PCNA Expression in cagA Strain H. pylori Gastritis: Immunohistochemical and *In situ* Hybridization Study

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Abstract

**Background**
Carriage of *Helicobacter Pylori* (H. Pylori) in the human stomach is associated with increased risk of peptic ulcer disease, distal gastric adenocarcinoma and gastric B-cell mucosa associated lymphoid tissue lymphoma. Several studies have shown increased evidence of increased cell proliferation in the gastric mucosa both in human carrying *H. Pylori*, and animal model of *H. Pylori* infection.

**Objective**
To study the immunohistochemical expression of Proliferating cell nuclear antigen (PCNA), as a proliferative marker in the gastric mucosa of patients infected with CagA *Helicobacter Pylori* demonstrated by *in situ* hybridization method.

**Methods**
Gastric antrum and corpus biopsies from 99 patients with dyspeptic symptoms (50 men, 49 women, and median age 40) were analyzed for *H. pylori*, presence of chronic inflammation, intestinal metaplasia, and atrophy according to updated Sydney system. *In situ* hybridization technique was done to detect cagA *H. Pylori*. Immunostaining for PCNA (Avidin-Biotin method) was performed on paraffin embedded tissue specimens.

**Results**
Forty four patients (44.44%) had *H. Pylori* cagA positive strain. Atrophy of gastric mucosa was present in 14 (14.14 %) patients. Intestinal metaplasia was present in 8 (8.08%) patients. The frequency of atrophy was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (p=0.041). The frequency of intestinal metaplasia was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (p=0.023). PCNA labeling index (LI) of the gastric glands was significantly higher in presence of atrophic alterations (p <0.001), intestinal metaplasia (p <0.001) and in cagA strain *H. Pylori* positive gastritis (p<0.001).

**Conclusion**
The rates of gastric glandular atrophy, intestinal metaplasia, and epithelial proliferation increase in the presence of *H. Pylori* infection, and are further increased when *H. Pylori* is of cag A strain.

**Key words**

Introduction

Carriage of *Helicobacter Pylori* in the human stomach is associated with increased risk of peptic ulcer disease, distal gastric adenocarcinoma and gastric B-cell mucosa associated lymphoid tissue lymphoma(1). In developed countries, strains of *Helicobacter Pylori* that carry the cag Pathogenesity Island, a 35-40 Kb DNA fragment encoding a series of virulence-related gene associated with an extracellular secretory apparatus, are associated with a greater risk of peptic ulcer and adenocarcinoma than strains that are negative.
for cag island \(^{(2,3)}\). Several studies have shown increased evidence of increased cell proliferation in the gastric mucosa both in human carrying \textit{Helicobacter Pylori} \(^{(4-10)}\) and animal model of \textit{Helicobacter Pylori} infection \(^{(11,12)}\). After eradication therapy, increased proliferation returns to normal levels, which suggests that \textit{Helicobacter Pylori} or the associated inflammatory response is responsible for the increased proliferation observed \(^{(4,6-9)}\).

Proliferating cell nuclear antigen (PCNA) is a 36 KDa intranuclear polypeptide protein whose expression is associated with DNA synthesis and cell proliferation \(^{(13,14)}\). It is a useful immunohistochemical marker of cell proliferation because its expression and distribution correlate with cellular proliferation rate and DNA synthesis \(^{(15)}\).

The aim of this study is to study the immunohistochemical expression of PCNA, as a proliferative marker in the gastric mucosa of patients infected with \textit{Helicobacter Pylori} demonstrated by \textit{insitu} hybridization method.

**Methods**

A total of 99 adult patients presented with dyspeptic symptoms referred to the OGD (oesophagastroduodenoscopy) unit at Al-Kadhimiya teaching Hospital in Baghdad with an age range of 19-70 years (median 40 years) for upper endoscopy between June 2009 and March 2010 were included. In this study patients who had received anti-ulcer agents or antibiotics for up to two months before the examination and those who had histories of gastric cancer, gastric or duodenal ulcer, or gastric surgery, were excluded. The study was approved by the committee of ethical approval in the College of Medicine, Al-Nahrain University.

