• *RAPID COMMUNICATION*•

Prevalence of *Helicobacter pylori* infection and intestinal metaplasia in subjects who had undergone surgery for gastric adenocarcinoma in Northwest Italy

Giorgio Palestro, Rinaldo Pellicano, Gian Ruggero Fronda, Guido Valente, Marco De Giuli, Tito Soldati, Agostino Pugliese, Stefano Taraglio, Mauro Garino, Donata Campra, Miguel Angel Cutufia, Elena Margaria, Giancarlo Spinzi, Aldo Ferrara, Giorgio Marenco, Mario Rizzetto, Antonio Ponzetto

Giorgio Palestro, Department of Oncology, University of Torino, Italy
Rinaldo Pellicano, Mario Rizzetto, Antonio Ponzetto, Department of Gastro-Hepatology, Ospedale S. Giovanni Battista (Molinette), Torino, Italy
Gian Ruggero Fronda, Mauro Garino, Donata Campra, Department of Surgery, Ospedale S. Giovanni Battista (Molinette), Torino, Italy
Guido Valente, Department of Pathology, University of Piemonte Orientale, Novara, Italy
Marco De Giuli, Department of Surgery, Ospedale S. Giovanni Antica Sede, Torino, Italy
Tito Soldati, Department of Surgery, Ospedale degli Infermi, Biella, Italy
Agostino Pugliese, Department of Infectious Diseases, University of Torino, Italy
Stefano Taraglio, Department of Pathology, Ospedale Maria Vittoria, Torino, Italy
Miguel Angel Cutufia, Department of Biology, Biochemistry and Genetics, University of Torino, Italy
Elena Margaria, Department of Pathology, Ospedale S. Giovanni Antica Sede, Torino, Italy
Giancarlo Spinzi, Gastroenterology Unit, Ospedale Valduce, Como, Italy
Aldo Ferrara, Gastroenterology Unit, Ospedale di Legnano, Legnano, Italy
Giorgio Marenco, General Medicine Unit, Ospedale Santa Corona, Pietra Ligure, Italy
Correspondence to: Professor Antonio Ponzetto, Department of Internal Medicine, University of Torino and Ambulatorio di Gastroenterologia, Ospedale S Giovanni Battista, Via Chiabrera 34, III piano, 10126 Torino, Italy. ponzetto@inwind.it
Telephone: +39-11-6336033   Fax: +39-11-6336033

Abstract

AIM: To investigate the seroprevalence of *Helicobacter pylori* (*H pylori*) infection and its more virulent strains as well as the correlation with the histologic features among patients who had undergone surgery for gastric cancer (GC).

METHODS: Samples from 317 (184 males, 133 females, mean age 69±3.4 years) consecutive patients who had undergone surgery for gastric non-cardia adenocarcinoma were included in the study. Five hundred and fifty-five (294 males, 261 females, mean age 57.3±4.1 years) patients consecutively admitted to the Emergency Care Unit served as control. Histological examination of tumor, lymph nodes and other tissues obtained at the time of surgery represented the diagnostic "gold standard". An enzyme immunosorbent assay was used to detect serum anti-*H pylori* (IgG) antibodies and Western blotting technique was utilized to search for anti-CagA protein (IgG).

RESULTS: Two hundred and sixty-one of three hundred and seventeen (82.3%) GC patients and 314/555 (56.5%) controls were seropositive for anti-*H pylori* ($P<0.0001$; OR, 3.58; 95%CI, 2.53-5.07). Out of the 317 cases, 267 (84.2%) were seropositive for anti-CagA antibody *vs* 100 out of 555 (18%) controls ($P<0.0001$; OR, 24.30; 95%CI, 16.5-35.9). There was no difference between the frequency of *H pylori* in intestinal type carcinoma (76.2%) and diffuse type cancer (78.8%). Intestinal metaplasia (IM) was more frequent but not significant in the intestinal type cancer (83.4% *vs* 75.2% in diffuse type and 72.5% in mixed type). Among the patients examined for IM, 39.8% had IM type I, 8.3% type II and 51.9% type III (type III *vs* others, $P = 0.4$).

CONCLUSION: This study confirms a high seroprevalence of *H pylori* infection in patients suffering from gastric adenocarcinoma and provides further evidence that searching for CagA status over *H pylori* infection might confer additional benefit in identifying populations at greater risk for this tumor.

