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Detection of DNA *H.pylori* and distribution of CagA genotype in cancerous and precancerous tissue

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Abstract:

Helicobacter pylori (*H. pylori*) has been recognized as the causative agent of chronic gastric inflammation, which can progress further to a variety of diseases such as peptic ulcer and adenocarcinoma. The major bacterial virulence markers of *H. pylori*, the cytotoxin-associated gene (CagA), may play a role in determining the clinical outcome of Helicobacter infections. Aim of this study to investigate the presence of *H.pylori* DNA within gastric epithelial cells in patients with *H.pylori* infection and to determine the prevalence of CagA among patients with cancerous and precancerous lesion. Methods: A total of 92 gastric biopsy samples, 25 *H.pylori* negative and 67 *H.pylori* positive patients. *H.pylori* DNA in gastric epithelial cells and CagA gene of *H. pylori* was assessed by using the in situ hybridization test. Results: In *H. pylori* positive group, the positive rates of *H.pylori* DNA in the gastric epithelial cells were progressively increased in chronic superficial gastritis, precancerous changes and gastric cancer groups($P>0.01$); The detection of CagA positive *H. pylori* was significantly higher in patients with gastric cancer compared to those with chronic superficial gastritis and atrophic gastritis($P<0.01$). Conclusion: The pathological progression from chronic superficial gastritis, precancerous changes to gastric cancer is associated with higher positive rates of *H.pylori* DNA presence in the gastric epithelial cells, and there was a significant increase in CagA-positive *H.pylori* among patients with gastric cancer.

Key world: *H. pylori, DNA, CagA, ISH*

Introduction:

Helicobacter pylori infection has a role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma and lymphoma (1, 2). It is estimated that *H.pylori* infects more than 50% of the world's population. The, possibilities include the presence of disease-specific strains, host genetics and environmental factors (3, 4) so; H. pylori infection increases the risk for gastric cancer depend primarily on the involves microbial virulence factors as the host response to the bacteria (5).

In 1994, an International Agency for Research on Cancer (IARC) Working Group conducted a systematic review and concluded that there was sufficient evidence in humans for the carcinogenicity of infection with *Helicobacter pylori*. However, there has been heterogeneity among populations concerning the riskof stomach cancer associated with this infection (6).

The gastric mucosa in high-risk populations have revealed a series of lesions, which apparently represent a changes from normal to carcinoma, the complete process taking at least two decades (7). This includes, in order of increasing severity, superficial gastritis (SG), chronic gastritis (CG), chronic atrophic gastritis (AG), intestinalmetaplasia (IM), and dysplasia. H. pylori have been shown to induceacute gastritis, which can progress to CG, AG, and IM (8).The *H.pylori* DNA must invade gastric epithelial cells first, and then exists chronically in gastric epithelial cell in an unknown manner before integration (9, 10).

The major H. pylori candidate virulence factors include the Cag pathogenicity island (PAI), The Cag pathogenicity island (Cag PAI) is a 40 kilobase segment of DNA, containing 31 genes, many of which encode components of bacterial se

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cretion system (11, 12, 13). The secretion system acts as a molecular syringe for delivery of bacterial products, including the Cag gene product and peptidoglycan component into eukaryotic cells (14). The CagPAI plays an important role in H. pylori pathogenesis, and is not expressed in all strains. CagA is a 121–145 kD immuno-dominant protein, encoded by one of the genes CagA within the Cag PAI. CagA-positive strains are more commonly associated with peptic ulceration, atrophic gastritis and gastric adenocarcinoma than CagA negative strains, (15).

The aim of this study was to investigate the presence of *H.pylori* DNA within gastric epithelial cells and the possible carcinogenic mechanism and to investigate the virulence factor (CagA) positivity, in relation to gastric cancer susceptibility.

Materials and Methods

Ninety two patients with *H.pylori* positive were confirmed by rapid urase test and histology. Forty fife male and 47 female; mean age 51.7, were referred to the gastrointestinal endoscopy unit at Al- Yarmook Teaching Hospital. None of whom had received non-steroidal anti- inflammatory drugs, participated in this study. There were 47 cases with chronic superficial gastritis, 28 with atrophic gastritis and 17 patients with gastric cancer.

Biopsy specimens were taken from the antrum of all subjects in this study, by using the forceps, from similar topographical sites at each endoscopy; biopsies were fixed in 10% formalin immediately after resection, embedded in paraffin and cut into 4 µl thick section for In situ hybridization study and routine histological examination.

