Antibody against *Helicobacter pylori* CagA and VacA and the risk for gastric cancer

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Abstract

***Aim*—*Helicobacter pylori* is associated with gastric cancer. Our aim was to investigate whether CagA or VacA seropositivity provides additional risk for gastric cancer.**

***Methods*—Sera from 110 gastric cancer patients were sex and aged matched with asymptomatic controls. *H pylori* status was determined by IgG enzyme immunoassay (HM-CAP EIA); CagA status was assessed by enzyme linked immunosorbent assay (ELISA) (OraVax) and immunoblotting (Chiron), and VacA status by immunoblotting using recombinant proteins as antigens.**

***Results*—*H pylori* infection was associated with an increased risk of gastric cancer (odds ratio (OR) = 2.19, 95% confidence interval 1.17 to 4.1). Subgroup analysis showed a significant association with intestinal type (OR = 2.94, 1.35 to 6.41), distal type (OR = 2.97, 1.39 to 6.33), early gastric cancer (OR = 3.74, 1.54 to 9.06), and age ≤ 55 years (OR = 8.33, 2.04 to 34.08), but not with diffuse type (OR = 0.83), proximal type (OR = 1.0), advanced gastric cancer (OR = 1.13), or age > 55 years (OR = 1.40). Serum CagA IgG and VacA antibody positivity was present in similar proportions in patients with and without cancer, with no significant differences in histological classification, clinical stage, or location (p > 0.3).**

***Conclusions*—*H pylori* infection causes chronic gastritis and is associated with the development of gastric cancer. Neither CagA nor VacA seropositivity added additional information or stratification.**

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Keywords: *Helicobacter pylori*; CagA gene; VacA gene; gastric cancer; virulence factors

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Helicobacter pylori infection is now accepted as the major cause of chronic gastritis. Several epidemiological studies have shown that *H pylori* infection is also linked to the gastritis associated diseases (gastric cancer and peptic ulcer).[1–3] In 1994, the International Agency for Research on Cancer categorised *H pylori* infection as a group I carcinogen.[4] In 1993, an estimated 0.4% of the 60 million Japanese who were infected with *H pylori* were diagnosed with gastric cancer.[5 6] Thus although gastric cancer is one of the commonest cancers in Japan, only a small percentage of individuals with *H pylori* infection ever develop it. One possible reason for differences in outcome of *H pylori* infection may relate to differences in virulence of *H pylori* strains.

Studies on bacterial virulence have focused primarily on two groups of putative bacterial virulence factors, the *cag* pathogenicity island (for which CagA antibody is a marker) and the vacuolating cytotoxin, VacA. Recent serological studies have shown that the CagA and VacA seropositivity were associated with an increased risk of developing atrophic gastritis and gastric cancer.[7–11]

In Japan, the predominant types of *H pylori* circulating in the population express CagA and VacA.[12–17] Two recent reports regarding the possible relation of serum anti-CagA antibody in gastric cancer in Japan reached opposite conclusions.[18 19] Katagiri *et al* reported that there was no relationship between CagA seropositivity and gastric cancer,[18] whereas Shimoyama *et al* reported the opposite.[19] Both studies used recombinant CagA protein as the antigen for the serological study but the source and structure differed. Katagiri *et al* used orv220, a 65 000 fragment CagA protein from OraVax Inc (Cambridge, Massachusetts, USA) whereas Shimoyama et al used a 25 500 fragment antigen from Immunobiological Research Institute Siena (IRIS, Siena, Italy). It is not clear whether their discrepant findings resulted from the use of different populations or different methods, or from differences in the antigens used to detect anti-CagA antibodies.

We used both CagA antigens to investigate whether CagA seropositivity was associated with increasing risk of gastric cancer in Japan. We also examined the relation between the VacA seropositivity and gastric cancer using a recombinant VacA antigen.

Methods

PATIENTS

Sera were obtained from 110 Japanese patients with histologically proven gastric cancer (80 men and 30 women, mean age 64.5 years, age range 42 to 84). Early gastric cancer was diagnosed pathologically by the growth of a tumour confined to the mucosa and submucosa of the stomach, as defined by the Japanese Research Society for Gastric Cancer.[20] The histological type of the malignancy was determined according to Lauren, as either intestinal or diffuse type.[21]

Two control groups were evaluated. First, each cancer patient was sex and aged matched with asymptomatic control (one control for each case) to assess the role of *H pylori* infection in gastric cancer (control 1). A second control (control 2) compared CagA antibody and VacA antibody status in *H pylori* positive

cases and required the addition of 29 *H pylori* antibody positive age and sex matched cases. None of the controls had histology of peptic ulcer disease or dyspepsia. None of the patients had been treated for *H pylori*, had received recent blood transfusions, non-steroidal anti-inflammatory drugs, proton pump inhibitors, or antibiotics within the previous three months.

