Helicobacter pylori Cytotoxin-Associated Genotype and Gastric Precancerous Lesions

Martyn Plummer, Leen-Jan van Doorn, Silvia Franceschi, Bernhard Kleeter, Federico Canzian, Jorge Vivas, Gladys Lopez, Didier Colin, Nubia Muñoz, Ikuko Kato

Background Helicobacter pylori infection is associated with the development of gastric cancer. Although infection with an H. pylori strain containing the cytotoxin-associated (cag A) gene (a marker for a pathogenicity island) may increase the risk of atrophic gastritis and gastric cancer, the relationship of variants in pathogenic H. pylori genes to the severity and progression of precancerous lesions is not well defined.

Methods Gastric biopsy specimens were obtained at enrollment from 2145 participants in a chemoprevention trial in Tachira State, Venezuela, and examined histologically to determine the severity of precancerous lesions. The presence of H. pylori DNA in gastric biopsies and the strain type according to presence or absence of the cagA gene were detected by polymerase chain reaction and specific probes. The relationship between H. pylori DNA and histologic diagnosis was analyzed by polytomous logistic regression. Rates of progression and regression of precancerous lesions were determined from biopsies from additional annual gastroscopies (mean follow-up = 3.5 years). All statistical tests were two-sided.

Results At enrollment, there was a strong association between cagA-positive H. pylori infection and the severity of gastric precancerous lesions, but cagA-negative H. pylori was associated only with chronic gastritis. Using individuals with normal mucosa or superficial gastritis as control subjects, the odds ratio for dysplasia was 15.5 (95% confidence interval [CI] = 6.42 to 37.2) in cagA-positive individuals compared with uninfected individuals and 0.90 (95% CI = 0.37 to 2.17) for individuals infected with cagA-negative H. pylori compared with uninfected individuals. Individuals infected with cagA-positive H. pylori appeared more likely to experience progression (and less likely to experience regression) of precancerous lesions than those infected with cagA-negative H. pylori, but the differences did not attain statistical significance.

Conclusions This large epidemiologic study shows a strong relationship between the presence of H. pylori DNA in gastric biopsies and the severity of precancerous lesions that is specific to cagA-positive strains. The association between H. pylori and gastric carcinoma may have been previously underestimated due to the poor accuracy of serologic H. pylori markers and lack of discrimination by cagA genotype.


Helicobacter pylori is a bacterium that colonizes the human stomach and can establish a long-term infection of the gastric mucosa (1). The seroprevalence of H. pylori antibodies is 80%–90% in developing countries (2) but lower in developed countries, especially among later birth cohorts (3). Persistent H. pylori infection often induces gastritis and is associated with the development of peptic ulcer disease, atrophic gastritis, and gastric adenocarcinoma (4). Although gastric adenocarcinoma is one of the most common cancers worldwide, with 930 000 cases per year (5), the absolute risk for this disease is small in comparison to the prevalence of H. pylori infection. Thus, gastric adenocarcinoma can only be a rare outcome of H. pylori infection. This may be due to several factors, such as age at first infection, environmental factors (e.g., diet), and genetic variation in both humans and H. pylori.

There is evidence to support the existence of distinct genetic lineages of H. pylori (6), and this genetic variation may play a role in its pathogenicity. Among genes related to pathogenicity, the cytotoxin-associated (cagA) gene and the vacuolating cytotoxin (vacA) genes of H. pylori have been studied most extensively. The cagA gene, which is not present in all strains, is considered to be a marker for the presence of a pathogenicity island of approximately 35 000 bp that encodes a type IV secretion system that transfers the

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CagA protein into the host cells (7). Infection with cagA-positive \textit{H. pylori} strains increases the risk for the development of atrophic gastritis and gastric cancer (8,9). The vacA gene encodes a vaculating cytotoxin that is excreted by \textit{H. pylori} and damages epithelial cells. The gene is present in all strains and contains two variable regions, designated s and m, variations in which are associated with the level of toxicity of the VacA protein. The s region (encoding the signal peptide) occurs as an s1 or s2 allele, and within type s1, s1a, s1b, and s1c subtypes have been identified. The m (middle) region occurs as m1 or m2 alliec types, and among type m2, two subtypes (m2a and m2b) have been identified. The vacA and cagA genotypes are strongly linked: the great majority of cagA-positive strains also contain the vacA s1 m1 allele, whereas most cagA-negative strains contain the vacA s2 m2 allele.

