The detection of a *Helicobacter pylori* antigen in some different types of gastrointestinal cancers patients

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Abstract: Aim: This work is concerned with the identification of *Helicobacter pylori (H. pylori*) antigen in sera of patients with some different gastrointestinal cancers (pancreatic cancer (PC); gastric cancer (GC) and colon cancer (CC)) and then estimating its impact in the incidence of these different types of cancers. Method: A total of 175 individuals constituted this study (Healthy=75; PC=31; GC=30 and CC=39). Western-blot and ELISA were used for identifying the target *H. pylori*-antigen. Results: A single immunoreactive band was shown at 58-kDa corresponding to *H. pylori*-antigen due to its binding with its respective antibody. The detection rate of *H. pylori*-antigen was found to increase in patients who have CC (64%) when compared to patients who developed GC (60%) or PC (48%) but without any significant difference. Additionally, *H.pylori*-antigen levels were determined in each type of cancer being maximum in patients who developed CC in comparison with other types of gastrointestinal cancers. As well, *H. pylori* was found to increase GC and CC-risk, with an estimated odds ratio=1.54 and 1.8 higher than that of GC (OR=0.96). *Conclusion*: Patients infected with *H. pylori* were 54% and 80% more likely to be susceptible to GC and CC, respectively, than those without *H. pylori*-infection.
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1. Introduction

Helicobacter pylori (H. pylori) is one of the most successful human pathogens that is responsible for a variety of gastro-duodenal pathologies in the developed and developing world (1). In Egypt, there were no sufficient studies performed to give information about the prevalence of *H. pylori* infection. Generally, *H. pylori* colonizes the stomach and induces chronic gastritis representing a key factor in the etiology of various gastrointestinal diseases (2). Gastric cancer continues to be a major global health problem. Approximately, 70% of all gastric cancer cases worldwide are directly attributable to prior *H. pylori* infection (3). So, the World Health Organization's International Agency for Research on Cancer classified this pathogen as a group I carcinogen. Hence, the evidence linking *H. pylori* to gastric cancer has continued to accumulate and strengthen (4, 5). In addition to its role in gastric cancer; *H. pylori* may be associated with other extra-gastric cancers. Thus, it would be important to know if *H. pylori* infection is a risk factor for other gastrointestinal cancers (6-8). It was reported that colon and pancreas are likely probable organs to be affected by *H. pylori* activity (9). Therefore, clinicians would need a rapid and reproducible, noninvasive assay for the screening of patients for the presence of *H. pylori*. For these reasons, we previously created a novel noninvasive test based on the detection of *H. pylori* circulating antigen in sera of individuals infected with *H. pylori* with a high degree of efficiency (10). In this work, we are concerned with the identification of *H. pylori*-antigen in sera of patients with some different gastrointestinal cancers (colon, gastric and pancreatic cancers) and then estimating if this pathogen has an impact in the incidence of these different types of cancers.

2. Material and methods

Patients

A total of 175 consecutive Egyptian individuals constituted this study. They were categorized into four groups. The first one included serum samples of seventy-five healthy volunteers used as a control group. This cohort comprised 60 males and 15 females with a mean (±SEM) age of 51.2 (±2.1) years. The second one included serum samples from thirty-one patients who had pancreatic cancer (PC). This cohort comprised 19 males and 12 females with a mean (±SEM) age of 56.5 (±1.9) years. The third group included thirty serum samples from patients with gastric cancer (GC). This cohort comprised 18 males and 12 females with a mean (±SEM) age of 55.3 (±2.4) years. The final group included thirty-nine serum samples from patients with colon cancer (CC). Colon cancer was diagnosed by

elevated Carcinoembryonic Antigen (CEA) tumor marker (11) followed by colonic endoscope and confirmed by colonic biopsy. Gastric cancer was diagnosed by upper gastrointestinal endoscope confirmed by gastric biopsy. Pancreatic cancer was investigated by measuring of Carbohydrate Antigen 19.9 (CA19.9) tumor marker (11) and radiological studies (chest X-ray, abdominopelvic CT scans with contrast enhanced triple phase helicals and Doppler studies). All serum samples were subjected to laboratory investigations including liver function tests (alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin and alkaline phosphatase (ALP)) which were measured on an automated biochemistry analyzer (Hitachi 917; Roche diagnostics). At the same time, tumor markers CEA and CA19.9 were detected, using a commercial ELISA kit, according to the manufacturer's instructions (CanAg CEA EIA and CanAg CA19.9 EIA, CanAg, Diagnostics AB, Gothenburg, Sweden).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out in 0.75 mm-thick, 12% vertical slab gels according to the method of Laemmli (12). Serum samples were mixed with the sample buffer (0.125 M Tris base, 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, and 0.1% bromophenol blue as a tracking dye). They were then separated and resolved as discrete bands as they migrate in an electric field through the "sieving" action of the acrylamide gel matrix. Standard molecular weights (BioRad Laboratories, CA) were run in parallel.

