

Short Communication***Helicobacter pylori* Strain Types and Risk of Gastric Cancer: A Case-Control Study¹**

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Abstract

The aim of this novel endoscopy clinic-based case-control study was to explore the influence of different *Helicobacter pylori* strain types on the risk of gastric adenocarcinoma using isolated bacterial strains, tissue samples, and sera. We included 72 cases with gastric adenocarcinoma and 324 age- and sex-matched controls. Histological characterization, culture, molecular typing of *H. pylori* genes by PCR (*cagA/vacA*), conventional IgG ELISA, and immunoblotting (Western blot) for the CagA and VacA proteins were performed. With four tests combined, *H. pylori* infection was detected in 57 (79%) cases and 213 (66%) controls. A positive association between *H. pylori* infection and gastric cancer risk was found [odds ratio (OR), 2.1; 95% confidence interval, 1.1–3.9]. Type I (OR, 1.8), intermediate (OR, 2.0), and type II (OR, 0.2) strains of *H. pylori* presented different serum antibody levels and different levels of association with gastric cancer. Our case-control study, based on molecular characterization and serology, provides further evidence that infection by more virulent strains of *H. pylori* and the presence of antibodies toward the CagA protein can be used as markers for an increased risk of gastric adenocarcinoma and that the strain types of *H. pylori* could be used in the future to determine disease outcome.

Introduction

Evidence for a positive association between *Helicobacter pylori* infection and gastric cancer risk is now quite strong (1–3). Only a small proportion of all infected individuals develop gastric cancer, and because those with duodenal ulcer seem to be protected (4), it has been postulated that certain circumstances might modify the relationship between *H. pylori* infection and gastric cancer (5). Factors of importance may include age at infection, characteristics

of the host such as gastric acid secretory capacity, and genetic and phenotypic characteristics of the infecting strain of *H. pylori* (6–9). The cytotoxin-associated gene (*cagA*), the corresponding protein (CagA), and the vacuolating cytotoxin (VacA) seem to be associated with an increased risk of a variety of diseases of the stomach (10–15). Isolated *H. pylori* strains have been classified into groups: (a) type I strains (highly virulent); (b) intermediate strains; and (c) type II strains (reduced virulence), depending on the expression of these proteins (7, 8, 16). However, it is difficult to obtain intragastric material in epidemiological studies. Therefore, most previous studies addressing the clinical consequences of strain variations have had limitations. The aim of our hospital-based epidemiological study was to further explore the influence of different *H. pylori* strain types on the risk of gastric adenocarcinoma.

Materials and Methods

The study base was patients who underwent gastroscopies at eight Swedish hospitals between September 1995 and August 1997. Cases were patients with newly diagnosed gastric adenocarcinoma. As controls, we recruited the next five consecutive patients without gastric adenocarcinoma, matched to cases for age (in 10-year age bands) and sex. A total of 72 gastric cancer cases and 324 controls were identified and included in the study. Among the controls, 114 presented with normal endoscopy (normal gastric mucosa, without macroscopic disease), 85 presented with gastritis, 54 presented with esophagitis, 21 presented with duodenal ulcer, 20 presented with gastric ulcer, 6 presented with polyps, 5 presented with lymphoma, and 19 presented with other gastric diseases.

Eight biopsies (antrum and corpus) were obtained from noncancerous gastric mucosa in both cases and controls (four biopsies for culture and four biopsies for histological examination). Ten ml of blood were collected, and the subjects were asked to complete a two-page self-administered questionnaire about sociodemographic characteristics, diet, and medical history. All subjects were included after providing informed consent, and the study was approved by the ethics committee of the Medical Faculty, Uppsala University (Uppsala, Sweden).

Biopsies were sent in Portagerm transport medium (bioMérieux sa, Marcy l'Etoile, France) to the laboratory, homogenized together, and cultured under standard conditions. All 155 *H. pylori* strains isolated by culture were used for DNA preparation. Frozen bacteria were treated with the Sputum Sample Preparation Kit (Roche Diagnostics). PCR assays for detection of the *cagA* gene (17) and the *s1*, *s2*, *m1*, and *m2* alleles of the *vacA* gene were performed (14, 18). *cagA*-negative strains were tested further by a specific PCR for the presence of the *cag* PAI³ using primer sets just outside the

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³ The abbreviations used are: PAI, pathogenicity island; OR, odds ratio, CI, confidence interval.

