CagA and VacA *Helicobacter pylori* antibodies in gastric cancer

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BACKGROUND: Infection with different genotypes of virulent *Helicobacter pylori* strains (cytotoxin-associated gene A [CagA]- and/or vacuolating cytotoxin A [VacA]-positive) can play a role in the development of atrophic gastritis, duodenal ulcer (DU) and gastric cancer (GC).
OBJECTIVE: To determine whether patients with GC and *H pylori*-negative histological staining had previously been infected with *H pylori* CagA- and/or VacA-positive virulent strains.
METHODS: Twenty-three GC patients with a mean (± SD) age of 68.14±9.8 years who tested *H pylori*-negative on histological staining took part in the study. Three control groups were included. The first group comprised 19 patients with past *H pylori* infection and DUs eradicated 10 years earlier, with a mean age of 58±18.2 years. *H pylori*-negative status for this group was determined every year with Giemsa staining, and follow-up testing occured 120±32 months (mean ± SD) after therapy. The subsequent control groups included 20 asymptomatic children, with a mean age of 7±4.47 years, and with *H pylori*-negative fecal tests; the final group contained 30 patients without clinical symptoms of *H pylori* infection, with a mean age of 68±11.6 years, who tested *H pylori*-negative by histological staining.
RESULTS: Prevalence of CagA and VacA seropositivity, respectively was 82.6% and 73.91% in GC patients; 84.2% and 84.2% in *H pylori*-negative DU patients; 25% and 5% in *H pylori*-negative children; and 36.6% and 16.6% in the patients without clinical symptoms on histological staining. CagA and VacA antibody positivity was not significantly different between GC patients and patients with DUs that had been eradicated 10 years earlier. Significant positivity was found between the children's group and the *H pylori*-negative (with past DUs) group (P<0.001). A statistically significant difference was found in age between groups (P<0.03).
CONCLUSIONS: Patients with GC, even when *H pylori*-negative at the time of the present study, may have been infected by *H pylori* before the onset of the disease, as confirmed by CagA and VacA seropositivity. These data reinforce the hypothesis that *H pylori* may be a direct carcinogenic agent of GC.

Key Words: *CagA; Gastric cancer;* Helicobacter pylori; *VacA*

Anticorps anti-*Helicobacter pylori* et gènes de virulence CagA et VacA dans le cancer de l'estomac

HISTORIQUE : L'infection causée par les gènes de virulence CagA (pour cytotoxin-associated gene A) et/ou VacA- (pour vacuolating cytotoxin A) d'*Helicobacter pylori* peut jouer un rôle dans le développement de la gastrite atrophique, de l'ulcère gastroduodénal (UGD) et du cancer de l'estomac.
OBJECTIF : Déterminer si les patients atteints de cancer de l'estomac chez qui on obtient une coloration histologique *H. pylori*-négative avaient déjà été infectés par des souches virulentes CagA- et/ou VacA-positives d'*H. pylori*.
MÉTHODES : Vingt-trois patients atteints de cancer de l'estomac âgés en moyenne de 68,14 ± 9,8 ans ± É.-T. et présentant un test de dépistage de *H. pylori*-négatif à la coloration histologique ont pris part à l'étude. Trois groupes témoins ont été inclus. Le premier groupe comprenait 19 patients qui avaient déjà été infectés par *H. pylori* et avaient souffert d'UGD éradiqué dix ans auparavant, âgés en moyenne de 58 ± 18,2 ans. Dans ce groupe, on a contrôlé annuellement le statut *H. pylori*-négatif (une coloration de Giemsa et un test de contrôle ont été effectués 120 ± 32 mois (moyenne ± É.-T.) après le traitement. Le groupe témoin suivant a inclus 20 enfants asymptomatiques âgés en moyenne de 7 ± 4,47 ans chez qui des tests fécaux *H. pylori*-négatifs avaient été réalisés. Le dernier groupe comprenait 30 patients normaux âgés en moyenne de 68 ± 11,6 ans ayant obtenu un résultat *H. pylori*-négatif à la coloration histologique.
RÉSULTATS : La prévalence de la CagA- et VacA-séropositivité a été respectivement de 82,6 % et de 73,91 % chez les patients atteints de cancer de l'estomac, de 84,2 % et de 84,2 % chez les patients atteints d'UGD *H. pylori*-négatif et de 25 %, de 5 % chez les enfants *H. pylori*-négatifs et de 36,6 % et 16,6 % chez les patients normaux, selon la coloration histologique. La positivité à l'égard des anticorps anti-CagA et VagA n'a pas été significativement différente entre les patients atteints de cancer de l'estomac ou d'UGD qui avaient subi un traitement d'éradication dix ans auparavant. Une positivité significative a été observée entre le groupe d'enfants et le groupe *H. pylori*-négatif (antécédents d'UGD) (p < 0,001). Une différence statistiquement significative a été observée quant à l'âge entre les groupes (p < 0,03).
CONCLUSION : Les patients atteints de cancer de l'estomac, même *H. pylori*-négatifs au moment de l'étude, peuvent avoir été infectés par *H. pylori* avant le déclenchement de la maladie, comme le confirme la CagA et la VacA-séropositivité. Ces données étayent l'hypothèse selon laquelle *H. pylori* pourrait être un agent cancérogène direct dans le cancer de l'estomac.