Histopathologic studies conducted in high-risk populations have identified a sequence of changes in the gastric mucosa that apparently represent a continuum from normal mucosa to carcinoma, with successive intervening stages of chronic gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia (10). In this study, we used data and biologic samples collected as part of a chemoprevention trial (11) on precancerous lesions of the stomach to investigate the relationships among \textit{H. pylori} infection, bacterial virulence-associated genotypes, and the severity of gastric precancerous lesions in a population at high risk for both \textit{H. pylori} infection and gastric cancer.

**Methods**

**Study Population**

The randomized trial that provided the infrastructure for this study has been described previously (11). Briefly, eligible subjects were participants in the gastric cancer control program of Tachira State, Venezuela, between 35 and 69 years of age, who had been referred for gastroscopy at the Cancer Control Center of Tachira State in San Cristobal, Venezuela, after an initial photofluoroscopic screening to detect abnormalities in the gastric mucosa. After they gave written informed consent, all subjects underwent gastroscopic examination with collection of gastric biopsies, blood, and urine specimens, and they were administered a questionnaire on sociodemographic and lifestyle variables by a trained interviewer. Subjects were then randomly assigned to receive either vitamin supplementation with beta-carotene, vitamin C, and vitamin E or placebo for 3 years. A follow-up physical examination and gastroscopy were performed annually. During the study recruitment period from July 1991 to February 1995, there were 4349 eligible subjects, of whom 2272 were invited to participate in the trial. Of these, 72 refused to participate.

**Gastroscopic and Histologic Examinations**

At each gastroscopic examination, seven biopsies were taken. Five of the biopsies (four from the antrum and one from the corpus) were for histologic assessment (11). One of three pathologists at the Cancer Control Center classified each of the five biopsies as indicative of normal mucosa, superficial gastritis, chronic gastritis, chronic atrophic gastritis, intestinal metaplasia, or dysplasia, according to previously described criteria (12). Biopsies indicative of intestinal metaplasia were additionally classified as intestinal metaplasia subtypes I, II, or III, according to Filipe and Jass (13). The most severe disease classification indicated by one of the five biopsies was used as the global diagnosis for a given subject at each examination. The two additional biopsies were from the midportion of the lesser curvature of the antrum and the midportion of the anterior wall of the antrum. These biopsies were frozen at −80 °C until used for \textit{H. pylori} genotyping.

**DNA Isolation from Gastric Biopsies and \textit{H. pylori} Genotyping**

Total DNA was extracted from gastric biopsy specimens after digestion with Proteinase K. Briefly, biopsies were incubated in 250 µL of a solution of 10 mM Tris–HCl (pH 8.0), 5 mM EDTA, 0.1% sodium dodecyl sulfate, and 0.1 mg/mL Proteinase K for at least 2 hours at 55 °C. Proteinase K was inactivated by incubation at 95 °C for 10 minutes. Ten microliters of the lysate was used for a multiplex polymerase chain reaction (PCR) that amplified part of the \textit{cagA} gene as well as the \text{s} region and \text{m} region from the \text{vacA} gene, as previously described (14,15). The PCR products were used to identify the \text{vacA} genotypes by reverse hybridization using a line probe assay that contained probes for the \text{vacA} sequences s1a, s1b, s1c, s2, m1, m2a, and m2b and \textit{cagA} (the reverse hybridization strip was from Labo Bio-medical Products, Rijswijk, The Netherlands). This assay has been demonstrated to be highly accurate for detection of the \text{vacA} and \text{cagA} genotypes (14,15). When only the presence of \textit{cagA} was assessed, the products of the same multiplex PCR were hybridized to the probes in a microtiter plate in a DNA enzyme immunoassay. Reverse primers used in amplification contained a biotin label at the 5′ end for capture of the reverse strand onto streptavidin-coated microtiter plates. Bound amplimers were denatured by alkaline treatment and probed simultaneously with two digoxigenin-labeled nucleotides designed for discrimination by \textit{H. pylori} that tested positive for a genetic marker for a set of pathogenic genes and severe precancerous lesions.

