) and serum PG I and II levels (CLIA Abbott; E-plate Eiken, LZ test Eiken).

Measurement

We used the ABC classification system as described previously [8, 9]. Fasting sera were obtained from all patients, and serum PG I and II levels (CLIA Abbott, E-plate Eiken; LZ test Eiken, Tokyo) and anti-*H. pylori* antibody titers (E-plate) were evaluated. In the PG test, a set of cutoff values ($\text{PG I} \leq 70 \text{ ng/ml}$ and $\text{PG I/II} \leq 3.0 \text{ ng/ml}$) was used for the diagnosis of atrophic gastritis. If PG levels were less than the cutoff values, they were considered as positive for the test. In the anti-*H. pylori* antibody titer test, the cutoff value for the diagnosis of *H. pylori* infection was more than

10.0 U/ml. Because the lower sensitivity limit is 3.0 U/ml in this kit, antibody titers between 3 and 9.9 U/ml are considered within the measurable range of a negative test result (“negative-high test”).

Endoscopic findings

The endoscopic grade of gastric mucosal atrophy was evaluated on the basis of the endoscopic classification system described by Kimura and Takemoto [13], which was used to classify cases into seven grades (C0, C1, C2, C3, O1, O2, O3). Information concerning the gastric cancer (size, endoscopic gross type, histological type, location, and depth of invasion) was recorded according to the Japanese Classification of Gastric Carcinoma [14]. For comparison, the histological type was divided into two groups in this report: differentiated type (tubular adenocarcinoma and papillary adenocarcinoma) and undifferentiated type (poorly differentiated adenocarcinoma and signet-ring cell carcinoma).

Statistical analysis

The statistical analysis was performed using the chi-square test and analysis of variance (ANOVA). For multiple comparisons, the Steel–Dwass method was used. When $p < 0.05$, differences were recognized as statistically significant. Statistical analysis was performed using JMP (SAS Institute).

Ethics

The protocol was approved by the ethics committee of Hiroshima University Hospital (no. Epd-1029-1).

Results

Clinicopathological characteristics in each group

Clinical and pathological data are summarized in Table 1. Among 109 patients in group A, only 7 (6.4%) were included in the *H. pylori*-uninfected group A (group AN). The *H. pylori*-infected group A (group AP) consisted of 102 (93.6%) of 109 patients, and all these patients had CAG that was visible endoscopically. In Table 1, p values are shown for comparisons between group AN and group AP, between group AN and group BCD, and between group AP and group BCD, respectively.

There was a male dominance in all groups. In group AN, the median age of the patients was younger than that in group AP ($p = 0.018$) and group BCD ($p = 0.086$). Group AP was slightly older than group BCD

Table 1 Clinicopathological characteristics of patients

	Group AN	<i>p</i> value vs. AP	<i>p</i> value vs. BCD	Group AP	<i>p</i> value vs. BCD	Group BCD
Sex (M/F)	5/2 (7)	0.810	0.87	77/25 (102)	0.83	136/47 (183)
Age (mean age $\pm$ SD, median)	57.3 $\pm$ 10.87, 61	0.018	0.086	69.5 $\pm$ 9.10, 70	0.077	66.4 $\pm$ 9.89, 67
Medical history		0.570	0.018		0.016	
Post <i>H. pylori</i> eradication	0			5		6
PPI using	2			10		0
Operation of stomach	0			1		0
Tumor size (mm, mean size $\pm$ SD, median)	10.4 $\pm$ 6.76, 9	0.048	0.0068	16.0 $\pm$ 8.07, 15	0.008	21.2 $\pm$ 14.32, 20
Macroscopic type ^a	<i>n</i> = 7	0.8041	0.65	<i>n</i> = 107	0.21	<i>n</i> = 201
0-I (protruding)	0			2 (1.87%)		5 (2.49%)
0-IIa (superficial elevated)	0			22 (20.6%)		65 (32.3%)
0-IIb (superficial flat)	0			0		3 (1.49%)
0-IIc (superficial depressed)	7 (100%)			80 (74.8%)		125 (62.2%)
Type 1 (mass)	0			1 (0.93%)		1 (0.50)
Type 2 (ulcerative)	0			1 (0.93%)		0
Type 3 (infiltrative ulcerative)	0			0		0
Type 4 (diffuse infiltrative)	0			1 (0.93%)		1 (0.50)
Type 5 (unclassifiable)	0			0		1 (0.50)
Histological type	<i>n</i> = 7	<0.0001	<0.0001	<i>n</i> = 107	0.87	<i>n</i> = 201
Differentiated	0			93 (86.92%)		176 (87.56%)
Undifferentiated	7 (100%)			14 (13.08%)		25 (12.44%)
Location	<i>n</i> = 7	0.31	0.55	<i>n</i> = 107	0.43	<i>n</i> = 201
Upper third	0			15 (14.02%)		22 (10.95%)
Middle third	1 (14.29%)			38 (35.51%)		59 (29.35%)
Lower third	6 (85.71%)			53 (21.50%)		119 (59.20%)
Unclassifiable	0			1 (0.93%)		1 (0.50%)
Depth of invasion	<i>n</i> = 7	0.99	1.00	<i>n</i> = 107	0.48	<i>n</i> = 201
M (mucosa)	6 (85.71%)			82 (77.36%)		169 (84.08%)
SM (submucosa)	1 (14.29%)			21 (19.81%)		29 (14.43%)
MP (muscularis propria)	0			0		0
SS (subserosa)	0			1 (0.94%)		1 (0.50%)
SE (serosa)	0			0		1 (0.50%)
SI (invades adjacent structures)	0			1 (0.94%)		1 (0.50%)
Unknown	0			2 (1.88%)		0