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

The use of Biotin – Labeled DNA probe for *H.pylori* / DNA (Maxim Biotic, USA) 303 bp, CagA (8 □g/10015 ML) 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 phosphatase) Conjugate is applied followed by addition of the substrate promo-chloro – indolyl – phosphatel / 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 CagAof *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% (16).

Statistical analysis:

The associations between the presences of H. pylori in different groups were assessed by the Chi-square test and using the ANOVA test to determine whether the means were equal among three groups. P value of <0.05was considered statistically significant.

Results:

The frequency distribution of H pylori infection, in subjects with CSG, AG and GC ispresented in Table -1. The percentages mean of presence the H. pylori significantly higher in GC cases than those in CSG and AG.

Table 1: The presence of H. pylori in subjects with gastric cancer and precancerous lesion.

Variables	CSG (n=47) NO %	AG (n=28) NO %	GC (n=17) NO %	Chi - Seq P value
Hp.infection	31 (65.95)	21 (75)	15 (88.23)	
Yes	16 (34.04)	7 (%)	2 (11.76)	0.001*
No				

Hp: H. pylori, CSG: chronic superficial gastritis, AG: atrophic gastritis, GC: gastric cancer. Highly significant difference (P<0.001)*

Based on ANOVA test analysis table -2, shows, the mean percentage of expression of *H.pylori* DNA in patients complaining gastrointestinal diseases and infected with *H.pylori* detected by in situ hybridization technique. The results revealed that there was significantly difference between chronic superficial gastritis, atrophic gastritis and gastric cancer($p<0.01$). But the *H.pylori* DNA was higher in gastric cancer than atrophic gastritis but statistically not significant ($p<0.44$). Figure two shows the brown dots that detect the presence of *H.pylori* DNA.

Table -2: The mean percentage of DNA among H. pylori positive patients.

Variable	Studied groups	No=67	Mean± SE	F test P value	Sig. between groups
H. pylori DNA	CSG	31	42.6 ± 2.1	< 0.01	CSG – AG*
	AG	21	67.3 ± 7.5		CSG – GC*
	GC	15	78.8 ± 2.6		AG - GC

