CagA seropositivity associated with development of gastric cancer in a Japanese population

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Abstract

Background/aims—Infection with *Helicobacter pylori* strains possessing the *cagA* gene is associated with increased risk of gastric cancer of the intestinal type. The aims of this study were to investigate whether CagA seropositivity is associated with increasing risk of gastric cancer in a Japanese population that has a much higher incidence of gastric cancer than western populations.

Methods—Eighty one gastric cancer patients and 81 sex and age matched endoscopically evaluated controls were studied. Histologically, 62 cancers were of the intestinal type and 76 were early gastric cancer. Serum CagA IgG antibodies were assayed by enzyme linked immunosorbent assay (ELISA) using purified recombinant CagA protein as antigen. Polymerase chain reaction (PCR) analysis for *cagA* in *H pylori* isolates (n = 80) showed that the CagA ELISA had a sensitivity of 83.3% (controls) and 72.5% (cancers).

Results—CagA seropositivity was 60% (49 of 81) in cancer patients and 44% (36 of 81) in controls. The odds ratio for the risk of cancer if CagA seropositive was 1.93 (95% confidence interval (CI) 1.01 to 3.68; $p < 0.05$). In the 57 *H pylori* positive cancer patients and their matched *H pylori* positive controls, the odds ratio for the risk of cancer if CagA seropositive was 2.2 (95% CI 1.04 to 4.65; $p < 0.05$).

Conclusions—These results suggest that CagA seropositivity is associated with increased risk of gastric cancer in Japanese populations.

(J Clin Pathol 1998;51:225-228)

Keywords: *Helicobacter pylori*; gastric cancer; CagA

Since the discovery of *Helicobacter pylori*, many studies have implicated infection with this bacterium in the pathogenesis of gastric diseases. *Helicobacter pylori* is strongly associated with atrophic gastritis and intestinal metaplasia,^{1,2} which are precursor lesions for gastric cancer,³ and infection is now a well established risk factor for the development of gastric adenocarcinoma.⁴⁻⁶ However, few patients with *H pylori* infection develop gastric cancer,⁷ and there has been recent interest in whether specific *H pylori* strains are associated with gastric carcinogenesis. The majority of *H pylori* strains can be divided into one of two groups (cag positive or cag negative), based on the possession of the cytotoxin associated gene A (cagA)^{8,9} and associated genes in the cag

pathogenicity island.¹⁰ CagA positive strains are associated with enhanced inflammatory responses,¹¹⁻¹⁵ which may increase the risk for the development of gastric cancer. Previous serological studies have also shown that infection with cagA positive strains is associated with an increased risk of developing atrophic gastritis^{16,17} and gastric cancer.^{18,19} However, few studies have assessed the relation between CagA seropositivity and gastric cancer in Japanese populations, which have a much higher incidence of gastric cancer than Western populations.

In this study, we investigated the association of CagA seropositivity with gastric cancer in a Japanese population using gastric cancer patients and sex and age matched controls who had received gastrointestinal endoscopy. We also investigated the sensitivity of the CagA enzyme linked immunosorbent assay (ELISA) in this elderly population by comparing the serological results with the possession of cagA in *H pylori* strains obtained from the studied patients.

Methods

PATIENTS

Patients scheduled for upper gastrointestinal endoscopy for routine screening for gastric cancer at the Hirosaki University Hospital between March 1995 and August 1996 were prospectively enrolled into the study. Patients were excluded if they had received anti-ulcer agents or antibiotics during the two months before the examination, or had previous histories of gastric tumours, gastric or duodenal ulcers, and gastric surgery. All subjects provided informed consent before their endoscopy and this study was approved by the ethics committee of Hirosaki University. A total of 162 patients were entered into the study. There were 81 patients with gastric cancer (mean (SD) age 62.5 (18.9); 50 men, 31 women) and age (± 3 years) and sex matched controls for each cancer patient (mean (SD) age 62.3 (19.3)). Control patients were selected randomly from those undergoing endoscopy for gastric cancer screening. Subjects on acid suppression agents and/or antibiotics were excluded. Subjects were considered to be eligible for inclusion as controls when their endoscopic diagnosis was normal or atrophic gastritis²⁰ was present without any evidence of ulceration, erosions, or neoplasia.