**Limitations**

Further study will be needed to determine if the association of a \textit{H. pylori} genotype with the risk of advanced precancerous lesions also holds for gastric cancer.

**Prior knowledge**

Infection with the bacterium \textit{Helicobacter pylori} is associated with the development of gastric cancer, but information on which specific strains of the bacterium are most strongly associated with precancerous lesions was lacking.

**Study design**

Gastric biopsy specimens from participants in a chemoprevention trial were used to assess the relationship between \textit{H. pylori} genotype and severity of precancerous lesions.

**Implications**

The risk of advanced precancerous lesions conferred by \textit{H. pylori} infection may have been underestimated previously due to failure to discriminate by \textit{H. pylori} genotype.

**Further study needed**

Further study will be needed to determine if the association of a \textit{H. pylori} genotype with the risk of advanced precancerous lesions also holds for gastric cancer.
to detect all cagA sequence variants (15,16). Probe binding was quantified by optical density. If the assay yielded a borderline optical density value (i.e., 75%–100% of the cutoff value), the multiplex PCR was repeated and the amplimers were retested. The presence of *H. pylori* (irrespective of cagA status) was determined using the same approach by probing the same multiplex PCR with an oligonucleotide that corresponded to the s region of vacA.

**Statistical Analyses**

Cross-sectional analysis of the relationship between *H. pylori* DNA and histologic diagnosis was conducted by polytomous logistic regression. Odds ratios (ORs) for the presence of precancerous lesions and 95% confidence intervals (CIs) were calculated for each level of histologic diagnosis using subjects with normal mucosa and superficial gastritis as control subjects. *H. pylori* DNA was considered as an exposure variable with three levels: uninfected, infected with cagA-negative *H. pylori*, and infected with cagA-positive *H. pylori*. All analyses were adjusted for age and sex, and all statistical tests were two-sided.

Study participants were followed for an average of 3.5 years (11). Follow-up analyses were conducted using baseline *H. pylori* DNA status to predict progression and regression of the global diagnosis for each subject between the first and last gastroscopy. A Poisson regression model was used with age, sex, baseline diagnosis, and treatment allocation as confounders.

The dose–response relationship between severity of gastric precancerous lesions and *H. pylori* status was represented graphically using floating absolute risks (17). This method ascribes a “floating” standard error to each category of the histologic diagnosis that is independent of the choice of control group. A confidence interval for the odds ratio between any two diagnostic categories can be calculated from the floating standard errors.

**Pilot Study to Determine Degree of Genetic Variation in the Study Population**

Before the study, there was some uncertainty about the prevalence of cagA-positive *H. pylori* strains and individual vacA alleles in the population we studied, and, thus, about the suitability of the population to assess the association of *H. pylori* genotypes with disease severity. The prevalence of *H. pylori* as determined by histologic assessment at baseline was 94% in our patient sample (12). In a case–control study conducted in the same population (18), seroprevalence of *H. pylori* antibodies in population control subjects ranged between 72% and 92%, depending on the assay used, and antibodies to the CagA protein were found in 78% of the control subjects. Genotypic information on this geographic region from other sources is limited (19). To obtain better information about the prevalence of cagA and of particular vacA alleles in the study population, we conducted a pilot study that analyzed biopsies for the presence of *H. pylori* and cagA and vacA genotypes. Frozen biopsies were selected from subjects in the study who had a single gastroscopy at baseline and subsequently dropped out of the study, including all subjects in this category with high-grade lesions (i.e., intestinal metaplasia type II or III and dysplasia, n = 103). To obtain some indication of the association of *H. pylori* genotypes with prevalence of disease, a control group (n = 103) matched by age and sex was selected among subjects with superficial and chronic gastritis, omitting the subjects with atrophic gastritis to provide maximum contrast with the subjects with high-grade lesions.

