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ORIGINAL ARTICLE

The Relationship Between Virulence Factors of *Helicobacter pylori* and Severity of Gastritis in Infected Patients

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Abstract The outcome of *Helicobacter pylori* infection has been related to specific virulence-associated bacterial genotypes. The best known genotypic virulence factors of *H. pylori* are cytotoxin-associated gene A (cagA) and vacuolating cytotoxin gene A (vacA). The objective of this study was to assess the relationship between *H. pylori* cagA and vacA status and histopathological findings. Esophagogastrodoedonoscopy was performed in 80 dyspeptic patients. Antrum and corpus biopsies were obtained for isolation of *H. pylori* and for histopathological assessment. The polymerase chain reaction was used to detect cagA and vacA genes of *H. pylori* using specific primers. Biopsy samples were stained with hematoxylin and eosin, and histopathological findings were graded using the "updated Sydney system". *H. pylori* from 57 of the 80 patients was incubated. Of the 57 patients, 44 were cagA positive. In the corpus biopsy specimens there was a significant relationship between the density of *H. pylori* colonization ($P = 0.02$) and chronic inflammation ($P = 0.02$) and cagA-positive genotypes. In the antrum specimens there was a significant relationship between cagA positivity and neutrophil activity ($P = 0.003$) and glandular atrophy ($P = 0.002$), but not with *H. pylori* density, chronic inflammation, and intestinal metaplasia. The odds ratio of cagA-positive vs. cagA-negative strains for the presence of glandular atrophy, irrespective of grading and of gastric localization, was 4.62 (95% CI, 1.18–18.08, $P = 0.041$). No significant relationships were observed between vacA s1 and s2 genotypes and histopathological parameters. Corpus neutrophil infiltration was found to be more severe in the m1 group than in the m2 group ($P = 0.004$). Other histopathological features showed no difference between m1 and m2 genotypes. In conclusion *H. pylori* strains showing cagA positivity are associated with more severe gastritis in some histological features but virulence factors of *H. pylori* do not appear to determine the overall pattern of gastritis.

Keywords *Helicobacter pylori* · Gastritis · Bacterial virulence factors · cagA · vacA

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Introduction

Helicobacter pylori is a spiral, microaerophilic, Gram-negative bacterium that permanently colonizes gastric epithelial cells in approximately 25% of the population in developed countries and 70–90% in developing countries [1]. Whereas most infected individuals are asymptomatic, chronic *H. pylori* infection in susceptible individuals is associated with a variable degree of mucosal damage ranging from mild gastritis and ulcer disease to gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [2, 3]. The clinical outcome of *H. pylori* infection has been associated with bacterial virulence factors, host gastric mucosal factors, and the environment [4].

The two best studied bacterial determinants of *H. pylori* infection are the presence of cytotoxin-associated gene A and vacuolating cytotoxin genotype. Cytotoxin-associated

gene A encodes a high-molecular-weight immunodominant protein. This cagA gene product is not itself a virulence factor but is part of a 40 kb cluster of genes (cag pathogenicity island), some of which contribute to pathogenicity [5]. A number of studies in Western countries have confirmed that infection with cagA-positive strains is associated with more severe gastritis and higher prevalence of peptic ulcer and gastric cancer [6, 7]. However, studies in Far Eastern countries demonstrate equally high prevalence of cagA-positive strains in patients with peptic ulcer, gastric cancer, and nonulcer dyspepsia and in control subjects [8]. In Turkey, cagA positivity and the clinical outcome of *H. pylori* infection has been studied. In these studies, a relationship has been demonstrated between peptic ulcer and gastric cancer and cagA positivity, similar to Western countries [9, 10].