Three tissue biopsies were obtained from each patient, two from the antrum and corpus and one from the corpus. Rapid urease test was performed on one of the antral biopsies. The medium used for the test was urea broth. It consists of urea, phenol red indicator and distilled water. One biopsy piece from each sample was inoculated immediately after collection into 1.5ml to 2ml of urea broth. It was incubated at 37\(^\circ\)C in the incubator for one and a half hour. The change in color of the broth from pale yellow to deep pink was taken as a positive reaction. The other biopsy specimens were paraffin embedded and processed. One section from each block was stained by H&E to study the histopathological features and grading of gastritis was done according to the updated Sydney system. One section was used for \textit{insitu} hybridization (ISH) method to identify Cag-A starin \textit{H. pylori}, and one section was stained immunohistochemically for PCNA (Dakocytomation-Mouse monoclonal primary antibody).

Two methods were used to identify \textit{H. Pylori} infection status; rapid urease test and histological sections stained with H&E stain. Patients were considered to be infected with \textit{H. pylori} if one or two of the tests were positive: rapid urease test, or histology. Patients were considered infection free when both of the two tests were negative.

Cell proliferation in gastric epithelium was examined by PCNA labeling indices. The number of positive cells/100 gastric mucosal epithelial cells was counted and was considered to be the proliferation index \(^{(16)}\).

\textit{In-situ} hybridization technique uses biotinylated cDNA probe (for \textit{H. Pylori} cagA gene detection) together with Maxim’s ISH detection kit. This complete hybridization and immune detection system, incorporates the biotin-streptavidin amplified technology to provide consistent results and maximum sensitivity to ensure economical and efficient use of the nucleic acid probes. A dark blue signal appears at specific site of the hybridized probe.

Statistical analysis was performed using SPSS 16 and Microsoft Excel 2007. Numeric variables were expressed as mean\(\pm\)SD. Chi-square test
was used to study association between two discrete variables. T-test was used to compare the mean of numeric variables. A P-value of less than 0.05 was considered significant.

Results

Histopathological assessment of gastritis: the assessment was done according to the revised Sydney system (17) (Figure 1).

Figure 1. Distribution of cases according to total Sydney score

The results were as follow:
1. Chronic inflammation was mild in 46 (46.46%) patients, moderate in 37 (37.37%) patients and severe in only 16 (16.16%) patients.
2. Active inflammation was present in only 34 out of 99 patients (34.34%), and it was mild in most of the cases (29 patients [29.29%]).
3. Atrophy of gastric mucosa was present in only 14 out of 99 patients (14.14%) and was of mild degree.
4. Intestinal metaplasia was present in 8 out of 99 patients (8.08%), and it was of mild degree.
5. H. Pylori infection was present in 69 out of 99 patients (69.69%) (44 were cagA positive), and it was of mild degree in most of the case.

Relation between various histopathological parameters and cagA H. pylori gastritis (cagA versus non cagA):
1. Chronic inflammation and CagA: The degree of chronic inflammation in the presence of cagA strain was significantly higher than that in the absence of cagA strain (mean score 2.11±0.65 versus 1.00±0.00; p<0.001) as shown in table 1.
2. Activity of inflammation and cagA: The activity of inflammation in the presence of cagA strain was significantly higher than that in the absence of cagA strain (mean score 0.90±0.64 versus 0.00±0.00; p<0.001) as shown in table 1.
3. Atrophy and cagA status: The degree of atrophy was significantly higher in cagA H. Pylori gastritis than non-cagA H. Pylori gastritis (0.22±0.42 versus 0.16±0.37; p=0.041). Also, the distribution of atrophy was more frequent among cagA H. Pylori gastritis than non-cagA H. Pylori gastritis (13/44 versus 1/25; p=0.011) as shown in tables 1 & 2.
4. Intestinal metaplasia and cagA: The degree of intestinal metaplasia was significantly higher in cagA H. Pylori gastritis than non-cagA H. Pylori gastritis (0.18±0.39 versus 0.00±0.00; p=0.023). Also, the distribution of intestinal metaplasia was more frequent among cagA H. Pylori gastritis than non-cagA H. Pylori gastritis (8/44 versus 0/25; p=0.044) as shown in tables 1 & 3.