**Results**

**Prevalence of cagA and vacA Alleles in the Study Population**

Only 15 of the 206 subjects in the pilot study were uninfected with *H. pylori*. The distribution of variants of s and m sequences of vacA according to cagA status among infected subjects with less severe (superficial or chronic gastritis) and more severe (intestinal metaplasia or dysplasia) lesions is shown in Table 1. The prevalence of cagA was 86% in the group with more severe lesions and 59% in the group with less severe lesions (P < .001 for difference by chi-square test). Among subjects with less severe lesions, the presence of cagA was strongly associated with the vacA genotype. None of the 55 cagA-positive subjects were infected with the vacA s2 genotype, and only five were infected with the vacA m2 genotype. Conversely, the majority of cagA-negative subjects were infected with a vacA s2 genotype or an m2 genotype (29/39 and 28/39, respectively). Among subjects with more severe lesions, 85 of 97 (88%) were infected with the cagA+/vacA s1 genotype. Identical results were found for the cagA+/vacA m1 genotype.

The pilot study provided sufficient evidence of genetic heterogeneity of *H. pylori* and a possible association with severity of disease to continue *H. pylori* genotyping in the rest of the population. However, in view of the finding that the presence of cagA was strongly associated with the vacA s1/m1 genotype, it was decided to analyze the remaining gastric biopsy specimens only for cagA.

**Cross-sectional Association of Precancerous Lesions and *H. pylori* Genotype**

Frozen gastric biopsies were available for 2145 of the 2200 subjects enrolled in the randomized trial. Eight subjects with unknown type of intestinal metaplasia, three subjects with borderline *H. pylori* DNA status, and three subjects with borderline cagA DNA status were excluded from the analysis because they could not be unambiguously classified. Among the remaining 2131 subjects, 10 (0.5%) were diagnosed with normal mucosa, 81 (4%) with superficial gastritis, 1022 (48%) with chronic gastritis, 326 (15%) with atrophic gastritis, 572 (26%) with intestinal metaplasia, and 120 (6%) with dysplasia. After *H. pylori* genotyping, 947 subjects (44%) were...
classified as infected with cagA-negative *H. pylori*, 852 (40%) as infected with cagA-positive *H. pylori*, and 332 (16%) as uninfected. The odds ratios for severity of histologic diagnosis by *H. pylori* infection status were calculated using subjects with normal mucosa and superficial gastritis as control subjects (Fig. 1). Distinct patterns of odds ratios according to severity of diagnosis were found for cagA-negative and cagA-positive *H. pylori*. When cagA-negative *H. pylori*-infected individuals were compared with uninfected individuals, the former had a statistically significantly elevated odds ratio for chronic gastritis (2.12; 95% CI = 1.28 to 3.53) but not for more advanced lesions. Conversely, when cagA-positive *H. pylori*-infected subjects were compared with uninfected ones, the OR was 4.33 (95% CI = 2.24 to 8.34) for chronic gastritis and 15.5 (95% CI = 6.42 to 37.2) for dysplasia. Thus, the odds ratios increased with severity of disease.

Follow-up Analysis of Progression of Precancerous Lesions

Due to withdrawals from the study, follow-up gastroscopies were available for only 1474 subjects. Rates of progression and regression of precancerous lesions during this follow-up period were calculated according to *H. pylori* status at baseline (Table 2). Progression rates per 1000 person-years were 76.5 for uninfected subjects, 76.0 for subjects infected with cagA-negative *H. pylori*, and 80.3 for subjects infected with cagA-positive *H. pylori*. The corresponding regression rates were 137.8, 149.2, and 126.9 per 1000 person-years, respectively. When the results were adjusted for age and sex, subjects with cagA-positive infection had slightly more progression (rate ratio = 1.19, 95% CI = 0.87 to 1.63) and less regression (rate ratio = 0.86, 95% CI = 0.64 to 1.15) than uninfected subjects. The rate ratios were not statistically significant (*P* = .28 for progression, *P* = .31 for regression by Wald test), but the number of events in the baseline category of uninfected subjects was small (43 progressions and 51 regressions), so this comparison had low power. When a direct comparison was made among infected subjects between cagA-positive and cagA-negative *H. pylori*, the rate ratio was 1.21 (95% CI = 0.94 to 1.57) for progressions and 0.81 (95% CI = 0.64 to 1.04) for regressions (*P* = .15 for progression, *P* = .09 for regression by the Wald test).