Type 1–5 advanced type, AN *Helicobacter pylori*-uninfected group, AP *H. pylori*-infected group, BCD classification of gastric cancer patients, PPI proton pump inhibitors [14]

n lesion numbers

^a 0-I, 0-IIa, 0-IIb, 0-IIc; superficial type, tumor confined to the mucosa or submucosa

($p = 0.077$). The mean tumor size of group AN was the smallest ($p = 0.048$ vs. group AP, $p = 0.0068$ vs. group BCD), and group AP had smaller tumor sizes than group BCD ($p = 0.08$). Concerning the macroscopic features, all patients in group AN showed 0-IIc (flat depressed type) morphology, and the prevalence of 0-IIc cancer was higher in group AP than in group BCD; however, there were no significant differences. Histologically, all tumors

in Group AN showed the undifferentiated type (poorly differentiated adenocarcinoma, 1; signet-ring cell carcinoma, 6) ($p < 0.0001$), whereas the differentiated type was the dominant type in group AP and group BCD. In terms of the histological type, there was no significant difference between groups AP and BCD ($p = 0.87$). There was no significant difference in tumor location or depth among the three groups.

Serum anti-*H. pylori* antibody titer in each group

All patients in group A (AN and AP) were negative for the anti-*H. pylori* antibody titer test (<10 U/ml). As demonstrated in Table 2, 6 (85.7%) of 7 patients in group AN showed titers below the sensitivity limit (<3 U/ml). However, 52 (51.0%) of 102 patients in group AP showed negative-high titers, between 3 and 9.9 U/ml. The prevalence of patients with negative-high titers of the anti-*H. pylori* antibody in group AP was greater than that in group AN; however, it was not statistically significant ($p = 0.33$).

Endoscopic evaluation of corpus atrophy

All 7 patients in group AN and 74 patients in group AP were subjected to endoscopic assessments of corpus atrophy based on the Kimura and Takemoto classification [13]. As shown in Table 3, the 7 patients in group AN showed C0/C1, indicating no atrophic change in the gastric corpus. The prevalence of atrophic gastritis was higher in group AP than in group BCD ($p = 0.025$). In group AP, C2/C3 was recognized in 14 patients (19%) and open type (O1–O3) was observed in 60 (81%) of 74 patients. In group BCD, C2/C3 was recognized in 16 patients (8.9%) and open type (O1–O3) was observed in 162 (91.1%) of 178.

Serum levels of PG in each group

We evaluated the serum PG levels in each group. As shown in Table 4, the median PG I levels in group AN, group AP, and group BCD were 71.6, 40.25, and 39.0 ng/ml, respectively. PG I levels in group AN were higher than those in groups AP and BCD; however, they were not significantly different ($p = 0.0577, 0.0592$). PG II levels in group AP (median, 8.35 ng/ml) were statistically lower than those in group BCD (median, 17.8 ng/ml) ($p < 0.0001$). PG I/II ratios in group AN (median, 5.9) were statistically higher than those in group AP (median, 4.41) and in groups BCD (median, 2.1) ($p = 0.0015, p < 0.0001$). The PG I/II ratios in group AP were statistically higher than those in group BCD ($p < 0.0001$).