In cancer patients and controls, two biopsy specimens were taken from both the antrum and the body of the stomach, one for *H pylori* culture and another for histology. Blood was also taken from each patient and serum was

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Accepted for publication 9 December 1997

stored at -20°C . Biopsy specimens were taken at least 2 cm away from tumours, embedded in paraffin wax, and stained with both haematoxylin-eosin and Warthin-Starry stains. Specimens were examined for the presence of *H. pylori* by an experienced pathologist (MT) without knowledge of the serological results. Full histological diagnosis of the tumour type and stage was undertaken on resected gastric mucosa. The gastric cancers were classified histologically according to Lauren system.²¹ Early gastric cancer was pathologically diagnosed by the growth of tumour confined to the mucosa and submucosa of the stomach as described previously.²²

H. pylori status was evaluated by serology, histology, and bacterial culture in each patient. Histologically, *H. pylori* was considered to be positive if either the antral or the corpus biopsy was positive. Patients were considered *H. pylori* positive if at least one detection method showed a positive result.

SEROLOGICAL ASSAYS

Serum samples were assayed for the presence of antibodies to *H. pylori* and CagA. *H. pylori* antibodies were measured using HELpTEST (AMARD, Kew, Victoria, Australia) according to the manufacturer's instruction.²³ Previous studies have shown the sensitivity and specificity of HELpTEST in Japanese patients to be 91.2% and 81.3%, respectively.²⁴ CagA antibodies were assayed by ELISA using a recombinant fragment of CagA (kindly provided by Dr A Covacci, IRIS, Siena, Italy) as antigen, as previously described by Xiang and colleagues.²⁵ Briefly, flat bottom 96 well microtitre plates were coated with 0.1 $\mu\text{g}/\text{well}$ antigen in 0.1 M bicarbonate buffer for 24 hours at 4°C . Plates were washed with phosphate buffered saline (PBS) containing 0.1% Tween-20 and blocked with 1% bovine serum albumin (BSA) in PBS-Tween for one hour at 26°C . Serum samples diluted 1/75 in 1% BSA/PBS were incubated in duplicate for 90 minutes at 26°C . Following incubation with goat antihuman IgG alkaline phosphatase conjugate (Sigma, Poole, Dorset, UK) for two hours at 26°C , bound antibodies were detected with p-nitrophenyl phosphate substrate (Sigma) at 1 mg/ml in diethanolamine- MgCl_2 buffer. Positive control serum diluted 1/50 to 1/3200 to generate a standard curve was included on each plate. The cut off for positivity was the mean + 2 SD of 30 patients who were histologically negative for *H. pylori* and seronegative by western blotting.

HELICOBACTER PYLORI CULTURE

Biopsy specimens were inoculated on to Skirrow blood agar and cultured for 3–5 days at 37°C . The bacteria were identified as *H. pylori* by colony morphology, and positive oxidase, catalase, and urease reactions. The clone picked strains were suspended in 1 ml PBS (pH 7.6) for DNA preparation.

DNA PREPARATION AND PCR ASSAY FOR caga

Aliquots of bacteria (1 ml) were centrifuged at $10\,000 \times g$ for five minutes. The supernatant