INTRODUCTION

Gastric cancer (GC) is the world's second leading cause of cancer-related mortality[1] but in some countries it represents the most common malignancy in males[2].

GC occurrence in many Italian regions is similar to that in Japan. In Italy, GC is usually discovered at a later stage and therapeutic approaches cannot save a majority of patients. As a consequence, mortality parallels incidence[3].

The most frequent histologic type of GC is adenocarcinoma, which is thought to originate from a continuing and active proliferation of gastric pits following the destruction of glands due to active inflammatory infiltration. The process that has been described by Correa[4] from an inflammatory setting (gastritis) through intestinal metaplasia (IM) and dysplasia, evolves to adenocarcinoma.

In 1994, the International Agency for Research on Cancer defined *H pylori* as a class I gastric carcinogen[5]. Evidence supporting a causal association has been demonstrated by epidemiological data[6], ecologic studies[1] and in experimental animal models[7]. Regarding the first aspect, in a prospective study including 1 526 Japanese subjects during a mean follow-up of 7.8 years (range 1.0-10.6 years), 2.9% of infected persons developed GC *vs* none among uninfected subjects[8]. A combined analysis of 12 case-control studies (with 1 228 GC cases considered) nested within prospective cohorts has found an association between non-cardia GC and *H pylori* infection of 5.9 (95% confidence interval [CI] 3.4-10.3)[9]. A meta-analysis of 21 case-control studies suggested that the risk of GC is increased by threefold in those chronically infected with *H pylori*[10]. More recently, another meta-analysis of case-control studies with age- and sex-matched controls was published by Huang *et al.*[11]. In this work, a comprehensive literature search identified 16 qualified studies with 2 284 cases and 2 770 controls. The authors found that *H pylori* and CagA (cytotoxin-associated gene A) protein seropositivity significantly increases the risk for GC by 2.28- and 2.87-fold, respectively.

There is still no final conclusion regarding the association between the infection and the malignancy due to marked geographic variations. Some studies have not found any correlation between seropositivity for *H pylori* antibodies (as an indicator of *H pylori* infection) and GC[12-14]. For example, in the study performed by Rudi *et al.*[12] in Germany, 58.6% of patients suffering from GC and 50.6% of control subjects have IgG antibodies against *H pylori*. In Gambia, though more virulent strains of *H pylori* are present, gastric atrophy and IM are rare[15]. Seropositivity for *H pylori* and the CagA antigen cannot explain the differences in the prevalence of precancerous gastric lesions in two Chinese populations with contrasting GC rates[16]. Recently, Wong *et al.*[17] found that the incidence in GC development is similar between the subjects receiving *H pylori* eradication treatment and those receiving placebo during a period of 7.5 years in a high-risk region of China. Furthermore, not all the stomach tumors are *H pylori* positive.

In previous local pilot studies in North Italy, a high prevalence of *H pylori* infection has been associated to the presence of GC[18,19]. To investigate the correlation in a vast area of Northwest Italy in more detail, we started a research network on gastric cancer and precursor lesions in 1993, which we named Metaplasia *H pylori* Histology (MHEPHISTO). In this multicenter survey, a prospective case-control study of patients who had undergone surgery for GC in Northwestern Italy was performed. The aim was to ascertain the seroprevalence of *H pylori* infection and its more virulent strains by searching for antibodies against the CagA protein and to establish the correlation with the subtypes of IM.

MATERIALS AND METHODS

Study population

Specimens from 317 (184 males, 133 females, mean age 69±3.4 years) consecutive patients who had undergone surgery for gastric non-cardia adenocarcinoma were included in the study. Five hundred and fifty-five patients (294 males, 261 females) consecutively admitted to the Emergency Care Unit of S. Giovanni Battista (Molinette) Hospital of Torino served as control with a mean age 57.3 ±4.1 years. Cases and controls came from the geographical area of Northwestern Italy.

Methods

Clinical diagnosis of malignancy was established by standard medical examinations including upper GI endoscopy, diagnostic ultrasound and computed tomography (CT) scan. Endoscopic ultrasound (EUS) served as a part of the routine examination.

Histological examination of tumor, lymph nodes and other tissues obtained at the time of surgery represented the diagnostic "gold standard". Pathologists with special interest and experience in GI pathology reviewed the histological sections. Appropriate forms were used to record the pathological findings. All the diagnostic criteria used for our survey were discussed and sample slides were reviewed by the pathologists before the study to minimize interobserver variations as far as possible.

