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Relation of CagA seropositivity to *cag*PAI phenotype and histological grade of gastritis in patients with *Helicobacter pylori* infection

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Abstract

AIM: Infection with *Helicobacter pylori* (*H pylori*) possessing the *cag* pathogenicity island (PAI) has been associated with severe clinical outcome and CagA-antibody has been used to indicate *cag*PAI-positive infection. The aim of this study was to examine the accuracy of CagA seropositivity to indicate the virulence of the *cag*PAI in Japan.

METHODS: Sixty isolates of *H pylori* cultured from gastric biopsies were examined by polymerase chain reaction assays for the presence of *cagA*, *cagE* and *VirD4*. Anti-CagA IgG antibody in matching sera was tested by both ELISA and immunoblot assay. Histological grade of gastritis was graded according to the updated Sydney System.

RESULTS: Amongst 53 patients infected with *cagA*+/*cagE*+/*VirD4*+ strain, 38 were CagA seropositive. There were four patients infected with strains possessing incomplete *cag*PAI. Two out of three patients with *cagA*+/*cagE*-/*VirD4*- infection were CagA seropositive, while a patient with *cagA*-/*cagE*+/*VirD4*+ infection was CagA seronegative. Accuracy of ELISA to predict bacterial possession of *cagA* was 61.7% whereas 58.3% for *cagE* and *VirD4*. The immunoblot assay showed relatively higher sensitivity and showed better accuracy. The lower grade of gastric mucosal inflammatory infiltration was seen in false CagA-seronegative patients.

CONCLUSION: Some serodiagnosis does not seem to have enough accuracy to indicate virulence of *cag*PAI, particularly in infection of strains with incomplete *cag*PAI. The degree of gastric mucosal inflammation may affect the results of CagA serodiagnosis.

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INTRODUCTION

Infection of *Helicobacter pylori* (*H pylori*) has been implicated in the pathogenesis of gastro-duodenal diseases. *H pylori* is highly diverse genetically, and certain genotypes have been associated with severe clinical outcome. To date, strains possessing the *cag* pathogenicity island (PAI) are the most-known virulent phenotype[1]. The *cag*PAI is a region of 40-kb DNA that contains about 30 genes and encodes a type IV secretion system[2]. The *cagA* gene is one of the genes within the *cag*PAI and the most investigated gene of *H pylori*[3,4]. The *cagA* gene encodes CagA protein, which is delivered into gastric epithelial cells where it is phosphorylated[2,5]. Phosphorylated CagA is considered to play a significant role in the gastric carcinogenesis in *H pylori*-infected mucosa[6]. On the other hand, other genes within the *cag*PAI are more relevant in gastric epithelial IL-8 production. Infection with *cag*PAI-positive *H pylori* strongly stimulates gastric epithelial cells to synthesize interleukin (IL)-8[1,7]. Other genes in *cag*PAI participate in gastric epithelial IL-8 production stimulated by *H pylori*[1,3,8]. IL-8 production is correlated to the degree of neutrophil infiltration in the gastric mucosa infected with *H pylori*[9]. Therefore, it is clinically important to know whether infected *H pylori* possesses whole *cag*PAI or not. *cagE* and an *Agrobacterium VirD4* homologue are one of the genes inside the cagI and cagII loci of *cag*PAI, respectively. Absence of these genes indicates that the strain does not have whole *cag*PAI.

The presence of anti-CagA antibody has been widely used to indicate *cag*PAI-positive infection. However, recent Western studies have shown that CagA serology was not able to predict severity of diseases, while genetic *cag* status was useful[10,11]. These results might be associated with diversity of the *cag*PAI. *cagA* is possessed by 50-70% of *H pylori* isolates in Western countries, while most strains possess *cagA* in Japan[12]. Therefore, association between CagA serology and the genetic diversity in the *cag*PAI would be different in Japan from those in Western populations. It is also necessary to understand why false seronegative results occur in patients infected with *cagA*-positive strain. Several factors, such as bacterial colonization or host recognition of CagA, are possible causes for false seronegativity.

In the present study, we examined the association between

CagA serology and the structure of the *cag*PAI to examine the accuracy of CagA serology to indicate the virulence of *cagA*- or *cag*PAI-positive *H pylori* in Japan. Furthermore, we compared the histologic grade of gastritis between CagA seropositive and seronegative patients who were infected with *cagA*-positive *H pylori* strain.