PAI.⁴ If the PAI was deleted in the tested strain, an amplified product of 562 bp was observed, and if the PAI was intact, no product appeared.

Biopsies were fixed in 4% buffered formalin, embedded in paraffin, cut into 4- μ m sections, and stained by H&E, Alcian blue-periodic acid-Schiff, and Giemsa stain. All sections were scored by one pathologist blinded to culture results and disease status. Antrum and corpus biopsies were scored separately for type, grade and localization of inflammation, intestinal metaplasia, and the grade of atrophy. Immunohistochemical staining was done with anti-*H. pylori* antibody (DAKO A/S, Glostrup, Denmark), and the *H. pylori* organisms were visualized using standard procedures. The density of bacterial colonization was also noted. After evaluation of noncancerous mucosa, the histological slides from the tumors were reviewed using the scheme of Laurén to classify the tumors as diffuse, intestinal, or mixed type of gastric cancer.

A conventional ELISA test, HM-CAP (Enteric Products, New York, NY), was used for the detection of serum IgG antibodies. Samples were scored negative, borderline, and *H. pylori* positive using numerical values. Samples with borderline values were retested once more. Immunoblotting (Western blotting) was performed on Helico Blot 2.0 strips (Genelabs Diagnostics, Singapore Science Park, Singapore), with results given as the presence or absence of certain protein bands (p116 or CagA, p89 or VacA, p35, p30, p26.5, and p19.5). Patients were classified as *H. pylori* negative or *H. pylori* positive in three different grades: (a) high positive (p116 and p89 positive); (b) intermediate (positive for p116 or positive for p89); or (c) low positive (presenting different combinations of at least two of p35, p30, p26.5, and p19.5). This classification corresponds to negative, type I, intermediate, and type II strains of *H. pylori*, respectively (16).

Subjects were considered positive for *H. pylori* when at least one of the four diagnostic methods, culture, immunohistochemistry, ELISA and immunoblotting, showed the presence of *H. pylori* infection. All available data from all four methods were combined, and a new variable, total *H. pylori* status, was created and used in the final analysis of gastric cancer risk. Subjects with missing test results were classified by the available tests.

Logistic regression was used for univariate and multivariate analyses of the relationship between *H. pylori* infection and gastric cancer. ORs and 95% CIs were computed from the model parameters and their standard errors. The results obtained using unconditional logistic regression differed little from those obtained with conditional logistic regression; therefore, only the former are presented here because they were more precise. The base model included sex, age, and hospital. We expected that *H. pylori*-associated gastric pathology was over-represented among the controls relative to the prevalence in the underlying catchment area population; therefore, we performed subanalyses restricted to controls with normal gastroscopy.

Results

Characteristics of the study subjects are presented in Table 1. Of the 396 study subjects, 270 (68%) were positive for *H. pylori* by at least one of the four methods. Cases and controls were similar with respect to their mean age and their distribution by sex. The prevalence of *H. pylori* was higher in cases than in controls but was similar in men (69%) and in

Table 1 Demographic features of cases and controls

Characteristics	No. of cases (%)	No. of controls (%)
Subjects included in the study	72	324
Subjects used for matched analysis ^a	49/72 (68)	211/324 (65)
<i>H. pylori</i> positive by four methods combined ^b	57/72 (79)	213/324 (66)
<i>H. pylori</i> positive by culture	29/72 (40)	126/324 (39)
<i>H. pylori</i> positive by immunohistochemistry	30/72 (42)	126/323 (39)
<i>H. pylori</i> positive by ELISA	43/65 (66)	136/310 (44)
<i>H. pylori</i> positive by immunoblotting	51/66 (77)	203/308 (66)
Female	20/72 (28)	114/324 (35)
Mean (SE) age (yrs)	72.7 (12)	70.9 (12)
Subjects included from Kalmar Hospital ^c	29/72 (40)	222/324 (68)
Questionnaire response rate	40/72 (56)	213/324 (66)

^a Cases matched by at least one control.

^b Culture, immunohistochemistry, ELISA, and immunoblotting.

