ABERRANT LINE-1 METHYLATION UPON HELICOBACTER PYLORI INFECTION

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ABSTRACT
Four histo-examination patterns of gastric biopsies used to study global genomic methylation status upon Helicobacter pylori infections by using LINE-1(long interspersed nucleotide element-1). H. pylori detection protocol in 120 samples was done by 23S ribosomal RNA gene. significant association (p= 0.03) have been showed between H. pylori and carcinogenesis progression comprising normal gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer, respectively. Methyl-specific PCR showed that the methylation status of LINE-1 was decrease during carcinogenesis progression (p=0.0179). Among 56 H. pylori infected cases 32(57.1%) were LINE-1 hypomethylated, and there is no significant association between H. pylori infection and LINE-1 hypomethylation during development of gastric cancer (p=0.26). The current study confirmed that H. pylori is causative agent of gastric cancer, but not directly by causes aberrant LINE-1 hypomethylation which linked to gastric cancer progression.

KEYWORDS: Helicobacter pylori, LINE-1, hypomethylation, carcinogenesis.

INTRODUCTION
Helicobacter pylori is Gram-negative, spiral shaped bacterium considered one of the most important human pathogen, infects the stomach of approximately 50% of the world’s human population. [3] The pathogenic potential of H. pylori is intricately linked to the presence of several virulence factors such as host adapting enzymes, cytotoxin associated gene A (CagA), vacuolating cytotoxin (VacA) and outer inflammatory protein (OipA) in addition to its genomic plastiCty. [2,3,4]
In 1994, H. pylori has been classified as a definite group 1 human carcinogen by the International Agency for Research on Cancer. [5]

Abundant evidences were elucidated the strong association between gastric chronic infection of H. pylori and the progression of gastric cancer, which accused as the causative agent of chronic atrophic gastritis, a precursor of gastric carcinomas via a series of steps comprising gastritis, atrophy, intestinal metaplasia, dysplasia and gastric cancer. [6] Otherwise the outcomes of acute infection of H. pylori are including peptic ulceration and mucosa associated lymphoid tissue (MALT) lymphomas. [7]

The cell progression to cancer requires the stepwise up regulation or down-regulation of critical genes. Which divided to; Genetically Mutation, LOH, translocation, small and deletions have each been contribute toward these alterations in gene expression. Epigenetic abnormalities are DNA methylation, histone modification and chromatin remolding. methylation of the promoter regions of genes is one genomic change which is associated with silencing of gene expression, usually tumor suppressor gene, global hypomethylation of repetitive sequence (LINE and Alu) lead to genomic instability and activate oncogenes, consequently this event can alter cell phenotype. [8,9]

Global DNA hypomethylation has been reported also, in repetitive sequence such as (LINE and Alu) which plays an important role in genomic instability and carcinogenesis. DNA methylation in the long interspersed nucleotide element-1, L1 (LINE-1) repetitive element is a good indicator of the global DNA methylation level because there are more than 500.000 copy number of LINE-1 in human genome. In some types of human neoplasms, LINE-1 methylation level is attracting interest as a predictive marker for patient prognosis. [10]

The aim of this study is to reveal the relationship between LINE-1 aberrant methylation status as indicator...
for global genomic methylation and development of gastric cancer upon *H. pylori* infection.

**MATERIALS AND METHODS**

The sources of samples were gastric biopsies (n=120). Samples were collected during the period 10/2/2015 to 20/3/2016. Upper gastrointestinal tract diseases patients were diagnosed clinically and the disease was evaluated by specialist physicians, presented with dyspepsia referred to the Esophago Gastroduodeno Scope Unit for upper endoscopy at many Iraqi hospitals. Three tissue biopsies were obtained from antrum. Rapid urease test was performed on one of the antral biopsies at the time of endoscopy.

The other biopsy specimens placed in 1 ml of formalin was examined under microscope after formalin embedding. Another biopsy specimen was preserved immediately at −60°C for molecular analysis. The gene of 23S rRNA was used to detection of *H. pylori*, amplification and melting conditions were optimized for the PCR using specific primer set forward GGTCTCAGCAAAGAGTCCCT and reverse CCCACCAAGCA TTGTCCT for 41 cycles at 57°C annealing temperature for 35 sec, the product was 493 bp.