The vacuolating cytotoxin A gene, which is another important virulence factor of *H. pylori*, encodes an 87 kD protein that induces vacuolation of epithelial cells [11]. The vacA gene is present in all strains of *H. pylori* and comprises two variable parts. The *H. pylori* strains have one of two types of vacA signal sequence (s1 and s2) and two types of mid region (m1 and m2) [12, 13]. The mosaic combination of s and m region allelic types determines the production of the cytotoxin and is associated with pathogenicity of the bacteria [3, 14]. As with cagA status, there are geographic differences between vacA status and the *H. pylori*-related diseases. In Western countries infection with vacA s1 strain is more common in patients with peptic ulcer than in those with chronic gastritis. However in Asian populations, the association between vacA diversity and clinical outcome is not established [15, 16]. In a study from Turkey, the vacA s1a genotype was detected in 66.7, 96.4, and 87.9% of isolates from patients with functional dyspepsia, duodenal ulcer, and gastric cancer, respectively [17]. The objective of this study was to research gastric histopathology in Turkish dyspeptic patients infected with *H. pylori* and to assess its relationship with bacterial virulence-associated cagA and vacA genotypes.

Materials and Methods

Patients

A total of 57 *H. pylori* isolates were obtained from gastric biopsies from Turkish patients who underwent upper gastrointestinal endoscopy. Patients with a history of gastric surgery, active gastrointestinal bleeding, or use of steroids, immunosuppressive drugs, antibiotics, bismuth compounds, or proton pump inhibitors were excluded. Patients with duodenal ulcer, gastric ulcer and gastric cancer were also excluded from the study. All patients provided written informed consent for this study. The ethical committee of Trakya University approved the study protocol.

H. pylori Culture, Preparation of Genomic DNA and Polymerase Chain Reaction (PCR)-Based Genotyping

The biopsy specimens were spread with an applicator and were cultured under microaerophilic conditions (GenBox Microaer; Biomerieux, France) on special culture media (Pyloriagar; Biomerieux) containing horse serum, antibiotic mixture, and polyvitex for up to 5 days. The organisms were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase, and urease reactions.

For *H. pylori* chromosomal DNA extraction, bacteria were harvested in phosphate buffered saline solution and centrifuged. The pelleted cells were resuspended in 200 µl proteinase K solution containing 5 mmol/l ethylenediaminetetraacetic acid, 0.5% sodium dodecylsulfate, and 10 mg/ml proteinase K in 10 mmol/l Tris–HCl and subsequently incubated at 50°C for 2 h. The DNA was extracted with phenol–chloroform–isoamyl alcohol by the standard procedure, and was finally precipitated by adding 1/10 vol 3 mol/l sodium acetate and 2.5 vol ethanol. After centrifugation, the pellet was washed with 70% ethanol and then dissolved in 50 µl TE buffer (10 mmol/l Tris–HCl, pH 8.0; 5 mmol/l EDTA, pH 8.0).

PCR reactions were performed in a volume of 100 µl containing 71.5 µl distilled water, 10 µl PCR buffer, 2 µl deoxynucleoside triphosphate, 0.5 µl Taq polymerase, 1 µl BSA, and 5 µl of both forward and reverse primers. CAGAF and CAGAR primers were used to amplify a 349-bp product from the middle conservative region of the cagA gene. VAG-F and VAG-R primers were used to amplify a 570-bp product for m1 and 645-bp products for m2 from the middle of the vacA gene. VA1-F and VA1-R primers were used to amplify a 259-bp product for s1 and 286-bp products for s2 from the signal sequence of the vacA gene [6, 16]. PCR primers used in this study are listed in Table 1. Thermal cycling for each set of primers was performed as previously described [16].

Histopathology

Two gastric biopsy specimens, one from the antrum and one from the corpus, were immersed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Only cases with adequate sized biopsy specimens of both antral and corpus mucosa were accepted for histological assessment by an experienced pathologist. The pathologists were unaware of the clinical information