^c The highest number of subjects included were from this hospital.

women (67%). Sixty-four cases had noncardia cancer, and eight cases had cardia cancer. By using the classification system of Laurén, 40 cases had intestinal type gastric adenocarcinoma, 25 had diffuse type gastric adenocarcinoma, 4 had mixed type gastric adenocarcinoma, and 3 were not possible to classify.

The OR for gastric cancer among *H. pylori*-infected subjects, compared with noninfected subjects, was 2.1 (95% CI, 1.1–3.9; Table 2). The *H. pylori* prevalence was 81% in noncardia cancer, 63% in cardia cancer, 78% in the intestinal type, 80% in the diffuse type, and 100% in the mixed type. Normal controls had a lower prevalence (62%) than controls with positive endoscopy (68%). When the analysis was performed on only those subjects tested by all four tests ($n = 372$), the increase in gastric risk was slightly higher with an OR of 2.2 (95% CI, 1.1–4.5). The association between gastric cancer risk and *H. pylori* infection was confirmed using both the normal controls and those with positive endoscopy, although a somewhat weaker association was observed in the latter group. The OR for gastric cancer risk was higher in cases with noncardia cancer than in cases with cardia cancer, and risk was highest using normal controls. The associations between *H. pylori* and risk for intestinal and diffuse gastric cancer, using different subsets of controls, did not reach statistical significance. The relative risk of gastric cancer was somewhat higher in females than in males when adjusted for age and hospital, but the ORs were statistically nonsignificant. When the association between *H. pylori* and risk of gastric cancer was adjusted for the presence of intestinal metaplasia and inflammation as well as age, sex, and hospital, the OR was 2.1 (95% CI, 1.0–4.3). Adjustment for other variables including histological variables, as well as childhood and adult living conditions, had little impact on this estimate. Among those 253 subjects who completed the questionnaire, 64% were positive for *H. pylori*, as compared with 76% of those who did not fill it out. Also, the median antibody titers and the OR for gastric cancer risk were different between responders (OR, 1.3) and nonresponders (OR, 4.4). This precluded meaningful use of variables from the questionnaire in our analysis.

In this study, 33% (83 of 254) of the subjects infected by *H. pylori* harbored type I strains, 54% of *H. pylori*-positive subjects (136 of 254) harbored intermediate strains, and 14% of *H. pylori*-positive subjects (35 of 254) harbored type II strains (Table 3). Cases and controls differed with respect to their distribution by strain types, but the intermediate strains were the most common strains in both groups. Type I and interme-

⁴ A. Sillén, personal communication.

Table 2 The association between *H. pylori* infection and risk of gastric adenocarcinoma (adjusted for sex, age, and hospital)

Type of gastric cancer	Cases	All included controls		Controls with normal mucosa	
	(no.)	(no.)	OR (95% CI) ^a	(no.)	OR (95% CI) ^a
All gastric adenocarcinoma					
<i>H. p</i> neg ^b	15	111	1 (Reference)	43	1 (Reference)
<i>H. p</i> pos	57	213	2.1 (1.1–3.9)	71	2.1 (1.0–4.6)
Noncardia cancer					
<i>H. p</i> neg	12	111	1 (Reference)	43	1 (Reference)
<i>H. p</i> pos	52	213	2.4 (1.2–4.8)	71	2.6 (1.1–5.8)
Cardia cancer					
<i>H. p</i> neg	3	111	1 (Reference)	43	1 (Reference)
<i>H. p</i> pos	5	213	0.7 (0.2–3.2)	71	0.7 (0.1–3.4)
Intestinal type					
<i>H. p</i> neg	9	111	1 (Reference)	43	1 (Reference)
<i>H. p</i> pos	31	213	1.9 (0.9–4.3)	71	2.1 (0.8–5.3)
Diffuse type					
<i>H. p</i> neg	5	111	1 (Reference)	43	1 (Reference)
<i>H. p</i> pos	20	213	2.2 (0.8–6.2)	71	2.1 (0.7–6.5)

^a Same subset of controls are used as reference for all groups of gastric cancer.

^b *H. p* neg, *H. pylori* negative; *H. p* pos, *H. pylori* positive.