Discussion

This study shows a very strong association between current infection with cagA-positive strains of *H. pylori* and the severity of gastric precancerous lesions in a Latin American population at high
risk of both *H. pylori* infection and gastric cancer. The prevalence of cagA-positive *H. pylori* infection was 75% in subjects with intestinal metaplasia type III or dysplasia compared with 17% in subjects with normal gastric mucosa. Conversely, infection with cagA-negative *H. pylori* was not associated with any lesion more severe than chronic gastritis.

Weaker associations were found in the follow-up analysis, in which *H. pylori* and cagA status at enrollment were used to predict progression and/or regression of gastric lesions. Although tests of these associations did not attain statistical significance at the 5% level, the direction of the associations was consistent with the cross-sectional findings. It would be expected that the magnitude of the rate ratio in a short follow-up study would be lower than the corresponding cross-sectional odds ratio. This is because the follow-up analysis was based on changes in the gastric mucosa over an average of 3.5 years, whereas the cross-sectional analysis accumulated changes over many decades, i.e., from first exposure to *H. pylori*, likely to occur in childhood, to middle age. In a multistep disease process like gastric carcinogenesis, small differences in progression rates can accumulate over time to create large differences in the prevalence of high-grade lesions.

The short follow-up time is one important limitation of the study. A second limitation is that the population sample included no cases of gastric cancer. An interpretation of the results in terms of gastric cancer incidence therefore would require an extrapolation based on the advanced precancerous lesions observed in a small fraction of the study participants.

A role for *H. pylori* in the etiology of gastric cancer has been recognized for at least a decade (4), and the importance of cagA as a marker of increased virulence has also long been recognized (8,9). However, it has been difficult to quantify the risk of gastric cancer associated with *H. pylori* infection. Most of the previous evidence that *H. pylori* infection is associated with gastric adenocarcinoma or its precursors has come from seroepidemiologic studies that detected antibodies against either *H. pylori* or the CagA protein. It is widely believed that retrospective assessment of *H. pylori* status in gastric cancer patients has low sensitivity due to a progressive loss of *H. pylori* infection as gastric atrophy develops. For this reason, prospective studies with a long follow-up period are considered to be the most reliable source of risk estimates for gastric cancer. In a meta-analysis of 12 prospective studies of gastric cancer, *H. pylori* infection was not associated with cancer of the gastric cardia but was associated with noncardia cancer. The strength of the relative risk increased with follow-up time, from 2.39 (95% CI = 1.82 to 3.12) when blood samples were collected less than 10 years before cancer diagnosis to 5.9 (95% CI = 3.41 to 10.3) when they were collected 10 years or more before diagnosis (20). Although the current study did not include gastric cancer patients, it demonstrated that *H. pylori* DNA remains detectable in the gastric mucosa of individuals with advanced precancerous lesions. Furthermore, there was a strong enrichment of cagA-positive strains with increasing severity of gastric lesions, from 25% in subjects with normal mucosa or superficial gastritis to 83% in subjects with dysplasia. This implies a role for *H. pylori* throughout the entire spectrum of precancerous lesions and is not consistent with a “hit-and-run” mechanism in which *H. pylori* causes chronic gastritis but then disappears when more severe lesions develop.

In this study, the risk of precancerous lesions conferred by *H. pylori* was specific to cagA-positive strains. Failure to distinguish between cagA-positive and -negative strains would have led to a substantial underestimation of the relative risk—when we recalculated the odds ratio for dysplasia comparing all *H. pylori*-infected subjects with uninfected subjects, the OR estimate was 4.2 (95% CI = 1.98 to 8.14), compared with 15.5 (95% CI = 6.42 to 37.3) when only cagA-positive *H. pylori*-infected subjects were compared with uninfected subjects. Thus, previous studies that did not assess cagA status may have underestimated the increased risk conferred by infection with *H. pylori* to an extent that would depend on the prevalence of this genetic marker in the populations studied. In our study, this prevalence was 41% (47% of all *H. pylori* infections), much lower than the seroprevalence of anti-CagA antibodies of 78% previously found in healthy control subjects in this population (18). The discrepancy may be due to the fact that in populations with a high prevalence of *H. pylori* infection, such as Venezuela, individuals may be infected with multiple *H. pylori* strains over time (19), and therefore serologic assessment by anti-CagA antibodies may not accurately reflect the *H. pylori* status of the gastric mucosa.