Helicobacter pylori infection is one of the most widespread infections in humans. It is highly prevalent, especially in developing countries. In the western world, the infection rate is gradually decreasing; however, the prevalence still ranges from 25% to 50% (1). Virtually everyone infected with *H pylori* develops lifelong chronic type B gastritis, but only a minority of infected individuals will develop a clinically relevant condition, such as gastric cancer (GC). Seroepidemiological studies

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have demonstrated a three- to sixfold increased risk for infected individuals of developing GC (2-4). Based on these and other findings, *H pylori* has been classified as a class I human carcinogen by the International Agency for Research on Cancer (5), although the exact nature and strength of its association with GC has remained debatable. However, recent meta-analyses (6-8) indicate a weaker RR (approximately two) than originally reported for seropositive individuals (2,3).

Although it is clear that *H pylori* infection increases the risk of these upper gastrointestinal diseases, it is still not fully understood why infected individuals develop one disease rather than another, emphasizing the importance of the host and other cofactors. It has been suggested that both the possession of the cytotoxin-associated gene A (CagA), and the production of a vacuolating cytotoxin encoded by the vacuolating cytotoxin A (VacA) gene, are linked to increased pathogenicity of *H pylori* strains (9,10). There is substantial evidence that *H pylori* infection, especially with the strains expressing the 128 kDa CagA protein, is associated with enhanced gastric inflammatory response and increased risk of developing atrophic gastritis (11-13), peptic ulcer (10,14,15) and even GC (2,16-18). However, conflicting results regarding the association between these virulence factors and clinical disease have been reported, and epidemiological papers suggest that not all tumours are *H pylori*-positive (19,20). The objective of the present study was to determine whether patients with GC who tested *H pylori*-negative by histological Giemsa staining were previously infected with *H pylori* CagA- and/or VacA-positive virulent strains.

PATIENTS AND METHODS

Twenty-three individuals with GC (16 men, seven women; mean [± SD] age 68.14±9.8 years) who tested negative for *H pylori* by histological Giemsa staining were included in the study.

The presence of *H pylori* in gastric antral and corpus mucosa was determined by Giemsa staining, and also by immunoglobulin (Ig) G *H pylori* antibodies detected at the same time as VacA and CagA antibodies in human serum.

Endoscopic mucosal biopsies were obtained from the antrum and corpus mucosa, far from the tumour site, for histological assessment of *H pylori*. The patients underwent two antral biopsy samplings taken from the lesser and greater curvature, approximately 2 cm from the pylorus if possible. Two further biopsies of the gastric body were taken from the major and minor curves, if possible. Multiple biopsies were obtained from the tumour lesion to establish the diagnosis according to the Lauren classification (21).

In the present study, three control groups were used; each group consisted of *H pylori*-negative subjects. The first control group comprised 19 individuals (14 men, five women; mean age 58±18.2 years) with past *H pylori* infection and duodenal ulcers (DUs) that were eradicated 10 years earlier. In this group, patients were tested for *H pylori*-negative status every year by endoscopy, and gastric biopsy specimens were collected in the same way as for the GC group. For histopathological examination and *H pylori* detection, two antrum and two corpus biopsy specimens were stained using hematoxylin and eosin, and Giemsa staining. In this group of patients, IgG *H pylori* antibodies were also detected at the same time as VacA and CagA antibodies in human serum. The follow-up period of observation after *H pylori* eradication therapy was (mean ± SD) 120±32 months (range 96 to 144 months). All histological specimens were assessed by the same pathologist (EC).

The second control group was composed of 20 asymptomatic children (10 boys, 10 girls; mean age 7±4.47 years) with *H pylori*-negative fecal tests.

The third control group was composed of 30 patients without clinical symptoms (17 men, 13 women; mean age 68±11.6 years). All patients tested *H pylori*-negative by histological staining; again for this group, mucosal biopsies were obtained from the antrum and corpus mucosa, and were stained with hematoxylin and eosin, and Giemsa staining. The endoscopic diagnosis of these patients was esophagitis in nine patients, gastritis in 17 patients, DU in two patients and normal mucosal pattern in another two patients. VacA, CagA and IgG *H pylori* antibodies were detected simultaneously.