Table 1 Primers used in PCR for amplification of cagA and vacA sequences

DNA region amplified	Primer	Primer sequence	PCR products (bp[a])
cagA	CAGAF	5′-GATAACAGGCAAGCTTTTGAGG-3′	349
	CAGAR	5′-CTGCAAAAGATTGTTTGGCAGA-3′	
vacA-m1	VAG-F	5′-CAATCTGTCCAATCAAGCGAG-3′	570
	VAG-R	5′-GCGTCAAAATAATTCCAAGG-3′	
vacA-m2	VAG-F	5′-CAATCTGTCCAATCAAGCGAG-3′	645
	VAG-R	5′-GCGTCAAAATAATTCCAAGG-3’	
vacA-s1	VA1-F	5′-ATGGAAATACAACAAACACAC-3′	259
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′	
vacA-s2	VA1-F	5′-ATGGAAATACAACAAACACAC-3′	286
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′	

[a] Base pair

of each patient. *H. pylori* density, chronic inflammation, neutrophil activity, glandular atrophy, and intestinal metaplasia were scored on an ordinal scale (0–3) using the criteria described in the updated Sydney classification system [18].

Statistical Analysis

Data were analyzed in Minitap release 13 (Licence Number: wcp1331.00197). Chi-square test and Fisher's exact test were used to assess the relationship between individual genotypes. Histopathological parameters were scored on ordinal scales (from 0 to 3) and analyzed by the Mann–Whitney test. Differences were regarded as statistically significant when the *P*-value was ≤ 0.05.

Results

Antral biopsies were obtained for *H. pylori* culture from 80 functional dyspeptic patients who had undergone upper gastrointestinal endoscopy with positive rapid urease test. *H. pylori* was isolated successfully from 57 of the 80 patients' specimens (71.2%). *H. pylori* was histopathologically demonstrated in just 53 of the 57 culture-positive patients. A total of 57 culture positive patients (30 men and 27 women), with a mean age 44.9 years (range 20–72 years) were included in our study.

A total of 44 of the 57 *H. pylori* isolates were cagA positive (77.2%). There was no association between cagA status and the mean ages or genders of the patients. In typing of the vacA gene m region, 23 *H. pylori* isolates were vacA m1 and 30 were vacA m2. In three isolates, genotyping could not be performed and in one isolate, both m1 and m2 genotypes were observed. These four isolates were excluded from the statistical analysis. Evaluation of the vacA signal region revealed that 32 isolates were s1 and 18 were s2, while seven showed more than one signal region. These seven isolates with multiple signal regions were excluded from the analysis.

H. pylori Genotypes and Histopathological Findings

Relationships between histological parameters determined in gastric biopsy specimens and *H. pylori* cagA status, and vacA s and m genotypes are shown in Tables 2–4, respectively.

H. pylori Density

The density of *H. pylori* was scored in antral and corpus biopsy specimens. The density scores of *H. pylori* in the corpus biopsies of the cagA-positive isolates were higher than in the cagA-negative isolates ($P = 0.02$). There was no significant relationship between cagA positivity and *H. pylori* density in the antrum. No relationships were found between vacA s or m regions and *H. pylori* density in either the antrum or corpus.

Neutrophil Activity

cagA-positive genotypes were strongly associated with higher activity in the antrum ($P = 0.003$) but not in the corpus. The activity was higher only in the corpus with the vacA m1 genotype ($P = 0.004$). No relationships were found between activity and vacA s genotypes in the antrum or corpus.

Chronic Inflammation

There was an association between cagA positivity and high inflammation scores in both corpus and antrum biopsy specimens, but only in corpus biopsies did statistical analysis show significance ($P = 0.017$). There was no relationship between vacA s or m genotypes and chronic inflammation in either corpus nor antrum biopsies.

Table 2 Relationship between histological parameters determined in gastric biopsy specimens and *H. pylori* cagA status