was discarded and the bacterial pellet was digested with 0.3 units of proteinase K at 55°C for two hours. DNA was extracted using phenol/chloroform/isoamylalcohol and precipitated. The concentration and quality of DNA samples were estimated by measuring A260/A280. The presence of cagA was determined by polymerase chain reaction (PCR) as described previously.²⁶ Briefly, 35 cycles of PCR including 40 seconds of denaturation at 94°C , 40 seconds of annealing at 50°C , and 60 seconds of polymerisation at 72°C were performed using a pair of primers: 5'-GAT AACAGGCAAGCTTTTGAGG-3' and 5'-CTGCAAAAGATTGTTTGCGAGA-3'. The final cycle included an extension step for 10 minutes at 72°C . Each PCR amplification was performed using a total of 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 200 μM each dNTP, 100 pmol of each primer, 0.5 units Taq polymerase (TaKaRa Taq; Takara Biochemicals, Tokyo, Japan), and 0.1 μg of prepared DNA as template. PCR amplification was performed in duplicate for each DNA sample and positive and negative controls were included in each assay. Aliquots (10 μl) of each PCR product were analysed by 3% agarose gel electrophoresis. Results were considered positive when the product, which was equivalent to the fragment described by Peek and colleagues,²⁶ was found.

STATISTICAL ANALYSIS

Odds ratio and 95% confidence intervals (CI) were calculated by conditional logistic regression analysis in which cases and controls are individually matched. Chi-square analysis was performed to compare the CagA seropositivity between intestinal and diffuse type cancer groups. The two tailed *t* test was used to compare the titres in the CagA ELISA.

Results

HELICOBACTER PYLORI STATUS AND HISTOLOGICAL ANALYSIS

H. pylori status was evaluated by serology, histology, and bacterial culture (table 1). *H. pylori* positivity by serology, histology, and bacterial culture was 84.0%, 82.7%, and 66.7%, respectively, in cancer patients, and 79.0%, 66.7%, and 59.3%, respectively, in control subjects. There was no significant difference in *H. pylori* positivity between cancer patients and control subjects. Histologically, 62 cancers were of the intestinal type and 76 were early gastric cancer. *H. pylori* positivity by serology, histology, and bacterial culture was 82.3%, 80.6%, and 64.5% in intestinal-type gastric cancer patients, and 89.5%, 89.5%, and 73.7%, respectively, in those with diffuse-type gastric cancer. *H. pylori* infection was defined as negative if all of the three tests showed negative results. Seventy cancer patients and 67 control subjects were considered to be *H. pylori* positive. In 67 *H. pylori* positive control subjects, the prevalences of glandular atrophy and intestinal metaplasia were 78% and 35%, respectively.

Table 1 Patient characteristics and *H pylori* status

	Mean age (range)	Sex (M/F)	Stage (E/A)	Serology positive	Histology positive	Culture positive
Cancers	62.5 (43–85)	50/31	76/ 5	84.0% (68/81)	82.7% (67/81)	66.7% (54/81)
Intestinal	63.0 (44–85)	43/19	59/ 3	82.3% (51/62)	80.6% (50/62)	64.5% (40/62)
Diffuse	60.7 (43–73)	7/12	17/ 2	89.5% (17/19)	89.5% (17/19)	73.7% (14/19)
Controls	61.8 (42–86)	50/31		79.0% (64/81)	66.7% (54/81)	59.3% (48/81)

E, early stage; A, advanced stage.

Table 2 *Helicobacter pylori* positivity and CagA seropositivity

	<i>H pylori</i> positive*	CagA positive
Cancers	86.4% (70/81)	70.0% (49/70)
Diffuse	89.5% (17/19)	70.6% (12/17)
Intestinal	85.5% (53/62)	69.8% (37/53)
Controls	82.7% (67/81)	53.7% (36/67)

*At least one of serology, histology, or culture was positive.

CAG A SEROPOSITIVITY

CagA seropositivity in cases and controls is shown in table 2. Sixty per cent (49 of 81) of cancer patients and 44% (36 of 81) of controls were CagA seropositive. In the *H pylori* negative cancers and controls, CagA seropositivity was observed only in one control subject. The odds ratio for the risk of gastric cancer if CagA seropositive was 1.93 (95% CI 1.01 to 3.68; $p = 0.041$). No difference was seen between the two types of carcinoma in CagA seropositivity, 60% of patients with intestinal-type cancer and 63% of patients with diffuse-type cancer were CagA seropositive. Among the CagA seropositive subjects, titres were similar in the two groups. In the *H pylori* positive cancer patients and controls, CagA seropositivity was 70% (49 of 70) and 54% (36 of 67), respectively (table 2). In 57 of the 70 *H pylori* positive patients with gastric cancer, the matched control was also *H pylori* positive. In the *H pylori* positive cancer patients and matched controls, CagA seropositivity was 74% (42 of 57) and 53% (30 of 57), respectively. The odds ratio for the risk of gastric cancer in *H pylori* positive patients if CagA seropositive was 2.2 (95% CI 1.04 to 4.65; $p = 0.039$).