MATERIALS AND METHODS

Patients

Patients scheduled for upper gastro-intestinal endoscopy for routine screening for gastric cancer at Hirosaki University Hospital were enrolled in the study. Patients were considered to be eligible for inclusion when their endoscopic diagnosis was normal or atrophic gastritis was present without any evidences of neoplasia. Patients were excluded if they had received at least one of acid suppression agents, anti-ulcer agents and antibiotics during the 2 mo before the examination or had previous histories of gastric tumors and gastric surgery. All subjects provided informed consent before their endoscopy and this study was approved by the ethics committee of Hirosaki University. Peripheral venous blood was obtained at the time of endoscopy and biopsy specimens were also taken from the antrum and the corpus of the stomach. Patients were eligible if *H pylori* was isolated from their biopsy specimens.

H pylori culture

Biopsy specimens were homogenized and cultured for 3-5 d on Skirrow blood agar at 37 ℃. The bacteria were identified as *H pylori* by colony morphology, positive oxidase, catalase, and urease reactions. The clone-picked strains were suspended in 1 mL phosphate-buffered saline (PBS, pH 7.6) for DNA extraction.

DNA preparation and PCR assay

One milliliter of aliquots of bacteria in PBS were centrifuged at 10 000 *g* for 5 min. After the supernatant was discarded, the bacterial pellets were digested with 0.3 units of proteinase K at 55 ℃ for 2 h and DNA was extracted with phenol-chloroform-isoamylalcohol. The concentration and quality of DNA samples were estimated by measuring $A_{260\,nm}/A_{280\,nm}$. The presence of the *cagA*, *cagE*, and *VirD4* homolog were determined by polymerase chain reaction (PCR) using the oligonucleotide primer pairs described previously[13-15]. In brief, after 5 min of denaturing at 94 ℃, 35 cycles of PCR, including denaturing at 94 ℃ for 40 s (*cagA*) or 30 s (*cagE* and *VirD4* homolog), annealing at 50 ℃ for 40 s (*cagA*) or 30 s (*cagE* and *VirD4* homolog), and polymerization at 72 ℃ for 1 min (*cagA*) or 2 min (*cagE* and *VirD4* homolog) were performed. The final cycle included an extension step for 10 min at 72 ℃. Each PCR amplification was performed using 0.1 μg of extracted DNA, which was added to 50 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 200 μmol/L each deoxynucleotide (dNTPs), 100 pmol (*cagA*) or 200 pmol (*cagE* and *VirD4* homolog), and 0.5 units of *Taq* polymerase (TaKaRa *Taq*, Takara Biochemicals, Tokyo, Japan). PCR amplification was performed in duplicate for each DNA sample. PCR products were electrophoresed in 3% (*cagA*) or 2% (*cagE* and *VirD4* homolog) agarose gel. Each sample was also tested for the presence of the 23S rRNA gene by PCR to confirm the existence of *H pylori* DNA as described previously[16].

Serological assays

Serum samples were assayed for the presence of antibodies to CagA. CagA antibodies were assayed by ELISA using a recombinant fragment of CagA as antigen[17]. In brief, flat-bottom 96-well microplates were coated with 0.1 μg/well antigen in 0.1 mol/L bicarbonate buffer for 24 h at 4 ℃. Plates were washed with PBS containing 0.1% Tween-20 and blocked with 1% bovine serum albumin (BSA) in PBS-Tween for 1 h at 26 ℃. Serum samples diluted 1/75 in 1% BSA/PBS were incubated in duplicate for 90 min at 26 ℃. Following incubation with goat anti-human IgG alkaline phosphatase conjugate (Sigma, Poole, Dorset, UK) for 2 h at 26 ℃, bound antibodies were detected with *p*-nitrophenyl phosphate substrate (Sigma) at 1 mg/mL in diethanolamine-MgCl₂ buffer. IgG antibodies to CagA were also measured using Chiron Diagnostics' Recombinant Immunoblot Assay (RIBA™). The relative intensity of antibodies to CagA was determined by comparing the intensity of CagA labeling to that of the human IgG internal control. The cut off for positivity was determined using sera of known *H pylori* status[18].

Histological grading of gastritis

The specimens were embedded in paraffin and stained with hematoxylin and eosin and also Warthin-Starry method. Mononuclear cell (MNC) infiltration, polymorphonuclear cell (PMN) infiltration, glandular atrophy, and the density of *H pylori* were graded into 0-3, according to the updated Sydney System by an experienced pathologist without the knowledge of bacterial genotype.

Statistical analysis

The differences of histological grade of gastritis were tested by Mann-Whitney *U* test. A *P* value of less than 0.05 was considered significant.