Western-blot

Serum samples which were separated on SDS-PAGE were then electrotransferred onto nitrocellulose membrane (0.45 mm pore size, Sigma) in a protein transfer unit (13). The nitrocellulose membrane was blocked using 5% nonfat dry milk dissolved in 0.05% M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and then incubated with rabbit anti-*H. pylori* lysate antibodies (ABC Diagnostics, New Damietta, Egypt) diluted in blocking buffer with constant shaking overnight. Nitrocellulose membrane was then washed in TBS three times, 20 min each followed by incubation separately for 2 hours with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted in TBS. After washing in TBS, the target antigens were visualized by incubating the nitrocellulose membrane filter in alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP]/nitro-blue tetrazolium [NBT]) in 0.1 M Tris buffer, pH 9.6, (Sigma) for 10 min. The reaction was stopped using distilled water.

Detection of 58-kDa *H. pylori* antigen using ELISA

Diluted serum samples in coating buffer (50 mM Carbonate/Bicarbonate buffer, pH 9.6), were tested (50 µL/well) bound on a 96-well polystyrene microtiter plates at 4°C overnight. After blocking with 0.2% nonfat milk in coating buffer (200 µL/well), mono-specific antibody diluted in PBS was added (50 µL/well) and incubated at 37°C for 2 hours. Then, goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma) diluted in 0.2% BSA in PBS-T20 was incubated at 37°C for 1 hour. The plate was washed with PBS + 0.5% Tween 20 after every step. The substrate was 1 mg/ml p-nitrophenyl phosphate and the intensity of the signal was determined by measuring the absorbance at 450 nm after 10 min using a microtiter plate reader (Σ 960, Metretech Inc, Germany). Color intensity was proportional to the amount of bound conjugate and therefore is a function of the concentration of *H. pylori* antigen present in the serum sample.

Statistical analysis

All statistical calculations were done by SPSS software v.15.0 (SPSS Inc., Chicago, IL) as well as Graph Pad Prism package; v. 5.0 (Graph Pad Software, San Diego, CA). Continuous variables were expressed as mean ± standard error of mean (SEM). Student's test was used to compare continuous variables while Chi-square test was used to compare proportions. A value of $P > 0.05$ was considered statistically significant.

3. Results

Identification of *H. pylori* antigen in patients with gastrointestinal cancers

SDS-PAGE and Western blotting were used as described previously in materials and methods section for identifying the target *H. pylori* antigen. As a result, a single band was shown at 58-kDa molecular weight corresponding to *H. pylori* antigen in sera of patients who had pancreatic cancer (PC), gastric cancer (GC) and colon cancer (CC). That's due to the binding with its respective mono-specific antibody. Similar 58-kDa band was recognized in serum taken from *H. pylori* culture positive individual while no specific reaction was observed in the serum taken from *H. pylori* culture negative individual as depicted in Figure 1.

The characteristics of patients who had some gastrointestinal cancers (pancreatic cancer, gastric cancer and colon cancer) were summarized in Table 1. The prevalence of 58-kDa *H. pylori* antigen was investigated in patients with some gastrointestinal cancers and depicted in Figure 2. As a result, the detection rate of 58-kDa *H.pylori* antigen was found to increase in patients who have colon cancer (64%) when compared to patients who developed gastric

cancer (60%) or pancreatic cancer (48%) but without any significant difference as shown in Figure 2. Next, the levels of 58-kDa *H. pylori* antigen were then determined in each type of cancer as seen in Figure 3. It was found that the levels of 58-kDa *H. pylori* were maximum in patients who developed colon cancer in comparison with other types of gastrointestinal cancers. Finally, the odds ratio (OR) was then used as a measure of association between 58-kDa *H. pylori* antigen and different groups of diseases. Patients with colon cancer were associated with higher odds ratio (OR=1.8) than patients with gastric cancer (OR=1.54) or pancreatic cancer (OR=0.96) as shown in Figure 4. This may indicate that *H. pylori*-infection increase the risk of gastric and colon cancers. Hence, patients who infected with *H. pylori* were 54% and 80% more likely to be susceptible to GC and CC, respectively, than those without *H. pylori*-infection.