Table 1. Correlation between various histopathological parameters and cag A H. pylori gastritis (cag A versus non cag A)

<table>
<thead>
<tr>
<th>Histological parameter</th>
<th>Cag A (present)</th>
<th>Non cag A (absent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean score±SD</td>
<td>No. = 44</td>
<td>No. = 25</td>
</tr>
<tr>
<td>Ch. inflammation</td>
<td>2.11±0.65</td>
<td>1.00±0.00*</td>
</tr>
<tr>
<td>Activity</td>
<td>0.90±0.64</td>
<td>0.00±0.00**</td>
</tr>
<tr>
<td>Atrophy</td>
<td>0.22±0.42</td>
<td>0.16±0.37***</td>
</tr>
<tr>
<td>Int. metaplasia</td>
<td>0.18±0.39</td>
<td>0.00±0.00***</td>
</tr>
</tbody>
</table>

PCNA immunohistochemical expression

PCNA LI of the corpus glands was significantly higher in presence of atrophic alterations (9.71±3.60) as compared to the non-atrophic
mucosa (0.75±1.47) (p <0.001). Also, PCNA LI of the antrum glands was significantly higher in presence of atrophic alterations (13.71±3.60) as compared to the non-atrophic mucosa (2.54±2.99) (p <0.001), as shown in table 4. PCNA LI of the corpus glands was significantly higher in presence of intestinal metaplasia (9.00±5.09) as compared to the non-atrophic mucosa (1.40±2.81) (p <0.001). Also, PCNA LI of the antrum glands was significantly higher in presence of intestinal metaplasia (13.00±5.09) as compared to the non-atrophic mucosa (3.34±4.15) (p <0.001) as shown in table 5.

The PCNA LI in the gastric antrum mucosa was significantly higher in cagA strain H. Pylori positive gastritis than cagA strain H. Pylori negative gastritis (7.79±4.47 and 2.6±3.51 respectively; p<0.001). The PCNA LI in the gastric corpus mucosa was significantly higher in cagA strain H. Pylori positive gastritis than cagA strain H. Pylori negative gastritis (4.00±4.52 and 0.96±2.18 respectively; p<0.001) as shown in table 6.

**Table 4. Comparison of PCNA LI between atrophic and non-atrophic mucosa**

<table>
<thead>
<tr>
<th></th>
<th>Atrophy</th>
<th>No.</th>
<th>Mean±SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA Corpus</td>
<td>Present</td>
<td>14</td>
<td>9.71±3.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>85</td>
<td>0.75±1.47</td>
<td></td>
</tr>
<tr>
<td>PCNA antrum</td>
<td>Present</td>
<td>14</td>
<td>13.71±3.60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>85</td>
<td>2.54±2.99</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5. Comparison of PCNA LI in the presence and absence of intestinal metaplasia**

<table>
<thead>
<tr>
<th></th>
<th>Metaplasia</th>
<th>No.</th>
<th>Mean±SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA Corpus</td>
<td>Present</td>
<td>8</td>
<td>9.00±5.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>91</td>
<td>1.40±2.81</td>
<td></td>
</tr>
<tr>
<td>PCNA antrum</td>
<td>Present</td>
<td>8</td>
<td>13.00±5.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>91</td>
<td>3.34±4.15</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6. Comparison between corpus and antrum mucosa in regard to PCNA LI**

<table>
<thead>
<tr>
<th></th>
<th>Cag A</th>
<th>No.</th>
<th>Mean±SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA LI Antrum</td>
<td>Positive</td>
<td>44</td>
<td>7.79±4.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>25</td>
<td>2.60±3.51</td>
<td></td>
</tr>
<tr>
<td>PCNA LI corpus</td>
<td>Positive</td>
<td>44</td>
<td>4.00±4.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>25</td>
<td>0.96±2.18</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

The sequence of events that have been suggested in the development of gastric carcinoma is chronic inflammation, mucosal atrophy, intestinal metaplasia, dysplasia and carcinoma. The incidence of the precancerous lesions (atrophy and intestinal metaplasia) is variable in different studies. The percentage of atrophy, in those studies, ranged from 9 to 15 %; while the percentage of intestinal metaplasia ranged from 35 to 42 %. In the
current study the percentages of intestinal metaplasia and atrophy were 8.8% and 14.14% respectively. Several studies have shown a significant positive association between \textit{H. pylori} infection and development of gastric atrophy \cite{21-24}, and this finding is in accordance with the result of the current study. Other studies claimed that infection with \textit{H. pylori} is responsible for higher rates of intestinal metaplasia \cite{21,24-26}.