Table 3 Association between type of *H. pylori* strain, CagA/VacA antibodies by immunoblotting, presence of *cagA/vacA* genes by PCR, and risk of gastric cancer

	No. of cases	No. of controls	Gastric cancer risk
			OR (95% CI)
By immunoblotting			
<i>H. pylori</i> neg ^a	15	105	1 (Reference)
Type II strain ^b	1	34	0.2 (0.0–1.8)
Intermediate strain ^c	34	102	2.0 (1.0–4.0)
Type I strain ^d	16	67	1.8 (0.8–4.0)
<i>H. pylori</i> neg ^a	15	105	1 (Reference)
CagA neg	2	39	0.4 (0.1–1.7)
CagA pos ^e	49	164	1.9 (1.0–3.7)
<i>H. pylori</i> neg ^a	15	105	1 (Reference)
VacA neg	34	131	1.6 (0.8–3.1)
VacA pos	17	72	1.8 (0.8–3.8)
By PCR			
<i>H. pylori</i> neg ^a	43	198	1 (Reference)
<i>cagA</i> neg	1	13	0.4 (0.0–3.2)
<i>cagA</i> pos	27	113	1.2 (0.7–2.1)
<i>H. pylori</i> neg ^a	43	198	1 (Reference)
<i>vacA s2</i>	1	2	2.3 (0.2–27)
<i>vacA s1</i>	27	124	1.1 (0.6–2.0)
<i>H. pylori</i> neg ^a	43	198	1 (Reference)
<i>vacA m2</i>	1	20	0.2 (0.0–1.8)
<i>vacA m1</i>	24	99	1.2 (0.7–2.2)

^a *H. pylori* neg, negative on all tests performed.

^b CagA and VacA negative.

^c CagA positive or VacA positive.

^d CagA and VacA positive by immunoblotting.

^e pos, positive.

diate strains were associated with increased gastric cancer risk, whereas type II strains were associated with a statistically nonsignificant reduction in risk. Although the magnitude of the association with gastric cancer was higher when cases were compared with normal controls than when cases were compared with controls with positive endoscopy, the patterns were similar to those described above. The ORs for noncardia cancer were 2.1 (95% CI, 0.8–5.6) for type I strains, 2.2 (95% CI, 0.9–5.4) for intermediate strains, and 0.3 (95% CI, 0.0–3.2) for type II strains when compared with normal controls. The distribution of strain types was similar for both intestinal and diffuse gastric

cancer. The type of the infecting strain was also associated with different median antibody titers, with type I strains giving the strongest median antibody response.

Infections with strains producing the CagA protein were associated with an elevated gastric cancer risk. In 86% (128 of 148) of the subjects, antibodies toward CagA were found, although 91% (135 of 148) of the strains were positive for the *cagA* gene. In total, 127 of 135 (94%) *cagA*-positive strains and 1 of 13 (8%) of *cagA*-negative strains seemed to produce CagA. The *cagA* gene was detected in 96% of the cancer strains and in 89% of the control strains. All but 1 of 24 *cagA*-positive cancer strains seemed to produce CagA. Among the control strains, a slightly lower proportion (93%) seemed to produce CagA. In 5 of 13 control strains tested, the *cag* PAI existed without giving a positive PCR reaction for the *cagA* gene.

No significant increase in gastric cancer risk was observed among subjects with antibodies toward the VacA protein. Of the 145 strains that had *vacA s1* allele, 66 (46%) of the infected subjects also had VacA antibodies, which were seen in fewer cases (36%) than controls (47%). Only strains from two controls and one case were found to have the *vacA s2* allele, of which one control strain seemed to produce VacA. The *vacA* allele combination *s1m1* was present in 92% (23 of 25) of the cancer strains and in 81% (97 of 119) of the control strains, *s1m2* was present in 4% (1 of 25) and 17% (20 of 119) of the cancer and control strains, respectively, and *s2m1* was present in 4% (1 of 25) and 2% (2 of 119) of cancer and control strains, respectively. No strains with the *vacA s2m2* combination of alleles were found.

Subjects infected by *cagA*-positive strains or with different *vacA* alleles had a risk of gastric cancer exceeding that in uninfected subjects, although the ORs were statistically nonsignificant. No significant associations between *cagA* positivity and *vacA* allele-type combinations *s1m1*, *s1m2*, *s2m1*, and *s2m2* and risk of gastric cancer were found.