Informed consent was obtained from all patients and the protocol was approved by the Hospital ethics committee.

Serum samples were stored at −80°C until serological testing.

ENZYME IMMUNOASSAY FOR *H PYLORI*

H pylori status was determined by IgG antibodies to *H pylori* using a commercially available enzyme immunoassay (EIA) kit (HM-CAP EIA, Enteric Products Inc). This assay system is reported to have 98.7% sensitivity and 100% specificity in the United States[22] and 100% sensitivity and 96% specificity in Japan in comparison with the urea breath test.[23] A cutoff optical density of 0.30 was chosen, according to Asaka *et al.*[23] Furthermore, a sensitivity and specificity of 96% and 98% have been found in 100 dyspeptic patients as determined by culture and histological analysis in our laboratory (data not shown). High titre antibody was defined by an optical density greater than 1.00.

ENZYME IMMUNOASSAY FOR CagA USING RECOMBINANT CagA ANTIGEN

Microtitre wells were coated with 1 μg/well orv220 antigen fragment of CagA (OraVax enzyme linked immunosorbent assay (ELISA), OraVax Inc) in 100 μl/well carbonate–bicarbonate buffer (100 mM, pH 9.6), overnight at 4°C. After washing and blocking with 200 μl/well 2.5% non-fat dried milk (NFDM) in phosphate buffered saline (PBS)–Tween (one hour, 37°C), serum samples diluted 1 in 100 in 100 μl/well NFDM/PBS–Tween were incubated at 37°C for one hour.

The second antibody, a goat antihuman IgG alkaline phosphatase conjugate, was then added at a dilution of 1:1000 at 37°C for one hour. After incubation with alkaline phosphatase substrate solution at 1 μg/ml in diethylamine–$MgCl_2$ buffer at room temperature for 20 minutes, the optical density was read at 405 nm. A standard curve of high titre positive and control serum samples was included on each plate. Results were expressed in ELISA units (0–100) determined from the standard curve. The cutoff was determined as 5.0 ELISA units. It was calculated as the mean plus 3 SD of the results obtained from 30 patients negative for *H pylori* (culture, histology, and urea breath test negative).

IMMUNOBLOTTING ASSAY FOR CagA AND VacA

Chiron recombinant immunoblot *H pylori* immunoassay (Chiron-RIBA, Chiron Corporation, Emeryville, California, USA) consisted of two different strips; one with a *H pylori* lysate (membrane derived antigens) and the other with the pathogenicity markers of this bacterium including VacA, CagA, and urease.

Membrane strips were incubated with 1:50 dilution of sera on a shaker for 4 to 4.5 hours at room temperature. After incubation, unbound serum components were removed by washing and aspiration. After washing, peroxidase labelled goat antihuman IgG conjugate was added to each strip and incubated for 9 to 11 minutes at room temperature. Substrate solution (hydrogen peroxide and 4-choro-1-naphthol) was added to each strip and agitated for 15 to 20 minutes at room temperature, after which the strips were washed twice with deionised water, dried, and mounted on non-absorbent paper. Serum positive and negative controls were included with each assay. Two levels of human IgG (level I, low control; level II, high control) were included on each strip as internal controls. *H pylori* reactivity was determined by comparing the intensity of the human IgG (level I and level II) internal control bands on each strip. The identity of the antigen bands was scored in relation to the intensities of the internal IgG controls as follows: score −, intensity of band is absent; ±, less than intensity of the level I IgG control band; 1+, equal to intensity of the level I IgG control band; 2+, greater than intensity of the level I IgG control band and less than intensity of the level II IgG control band; 3+, equal to intensity of the level II IgG control band; 4+, greater than intensity of the level II IgG control band.

A cutoff for seropositivity was defined as comparing + and ± data obtained from 30 *H pylori* negative controls. As a result, ± data are regarded as positive for CagA antibody and negative for *H pylori* status and anti-VacA antibody.

Results are given as odds ratios (OR) and 95% confidence intervals (CI).

Results

Seventy three patients had early gastric cancer and 37 had advanced gastric cancer.

Histologically, 88 patients had intestinal type adenocarcinoma and 22 had diffuse type cancer. Eighty seven gastric cancers had distal type (antrum to body) and 23 had proximal type (cardia and fundus).