Parameter	Localization	cagA positive[a]		cagA negative[a]		*z*	*P*[b]
		Mean ± SD	Median (range)	Mean ± SD	Median (range)		
H. pylori density	Antrum	1.50 ± 0.85	1 (0–3)	1.55 ± 1.04	2 (0–3)	−0.2	0.84
	Corpus	**1.33 ± 0.86**	**1 (0–3)**	**0.70 ± 0.95**	**0.5 (0–3)**	**−2.2**	**0.02**
Neutrophil infiltration	**Antrum**	**0.75 ± 0.81**	**1 (0–3)**	**0.08 ± 0.28**	**0 (0–1)**	**−2.9**	**0.003**
	Corpus	0.30 ± 0.60	0 (0–2)	0.15 ± 0.38	0 (0–1)	−0.7	0.497
Chronic inflammation	Antrum	1.33 ± 0.71	1 (0–3)	1.00 ± 0.82	1 (0–3)	−1.6	0.11
	Corpus	**1.02 ± 0.64**	**1 (0–3)**	**0.53 ± 0.78**	**0 (0–2)**	**−2.4**	**0.017**
Glandular atrophy	**Antrum**	**0.93 ± 0.87**	**1 (0–3)**	**0.31 ± 0.63**	**0 (0–2)**	**−2.4**	**0.02**
	Corpus	0.31 ± 0.72	0 (0–3)	0.31 ± 0.72	0 (0–2)	−0.1	0.89
Intestinal metaplasia	Antrum	0.26 ± 0.77	0 (0–3)	0.17 ± 0.58	0 (0–2)	−0.4	0.72
	Corpus	0.19 ± 0.67	0 (0–3)	0.23 ± 0.83	0 (0–3)	−0.2	0.87

[a] The histopathological parameters were scored as: 0, none; 1, mild; 2, moderate; 3, severe

[b] Median scores were compared with the Mann–Whitney test. Mean scores are presented for reader orientation

Bold values indicate statistically significant differences between cagA-positive and cagA-negative groups

Table 3 Relationship between histological parameters determined in gastric biopsy specimens and *H. pylori* vacA s region genotypes

		Antrum					Corpus				
		Hp	PNL	CI	GA	IM	Hp	PNL	CI	GA	IM
vacA s1	Mean[a]	1.64	0.63	1.23	0.63	0.23	1.28	0.19	0.81	0.20	0.13
	Median (range)	2 (0–3)	0 (0–2)	1 (0–3)	0.5 (0–2)	0 (0–3)	1 (0–3)	0 (0–2)	1 (0–2)	1 (0–1)	1 (0–3)
vacA s2	Mean	1.18	0.50	1.17	0.78	0.28	1.07	0.44	1.06	0.56	0.39
	Median (range)	1 (0–2)	0 (0–2)	1 (0–2)	0.5 (0–2)	0 (0–3)	1 (0–3)	0 (0–2)	1 (0–3)	1 (0–3)	1 (0–3)
P[a]		0.11	0.56	0.95	0.63	0.89	0.36	0.15	0.39	0.38	0.27

[a] Median scores were compared with the Mann–Whitney test. Mean scores are presented for reader orientation

Hp, *H. pylori* density; PNL, polymorphonuclear leucocyte infiltration; CI, chronic inflammation; GA, glandular atrophy; IM, intestinal metaplasia

Table 4 Relationship between histological parameters determined in gastric biopsy specimens and *H. pylori* vacA m region genotypes

		Antrum					Corpus				
		Hp	PNL	CI	GA	IM	Hp	PNL	CI	GA	IM
vacA m1	Mean[a]	1.58	0.78	1.27	0.96	0.38	1.10	**0.52**	1.09	0.41	0.27
	Median (range)	2 (0–3)	1 (0–2)	1 (0–2)	1 (0–2)	0 (0-3)	1 (0–3)	**0 (0–2)**	1 (0–3)	0 (0–3)	0 (0–3)
vacA m2	Mean	1.50	0.50	1.30	0.73	0.10	1.30	**0.07**	0.76	0.17	0.07
	Median (range)	1 (0–3)	0 (0–3)	1 (0–3)	0 (0–3)	0 (0–2)	1 (0–3)	**0 (0–1)**	1 (0–2)	0 (0–1)	0 (0–1)
P[a]		0.63	0.14	0.90	0.27	0.35	0.34	**0.004**	0.12	0.54	0.71

[a] Median scores were compared with the Mann–Whitney test. Mean scores are presented for reader orientation

Hp, *H. pylori* density; PNL, polymorphonuclear leucocyte infiltration; CI, chronic inflammation; GA, glandular atrophy; IM, intestinal metaplasia

Bold values indicate statistically significant differences between cagA-positive and cagA-negative groups

Glandular Atrophy

There was a strong association between the presence of glandular atrophy in the antrum and individual cagA-positive genotypes, compared to the cagA-negative genotypes ($P = 0.02$). The same relationship could not be observed in the corpus biopsy specimens. No association was found between vacA genotypes and the presence of glandular atrophy.