Surgical specimens were immersed in paraffin for routine pathological examination. Microtome sections (7-8 μm thick) were stained with hematoxylin and eosin as well as high iron diamine/alcian blue to identify sialo-and sulfomucins. Adenocarcinoma was diagnosed when the malignant cells invaded the lamina propria in single cells, glandular or solid nest arrangements, usually accompanied with fibrosis of the surrounding tissue. Carcinomas were classified histologically as either intestinal or diffuse type in accordance with Lauren's classification[20]. Intestinal metaplasia classified according to Filipe *et al.*[21] was defined as metaplastic transformation of gastric glandular and surface epithelium towards intestinal mucosal elements including goblet, absorptive, and Paneth cells.

Personnel not aware of the histological diagnoses performed serological testing. A commercial enzyme

Table 1 Seroprevalence of anti-*H pylori* antibodies among patients with gastric cancer and controls

Parameters	Gastric cancer (%)	Controls (%)
H pylori positive	261 (82.3)	314 (56.5)
H pylori negative	56	241
Anti-CagA positive	267 (84.2)	100 (18)
Anti-CagA negative	50	455

Table 2 Features of patients suffering from gastric cancer based on tumor subtype

Parameters	Intestinal type carcinoma (%)	Diffuse type carcinoma (%)
H pylori positive	138/181 (76.2%)	67/85 (78.8%)
Intestinal metaplasia	151/181 (83.4%)	64/85 (75.2%)

immunosorbent assay (ELISA, Helori-test® Eurospital, Trieste, Italy) was used to detect serum anti-*H pylori* (IgG) antibodies. The assay sensitivity and specificity were 94% and 87%[22]. Briefly, calibrators, positive and negative controls and diluted (1:200) serum samples were added to the wells coated with purified *H pylori* group-specific antigens. Plates were incubated for 60 min at 37 ℃, and then the liquid was removed completely and washed thrice with 200 μL/well of washing solution. One hundred microliters of anti-IgG conjugate was pipetted into each well. The wells were incubated for 60 min at 37 ℃, washed thrice with 200 μL/well of washing solution and 100 μL of chromogenic substrate was added to each well. The wells were again incubated for 30 min at 37 ℃, the reaction was stopped by adding 25 μL of the stopping solution. Reading was performed at 405 nm and the mean optical density was expressed as a percentage of the optical density of positive control serum assayed on the same plate. To detect the presence of serum IgG against *H pylori* CagA protein, Western blotting technique was used. *H pylori* CCUG 17874 (type strain, CagA positive, CagA mass = 128 ku) was cultured in *Brucella* broth containing 0.2% cyclodextrin at 37 ℃ in a microaerobic environment for 48 h. At the end of incubation, to exclude the presence of contaminants, the broth culture was subcultured onto plain blood agar plates and examined under optical microscope after staining with carbol fuchsin. The broth culture was centrifuged and the pellet was washed twice with phosphate buffered saline (PBS, pH 7.4) at 4 ℃. A whole cell suspension containing approximately 10^{10} bacteria was lysed and denatured in Laemmli buffer at 95 ℃ for 5 min, then run electrophoretically on 10% polyacrylamide gel with sodium dodecyl sulfate. Proteins were transferred onto nitrocellulose sheets saturated with 3% defatted milk in PBS with 0.1% Triton X (PBSMT). Strips were cut and serum samples were assayed at the dilution of 1:100 in PBSMT. After overnight incubation with constant agitation at room temperature, strips were washed with PBSMT and then incubated with an anti-human IgG serum conjugated with peroxidase at room temperature for 90 min. After the washings, the reaction was visualized by adding the substrate (H_2O_2 in a solution of 4-chloro-1-naphthol in Tris buffer 0.05 mol/L, pH 6.8). We used serum samples from patients infected respectively with CagA positive and negative *H pylori* strains as positive and negative controls with or without antibodies to CagA. Specific polyclonal antisera against CagA (kindly donated by Biocine-Chiron, Siena) was used as further controls.

The seroprevalence of anti-*H pylori* as well as the distribution of anti-CagA seropositivity in cases and controls were compared using the χ^2 test. Odds ratio (OR) and 95%CI assessing the risk of GC associated with *H pylori* infection were calculated using the Mantel-Haenszel method. $P<0.05$ was considered statistically significant.