H PYLORI AND GASTRIC CANCER

H pylori infection was associated with an increased risk of gastric cancer (OR = 2.19, 95% CI 1.17 to 4.1; table 1). Subgroup analysis showed a significant association with intestinal type (OR = 2.94, 95% CI 1.35 to 6.41), distal type (OR = 2.97, 95% CI 1.39 to 6.33), early gastric cancer (OR = 3.74, 95% CI 1.54 to 9.06), and age (≤ 55 years) (OR = 8.33, 95% CI 2.04 to 34.08), but not with diffuse type (OR = 0.83), proximal type (OR = 1.0), advanced gastric cancer (OR = 1.13), or age > 55 years (OR = 1.40) (table 1). The majority of patients with distal type early gastric cancer (57 of 60 patients; 95%) were *H pylori* antibody positive (OR = 8.14, 95% CI 2.25 to 29.46).

Sixty per cent (39 of 65) *H pylori* positive patients with early gastric cancer had high titre of *H pylori* antibody (OR = 2.11, 95% CI 1.05 to 4.25), compared with only eight of 25 patients (32%) with advanced gastric cancer

Table 1 Relation between gastric cancer and seropositivity of anti-H pylori IgG antibody

	No	Proportion of positive results (%)		Odds ratio	95% CI	p Value
		Case	Control			
All patients	110	81.8	67.3	2.19	1.17 to 4.10	<0.01
Distal type	87	86.2	67.8	2.97	1.39 to 6.33	0.01
Proximal type	23	65.2	65.2	1.00	0.29 to 3.36	NS
Intestinal type	89	87.5	70.5	2.94	1.35 to 6.41	0.01
Diffuse type	22	59.1	63.6	0.83	0.24 to 2.78	NS
Early stage	73	89.0	68.5	3.74	1.54 to 9.06	0.005
Advanced	37	67.6	64.9	1.13	1.54 to 9.06	NS
Age ≤ 55 years	28	89.3	50.0	8.33	2.04 to 34.08	0.005
Age > 55 years	82	79.3	75.6	1.40	0.68 to 2.89	NS

CI, confidence interval.

Table 2 Relation between gastric cancer and seropositivity of anti-CagA antibody (OraVax)

	No	Proportion of positive results (%)		Odds ratio	95% CI	p Value
		Case	Control			
All patients	90	83.3	81.1	1.16	0.54 to 2.50	NS
Distal type	75	84.0	81.3	1.20	0.52 to 2.81	NS
Proximal type	15	80.0	80.0	1.00	0.17 to 5.99	NS
Intestinal type	77	83.1	79.2	1.29	0.57 to 2.91	NS
Diffuse type	13	84.6	92.3	0.45	0.04 to 5.79	NS
Early stage	65	87.7	80.0	1.78	0.68 to 4.64	NS
Advanced	25	72.0	84.0	0.49	0.12 to 1.95	NS
Age ≤ 55 years	25	84.0	76.0	1.66	0.41 to 6.79	NS
Age > 55 years	65	83.1	83.1	1.00	0.40 to 2.50	NS

CI, confidence interval.

Table 3 Relation between gastric cancer and seropositivity of anti-CagA antibody (Chiron)

	No	Proportion of positive results (%)		Odds ratio	95% CI	p Value
		Case	Control			
All patients	90	73.3	71.1	1.12	0.58 to 2.15	NS
Distal type	75	74.7	72.0	1.15	0.55 to 2.37	NS
Proximal type	15	66.7	66.7	1.00	0.22 to 4.57	NS
Intestinal type	77	74.0	74.0	1.00	0.49 to 2.07	NS
Diffuse type	13	69.2	61.5	1.41	0.28 to 7.13	NS
Early stage	65	81.5	72.3	1.69	0.74 to 3.88	NS
Advanced	25	52.0	68.0	0.51	0.16 to 1.61	NS
Age ≤ 55 years	25	84.0	80.0	1.31	0.31 to 5.60	NS
Age > 55 years	65	69.2	67.8	1.07	0.51 to 2.26	NS

CI, confidence interval.

Table 4 Relation between gastric cancer and seropositivity of anti-VacA antibody (Chiron)

	No	Proportion of positive results (%)		Odds ratio	95% CI	p Value
		Case	Control			
All patients	90	82.2	81.1	1.08	0.51 to 2.29	NS
Distal type	75	80.0	81.3	0.92	0.41 to 2.07	NS
Proximal type	15	93.3	80.3	3.50	0.32 to 38.26	NS
Intestinal type	77	82.8	83.1	0.91	0.40 to 2.10	NS
Diffuse type	13	84.6	69.2	2.44	0.36 to 16.56	NS
Early stage	65	84.6	81.5	1.25	0.50 to 3.13	NS
Advanced	25	76.0	80.0	0.79	0.21 to 3.03	NS
Age ≤ 55 years	25	92.0	88.0	1.57	0.24 to 10.31	NS
Age > 55 years	65	78.5	78.5	1.00	0.43 to 2.31	NS

CI, confidence interval.