Bisulfite conversion of genomic DNA was conducted according Herman et al., (1969) [12]. One microgram of genomic DNA was denatured in 0.2 M NaOH for 10 minutes at 37°C. The denatured DNA was diluted in 500 µl of freshly prepared solution of 10 mM hydroquinone and 3 M sodium bisulfite and incubated for 16 h at 50°C. After incubation, the DNA sample was desalinized through a column (Wizard DNA Clean-Up System; Promega, Madison, WI, USA), treated with 0.3 M NaOH for 10 minutes at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was suspended in 40 µL of H2O.

Two sets of primers described by Gangshi et al., (2013)(13) were used for LINE-1 methyl-specific PCR (MSP): for methylated cases (CGCCGAGTCAAGTGGGC) forward and (ACCCGATTTCACCAATCGACC) reverse (116 bp product) treated by 40 reaction cycle at 60°C annealing temperatures, while unmethylated cases detected by (TGTGTGTGAGTGAAGTGGTG) forward and (ACCCAAATTTCACACACACCATCA) reverse (111 bp product) treated by 40 reaction cycle at 57°C annealing temperatures.

**Statistical analysis**

The association relationships were carried out by Fisher’s exact test according to ©2016 GraphPad Software (http://graphpad.com/quickcalcs/contingency1.cfm). P-value was calculated with Two-tailed as recommended in this software.

**RESULTS AND DISCUSSION**

One hundred twenty patients were enrolled in this study (53 men and 67 women). Four groups were identified according to tissue differentiation during disease development: normal gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer in 31(25.8%), 30(25%), 32(26.6) and 27 (22.5%) respectively.

During development of intestinal-type adenocarcinoma, Series of well-defined histological progression steps have been identified in several examinations, first described in 1975 [14]. Initiated by the transition from normal mucosa to chronic superficial gastritis, which followed by multifocal atrophic gastritis then intestinal metaplasia, finally the tissue could be transform to adenocarcinoma. [15]

Specific primers sequences were designed for 23S ribosomal RNA gene (493bp) for the purpose of PCR assay, Figure (1). Accordingly, 56 (46.6%) of patients were consider *H. pylori* infected in this study, while 64 (53.3%) patients were PCR negative.

![Figure 1: Gel electrophoresis analysis of PCR product of H. pylori 23S ribosomal RNA gene (493bp), lane1:size marker, lane 4: negative control, whereas other lanes were posetive result, using 5% polyacryl amide gel at 6 volt/cm for 1 hour](image)
Patients with gastric cancer pattern showed the highest frequency of *H. pylori* infected (63%), while lower rate was observed in normal patients (29%), Figure (2). Previous studies revealed that *H. pylori* prevalence increased with development of carcinogenesis, severe atrophic gastritis, corpus-predominant gastritis, or both, along with intestinal metaplasia are at high risk for intestinal-type gastric cancer with incidence of *H. pylori* infections.\(^\text{[16]}\) It has been shown that intestinal-type gastric cancer developed in *H. pylori*-positive patients who have severe multifocal atrophic gastritis in association with intestinal metaplasia.\(^\text{[15]}\) So, progression of atrophic gastritis can be causes by *H. pylori* infection.

The relationship between *H. pylori* and total patterns of patients considered statically significant (p= 0.03). This results confirm the previous studies which proved that *H. pylori* is the causative agent of gastric cancer progression.\(^\text{[3]}\)

For several years, the relationship between *H. pylori* and intestinal type gastric carcinoma was a matter of debate among scientists. However, abundant studies in last two decades provided clear evidences that this bacterium significantly increases gastric cancer risk, one of such result revealed in a study of *H. pylori* infection in 1,526 Japanese patients, by comparing to non-infected patients. Another study showed that about 3% of *H. pylori* infected patients developed intestinal type gastric cancer.\(^\text{[17]}\)

Corresponding studies demonstrated that eradication of *H. pylori* was decreases the risk of intestinal type gastric carcinoma in patients without premalignant lesions. That provided a clear evidence that *H. pylori* have an effect on early stages of gastric carcinogenesis.\(^\text{[18, 19]}\) Other studies revealed that the eradication of *H. pylori* by antibiotics attenuated the progression of gastric cancer.\(^\text{[20, 21]}\) Taken together with the current study with other they support an unequivocal role for *H. pylori* in the development of gastric cancer.