In *H pylori* positive subjects, there was no difference in IgG antibody titres to conserved *H pylori* antigens (HELpTEST) in the CagA seropositive and seronegative groups.

SENSITIVITY OF THE CAG A ELISA

H pylori strains isolated from 40 cancer patients and 40 controls were randomly selected to be tested for the presence of cagA by PCR. All of the strains tested from the cancer patients were cagA positive. Twenty nine of these patients were CagA seropositive and the sensitivity of the CagA ELISA in the cancer patients was therefore 72.5%. In the control subjects, 36 of 40 *H pylori* strains were cagA positive. Thirty of these 36 control subjects were CagA seropositive. The sensitivity of the CagA ELISA was 83.3% in the control group for detecting cagA infection. The four controls with negative PCR results were seronegative for CagA antibodies.

Discussion

This is the first study to investigate the association between gastric cancer and CagA sero-

positivity by a case control study using individually sex and age matched control subjects who had undergone endoscopy. The results show that CagA seropositivity is associated with increased risk of gastric cancer in an indigenous Japanese population, consistent with previous studies in North American,¹⁹ European,²⁷ and emigrant Japanese patients.¹⁸ Recently, considerable interest has focused on the strain diversity of *H pylori*. Many studies have shown that cagA positive strains are more virulent than cagA negative strains and that infection of cagA positive strains results in a more intense gastritis.^{11–14} In part, this is likely to result from the triggering of epithelial chemokine responses, which is dependent on multiple genes in the cag pathogenicity island.¹⁰ Reactive oxygen and nitrogen species released as a consequence of the inflammation contribute to epithelial cell injury and DNA damage. Correa *et al* suggested that epithelial cells with DNA damage too extensive to repair may undergo apoptosis, and that the loss of glands in atrophic gastritis may result from large scale induction of apoptosis as the result of such DNA damage.²⁸ Infection with cagA positive strains is considered to be associated with increased risk of atrophic gastritis and gastric cancer.^{16–19}

Few studies have investigated the relation between infection with CagA and/or VacA positive strains and gastric cancer in Japanese populations that have high incidences of gastric cancer.^{29–31} Kikuchi *et al* investigated CagA seropositivity in Japanese gastric cancer patients under the age of 40, together with age matched controls.³⁰ They showed lack of an association between CagA seropositivity and gastric cancer, conflicting with our results, but many of these young patients had diffuse gastric cancer. Previous studies^{18 19} reported that although CagA seropositivity was associated with increased risk of intestinal-type gastric cancer, there was no association with the diffuse type of gastric cancer. In a North American population, the odds ratio was 3.3 for the risk of gastric cancer in *H pylori* positive subjects if CagA seropositive.¹⁹ This odds ratio is similar to the results obtained in the present study in a Japanese population and an earlier study in emigrant Japanese.¹⁸

We also investigated the sensitivity of the CagA ELISA for detecting infection with CagA positive strains in Japanese patients with and without cancer. The sensitivities of the assay were 72.5% in cancer patients and 83.3% in controls. The patients in this study had a mean age of 62 years and the sensitivity of *H pylori* ELISA is known to be lower in the elderly.³² Higher sensitivity in the CagA ELISA has been observed in younger patient groups (Crabtree

JE, unpublished data, 1997). Although there was no significant difference in the CagA ELISA sensitivity between cancer patients and controls, our results suggest that an underestimation of CagA infection may occur in cancer patients. Extensive intestinal metaplasia will reduce bacterial load and this may well influence systemic immune responses to *H. pylori*. Patients with gastric cancer can have a gastric IgA response to *H. pylori* in the absence of systemic IgG antibodies to *H. pylori*.²⁷ In some cancer patients, secondary events associated with cancer might also contribute to false negative serological results. Despite the reduced sensitivity of the ELISA in cancer patients, CagA seropositivity was associated with increased risk of gastric cancer.