Discussion

In Japan, as prevention against gastric cancer death, a mass screening program using radiographic or endoscopic examination has been conducted. *H. pylori* infection and CAG are well-known risk factors for gastric cancer. *H. pylori* infection rates have decreased recently in Japan; the identification of individuals at a high risk for gastric cancer enables establishing efficient prevention strategies.

Table 2 Anti-*Helicobacter pylori* antibody titer

Anti- <i>H. pylori</i> antibody titer (U/ml)	Group AN (n = 7)	p value vs. AP	p value vs. BCD	Group AP (n = 102)	p value vs. BCD	Group BCD (n = 183)
<3	6 (85.7%)	0.33	<0.0001	50 (49.0%)	<0.0001	4 (2.0%)
$3 \leq, <10$	1 (14.3%)			52 (51.0%)		6 (3.0%)
$10 \leq$	0			0		173 (94.5%)

n lesion numbers

Table 3 Endoscopic evaluation of corpus atrophy

	Group AN (n = 7)	p value vs. AP	p value vs. BCD	Group AP (n = 74)	p value vs. BCD	Group BCD (n = 178)
Closed type						
C0	6 (85.71%)	<0.0001	<0.0001	0	0.0255	0
C1	1 (14.29%)			0		0
C2	0			4 (5.41%)		1 (0.56%)
C3	0			10 (13.51%)		15 (8.43%)
Open type						
O1	0			16 (21.62%)		46 (25.84%)
O2	0			30 (40.54%)		94 (52.81%)
O3	0			14 (18.92%)		22 (12.36%)

n lesion numbers

Table 4 Serum levels of pepsinogen (PG)

	Group AN (<i>n</i> = 7)	<i>p</i> value vs. AP	<i>p</i> value vs. BCD	Group AP (<i>n</i> = 102)	<i>p</i> value vs. BCD	Group BCD (<i>n</i> = 183)
PG I (ng/ml)		0.0577	0.0592		0.2236	
Average	123.3			51.8		46.4
SD	138.1			44.2		41.0
Median	71.6			40.25		39.0
PG II (ng/ml)		0.4246	0.6625		<0.0001	
Average	19.6			11.6		22.1
SD	20.14			9.5		20.7
Median	12.8			8.35		17.8
PG I/II ratio		0.0155	<0.0001		<0.0001	
Average	6.20			5.03		2.27
SD	0.80			3.17		1.32
Median	5.90			4.41		2.10

n lesion numbers

The serological risk prediction system combining the PG test and anti-*H. pylori* antibody testing is widely available to large populations. In this system, subjects are classified into four groups (A, B, C, D), and CAG as a high-risk group for gastric cancer is diagnosed using the PG test and anti-*H. pylori* antibody serology. In particular, group A patients (*H. pylori* negative and PG negative) should have an *H. pylori*-uninfected status.

It seems plausible that the ABC classification system in combination with radiographic or endoscopic examinations would contribute to the establishment of a more efficient screening program for gastric cancer by concentrating on the high-risk population. However, the ABC classification system has still unfortunately lacked sufficient evidence to reduce mortality from gastric cancer. Therefore, the Japanese guidelines for gastric cancer screening in 2014 have not yet recommended the introduction of the ABC classification system into the population-based screening program [15]. In these guidelines, nevertheless, endoscopic examinations as well as a radiographic screening are newly recommended as elements of population-based screening. In the near future in Japan, endoscopic screening for gastric cancer would increase in a population-based program. The ABC classification system is important for the effective selection of subjects to undergo endoscopic screening. However, the diagnostic accuracy used to discriminate the *H. pylori*-uninfected population that is truly at low risk is a limitation of the screening process.