Blood samples of all subjects recruited for the present study were collected. After centrifugation at 37°C, all serum samples were stored at –20°C and transported to the clinical immunology laboratory at Rivoli Hospital (Turin, Italy), where tests were performed.

CagA and VacA antibodies from all patients were detected by the Western blot technique, using a commercially available kit (Immunoblot Helicobacter IgG, Mikrogen, Germany). This test detects antibodies for proteins from different *H pylori* strains (22).

For IgG detection of *H pylori* in human serum, a two-step immunometric assay was applied, using a commercially available kit (Immulite 2000 *H pylori* IgG, Diagnostic Products Corporation, USA).

A commercially available kit (Premier Platinum HpSA enzyme immunoassay, Meridian Bioscience Inc, USA) was used to detect *H pylori* antigens in human stools.

Data are expressed as mean ± SD and as percentages of totals. The χ^2 test was used for statistical analysis. Differences were considered to be statistically significant at $P<0.05$.

RESULTS

The prevalence of CagA, VacA and IgG seropositivity for *H pylori* antibodies was 82.6%, 73.91% and 56.5%, respectively in GC patients; 84.2%, 84.2% and 47.3%, respectively in *H pylori*-negative DU patients; 25%, 5% and 10%, respectively in *H pylori*-negative children; and 36.6%, 16.6% and 40%, respectively in patients without clinical symptoms who tested *H pylori*-negative by histological staining. A statistically significant difference was found in serum CagA and VacA antibodies, versus IgG *H pylori* antibodies in *H pylori*-negative DU patients ($P=0.03$). In the GC group, a statistically significant difference was found in CagA versus IgG detection ($P=0.01$), whereas in VacA versus IgG detection, there was no statistically significant difference ($P=0.07$).

Data on VacA, CagA and IgG antibodies against *H pylori* in GC patients, DU patients, asymptomatic children and patients without clinical symptoms are shown in Table 1.

There were no significant differences between sexes in serum CagA, VacA and IgG antibody positivity in *H pylori*-negative GC patients, or in patients with past *H pylori* infection and DU eradicated 10 years earlier.

A statistically significant difference was found in serum CagA, VacA and IgG antibodies in the GC group versus in children ($P<0.001$). No significant difference was found in the

TABLE 1
Prevalence of VacA, CagA and immunoglobulin (Ig) G in gastric cancer patients, and in *Helicobacter pylori*-negative control patients

Groups	CagA+/total patients (%)	VacA+/total patients (%)	IgG+/total patients (%)
Gastric cancer	19/23 (82.6)	17/23 (73.9)	13/23 (56.5)
Control			
Duodenal ulcer	16/19 (84.2)	16/19 (84.2)	9/19 (47.3)
Children	5/20 (25)	1/20 (5)	2/20 (10)
Patients, no symptoms	11/30 (36.6)	5/30 (16.6)	12/30 (40)

+ Positive; CagA Cytotoxin-associated gene A; VacA Vacuolating cytotoxin A

GC group versus patients with past *H pylori* infection and DUs eradicated 10 years earlier (P=0.858).

CagA and VacA antibody positivity was found to be significantly different between the GC and *H pylori*-negative (with past DUs) groups, versus children and patients without clinical symptoms (P<0.001).

Moreover, a highly significant difference was found in serum CagA and VacA antibodies in the asymptomatic children and patients without clinical symptoms, versus patients with past *H pylori* and DUs eradicated 10 years earlier (P<0.001). The statistical significance for IgG was P<0.018 between asymptomatic children and patients with past *H pylori* and DUs eradicated 10 years earlier; there was no statistically significant difference between children and patients without symptoms.

No statistically significant difference was found among CagA, VacA and IgG antibodies against *H pylori* in DU and GC patients whose *H pylori* infection was eradicated 10 years earlier. The respective P values were P=0.905, P=0.420 and P=0.920.

A weak statistically significant difference was also found in age among groups (P<0.03).

Figure 1 shows a sample of similar patterns of CagA, assessed by Western blot in 1992 and 2002, from a patient with past *H pylori* and DUs who tested negative to histological Giemsa staining for *H pylori*.