We investigated the relationship between cagA positivity and atrophy in any localization and degree. Atrophy was observed in any localization and degree in 69.8% of

the cagA-positive and 33.3% of the cagA-negative cases. The odds ratio of cagA-positive vs. cagA-negative strains for the presence of glandular atrophy, irrespective of grading and of gastric localization, was 4.62 (95% CI, 1.18–18.08, $P = 0.041$).

Intestinal Metaplasia

The presence of intestinal metaplasia was not associated with cagA or vacA genotypes in the corpus or antrum biopsies.

All histological parameters determined in antral and corpus biopsy specimens according to the updated Sydney system and *H. pylori* cagA status are shown in Figs. 1 and 2.

Discussion

H. pylori infection results in chronic gastritis and, eventually, diseases, such as peptic ulcer, gastric cancer, and MALT lymphoma [1, 19, 20]. Genotypic alterations of *H. pylori* are thought to be responsible for the various clinical manifestations and for infection without symptoms or with symptoms of gastric carcinoma and MALT lymphoma. In our study, the presence of cagA, which is thought to be associated with severe diseases, was investigated in patients with functional dyspepsia and compared with histological findings. The prevalence of cagA expression in *H. pylori* strains is reported as 60% with geographic variation. The positivity of cagA shows variation within nationalities and states. cagA positivity is reported as 61.7–80.4% in our country; in our studies cagA positivity was 77.1% in patients with functional dyspepsia [21, 22].

Although an association between *H. pylori* infection and chronic gastritis is clear, development of severe gastric diseases is rare. These variations in the clinical consequences are because of factors such as duration of the infection, inflammatory response of the patient, virulence of *H. pylori* strains, etc. Infection with less virulent strains is associated with mild symptoms whereas infection with more virulent strains is thought to be associated with more severe gastric inflammation and, eventually, peptic ulcer, gastric adenoma, and MALT lymphoma. Gastric gland atrophy, intestinal metaplasia, and dysplasia may develop after prolonged *H. pylori* infection. Compared with the rest of the population, the risk of development of gastric cancer in patients with duodenal ulcer is low. Two different pathways—duodenal ulcer or gastric ulcer with atrophy, intestinal metaplasia and cancer—may be associated with *H. pylori* infection. The relationship between *H. pylori* genotypes and infection with gastric inflammatory response varies within nationalities. In Western countries it has been demonstrated that more severe gastric inflammation develops after infection with cagA-positive strains; in Asian countries there is no such difference. Studies from Turkey on cagA positivity and the histopathological findings of gastritis led to conflicting results. Demirturk et al. suggested that cagA positivity is associated with more severe glandular atrophy, inflammation, and activity, whereas Saruc et al. demonstrated a relationship between cagA positivity with inflammation, *H. pylori* density, and intestinal metaplasia but not with glandular atrophy [21, 23]. In our study, cagA-positive patients showed more severe neutrophil infiltration and chronic inflammation in both the antrum and the corpus. Nevertheless, only the activity of the gastritis in the antrum and the chronic inflammation in the corpus were statistically significant.

