Cytotoxicity–Associated gene (CagA) Producing *Helicobacter pylori* Increased Risk of Developing Colorectal Carcinoma in Iraqi Patients.

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Abstract:

Infection with *Helicobacter pylori*, particularly with strains positive for cytotoxicity–associated gene (CagA) gene, increases the risk of gastric adenocarcinoma and it may be associated with carcinogenesis in extra gastric target organ. The aim of this study to explore the possible association between CagA positive *H. pylori* and colorectal carcinoma and determine the association with clinico-pathological features.

Paraffin embedded tumor specimens from (49) patients with colorectal adeno-carcinoma and (30) healthy control were assessed by *in situ* hybridization for the expression of *H. pylori* CagA mRNA.

Statistical analysis of *H. pylori* CagA positive expression revealed highly significant difference in colorectal carcinoma patients than in control group. There was no relationship between *H. pylori* CagA positive expression and range of clinicopathological features.

Among patients infected with *H. pylori* CagA positive is associated with increased risk for colonic cancer.

Keywords: *H. pylori, CagA, colorectal cancer.*

الخلاصة:

أجريت هذه الدراسة على المرضى الوافدين الى وحدة الناظور/قسم الباطنية التابع لمستشفى اليرموك التعليمي. شملت الدراسة 127 عينة دم وخزعة نسيجية من أشخاص كانوا يعانون من امراض في المعدة. لوحظ أن 64 من المرضى مصابين بالتهاب المعدة المزمن و30 مصابين بسرطان المعدة، كما ان هناك 33 شخص كانوا بحالة صحية سليمة وغير مصابين ببكتريا *Helicobacter pylori* واعتبروا كمجموعة سيطرة.

اخضعت عينات الخزع النسيجية التي اخذت من الغار المعدي لكل شخص مريضا كان ام سليما التحليل اليوريز السريع والفحص النسيجي. تم فحص المصول للكشف عن وجود اجسام مضادة نوع IgG لبكتريا *H.pylori* وحساب مدى ارتفاع مستوى Pepsinogen I . II بواسطة مقايسة الممتز المناعي المرتبط بالانزيم (ELISA) كطريقة مصلية.

بينت الدراسة ان هناك ارتفاع معنوي $p<0.05$ ل pepsinogen I,II في مصل المرضى عند وجودالبكتريا وقلة النسبة PGI/PGII مقارنة بالاشخاص غير المصابين، كما وظهر انخفاض معنوي $p<0.01$ لمستوى SPGI في الاشخاص المصابين بسرطان المعدة مع ارتفاع معنوي $p<0.05$ لمستوى SPGII وقلة النسبة SPGI/SPGII بشكل ملحوظ مقارنة بالاشخاص المصابين بالتهاب المعدة المزمن والاشخاص الاصحاء الغير مصابين بالبكتريا.

Introduction:

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. The morbidity and mortality increased quickly in both developing and developed countries [1].

Colon cancer is a manifestation of a number of inherited cancer predisposition syndromes, including familial adeno-matous polyposis, hereditary non-polyposis colorectal cancer, and personal or family history of colorectal cancer and/or polyps and inflammatory bowel disease [2]. Other factors such as obesity, smoking, alcohol consumption, diet rich in high fat, red and processed meats and

inadequate intake of dietary fiber, fruits and vegetables are also associated with increased colon cancer risk [2,3]. Recent publications reported that *Helicobacter pylori* associated with colorectal carcinoma. However, this is quite controversial for different studies hold various results [3,6]. *Helicobacter pylori* are Gram-negative bacterium. Its infection has been recognized as a major risk factor for gastric cancer by the International Agency for Research on Cancer in 1994[7,8], and infected people are also at risk of developing a rare B cell tumor, so-called gastric mucosal-associated lymphoid tissue lymphoma [9]. *Helicobacter pylori* is one of the most genetically diverse bacterial species, some strains may be substantially more virulent than others [10].

The virulent strains have a unique CagA pathogenicity island, a 40 kilobase-pair segment of DNA comprising a collection of approximately 30 genes. Infection with *H. pylori* has been identified as a risk factor for gastric adenocarcinoma[1,11]. Subjects infected with *H. pylori* who have CagA are 5.8-fold more likely to develop gastric cancer[10]. It is role in colorectal cancer development remains unclarified. Some authors [9,12], shown a series of patients with colonic adenoma or cancer, reported an increased prevalence of *H. pylori* infection, whereas other[13], noted no such association.