In this study, *H. pylori* positivity by histology and culture in cancer patients was much higher than that reported in a recent European study.³³ In another study, there was a higher rate of histologically detectable *H. pylori* in early than in advanced gastric cancer, suggesting a loss of infection in advanced cancer.³⁴ We also found a higher rate of *H. pylori* positivity by both serology and bacterial culture in early than in advanced gastric cancer (unpublished data, 1998). Therefore, it is likely that the high percentage of *H. pylori* positivity in cancer patients seen in this study reflects the high proportion of patients with early stage gastric cancer.

In conclusion, our case controlled study shows that CagA seropositivity is associated with increased risk of gastric cancer in a Japanese population. These results support the concept that infection by strains with the cag pathogenicity island is relevant to inflammation in the gastric mucosa and to the gastric oncogenic process.

We thank Dr Y Saito for preparing *H. pylori* strains, Professor D Forman for his statistical advice and analysis, and Mrs S Perry for her technical assistance. Work in our laboratory is undertaken with financial support from the Yorkshire Cancer Research Campaign, European Commission (contract number IC18CT950024), and the Northern and Yorkshire Regional Health Authority.

- 1 Kuipers EJ, Uytendaele AM, Pena AS, et al. Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet* 1995;345:1525-8.
- 2 Siurala M, Sipponen P, Kekki M. Chronic gastritis: dynamic and clinical aspects. *Scand J Gastroenterol* 1985;20(suppl 109):69-76.
- 3 Imai T, Kubo T, Watanabe H. Chronic gastritis in Japanese with reference to high incidence of gastric carcinoma. *J Natl Cancer Inst* 1971;47:179-95.
- 4 Parsonnet J, Friedman GD, Vandersteen DP, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991;325:1127-31.
- 5 Nomura A, Stemmermann GN, Chyou P, Kato I, Pérez-Pérez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma in a population of Japanese-Americans in Hawaii. *N Engl J Med* 1991;325:1132-6.
- 6 Forman D, Newell DG, Fullerton F, et al. Association between infection with *Helicobacter pylori* infection and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991;302:1302-5.
- 7 Taylor DN, Blaser MJ. The epidemiology of *Helicobacter pylori* infection. *Epidemiol Rev* 1991;13:42-59.
- 8 Covacci A, Censini S, Bugnoli M, et al. Molecular characterization of the 128-kDa immunodominant antigen