** = significant difference ($p<0.01$)*

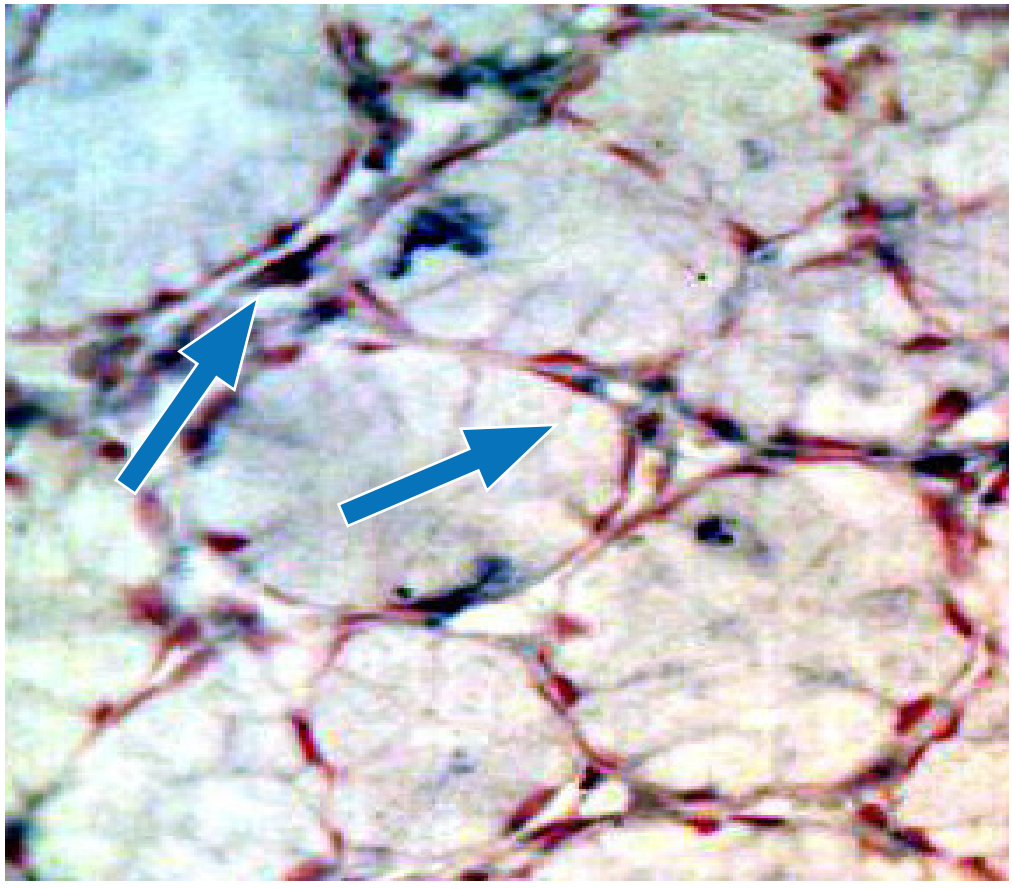

Figure 1: Detection of H.pylori DNA, in patients with gastrointestinal disease by in situ hybridization. Staining of H.pylori DNA by BCIP/ NBT (blue-black) counterstained with nuclear fast red. Tissue from patients with antral gastritis shows positive H.pylori DNA by hybridization signals

Table -3 shows the expression of H. pylori CagA in the gastric epithelial cells. It was significantly higher in gastric cancer than in the chronic superficial gastritis and atrophic gastritis ($p< 0.01$). Figure -1 reveals the expression of H. pylori CagA were dark brown staining in the tissue.

Table 3: Comparison between the mean percentages of CagA in H.pylori– positive patients with gastrointestinal diseases.

variable	Studied groups	No =67	Mean± SE	F test P Value	Sig. between groups
Cag A	CSG	31	54.8± 1.9	< 0.01	CSG – AG**
	AG	21	75.2± 2.2		CSG – GC**
	GC	15	92.5±2.5		AG - GC**

*= *significant difference ($p<0.01$)*

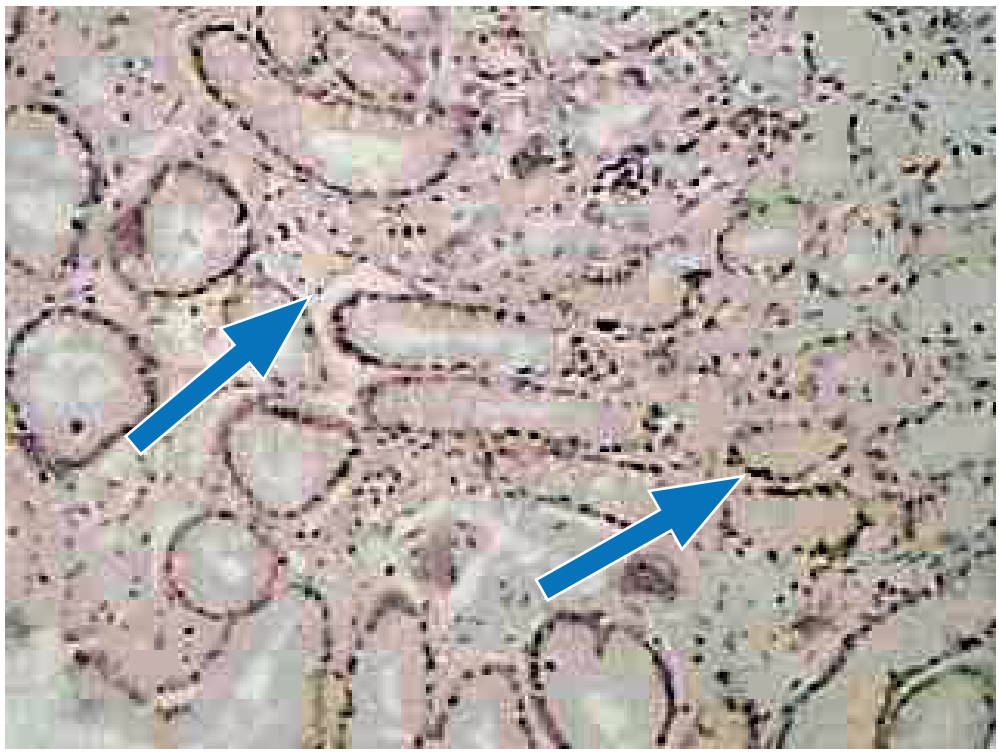

Figure 3: Detection of CagA, in patients with gastroduodenal disease by in situ hybridization. Staining of CagA mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from patients with antral gastritis shows positive CagA by hybridization signals.