The aim of the present study was to examine whether *H.* pylori especially positive Cag A statistically associated with colorectal cancer in Iraqi patients and possible correlation with colorectal cancer stage.

Materials and Methods:

Forty nine patients with colorectal adenocarcinoma infected with *H. pylori* (mean age 51.7 years and range 25- 80). The control group include 30 colorectal normal mucosa and uninfected with H. pylori (mean age 48.3 years and range between 22-78) were involved in this study.

The patients were referred to the gastrointestinal endoscopy unit at Al- Yarmook Teaching Hospital, non of whom had received non-steroidal anti-inflammatory drugs. Informed consent was obtained from all subjects.

Biopsy specimens were taken from all subjects in this study, by using the same size forceps, from similar sites at each endoscopy; biopsies were fixed in 10% formal buffer saline for histological examination and for bacteriological investigation (rapid urease test[14]).

Diagnosis of *H. pylori* infection Rapid urease test (RUT):

One biopsy was inoculated on urea agar slant, and then incubated at 37°C for 15 min. to 1hr. Slant was examined for color change from yellow to pink if the bacteria secret urease and hydrolyse the urea to ammonia and raises the pH of the medium which change the color.

Histology:

The colorectal biopsy specimens were embedded in paraffin and stained with haematoxylin–eosin (H&E) and Giemsa stained for *H. pylori* determination.

***In situ* hybridization (ISH) for detection of *H. pylori*/CagA gene:**

The use of Biotin – Labeled DNA probe for *H. pylori*/303 bp, CagA (8 µg/100 µl) litter dd H2O (Maxim Biotech, Inc., U.S.A).

In situ hybridization (ISH) is a technique makes use of the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell. For detection of this markers , the biotinylated DNA probe hybridize to the target sequence (*H. pylori* DNA/CagA mRNA sequence) then a streptavidin-AP (streptavidin-alkaline phosphates') Conjugate is applied followed by addition of the substrate promochloro unduly– phosphate/ nitro-blue tetrazolium (BCIP/NBT) which yield an intense blue–black signal appears at the directly specific site of the hybridized probe. This strepteividin–Ap

conjugate like the biotinylated probe provides a rapid and highly sensitive detection method. Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in both positive test tissues. Evaluation of the in situ staining was done with assistance of a histopathologist.

Scoring:

A scoring system that includes evaluation of the staining percentage of stained gastric cells was employed for the expression of DNA and CagA of *H. pylori*. Counting the number of the positive cells in the gastric tissue which gave a blue-black nuclear staining under the light microscope. The extent of the ISH signaling the cells of the examined tissue was determined in 10 fields under high power microscope (100X). In each field, the total staining score divided by the number of whole cell per field in 10 fields, so the percentage of positively stained cells in the 10 fields was calculated for each case by taking the mean of the percentage of the positively stained cell in the 10 fields. Tissues were regarded as *H. pylori* DNA and CagA positive when their ISH signaling scores were ≥ 5% [15].

Statistical analysis

Student test (t-test) was used for the quantitative data. The associations between *H. pylori* in different groups were assessed by the Chi-square test. *p* value of <0.05was considered statistically significant.

Results:

The prevalence of *H pylori* infection, in subjects with colorectal carcinoma is presented in Table-1. There was 62.54% of patients had *H.pylori*. The expression of CagA was detected by *in situ* hybridization technique. From 31 patients complaining colorectal carcinoma and infected with *H. pylori* who were tested for CagA, 20(64.51%) were found to be positive CagA and 11 (35.48%) patients have CagA negative (Table-2). Figure-1 reveals the expression of *H. pylori* CagA were dark brown staining in the tissue. Table -3 shows the expression of *H. pylori* CagA in the colon epithelial cells. It was significantly higher in colorectal cancer than in the control group (*p*<0.01).

Table-1: The prevalence of *H.pylori*infection in patients with gastroduodenal diseases.