Group A subjects are regarded as *H. pylori* uninfected and as having a low risk for gastric cancer development. Therefore, it is likely that these subjects may be excluded from the gastric cancer mass screening system [16]. Indeed, in our study, we found that only seven patients with gastric cancer were subclassified into group AN. As demonstrated in previous reports, gastric cancer patients in

group AN were younger than those in other groups, and the histological features of their gastric cancer were predominantly the undifferentiated type [11]. Recently, fundic gland-type cancer was reported to be a representative feature of *H. pylori*-uninfected gastric cancer [17]. However, fundic gland-type cancer was not included in our recent study, suggesting this is not a major part of *H. pylori*-uninfected gastric cancer. Concerning endoscopic or serum markers, none of the seven patients showed endoscopic atrophy in the corpus, and six of seven were below the measurement sensitivity (<3.0) in anti-*H. pylori* antibody titers.

On the other hand, Boda et al. reported that more than 10% of gastric cancer patients were classified into group A, suggesting that some patients with gastric cancer risk may be included in group A [12]. Recently, Itoh et al. also reported the aberration of high-risk subjects in group A by using radiologic findings [18]. In the present study, in the multicenter study we conducted in Japan, we clarified that (1) some gastric cancer patients were found in group A and (2) that only approximately 7% of all gastric cancer patients in group A were regarded as *H. pylori* uninfected. These findings suggest that some high-risk patients for gastric cancer may be included in group A, even though testing negative for *H. pylori* and CAG, and this is a crucial issue for applying the ABC system for a cancer mass screening program. False-negative results of anti-*H. pylori* antibody or PG tests may provide a reason for this discrepancy. It is well known that PG levels are influenced by use of PPIs [19]. In spite of careful by interviewing, some PPI users may have been included in this study. Serum PG levels will increase with PPI use or renal failure, which must not be neglected in the medical interview.

As already mentioned, the majority of group A patients with gastric cancer were classified into group AP. The

clinicopathological features of group AP were as follows: compared with group AN, they were older, had predominantly differentiated-type cancer, endoscopically visualized CAG, and a lower PG I/II ratio. Also, as compared with the BCD group, the depressed-type tumor was dominant, PG II levels were lower, and PGI/II ratios were higher. Group AP had CAG and mild inflammation without anti-*H. pylori* antibody. These results strongly suggest that these characteristics resemble those typical of patients after successful eradication therapy of *H. pylori*. Group AP subjects might already have unrecognized *H. pylori* eradication.

In subjects who have previously undergone eradication therapy, anti-*H. pylori* antibody and PG tests show negative results; therefore, these must be excluded from the serological predictive test. To decrease the proportion of patients in group AP, a medical interview discussing eradication is important. However, patients with natural or accidental *H. pylori* eradication (by antibiotics used for infectious diseases other than *H. pylori* infection) may not be recognized using a standard interview. The prevalence of accidentally eradicated patients among healthy Japanese subjects is unknown, but we should recognize that there may be a certain number of such patients. To identify these subjects, endoscopic or radiologic evaluation of their gastric mucosal status might be helpful.

The precise diagnosis of patients in group AN (*H. pylori*-uninfected group) is necessary to reduce the false-negative evaluation of gastric cancer risk. A previous report suggested that the subjects whose anti-*H. pylori* antibody titers were between 3 and 9.9 should be treated as having a high risk [18]. In addition, these subjects should receive gastrointestinal endoscopic examinations. Also, in the PG test, an advanced cutoff level [PGII $\geq$ 10 or PG I/II ratio $\leq$ 5] was reported to improve the accurate diagnosis of *H. pylori*-induced CAG [20].

There are some limitations to this study. The use of PPIs may be the most likely concern. Another problem is the determination of *H. pylori* status in our subjects. For the precise determination of *H. pylori* status, more than two tests should be carried out in each subject. In this study, we mainly used the serum anti-*H. pylori* antibody titer test and the endoscopic findings.

When we introduce the ABC classification system for gastric cancer mass screening, all should be aware of the presence of gastric cancer patients in group A who are wrongly judged as having no risk for gastric cancer development. In group A patients with gastric cancer, only 7% of patients were *H. pylori* uninfected. This is a crucial problem in the current ABC classification system, and a revised method should be proposed as soon as possible.

Compliance with ethical standards

Conflict of interest No author has conflicts of interest or financial arrangements that could potentially influence the described research.

Competing interests The authors have no competing interests.

Human rights statement and informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Declaration of Helsinki of 1964 and later versions. Informed consent or a substitute for it was obtained from all patients before they were included in the study.

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