Table 7. Correlation between Sydney score and PCNA expression

<table>
<thead>
<tr>
<th>Histological parameter</th>
<th>PCNA antrum</th>
<th>PCNA corpus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic inflammation</td>
<td>( r ) 0.401</td>
<td>( r ) 0.392</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>( P ) &lt;0.001</td>
</tr>
<tr>
<td>Activity</td>
<td>( r ) 0.556</td>
<td>( r ) 0.444</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>( P ) &lt;0.001</td>
</tr>
<tr>
<td>Atrophy</td>
<td>( r ) 0.787</td>
<td>( r ) 0.856</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>( P ) &lt;0.001</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>( r ) 0.532</td>
<td>( r ) 0.567</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>( P ) &lt;0.001</td>
</tr>
<tr>
<td>\textit{H. pylori} score</td>
<td>( r ) 0.550</td>
<td>( r ) 0.365</td>
</tr>
<tr>
<td></td>
<td>( P ) &lt;0.001</td>
<td>( P ) &lt;0.001</td>
</tr>
</tbody>
</table>

Again this finding is in accordance with the result of the current study. Some studies concluded that cag A strain is the main pathogen behind the higher rates of gastric atrophy and atrophic gastritis \cite{21}, which again supports the result of the current study. Also those studies had attributed the higher rates of intestinal metaplasia to cag A strain \cite{21}. This is also in accordance with the finding of the current study. All of the eight cases of intestinal metaplasia were seen in chronic atrophic gastritis, and no intestinal metaplasia was present in chronic non-atrophic gastritis. According to these data, it can be concluded that \textit{H. pylori} existence is an important factor for the development of atrophy and that atrophy can cause intestinal metaplasia. This finding is in accordance with Derya \textit{et al} \cite{16}.

PCNA:

The current study has revealed that severity of gastritis and presence of \textit{H. pylori} has different impacts on gastric epithelial cell proliferation. A significantly higher proliferation activity was established in the gastric mucosa in atrophy. These findings are in accordance with some other studies \cite{23,27}, which suggest higher proliferative activity of epithelial cells of gastric mucosa in the presence of atrophy.

Figure 2. \textbf{A}. Positive immunohistochemical expression of PCNA (10X). \textbf{B}. Positive immunohistochemical expression of PCNA (40X).

There are data from other authors, which show enhancement of proliferation index with increasing degrees of gastritis both in the antrum and in corpus mucosa \cite{28,29}. Our data are in concordance with the study of Lynch \textit{et al}. who have shown also a strong correlation between
epithelial proliferation and chronic inflammatory cell infiltrate \cite{10}.

Increased proliferative activity in the atrophic gastric mucosa suggests that the gland cell populations maintain an active cell turnover despite reduction in cellular mass as established through grading criteria. Some studies showed a positive correlation between the severity of chronic inflammation and the expression of proliferative marker (PCNA) \cite{30}. This is in accordance with findings of the current study.

Steven et al showed an increased rate of gastric epithelial cell proliferation in the presence of \textit{H. pylori} infection than in the absence of infection; and that the rate is further increased in the presence of cag A stain \textit{H. pylori} \cite{31}. These data again are in accordance with the results of the current study. Indeed an increase in mucosal cell proliferation increases the likelihood of a neoplastic clone of epithelial cells emerging where there is chronic epithelial cell injury associated with \textit{H. pylori} gastritis and in this way may play a part in gastric carcinogenesis.

**Conclusions**

The rates of gastric glandular atrophy, intestinal metaplasia, and epithelial proliferation increase in the presence of \textit{H. pylori} infection, and are further increased when \textit{H. pylori} is of cag A strain.

**References**


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