***H.pylori* status**	**Gastroduodenal disease (No. 127)**
***H.pylori*- positive**	94 (74.01%)
***H.pylori*-negative**	33 (26.0%)

***p*<0.05**

Table -2: Expression of CagA mRNA in *H.pylori*– positive patients with colorectal carcinoma

	CagA status	**No. (%)**
***H. pylori* positive**	positive	20 (64.51%)
	negative	11 (35.48%)
	Total	31 (100%)

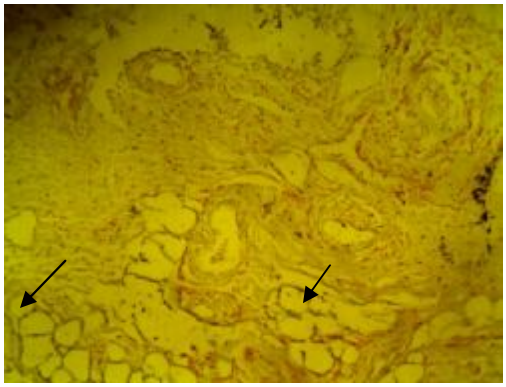

Figure- 1: Detection of CagA, in patients with colon carcinoma by *in situ* hybridization. Staining of CagA mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from patients with colon carcinoma shows positive CagA by hybridization signals.

Table-3: Comparison of mean percentage of CagA mRNA among studied group.

Studied groups	N	Mean± SE	Comparison of significant
			P-value
Controls	30	0.3 ± 0.02	(*P*<0.01)
colorectal carcinoma with *H. pylori*	31	34.2± 2.9	
Total	61		

Table-4: Comparison of *H. pylori* CagA mRNA by ISH among studied variables in colorectal carcinoma patients.

Variables	Cag A expression		Cag A Positive rate (%)	Chi sqaure *p*- value
	- n=11	+ n=20		
Tumor site				
Colon	4	11	55	>0.05 NS
Rectum	7	9	45	
Tumor Type				
Non-mucinous	5	10	50	>0.05 NS
mucinous	6	10	50	
TNM Staging				
T1,2	4	9	55	>0.05 NS
T3,4	7	11	45	

Ns: No significant difference (*P*>0.05), TNM: Tumor, lymph node, Metastasis.

Discussion:

Helicobacter pylori carriage has been associated with increased risk for both gastric and extragastric malignancy[3].

In this study *H. pylori* CagA was significantly higher in colorectal carcinoma patients than in cancer-free controls. The current study is in agreement with other studies, which showed that *H. pylori* that expressing CagA, was significantly higher in colorectal cancer patients than in controls [16, 17], and with the results of the research groups of Shmuely *et al* and Hartwich *et al*, who reported that CagA positive is associated with increased risk for both gastric and colonic cancer. But the results of this study could not determine a statistical association between its presence and the different stages of colorectal adenocarcinomas.

Some studies have not been able to substantiate an association between *H. pylori* and colorectal neoplasia [13,18,19]. and or, that these organisms can colonies the colon[19]. The interpretation of different results, possibly by using different methods to assess the expression of CagA positive *H. pylori* in patients, such as ELISA method, which demonstrated the anti CagA IgG antibodies that can persist for months after eradication of the bacterium with antimicrobial drugs. Some studies assessed the CagA by PCR, so, one possible problem is the reliability of the CagA PCR assay because a correct design of primers is very important because of strain genomic diversity. Therefore, the different sets of CagA primers give different results, and this will be attributed to divergence in the primer target sequences. *H. pylori* induced hyperga strinemia, either alone or in combination with alterations to the normal gastro-intestinal flora, represents a plausible mechanism whereby colonization with this organism could promote colorectal carci-nogenesis [20].

Occurring hypergastrinemia in cases of atrophic gastritis, as described above, the increase of the intraluminal ammonia products, which can trigger intracellular tumorigenic mechanisms, and finally the promotion of systemic inflammation, through overexpression of proinflammatory cytokines (IL-1,IL-8, TNF-α,etc) and growth factors (EGF,TGF-α, etc)[8, 21].

It is accepted that *H. pylori* strains that express the cytotoxicity-associated gene (CagA+) are associated to even greater increase of local and systemic inflammation, rising speculations regarding a possible correlation of increased expression of CagA positive strains in colorectal cancer patients, compared to CagA negative strains[21, 22].

Cytotoxicity- associated gene strains induce gastric cell proliferation that is not accompanied by a parallel increase in apoptosis, thereby increasing the risk for malignant transformation[10]. This local effect in the stomach occurs also in the colon. Fecal shedding of viable *H. pylori* and *H. pylori* antigen occurs under certain circumstances [23, 24, 25]. This indicates that *H. pylori* moves through the intestinal tract and makes contact with colonic mucosa. CagA strains may locally activate colonic carcinogenesis by inducing the expression of cytokines such as interleukin-8, which is known to be associated with both gastric and colorectal cancer [26].

In conclusion CagA positive *H. pylori* infections are associated with higher risk for colorectal cancer than are CagA negative *H. pylori* infection using in situ hybridization method.

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