RESULTS

H pylori strains and serum samples were obtained from a total of 60 patients (34 males and 26 females, mean age 62.1±11.7 years). Fifty-six of the sixty *H pylori* isolates had *cagA* and three of them did not have both *cagE* and *VirD4*. Four strains did not possess *cagA* and three of them were also negative for *cagE* and *VirD4*. There were four strains, which had incomplete *cag*PAI; three strains were *cagA+/cagE-/VirD4-* and another strain was *cagA-/cagE+/VirD4+*. Antibodies to CagA was positive in 33 patients (55.0%) by ELISA and in 46 patients by immunoblot assay (76.7%).

Table 1 shows the results of CagA serology and diversity of *cag*PAI. Although the sensitivity was relatively low, ELISA did not detect anti-CagA antibody in *cagA*-negative strains and the accuracy of ELISA to predict bacterial possession of *cagA* was 61.7%. In six patients infected with *cagE* and *VirD4*-negative strain, two were CagA seropositive. Thus, the accuracy of CagA ELISA to define *cagE* and *VirD4* positivity was only 58.3%. The immunoblot assay showed relatively

Table 1 CagA serology and bacterial phenotypes

	cagA		*cagE* and *VirD4*	
	Positive	Negative	Positive	Negative
ELISA				
Positive	33	23	31	23
Negative	0	4	2	4
Accuracy (%)	61.7		58.3	
Immunoblot				
Positive	44	12	43	11
Negative	2	2	3	3
Accuracy (%)	76.7		76.7	

higher sensitivity and showed better accuracy (76.7%) than ELISA. However, the high sensitivity of immunoblot assay was associated with false positive results. There were seven patients infected with *H pylori* without intact *cag*PAI, and their individual results of CagA serology and diversity of *cag*PAI were shown in Table 2. In two patients with *cagA* positive infection, both serological assays determined *cagA*-positivity correctly, while the strains did not possess both *cagE* and *VirD4*. In a patient with *cagA*-/*cagE*+/*VirD4*+ infection, immunoblot assay showed a positive result.

Table 2 CagA serology in seven patients with *H pylori* without intact *cag*PAI

	*cag*PAI		Serology	
(Age, sex)	*cagA*	*cagE* and *VirD4*	ELISA	Immunoblot
Patient A (73, F)	Positive	Negative	Positive	Positive
Patient B (83, M)	Positive	Negative	Positive	Positive
Patient C (74, F)	Positive	Negative	Negative	Negative
Patient D (64, F)	Negative	Positive	Negative	Positive
Patient E (62, F)	Negative	Negative	Negative	Positive
Patient F (51, F)	Negative	Negative	Negative	Negative
Patient G (69, M)	Negative	Negative	Negative	Negative

Fifty-five patients who were infected with *cagA*-positive strain were divided by the results of CagA serology. The differences of histological grade of gastritis are shown in Table 3. Bacterial colonization score was not different between the groups. The grade of glandular atrophy, which was associated with bacterial colonization, was also not different. Although the grade of PMN infiltration was not different, the difference of MNC infiltration was statistically significant in both the antrum and the corpus between CagA seropositive and seronegative patients when immunoblot assay was used ($P<0.05$ and $P<0.01$, respectively). On the other hand, there was no correlation between *cag*PAI genotype and histological features of gastritis in this series of patients because the number of patients infected with cagPAI-negative strain was too small.

DISCUSSION

Since early studies had demonstrated the association between gastric inflammation and the presence of CagA, a number of studies have implicated the virulence of this protein. CagA is now recognized as a protein encoded by the *cagA* gene, which is one of the genes inside the *cag*PAI. Recent

Table 3 Grade of gastritis in CagA seropositive and negative patients

		ELISA		Western blot	
	Grade	Positive	Negative	Positive	Negative
Antrum MNC[a]	0	0	0	0	0
	1	4	4	4	4
	2	19	17	30	6
	3	9	2	10	1
PMN	0	9	8	13	4
	1	17	13	23	7
	2	6	2	8	0
	3	0	0	0	0
H pylori	0	1	2	2	1
	1	14	7	17	4
	2	7	6	11	2
	3	10	8	14	4
Atrophy	0	4	4	4	4
	1	11	2	12	1
	2	9	11	16	4
	3	8	6	12	2
Corpus MNC[b]	0	1	2	1	2
	1	3	4	4	3
	2	23	14	31	6
	3	5	3	8	0
PMN	0	6	6	7	5
	1	20	12	28	4
	2	6	5	9	2
	3	0	0	0	0
H pylori	0	1	2	3	0
	1	8	6	10	4
	2	5	7	8	4
	3	18	8	23	3
Atrophy	0	4	2	5	1
	1	13	11	20	4
	2	10	5	14	1
	3	5	5	5	5

MNC, mononuclear cells; PMN, polymorphonuclear cells. Histological grades are 0: none, 1: mild, 2: moderate, and 3: marked. [a]$P<0.05$, [b]$P<0.01$ (when seropositivity was defined by Western blot).