A small number of previous studies of gastric cancer have reported results of genotyping of cagA of *H. pylori* in gastric biopsy

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**Table 2. Progression and regression of precancerous lesions during follow-up, according to *Helicobacter pylori* DNA status at enrollment**

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<th>Progressions by <em>H. pylori</em> DNA status</th>
<th>cagA</th>
<th>N</th>
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* Rate ratio relative to uninfected subjects, controlling for age, sex, vitamin supplementation, and initial diagnosis. cagA = cytotoxin-associated gene; CI = confidence interval.
specimens. A case–control study of distal gastric cancer in Brazil, in which all subjects were infected with *H. pylori* (21), reported an OR of 11.9 (95% CI = 4.8 to 29) for cagA DNA and one in Portugal (22) reported an OR of 15 (95% CI = 7.4 to 29). Weaker associations of the presence of cagA and gastric cancer were found in case–control studies conducted in Italy (OR = 2.94, 95% CI = 1.56 to 5.52) and Spain (OR = 3.7, 95% CI = 1.33 to 12.3) (23,24).

In addition, a number of gastroscopic surveys, including one conducted in the control population of the case–control study in Portugal (25), have found an association between cagA DNA and gastric atrophy and intestinal metaplasia. In comparison with previous studies, ours is unique in applying this detection method to a large number of subjects with a broad range of gastric lesions and in obtaining and analyzing follow-up data on the evolution of the disease. We also used a highly sensitive PCR-based assay that has shown to be very accurate in the detection of current infection with distinct *H. pylori* genotypes with a great diversity of geographic origins (15).

It has been reported that the variation in the virulence of *H. pylori* strains is insufficient to fully explain the variation observed in the infection outcome and incidence rates of noncardia gastric cancer worldwide (26). It has been hypothesized that this variation is therefore dependent on the genetics of the host and environmental factors and that a “virulent” *H. pylori* strain will cause the most damage when infecting a susceptible host. Polymorphisms in human genes encoding interleukin 1 beta (27) and other pro-inflammatory cytokines (26) have been shown to be associated with gastric cancer risk. In previous analyses of host genotype in the current study population, we observed that three of 13 polymorphisms examined, including one in the interleukin 8 gene, were associated with the prevalence of severe gastric lesions (intestinal metaplasia or dysplasia) (28–30). None of the associations of genetic polymorphisms with gastric cancer risk reported by us or by others (31) came close to the strength of the association for cagA-positive *H. pylori* that we observed (ORs greater than 15 for intestinal metaplasia type III and dysplasia). Therefore, accurate testing for cagA in large epidemiologic studies may furnish insight into the variation in noncardia gastric cancer worldwide.

In our study, additional genotyping of the *vacA* gene would not have greatly improved our ability to distinguish high-risk from low-risk strains, because the pilot study showed a very strong correlation between the presence or absence of cagA and *vacA* genotypes, in agreement with previous studies (16). Previous analyses in the population studied here have identified years of education, refrigerator use in childhood, smoking, and alcohol drinking as risk factors for precancerous gastric lesions. When sensitivity analyses were conducted to examine the effect of controlling for these potential confounders, the results were not substantially different from those obtained when controlling for only age and sex.

In conclusion, our study adds to the emerging body of evidence that the strength of the association between *H. pylori* and gastric carcinoma has been underestimated (32,33). In a recent review of infection-related cancer, the proportion of all gastric cancers worldwide attributable to *H. pylori* was estimated to be 63% (5). Our results imply that, in populations with a high prevalence of cagA-positive *H. pylori*, the attributable fraction may be even higher.

References


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