Discussion

In this study, *H. pylori* infection was associated with an increased risk of gastric cancer. The association was weaker when cases were compared with controls presenting with positive endoscopy (*i.e.*, with gastric diseases, some of which are known to be associated with *H. pylori* infection). Our results,

although based on a relatively small number of cases, show that the prevalence of *H. pylori* infection is higher among those with noncardia cancer than among those with cardia cancer, and the associated risk was therefore higher for those with noncardia cancer, confirming the results of others (19). It also seems that the excess risk is confined to those infected with more virulent *H. pylori* strains, namely, type I strains (CagA and VacA positive), which could be of importance in future treatment strategies.

One of the major limitations of our study is that we had to restrict our study base to individuals having endoscopy. We wanted to analyze not only serological responses to *H. pylori* but also genes in the isolated strains, thus requiring endoscopy in both cases and controls. The hospital-based controls were probably more similar to the cases than population controls would have been with respect to *H. pylori* infection, which may have resulted in an underestimation of the true risk of gastric cancer in *H. pylori*-positive persons. The difference between the ORs for gastric cancer risk when cases were compared with normal controls and with controls with other gastric diseases illustrates this in our study. Spontaneous seroreversion may also have occurred over time in a higher proportion of gastric cancer cases than controls, with a further underestimation of the association. We tried to avoid this by using four laboratory tests combined (culture, immunohistochemistry, ELISA, and immunoblotting), thereby giving the highest possible prevalence of past and present *H. pylori* infection. Because clearance of the infection might occur as a consequence of the carcinogenic process, any indication of a positive result in any of the tests is more likely to reflect a previous infection than a technical error.

Our finding of a CagA-positive antibody reaction in a patient with a *cagA*-negative strain might indicate misclassification of the strain, the antibody, or both. It might also be possible that the subject harbored an infection with multiple strain types or two subpopulations with different *cagA* status or that the subject might have had a previous CagA-positive *H. pylori* infection (20). CagA/cagA- and VacA-positive strains are positively associated with gastric atrophy, intestinal metaplasia, and gastric cancer risk (10–16). In our study, antibodies toward VacA and CagA were associated with a 2-fold increase in gastric cancer risk compared with *H. pylori*-negative controls, which corresponds well with previous results. The *vacA* *s1* allele, which is thought to be responsible for the production of the vacuolating cytotoxin, was associated with antibodies toward VacA in about half of the subjects. This appears to be a relatively low correlation, which could reflect either problems in discriminating between the *vacA* *s1/s2* alleles by PCR or the sensitivity of the immunoblotting (14, 21–23).

Established risk factors for gastric cancer other than *H. pylori* infection include low socioeconomic status, low intake of fruit and vegetables, smoking, and early age at acquisition of the infection (2, 24). When these factors were examined in our study, none was significantly associated with risk. However, we had a low response rate for completion of the questionnaire, which made it difficult to use the available data for tests of these possible confounders. Of the histological parameters tested, the presence of intestinal metaplasia and inflammation was significantly associated with increased risk in our model, but the risk estimate for *H. pylori* did not change after adjustments for these two variables. This finding was surprising, given that both have been perceived as almost obligatory steps in the causal pathway to gastric adenocarcinoma, and adjustment would therefore tend to cancel the association.

A relationship between *H. pylori* strain type and the possibility of developing either duodenal ulcer or gastric cancer has

been proposed (5). Bacterial factors seem to determine the magnitude of the risk of developing gastric diseases, whereas host and environmental factors may be responsible for the nature of the disease, complicating chronic infection (13). In another study, it was indicated that *cagA*-positive *vacA* *s1* strains might not be specific markers for gastric cancer alone (23). Other factors in the environment, the host or the bacteria that play a role in the development of gastric adenocarcinoma, may still not have been found. Some infections may also be beneficial to the host; it has been suggested that *H. pylori* may protect the stomach against other p.o. ingested pathogens and may also reduce the risk of diseases in the lower esophagus and cardia (25). Our study indicates that certain bacterial strains are also of critical importance for negative associations with risk.

In conclusion, our case-control study provides further evidence that infection by more virulent, highly pathogenic strains of *H. pylori* and the presence of antibodies toward the CagA protein can be used as markers for an increased risk of gastric adenocarcinoma, especially in those with noncardia cancer.

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