RESULTS

All patients selected were suffering from gastric non-cardia adenocarcinoma (intestinal type in 181, diffuse type in 85 and mixed type in 51). Among these, 261 out of 317 (82.3%) were seropositive for IgG anti-*H pylori* compared to 314 out of 555 (56.5%) controls ($P<0.0001$; OR, 3.58; 95%CI, 2.53-5.07) (Table 1). Moreover, out of the 317 cases, 267 (84.2%) were seropositive for anti-CagA antibody *vs* 100 out of 555 (18%) controls ($P<0.0001$; OR, 24.30; 95%CI, 16.5-35.9) (Table 1).

There was no difference between the frequency of *H pylori* in intestinal type and diffuse type carcinoma. Overall, *H pylori* occurred in 138 out of 181 patients (76.2%) suffering from the former compared to 67 out of 85 (78.8%) suffering from the latter ($P = NS$) (Table 2). Intestinal metaplasia was more frequently seen in the intestinal type cancer (151/181, 83.4% *vs* 64/85, 75.2% in diffuse type and 37/51, 72.5% in mixed type) but the difference was not statistically significant ($P = NS$, Table 2). Among the patients examined for IM, 72 out of 181 (39.8%) had IM type I, 15 out of 181 (8.3%) type II, and 94 out of 181 (51.9%) type III, (type III *vs* others $P = 0.4$). Furthermore, among the patients with IM of either body or antral mucosa, 117 out of 151 (77.4%) with intestinal type carcinoma were positive for *H pylori* compared to 59 out of 85 (69.4%) with diffuse type carcinoma ($P = 0.17$).

DISCUSSION

Gastric carcinogenesis involves a slow but continuous stepwise evolution from superficial gastritis and glandular atrophy to metaplasia and dysplasia and finally to adenocarcinoma[23]. The process of carcinogenesis which may well extend over decades provides an excellent opportunity for early detection and intervention to prevent further progression of the sequence of events preceding the development of the neoplasma. This is especially true because *H pylori* (which can be readily treated) is known to be the main factor though not the only[24] etiological agent

and initiating carcinogen.

On the other hand, the prognosis of GC is poor. In most industrialized countries, only around 10% subjects survive for 5 years. The sole exception is Japan where this malignancy is often identified at an early stage and in younger and fitter patients[26]. In Italy, 10-year survival is 12.1% in all GC patients and 20.8% in resected cases. However, though the survival is good when the diagnosis is performed at an early stage, only a few cases are diagnosed at stages when cure by radical surgery is more probable[3].

Regarding the biological plausibility for a causal role, a higher intragastric pH after the development of atrophic gastritis provoked by *H pylori* may favor the production of carcinogens[26]. The generation of reactive oxygen species and increased level of inducible nitric oxide synthase may in turn cause genetic alterations leading to cancer[27]. Nardone *et al.*[28] demonstrated by morphometric and immunohistochemical techniques that *H pylori* infection seems to be responsible for genomic instability in patients with chronic atrophic gastritis and eradication of *H pylori* can reverse inflammation, atrophy, metaplasia, and genomic instability.

CagA gene is situated at the end of the large region of the genome identified to be a pathogenicity island (PAI). The strains of *H pylori* expressing CagA protein are considered more virulent, being linked with an increased risk of duodenal ulcer and GC[23]. Moreover, CagA status is associated with a higher prevalence of p53 mutation in gastric adenocarcinoma[29].

This multicenter study showed a significant association between *H pylori* infection in particular by its more virulent strains and the presence of GC. In addition, the results confirmed that type III IM was most frequently associated with *H pylori* infection. These data suggest that CagA status is a helpful parameter in defining a subgroup of *H pylori*-infected patients at increased risk of developing gastric adenocarcinoma. The difference between the rate of *H pylori* infection and more virulent strains can be explained by the fact that CagA antibodies persist for a longer time than *H pylori* IgG surface antibodies. Hence, relying on *H pylori* IgG antibodies alone might misclassify a significant proportion of patients who once had the infection[11].

The established epidemiological association does not prove that there is a direct causal relationship. Therefore, to further confirm a causal role, we are going to evaluate the effect of *H pylori* on the morphological changes of gastric mucosa in patients with precancerous gastric lesions.

In conclusion, seroprevalence of *H pylori* infection is high in patients suffering from gastric adenocarcinoma, which provides further evidence that searching for CagA status over *H pylori* infection might confer additional benefit for identifying populations at greater risk for this tumor.

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