studies have shown that CagA protein is delivered into gastric epithelial cells through the type IV secretion system. Phosphorylated CagA forms a physical complex with a tyrosine phosphatase SHP-2 and perturbs cellular functions by deregulating SHP-2 activity[8]. Other genes inside the *cag*PAI, which encode the type IV secretion system, play a role in gastric mucosal inflammation. VirD4 is recognized as a possible adaptor to guide CagA into the secretion system and necessary for the virulence of CagA[19]. On the other hand, there are other virulent functions, which are independent of CagA-VirD4. Inactivation of *cagE* but not *cagA* causes a marked reduction of the ability of *H pylori* to stimulate gastric epithelial IL-8 production[7,20]. A recent study also showed the association between *cagE* status and NF-κB activation *in vivo*[21]. Although genes inside the *cag*PAI have different pathogenic mechanisms, presence of anti-CagA antibody has been only one serological test to indicate the virulence of *cag*PAI. Furthermore, CagA serology has been usually used without examining the structure of *cag*PAI. Although most Japanese *H pylori* isolates possess *cag*PAI, some strains lack *cag*PAI totally or partially[14,22]. Therefore, it was necessary to examine whether CagA serology can indicate the structure of *cag*PAI accurately in Japan.

In the present study, only 3 out of 60 (5%) *H pylori*

isolates lack all of tested genes inside the *cag*PAI. The proportion of *cag*PAI possession was consistent with previous studies in Japan[23]. CagA ELISA was negative in these three patients, while immunoblot assay showed a false positive result in one patient. Immunoblot assay has been shown to have a better sensitivity than ELISA to detect antibody to CagA[24] and better sensitivity was also observed in the present results. However, high sensitivity sometimes cause false positive results. In another patient, who was infected with *H pylori* of *cagA-/cagE+/VirD4+* genotype, anti-CagA antibody was detected by only immunoblot assay. However, it is possible that PCR might demonstrate the presence of *cagA* in this patient, if *cagA* was examined by different primers. In three patients infected with *H pylori* of *cagA+/cagE-/VirD4-* genotype, both ELISA and immunoblot assay were positive in two patients. Thus, both serological methods may be inadequate to indicate the virulence of the genes inside the *cag*PAI other than *cagA*.

In 53 patients infected with *H pylori*, which possess all the three genes tested, CagA seropositivity was significantly lower by ELISA (58.5%) than immunoblot assay (79.2%; *P*<0.05). The low sensitivity is a major cause of the low accuracy of CagA ELISA in this study. The recombinant CagA antigen used in ELISA may affect sensitivity. Basso *et al*[25], compared four different ELISA methods to detect serum anti-CagA antibody and showed different sensitivity, which varied from 79% to 100%. The difference of CagA antigen has caused controversial results of the association between CagA serology and gastric cancer in Japanese patients infected with *H pylori*. Three studies have examined the association by ELISA using different CagA antigen and two of them found significant association while another did not[17,26,27]. Therefore, before examining anti-CagA antibody by ELISA, sensitivity and specificity should be carefully investigated. Furthermore, when infected *H pylori* is *cagA* positive but lacks *cag*PAI partially, it is more questionable to indicate the virulence of the *cag*PAI by ELISA.

We also studied the association between histological gastritis and CagA seropositivity in patients infected with *cagA*-positive *H pylori*. Significantly lower degree of MNC infiltration was observed in CagA seronegative patients, whereas both bacterial density and glandular atrophy were not different between groups. Thus, recognition of CagA by host, but not CagA expression by bacteria, could be a relevant factor for CagA seropositivity. Prevalence of anti-*H pylori* antibody was similar in the two groups (anti-*H pylori* antibody was negative in only one CagA seropositive patient). It follows that host recognition of *H pylori* did not affect gastric mucosal inflammatory infiltration in patients infected with *cagA*-positive *H pylori*. The results were in consistent with general understanding that CagA seropositivity is associated with enhanced gastritis.

In conclusion, immunoblot assay showed a better sensitivity to indicate bacterial possession of *cagA* than ELISA in this series of patients. However, in this study, both ELISA and immunoblot assay for anti-CagA antibody were unreliable to indicate the virulence of *cag*PAI, particularly in strains, which lack genes inside the *cag*PAI other than *cagA*. Although, in Asia, some serological tests have shown that the CagA seropositivity in patients with *H pylori* infection up to 90%,

serodiagnosis of CagA requires careful evaluation of their accuracy before use. Analysis of bacterial genotype via isolation of *H pylori* from endoscopic gastric biopsy specimens is recommended to determine the presence of genes inside the *cag*PAI and its virulence.

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