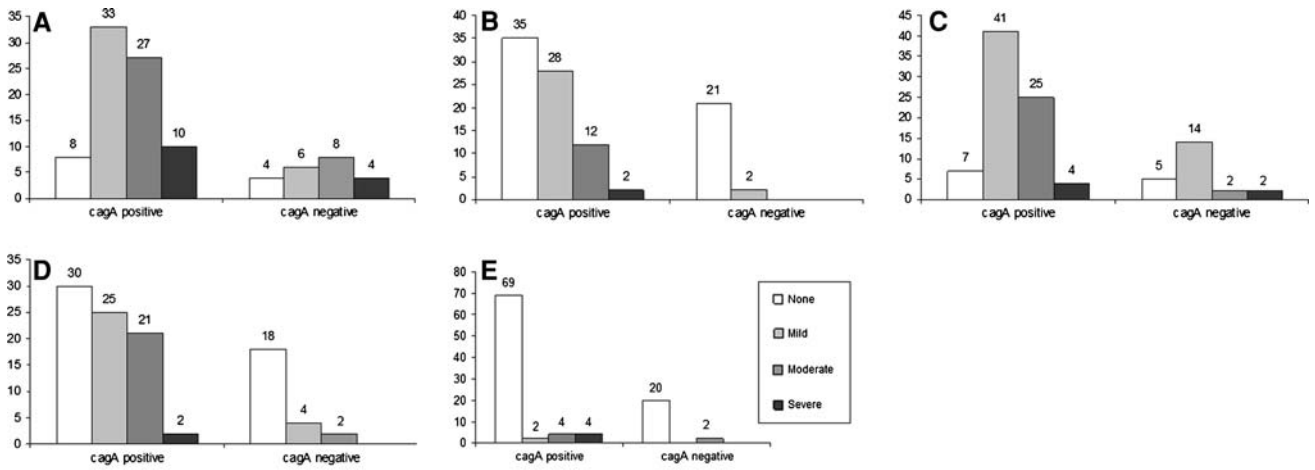

Fig. 1 Histological parameters determined in antral biopsy specimens and *H. pylori* cagA status. *H. pylori* density (**a**), neutrophil activity (**b**), chronic inflammation (**c**), glandular atrophy (**d**), and intestinal metaplasia (**e**) are illustrated

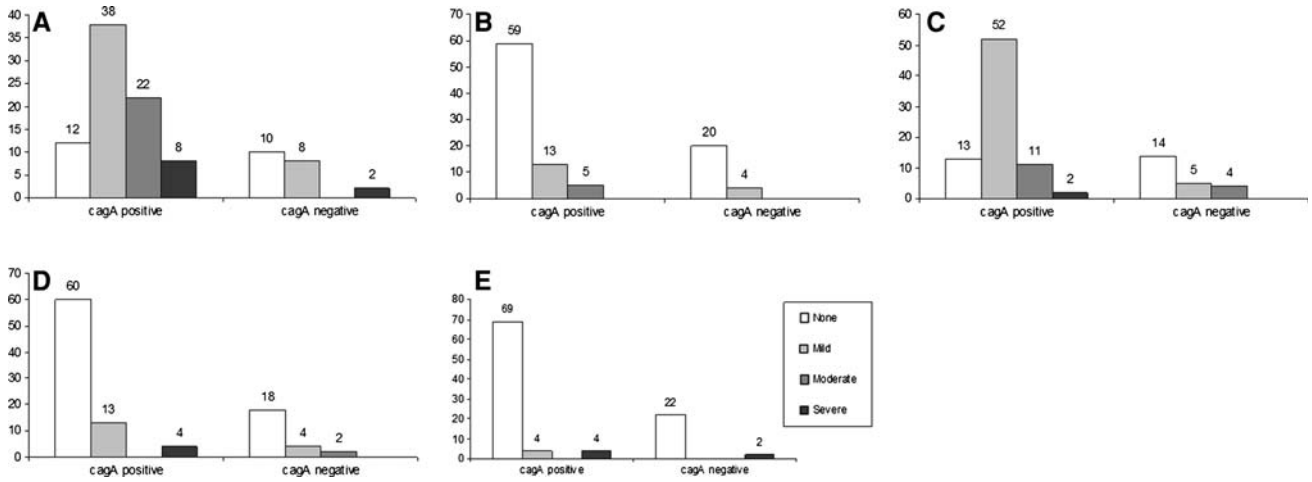

Fig. 2 Histological parameters determined in corpus biopsy specimens and *H. pylori* cagA status. *H. pylori* density (**a**), neutrophil activity (**b**), chronic inflammation (**c**), glandular atrophy (**d**), and intestinal metaplasia (**e**) are illustrated

The rate of the glandular atrophy and the *H. pylori* density were found to be significant in cagA-positive subjects.