DISCUSSION:

The rate of *H.pylori* DNA in the gastric epithelial cells was progressively increased in chronic superficial gastritis, atrophic gastritis and gastric cancer, respectively in the *H.pylori* positive group, although there was no significance difference between atrophic gastritis and gastric cancer. So the progression from chronic superficial gastritis to precancerous changes and to gastric cancer was associated with the presence of *H.pylori* DNA in the gastric epithelial cells. *H.pylori* DNA was also located in the cytoplasm of gastric epithelial cells and can be seen to invade gastric mucosa by electron or immunoelectron microscopy(17).Yang et al(18) found that *H.pylori* could be engulfed and degraded by the human gastric cancer cell line SGC-7901 using transmission electron microscopy.This may indicate that *H.pylori* DNA and the genome of the host cell may affect each other, as *H.pylori* DNA is integrated into genome of the host cell. As a result this may change the structure and function of the host cell genome, and thus destroy the stability of the genome(19, 20). The *H.pylori* DNA invaded the gastric epithelial cells, it could enter the nucleus when the karyotheca disappears during the metaphase of mitosis, may induce transformation or malignancy of the normal cell. (21).

CagA-positive strains have been reported as being more virulent with respect to atrophic gastritis, and gastric cancer development (22). CagA-positive *H.pylori* strains caused more severe inflammation in gastric mucosa than did CagA-negative strains (23).

Similarly, other studies showed that CagA among H. pylori infected patients was significantly greater in gastric cancer patients than in CSG and AG (24, 25). The associations with the subset of more aggressive tumors and the consistency of the data with our hypothesis suggest that the effect is real. This positive effect is biologically plausible for several reasons: infection with CagA positive strains has been associated with enhanced epithelial cell injury, and injury to surface gastric epithelial cells may promote or possibly initiate oncogenesis; infection with CagA strains is associated with higher degrees of gastric inflammation (26) These may contribute to epithelial injury Infection with cytotoxin-producing strains, as assessed by presence of serum neutralizing antibodies, may be associated with the presence of gastric cancer (27). The hypothesize that the enhanced intensity of inflammation induced by the CagA strain results in accelerated mucosal damage with loss of epithelial structures and subsequent atrophy and eventually metaplasia (28, 29).

The mechanisms by which CagA modify the activity of epithelial cells is explaining by serving as scaffolding protein able to interact and modify the function of a variety of molecules involved in cell to cell interaction, cell motility, and proliferation (30).

Our study suggests that *H.pylori* DNA exists in gastric epithelial cells in patients with *H.pylori* infections. The pathological progression from chronic superficial gastritis, atrophic gastritis to gastric cancer is associated with higher positive rates of *H.pylori* DNA presence in the gastric epithelial cells and the presence of CagA in a strain may only be a marker for particular phenotype that itself is relevant to inflammation or to the oncogenic process. In terms of the CagA genes that encode potential virulence factors that express CagA-producing H. pylori increases the risk of gastric cancer.

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الكشف عن الحامض النووي لبكتريا *H. pylori* وتوزيع جين CagA في النسيج السرطاني وماقبل السرطاني

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الخلاصة:

بكتيريا *Helicobacter pylori* هي احد العوامل المسببة لالتهابات المعدة المزمنة وسرطان المعدة. عامل الضراوة الموجود في هذه البكتريا هو CagA الذي يلعب دور في امراضية هذه البكتريا الهدف من الدراسة: هو التحقق من وجود الحامض النووي لبكتريا *H. Pylori* ضمن الخلايا الطلائية المعدية في المرضى المصابين بهذه البكتريا وتقرير انتشار بكتريا *H. pylori* الحاملة لجين CagA في مرضى سرطان المعدة وماقبل سرطان المعدة. طريقة العمل: من مجموع 92 نموذج مأخوذ من المعدة من مرضى يعانون من امراض المعدة 25 منهم سالبين لبكتريا *H. pylori* و موجبين لبكتريا *H.pylori* حيث تم تحديد الحامض النووي لبكتريا *H. pylori* و وجود جين CagA بطريقة التهجين الموضعي.النتائج: في المجموعة الموجبة لبكتريا *H. pylori* نسبة ظهور الحامض النووي في الخلايا الطلائية للمعدة تزداد تقدميا في التهاب المعدة السطحي والتغيرات ماقبل سرطان المعدة وصولا الى سرطان المعدة ($P<0.01$). اما وجود بكتريا *H. pylori* الحاملة لجين CagA تكون اعلى معنويا في سرطان المعدة مقارنة بالتهاب المعدة المزمن وضمور المعدة.الاستنتاج: التقدم المرضي من التهاب المعدة السطحي والتغيرات ماقبل سرطان المعدة الى سرطان المعدة يتشارك مع النسبة العالية لوجود الحامض النووي لبكتريا *H. pylori* في الخلايا الطلائية للمعدة. وهناك زيادة معتدة لبكتريا *H. pylori* الموجبة لجين CagA ضمن مرضى سرطان المعدة.