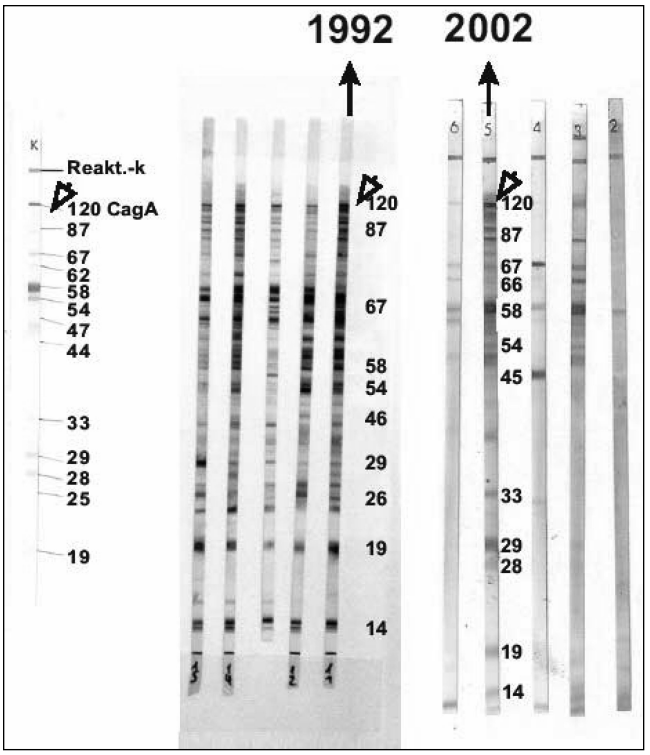

Figure 1) *Western blot of cytotoxin-associated gene A (CagA) antibodies in the sera of a duodenal ulcer patient whose* Helicobacter pylori *infection was eradicated 10 years earlier, and who tested negative to histological Giemsa staining with consistent patterns of CagA in 1992 and 2002*

DISCUSSION

H pylori infection causes both intestinal and diffuse types of gastric adenocarcinoma. Its carcinogenic effects have been attributed to induction of inflammation. The phenotype of *H pylori* that expresses CagA causes higher degrees of acute and chronic inflammation than the CagA-negative condition. Recent serological studies have shown that CagA and VacA seropositivity is associated with an increased risk for atrophic gastritis and GC (12,17,19). The *H pylori* strain possessing CagA-enhanced gastric epithelial proliferation and apoptosis induces tyrosine phosphorylation of the CagA protein (20,23), causing gastric cells to produce high levels of interleukin-8, which plays a crucial role in the inflammatory cell response to infection (24).

Whereas some studies on GC patients were unable to find an association between VacA and CagA antibodies, and GC (25), other studies demonstrated a significant association between VacA and CagA antibodies, and GC (26). Some authors found no difference in the prevalence of anti-CagA antibodies between *H pylori*-positive DU patients and patients without DUs and GC (27,28).

Although GC patients tested negative for *H pylori* at the time of the present study, they tested positive for serological CagA, VacA and IgG antibodies. The presence of CagA, VacA and IgG antibodies against *H pylori* in these patients likely indicates *H pylori* infection before the appearance of GC. In fact, CagA-positive *H pylori* strains seem to induce a high immune response with a markedly higher frequency of IgA. Although literature is scarce, existing clinical data indicate that CagA antibodies persist longer after eradication treatment than antibodies detected by IgG ELISA; a stronger association emerged when antibodies to CagA were used as markers of *H pylori* exposure (29). It therefore seems reasonable, as other studies report (30), to assume that the addition of immunoblotting would result in a more correct representation of prior exposure than the use of IgG ELISA alone.

These data were also confirmed by the present study, in which patients with previous DUs eradicated 10 years earlier, who were known to be *H pylori*-negative at the time of the histological biopsy of the antrum and corpus, and were followed up for a long period of time (120±32 months) tested positive for VacA and CagA proteins more often than for IgG.

This was also confirmed by the high level of CagA and VacA than IgG antibodies for *H pylori* in the patients with GC and in asymptomatic children.

These data reinforce the hypothesis that CagA and VacA proteins induce a strong mucosal and systemic immune response and may represent an immunological memory from previous contact with the bacteria. The former DU patients

showed seropositivity to CagA and VacA without any difference of prevalence versus GC patients, as some studies report. Seropositivity for CagA, VacA and IgG for *H pylori* in GC patients can be explained theoretically by a previous infection that was cured. Because several decades may pass between initiation and detection of GC, and the precancerous microenvironment promotes spontaneous eradication (31), substantial misclassification of relevant exposure to *H pylori* is likely to occur in case control and short-term follow-up studies. A declining association between *H pylori* and GC risk with advancing age could possibly be explained by increased exposure misclassification with advancing age.

Although the number of patients included in the present study was somewhat limited, it nevertheless reinforces the idea that CagA and VacA induce a strong mucosal and systemic immune response, and may represent an immunological memory from previous contact with the bacteria. *H pylori* can be considered a direct carcinogenic agent of GC, although it is not clear why some people develop cancer, and others only develop DUs or no pathology at all. Further studies are necessary for a better understanding of the pathogenic mechanism.

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