Methyl-specific PCR was used to discriminate between methylated and unmethylated LINE-1. MSP product was 116 bp for methylated cases and 111 bp for unmethylated cases, Figure (3).

**Figure (2): Distribution of patients according PCR assay upon disease progression.**

**Figure (3): Gel electrophoresis of MSP product of LINE-1 using 5% polyacryl amide gel at 6 volt /cm for 1 hour**

M: Product amplified with methylated-specific primer (161bp)

Lane 1: Posetive methylated control

Lane 5: Posetive unmethylated control

Lane 2, 3 and 4: Samples

**Association between methylation status of LINE-1 and progression of disease.**

The present study showed that methylated cases of LINE-1 were 75(62.5%), methylated status was decreased within the disease progressing starting from normal, atrophic gastritis, intestinal metaplasia to cancer.
80.6%, 60%, 59.3% and 48% respectively Figure (4). Global hypomethylation at repetitive sequences cause genomic instability. Its known that both types of DNA methylation (hyper and hypo) changes were implicated in the development and progression of cancers.\textsuperscript{22, 23}

Figure (4): Distribution of patients according LINE-1 methylation upon disease progression, M= methylated frequency U= unmethylated frequency

The relationship between increasing of LINE-1 hypomethylation and progression of disease was considered statistically significant (p= 0.0179), Table (1).

Table (1): Distribution with statistical analysis of patients between diseases patterns and LINE-1 methylation

<table>
<thead>
<tr>
<th>Cases</th>
<th>LINE M</th>
<th>LINE U</th>
<th>p-value (normal vs each pattern)</th>
<th>p-value (normal vs total patterns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal gastritis</td>
<td>25</td>
<td>6</td>
<td>0.0971</td>
<td>0.0179</td>
</tr>
<tr>
<td>Pattern 1</td>
<td>18</td>
<td>12</td>
<td>0.0994</td>
<td></td>
</tr>
<tr>
<td>Pattern 2</td>
<td>19</td>
<td>13</td>
<td>0.0132</td>
<td></td>
</tr>
<tr>
<td>Pattern 3</td>
<td>13</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M corresponding to methylated cases, U corresponding to unmethylated cases.

In comparison with other studies\textsuperscript{13} showed that LINE-1 was completely methylated in normal gastric mucosa cell line GES-1, but was partially methylated in gastric cancer cell lines.

By using COBRA- LINE-1 analysis method for detection of methylation status of LINE-1 in multistage carcinogenesis of the eight invasive cancers, LINE-1 shows hypomethylation level progressively increased from normal epithelium (59.4%) to carcinoma in situ (64.3%) and squamous cell carcinoma (66.3%) (p= 0.005), this confirmed the association in the current study between LINE-1 hypomethylation and carcinogenesis of cancer (p=0.0179). Another study revealed that the carcinoma in situ portion showed a significantly greater LINE-1 hypomethylation level (62.06 %) compared to normal epithelium cells (60.03 %) (p= 0.03). (P = 0.2).\textsuperscript{24}

Association between LINE-1 methylation status upon Helicobacter pylori infection

The current study revealed that 57% of cases were LINE-1 methylated among 56 suspected H. pylori infected.\textsuperscript{5}. While H. pylori non-infected cases showed that 67% of it were methylated.
Despite the increase of LINE-1 hypomethylation that has a good association with disease progress as describe previously, it does not have a good linkage with \textit{H. pylori} infection upon carcinogenesis of gastric cancer. Figure (6) shows a few differences between LINE-1 hypomethylation of \textit{H. pylori} infected and non-infected patients distributed within the studied patterns. So, the association between \textit{H. pylori} infection with hypomethylation of LINE-1 during disease progressing was statistically considered not significant (p=0.26) table(2).