Figure 1. Prevalence of 58-kDa *H. pylori* antigen among patients with gastrointestinal cancers. Identification of 58-kDa *H. pylori* antigen based on Western blot analysis using mono-specific antibody corresponding to 58-kDa *H. pylori* antigen in sera from patients with some gastrointestinal cancers. Lane 1: serum sample from healthy individual used as negative control. Lane 2: serum sample from *H. pylori* culture positive individual used as positive control. Lanes 3-4: selected serum samples from pancreatic cancer patients. Lanes 5-6: selected serum samples from gastric cancer patients. Lanes 7-8: selected serum samples from colon cancer patients. The molecular mass (M_r) protein markers (not shown but indicated by arrows) are phosphorylase B (M_r =97.4 kDa), bovine serum albumin (M_r =66.2 kDa), glutamate dehydrogenase (M_r =55.0 kDa), ovalbumin (M_r =42.7 kDa), aldolase (M_r =40.0 kDa), carbonic anhydrase (M_r =31.0 kDa), and soybean trypsin inhibitor (M_r=21.5 kDa).

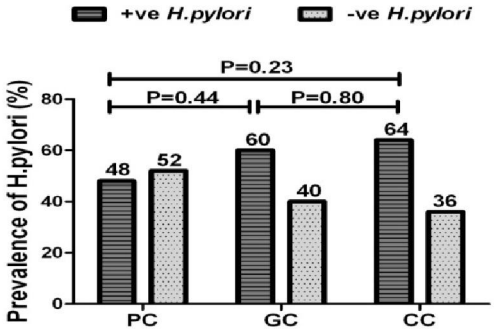

Figure 2. Prevalence of 58-kDa *H. pylori* antigen among patients with pancreatic cancer (PC), gastric cancer (GC) and colon cancer (CC) showing an increase in its detection rate in CC compared to GC and PC.

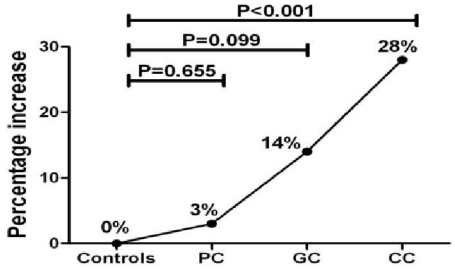

Figure 3. The levels of 58-kDa *H. pylori* antigen were then determined in each type of Cancer. Changes in 58-kDa *H. pylori* antigen concentration between patients who had different gastrointestinal cancers versus healthy individuals (Results are expressed as percentage increase as compared to controls that was set to 0%).

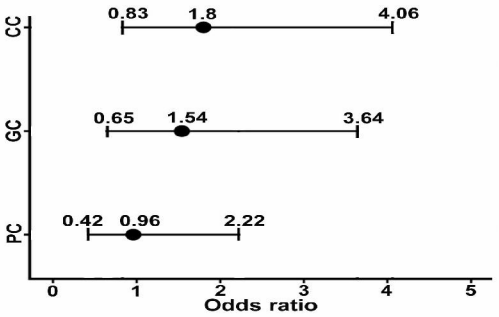

Figure 4. Odds ratio (OR) and 95% confidence intervals showing the risk of 58-kDa *H. pylori* antigen on other gastrointestinal cancers.

Table 1. Characteristics of patients who have pancreatic (n=31), gastric (n=30) and colon (n=39) cancers

Variable	Pancreatic cancer (31)	Gastric cancer	Colon cancer	*P value
Sex (M/F)	19/12	18/12	19/20	0.500
Age (years)	56.5±1.9	50.3±2.4	51.6±1.9	0.113
ALT (U/L) [a]	31.9±5.9	38.2±6.1	25.5±2.9	0.508
AST (U/L) [a]	34.2±5.6	36±4.8	34.9±4.1	0.971
Bilirubin (mg/dl) [a]	1.7±0.4	1.5±0.2	1±0.2	0.429
Albumin (g/L) [a]	36±2.3	32±2.9	33±2.5	0.395
ALP (U/L) [a]	219±44	182±47	228±48	0.790
CEA (U/L) [a]	49±34	33±21	230±220	0.847
CA19.9 (U/L) [a]	988±394	3337±1663	88±37	0.003

Continuous variables were expressed as mean ± SEM. [a] Reference values: aspartate aminotransferase (AST) up to 40 U/L; alanine aminotransferase (ALT) up to 45 U/L; total bilirubin up to 1 mg/dL; albumin 38-54 g/L; alkaline phosphatase (ALP) 22-92 U/L; Carcinoembryonic antigen (CEA) up to 5 U/L; Carbohydrate antigen 19.9 (CA19.9) up to 37 U/L. *P>0.05 is considered not significant; P< 0.05 is considered significant; P< 0.0001 is considered extremely significant.