- of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci USA* 1993;90:5791-5.
- 9 Tummuru MKR, Cover TL, Blaser MJ. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence for linkage to cytotoxin production. *Infect Immun* 1993;61:1799-809.
- 10 Censini S, Lange C, Xiang Z, et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* 1996;93:14648-53.
- 11 Crabtree JE, Farmery SM. *Helicobacter pylori* and gastric mucosal cytokines: evidence that CagA-positive strains are more virulent. *Lab Invest* 1995;73:742-5.
- 12 Crabtree JE, Taylor JD, Wyatt JJ, et al. Mucosal IgA recognition of *Helicobacter pylori* 120 kDa protein, peptic ulceration, and gastric pathology. *Lancet* 1991;338:332-5.
- 13 Peek RM, Miller GG, Tham KT, et al. Heightened inflammatory response and cytokine expression in vivo to cagA+ *Helicobacter pylori* strains. *Lab Invest* 1995;73:760-70.
- 14 Yamaoka Y, Kita M, Kodama T, Sawai N, Imanishi J. *Helicobacter pylori* cagA gene and expression of cytokine messenger RNA in gastric mucosa. *Gastroenterology* 1996;110:1744-52.
- 15 Crabtree JE. Gastric mucosal inflammatory responses to *Helicobacter pylori*. *Aliment Pharmacol Ther* 1996;10(suppl 1):29-37.
- 16 Kuipers EJ, Pérez-Pérez GI, Meuwissen SGM, Blaser MJ. *Helicobacter pylori* and atrophic gastritis: importance of the cagA status. *J Natl Cancer Inst* 1995;87:1777-80.
- 17 Beales ILP, Crabtree JE, Scunes D, Covacci A, Calam J. Antibodies to CagA protein are associated with gastric atrophy in *Helicobacter pylori* infection. *Eur J Gastroenterol Hepatol* 1996;8:645-9.
- 18 Blaser MJ, Pérez-Pérez GI, Kleanthous H, et al. Infection of strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* 1995;55:2111-5.
- 19 Parsonnet J, Friedman GD, Orentreich N, Vogelstein H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut* 1997;40:297-301.
- 20 Kimura K, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. *Endoscopy* 1969;1:87-97.
- 21 Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. *Acta Pathol Microbiol Scand* 1965;64:31-49.
- 22 Kodama A, Inokuchi K, Soejima K, Matsusaka T, Okamura T. Growth patterns and prognosis in early gastric carcinoma. *Cancer* 1983;51:320-6.
- 23 Schembri MA, Lin SK, Lambert JR. Comparison of commercial diagnostic tests for *Helicobacter pylori* antibodies. *J Clin Microbiol* 1993;31:2621-4.
- 24 Yamamoto I, Fukuda Y, Mizuta T, et al. Evaluation of nine commercial serodiagnostic kits for the detection of *Helicobacter pylori* infection in Japan. *Res For Dig Dis* 1994;1:75-85.
- 25 Xiang Z, Bugnoli M, Ponzetto A, et al. Detection in an enzyme immunoassay of an immune response to a recombinant fragment of the 128 kilodalton protein (CagA) of *Helicobacter pylori*. *Eur J Clin Microbiol Infect Dis* 1993;12:739-45.
- 26 Peek RM, Miller GG, Tham KT, et al. Detection of *Helicobacter pylori* gene expression in human gastric mucosa. *J Clin Microbiol* 1995;33:28-32.
- 27 Crabtree JE, Wyatt JJ, Sobala GM, et al. Systemic and mucosal humoral responses to *Helicobacter pylori* in gastric cancer. *Gut* 1993;34:1339-43.
- 28 Correa P, Miller MJ. *Helicobacter pylori* and gastric atrophy-cancer paradoxes. *J Natl Cancer Inst* 1995;87:1731-2.
- 29 Hirai M, Azuma T, Ito S, Kato T, Kohli Y, Fujiki N. High prevalence of neutralizing activity to *Helicobacter pylori* cytotoxin in serum of gastric-carcinoma patients. *Int J Cancer* 1994;56:56-60.
- 30 Kikuchi S, Forman D, Covacci A, Crabtree JE. Diffuse gastric cancer in young *H. pylori* positive Japanese patients: lack of association with CagA status. *Gut* 1996;39(suppl 2):A68.
- 31 Ito S, Azuma T, Murakita H, et al. Profile of *Helicobacter pylori* cytotoxin derived from two areas of Japan with different prevalence of atrophic gastritis. *Gut* 1996;39:800-6.
- 32 Crabtree JE, Shallcross TM, Heatley RV, Wyatt JJ. Evaluation of a commercial ELISA for serodiagnosis of *Helicobacter pylori* infection. *J Clin Pathol* 1991;44:326-8.
- 33 Menegatti M, Vaira D, Holton J, et al. Serological response to *Helicobacter pylori* in gastric and non-gastric cancer. *Clin Sci* 1996;91:219-23.
- 34 Caruso ML, Fucci L. Histological identification of in *Helicobacter pylori* early and advanced gastric cancer. *J Clin Gastroenterol* 1990;12:601-2.