A recent study, using the immunohistochemical evaluation of 5-mC, reveals a gradual decrease in the global DNA methylation from \textit{H. pylori}-negative normal gastric biopsy to \textit{H. pylori}-positive chronic gastritis lesions, \textit{H. pylori}-positive chronic atrophic gastritis and gastric cancer tissues, which suggested that the global DNA hypomethylation could be implicated in \textit{H. pylori}-related gastric cancer at an early stage.\textsuperscript{[25]}

<table>
<thead>
<tr>
<th>Cases</th>
<th>23S H. pylori</th>
<th>LINE M</th>
<th>LINE U</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal gastritis</td>
<td>+9</td>
<td>7 (77.7%)</td>
<td>2 (22.2%)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>-22</td>
<td>18 (81.1%)</td>
<td>4 (18.1%)</td>
<td></td>
</tr>
<tr>
<td>Atrophic gastritis</td>
<td>+11</td>
<td>7 (63.6%)</td>
<td>4 (36.3%)</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>-19</td>
<td>11 (57.8%)</td>
<td>8 (42.1%)</td>
<td></td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>+19</td>
<td>10 (52.6%)</td>
<td>9 (47.3%)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>-13</td>
<td>9 (69.2%)</td>
<td>4 (30.7%)</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>+17</td>
<td>8 (47%)</td>
<td>9 (53%)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>-10</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
The current results corresponded with a previous assessment of LINE-1 methylation status related to *H. pylori* infection. It was shown that the incidence of the loss of heterozygosity (LOH) by aberrant methylation was 83% in gastric epithelial dysplasia and the aberrant methylation frequencies were not significantly associated with *H. pylori* but corresponded to disease progression.[26]

In comparison with another study, correlational analyses were performed on each type of multistep gastric lesions according to the *H. pylori* status. LINE-1 methylation levels were significantly higher in *H. pylori*-positive chronic gastritis tissue patients than in *H. pylori*-negative chronic gastritis tissue patients (p = 0.038). But another corresponding study revealed that *H. pylori* infection did not affect LINE-1 methylation levels in intestinal metaplasia, gastric adenoma, or gastric cancer.[27] In high-risk gastritis patients who were *H. pylori* positive, no difference was found between the CpG methylation levels with the present and absent *H. pylori* (P=0.842).[28] This corresponded with the finding of this study.

Hypomethylation potentially promotes cancer via a number of mechanisms, including activation of proto-oncogenes, chromosome instability, reactivation of transposable elements, and loss of imprinting.[29,30] The overall level of genomic DNA methylation was reported to reduce by approximately 10% in colonic neoplasia.[31] It has been reported that methylation status of the well characterized tumor oncogenes, c-Ki-ras and n-Ha-ras was found to be hypomethylated in tumor cells, when compared to their adjacent normal cells in several types of cancers analyzed, and that may also become more pronounced in the later stages of tumor progression.[32]

There is an evidence that LINE-1 hypomethylation could induce the expression of the MET oncogene in cancer and normal tissues.[33] In addition to the activation of oncogenes, LINE1 hypomethylation is also associated with hypermethylation in certain genes, such as CDH1, CDH13, and PGP9.5 genes.[34,35]

Advances in the molecular medicine have not only clarified the carcinogenesis progression of gastric cancer, but also offered new novel approaches regarding prevention, prognosis, diagnosis and therapeutic intervention, taking advantages from the gradient alteration of DNA methylation status upon disease progression.

<table>
<thead>
<tr>
<th>Total (120)</th>
<th>(50%)</th>
<th>(50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>56 +</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td>(57.1%)</td>
<td>(42.8%)</td>
<td></td>
</tr>
<tr>
<td>64 -</td>
<td>43</td>
<td>21</td>
</tr>
<tr>
<td>(67.1%)</td>
<td>(32.8%)</td>
<td></td>
</tr>
</tbody>
</table>

M corresponding to methylated cases, U corresponding to unmethylated cases.

REFERENCES