(early gastric cancer *v* advanced gastric cancer, p = 0.031).

VIRULENCE FACTORS AND GASTRIC CANCER

The presence of IgG antibody to CagA was similar in gastric cancer and control subjects using both the OraVax ELISA and the Chiron RIBA (83.3% *v* 81.1% with OraVax ELISA, 73.3% *v* 71.1% with Chiron immunoblots, for gastric cancer *v* *H pylori* positive controls, respectively) (p > 0.9; tables 2 and 3). The results were concordant in 86% (both positive or both negative in 64 and 13, respectively, among the cancer cases). The results with the two CagA antibody tests were discordant in 14%, with the OraVax ELISA yielding the higher estimate (OraVax+/Chiron− in 11 and OraVax−/Chiron+ in two). Nevertheless, the conclusions of the study were unchanged, irrespective of which CagA antigen was used.

VacA IgG antibody was positive in 82.2% of gastric cancers and 81.1% of controls (table 4). There were no significant differences in histological classification, clinical stage, or location of the tumour and CagA or VacA status.

Discussion

This study confirmed that *H pylori* infection is associated with an increased risk of gastric cancer.[24-27] We found a significant association between *H pylori* infection and intestinal type, distal type, early gastric cancer, and age ≤ 55 years. *H pylori* infection is expected to have a strong relation to intestinal type gastric cancer, as chronic atrophic gastritis was recognised as a risk factor for gastric cancer for decades before the identification of *H pylori* as a cause of gastritis.[28 29] It is currently thought that *H pylori* infection has an indirect relation to gastric cancer and is largely responsible for the development of atrophic gastritis and the precursor lesion, intestinal metaplasia.[30] In Japan, the prevalence of atrophic gastritis is extremely high, with a 90% incidence in people over 60 years age.[31]

There are only a few studies of the relation between *H pylori* and both early gastric cancer and advanced gastric cancer.[24 26 32] Our results are in agreement with those of Asaka *et al*,[24] who reported that *H pylori* IgG antibody titres were significantly higher in early cancer than in advanced cancer. The lower frequency of high titre IgG antibody in advanced cancer may be the result of a decrease in antibody titre, because of the increasing extent of intestinal metaplasia associated with transition from the intestinal type of early gastric cancer to advanced cancer, such that the local environment is no longer ideal for growth of *H pylori*.[24 33 34]

There are many reports using recombinant CagA proteins used in this study,[9 10 18 19 35] but no studies comparing them. This study suggests that numerical results from studies using different antigens and different protocols may not be comparable. Epidemiology studies by Blaser *et al*[9] and Parsonnet *et al*[10] used the OraVax antigen, whereas the Eurogast Study Group[35] used IRIS antigen, which is also used in the Chiron test. It is not clear which, or if either, antigen provides the better assessment of the prevalence of infection with *H pylori* that contain the *cag* pathogenicity island. Overall, the conclusions of this study were the same (that is, that there was no relation between CagA antibody status and gastric cancer), irrespective of which antigen was used. Nevertheless, the proportion with positive tests differed, with the OraVax ELISA yielding the higher estimate. The reason for the difference is unclear. It is possible that the ELISA may detect conformational and linear epitopes whereas the immunoblot is likely to detect only

linear epitopes. Whatever the cause, even the OraVax test—which provided the highest rate of anti-CagA antibody—yielded a lower frequency in Japanese patients than evaluation of CagA status by polymerase chain reaction or immunoblotting of *H pylori* isolates obtained from patients with different *H pylori* associated diseases,[12–17] suggesting that data derived using this test may not accurately reflect the true prevalence of CagA positive *H pylori* in a population. To obtain reliable data it would appear prudent for investigators interested in the true prevalence of CagA or VacA positive *H pylori* in a disease or region to validate the assays in their geographical region.

Several other variables can influence the outcome of studies evaluating the possible role of putative virulence factors in various *H pylori* related diseases. For example, different proportions of different histological types (such as advanced type *v* early type) could easily lead to different outcomes, and such unrecognised differences may be responsible for the different results obtained by the studies of Katagiri *et al* and Shimoyama *et al*.[18 19] It appears unlikely that differences in the test used were responsible for the difference in results they obtained.

Rudi *et al* reported that VacA antibody was associated with an increased risk of gastric cancer (OR = 1.74, 95% CI 1.08 to 2.78).[11] However, there was no relation between the VacA and gastric cancer in this study. The two studies used different procedures and evaluated patients from different countries. Whether the differences are procedural or geographical will require additional investigation.

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