4. Discussion

As we previously stated, clinicians would need a rapid and reproducible, noninvasive assay for the screening of patients for the presence of *H. pylori*. No doubt that the use of antibodies-based methods can lead to ambiguous results especially since antibodies can be detectable a long time after the bacteria have ceased to colonize the gut (14). More efforts are directed toward identifying different *H. pylori* antigens using western blot technique e.g. 125 kDa CagA antigen and 87 kDa VacA antigen (15). In this respect, Attallah *et al.* (10) developed a novel non-invasive immunoassay based on the detection of *H. pylori* circulating antigen (HpCA) in serum samples from patients infected with *H. pylori*. This antigen was identified at 58 kDa. HpCA has been detected by ELISA with high degrees of sensitivity, specificity, and efficiency (>90%). Moreover, results obtained by ELISA showed no significant difference (P > 0.05) from results of *H. pylori* culture of gastric biopsy specimens. Herein, we preferred to use *H. pylori* antigen based method in order to examine the association between *H. pylori* infection and different gastrointestinal cancers. Many studies have failed to demonstrate an association between *H. pylori* and neoplastic colorectal lesions (adenomas and carcinomas) (16). However, there were sufficient published data, supportive of increased *H. pylori* seroprevalence in colorectal cancer patients (17, 18). Our results showed that 64% of colon cancer were tested positive for the presence of 58-kDa *H. pylori* antigen. Recently, similar results were showed by Strofilas *et al.* (19) who found the prevalence of *H. pylori* IgG antibodies in 71% of colorectal cancer patients compared with 65% in controls without significant difference. In addition, Jones *et al.* (18) detected *H. pylori* antibodies in colonic tissue in some types of colon neoplasms however; they can not infer causality from these results. So, the involvement of *H. pylori* in the pathogenesis and progression of colon cancer remains uncertain. In turn, the present study showed that 60% of patients who had gastric cancer were tested positive for the presence of 58-kDa *H. pylori* antigen. Serological association was reported between *H. pylori* and gastric cancer in many previous studies (20, 21); however no significant association was found in other studies (22, 23). Moreover, the decline in *H. pylori* IgG antibody level considered as a risk factor for gastric cancer (24). Forman and many other scientists considered that many cancer cases would have been likely to have severe atrophic gastritis and intestinal metaplasia, conditions that favor the loss of *H. pylori* colonization, and subsequently, loss of seropositivity (25). So that, patients with gastric cancer may be *H. pylori* seronegative even though they have been infected in the past (26). Numerous studies hint that *H. pylori* can trigger carcinogenesis. It seems *H. pylori* triggers a proliferative response in the epithelium, perhaps related to the delivery of CagA into the epithelial cells, whereas components of *H. pylori* such as CagA and VacA cause increased apoptosis to balance the proliferative effect. Although these events are not necessarily carcinogenic, the associated inflammation, reactive oxygen species, nitric oxide, HCl, and ammonia may generate known carcinogens, such as nitrosodimethylamine. Ultimately, mutations occur (e.g. in the p53 gene), which give *H. pylori* infected epithelial cells a proliferative advantage. The final steps toward malignancy might not require the continued presence of *H. pylori* (27). Furthermore, our study results were not supportive of a positive

correlation between *H.pylori* and pancreatic cancer, as 58-kDa *H. pylori* antigen was detected in 48% of pancreatic cancer patients. Similar conclusions with our results have reached the study groups of Lindkvist *et al.* (28) in which *H. pylori* IgG antibodies were positive in 45% of pancreatic cancer cases and de Martel *et al.* (29) who found *H. pylori* IgG antibodies in 49% of pancreatic cancer cases. Indeed, studies focusing on the relationship between *H. pylori* infection and pancreatic cancer risk have yielded conflicting results (9). Some previous studies show an association between *H. pylori* infection and pancreatic cancer (30), while others disproves this association (28). In pancreas histopathologic specimens investigated, *H. pylori* was absent from both malignant and normal pancreatic tissue (28). Takayama *et al.* (31) suggested that *H. pylori* infection of human pancreatic cells may enhance their malignant potential in a similar fashion to the gastric cell carcinogenesis. However; this correlation has not been confirmed.

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