Atrophic gastritis and intestinal metaplasia are important aspects of the development of gastric cancer. The association between *H. pylori* and chronic superficial gastritis, atrophic gastritis, and intestinal metaplasia is already known. Because prolonged *H. pylori* colonization is related to distal gastric adenocancer and *H. pylori* is accepted as a carcinogen type-1, it is reported in the Maastricht-III Concensus that patients with *H. pylori* infection with atrophic gastritis should definitely receive therapy [24]. Furthermore, despite the fact that most of the population is infected with *H. pylori*, the development of gastric adenocancer is rare.

Modes of treatment of *H. pylori* vary, because the infection is frequently asymptomatic yet is also related to severe outcomes, such as gastric cancer. Especially in patients with functional dyspepsia, the decision to eradicate *H. pylori* remains unclear. According to the Maastricht-2 report, patients with functional dyspepsia should certainly not receive eradication therapy. Nevertheless, patients with severe gastritis (atrophia, etc.) should receive medication. The willingness of the patient to undergo medication is accepted as an indication, because *H. pylori* type-1 is known to be a carcinogen and the prevalence is increasing within the population [25]. There are no proven data that eradication therapy is effective for the symptoms of the patients with functional dyspepsia. As a result, more objective data are needed for the decision to give medication to patients with functional dyspepsia.

In our study, relation with cagA positivity and atrophy in the gastric antrum or corpus is demonstrated in patients with functional dyspepsia and *H. pylori* infection (odds ratio: 4.62; 95% CI, 1.18–18.08, $P = 0.041$). It has recently become possible to detect antibodies for cagA proteins with ELISA methods [8, 26]. Along with isolation of *H. pylori*, demonstration of cagA positivity with non-invasive methods could be helpful in some patient groups. Studies and data are needed for evaluation of sensitivity and specificity of the non-invasive methods for showing functional cag-PAI.

The variability of the vacA s and m regions is thought to have effects over the secretion of vacuolating toxin. s1/m1 strains produce huge amounts of toxins and are highly detrimental. s2 strains do not produce such toxins. In many studies, the variability of the s genotypes is associated with disease formation but not proven. In our study, we did not find any statistically significant relationships between vacA signal region genotype and *H. pylori* density in the mucosa of the antrum and the corpus, neutrophil infiltration, chronic inflammation, glandular atrophy, and intestinal metaplasia. vacA signal region genotype and gastric inflammatory alterations show regional variations. Warburton et al. reported no relationship between vac s1 and s2 regions and histological findings, similar to our results [27]. This finding is controversial, because it is known that strains with s1 genotype produce much greater amounts of toxins. Subtypes of vacA s1 region (s1a, s1b) may be closely related to histological parameters. Nevertheless, Atherton et al. suggested that s1a genotype is associated with peptic ulcer and more severe gastritis [28].

There are limitations to our study. For example, genotyping was performed only on *H. pylori* strains obtained from antrum biopsies. For this reason, there may be no absolute relationship between the histopathological changes of the corpus mucosa and *H. pylori* genotypes, for there may be different strains in the corpus and the antrum. However, the false-negative rates are lower in the biopsies

obtained from the antrum, because the antrum is accepted as the major region of settlement of *H. pylori*.

Besides the cagA and vacA genotypes, variations of acid production, the genetics of the infected subject, tobacco and alcohol intake, and other virulence factors of *H. pylori* may affect both clinical outcomes and the histopathological findings. More studies are needed to evaluate other factors besides the *H. pylori* genotypes, because *H. pylori* is thought to be a cause of serious diseases.

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