Clinical Relevance of CagA EPIYA Motifs In Helicobacter pylori Among the Dyspeptic Patients in Kenya

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ABSTRACT

Introduction: The Helicobacter pylori cytotoxin-associated gene A (cagA) has been associated with disease severity and its oncogenic potential has been linked to its polymorphic EPIYA motifs whose combinations differ geographically. The aim of this study was to determine the frequency of cagA-positive Helicobacter pylori isolated from dyspeptic patients in Kenya and to assess the association of cagA EPIYA motif patterns with clinical outcome. Materials and Methods: H. pylori positivity was determined by histology and molecular diagnostic method directly from the gastric pathologies. The cagA presence and EPIYA Motif patterns were analyzed by polymerase chain reaction from the H. pylori positive samples. Results: The cagA gene was identified in 48.75% of the H. pylori patients. The cagA per se was not significantly associated with the gastroduodenal diseases. The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The presumed virulent ABCCC pattern was rare (5.13%). Increase in the number of EPIYA C (more than one C repeat) repeats was significantly associated gastric cancer (OR=6.577 95% CI 1.620-26.704, p=0.008). Conclusion: All the Kenyan cagA EPIYA patterns were of Western type. We found that infection by H. pylori cagA strains with multiple EPIYA C repeats to be associated with gastric cancer but not peptic ulcer in Kenya; but the low prevalence of these strains might contribute to the low incidence of gastric cancer in this country. Determining the EPIYA motifs in CagA, rather than detecting cagA gene alone, would be a better marker for assessing the risk of serious gastric pathology.

KEYWORDS: Helicobacter pylori cagA genotype, EPIYA Motif, Gastroduodenal diseases

INTRODUCTION

The development of the gastroduodenal disease such as gastritis, peptic ulcer, gastric cancer and gastric MALT are associated with Helicobacter pylori infection.[1] Gastric colonization with Helicobacter pylori affects at least half of the world’s population.[2] However, the factors that lead to few individuals to develop the associated diseases while others remain asymptomatic are unknown.

One of the virulent determinants of Helicobacter pylori is cagA Pathogenicity Island. Many studies have associated cagA positive strains with peptic ulcer and gastric cancer.[3,4] The cagA gene is part of the this PAI that codes a type IV secretion system, through which an effector protein, cagA, enters into the host gastric epithelial cells [5] where it is phosphorylated by the Src kinases on the tyrosine residue, the phosphorylation motifs in the carboxi-terminal variable region of the protein.[6-10] The tyrosine phosphorylation motifs contain the Glu-Pro-Ile-Tyr-Ala (EPIYA) sequence.[6] The cagA has a structural diversity in the EPIYA-repeat segment of which four types are described as A, B, C, and D.[8,11] On the basis of the EPIYA-repeat the cagA protein is classified into two types; the western and East Asian types. Both types have A and B however, they differ in that EPIYA C is specifically found in the Western type and often duplicated [8, 11] and EPIYA D in the East Asian type. [12]

Phosphorylated cagA forms a physical complex with SHP-2 phosphate triggering abnormal cellular signals leading to deregulation of cell growth, cell to cell contact and cell
migration, elongation of epithelial cells and increase of epithelial cell turnover enhancing the risk of damaged cells acquiring precancerous genetic changes. [8, 11] The EPIYA C and D are the main sites of phosphorylation of the cagA. Higashi et al [8] associated EPIYA D and Multiple EPIYA C repeats with increased SHP- Phosphate activity induced by cagA. Jang et al [13] presented epidemiological evidence that there is a significant association between the development of cancer and infection with H. pylori strains carrying EPIYA ABD cagA genotype in South Korea; an association that earlier been made by elsewhere. [14] Studies have also shown that strains with cagA with higher number of EPIYA C segment were closely associated with gastric cancer than strains with cagA with one EPIYA C segment only. [15, 16]

Further, we evaluated the prevalence of the cagA positive strains in Kenya and whether it is associated with disease. The EPIYA Pattern of the cagA and strain type of Helicobacter pylori was established and evaluated whether increased number of cagA EPIYA C phosphorylation motifs is associated with gastroduodenal diseases. Further the data obtained helped us to show the interrelationship between these virulence factors with severity of epithelial damage, gastritis, mucosa infiltration and intestinal metaplasia.

MATERIALS AND METHODS

Selection of the Subjects: The present Patients and Gastric biopsy samples

The study population included one hundred and twenty seven (127) patients with past and present history of dyspepsia and who had been referred for endoscopy at Kenyatta National Hospital (KNH), a national referral hospital in Kenya. Permission to carry out the study was obtained from the Kenyatta National Hospital Scientific and Ethical Review committee. It was conducted according to the ethical guidelines of the declaration of Helsinki, 2000. [17] After fulfilling the inclusion criteria and obtaining a written consent the patients provided the demographic data before undergoing routine endoscopy. Patients underwent a detailed history and physical examination. Esophageal gastro duodenoscopy (EGD) was performed under conscious sedation with intravenous midazolam. The lining of the esophagus, stomach and duodenum was observed. Two (2) antral and two (2) corpus biopsies about 2-3 mm in length, were obtained for routine histology and molecular testing (DNA extraction). The biopsy specimens for molecular analysis were put in a 2ml DESS (DMSO/ EDTA/ NaCl) solution and stored at −80°C until use, while samples for histological analysis were immediately fixed in buffered formalin.

Histopathology

The biopsies were processed in a tissue processor and ultramicrotomy was done in a microtome to produce 4-6 µm sections. The sections were deparaffinized and hydrated to distilled water. They were then stained in freshly prepared Giemsa working solution for 30 minutes – 1 hour; dehydrated in three changes of absolute alcohol and cleared in xylene for three changes. They were mounted with resinous medium and a cover slip placed. The slides were examined microscopically by using x400 and at least five high power fields examined by a pathologist. Histopathological findings were recorded and a histopathological classification of gastritis was used using updated Sydney system [18] which had a scale of 0 – 3 for scoring the features of chronic gastritis, corresponding to none, mild, moderate or severe respectively.

PCR-based genotyping of the cagA genes and gel electrophoresis

The samples were removed from DESS (DMSO/ EDTA/ NaCl) solution and soaked in 2 mL TE (10 mM Tris, 1 mM EDTA) for 2 hours. The biopsies were then collected in a microcentrifuge tubes containing 120 ul of sterile phosphate buffered saline and vortexed vigorously for 2 min. The tubes were then boiled in a water bath at 95 degrees centigrade for 15 min, cooled in ice, and centrifuged at 13,000 X g for 1 min. The supernatant was transferred to another tube and then stored at 2-8°C. About 3 ul of the supernatant containing the DNA template was added in 25 ul volumes containing 10 pmol of primers cag5c-F (5- GTTGATAAAGGCACTGCGTT-3) and cag3c-R (5- GGTGTTATGATATTTTCTCATAA-3), [19] 200 µM each dNTP is in 10 mM Tris-HCl, (pH 9.0 at room temperature), ~2.5 units of puReTaq DNA polymerase (GE Healthcare UK Limited), 50 mM KCl, and 1.5 mM of MgCl2 in a reaction standard PCR buffer (GE Healthcare UK Limited). Products were amplified under the following conditions: 3 min at 94°C for initial denaturation followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final round of 10 min at 72°C, in a thermal cycler. All the PCR products were electrophoresed in 1.5% gel and visualized under UV light as described elsewhere. [19]

Amplification of the 3-Variable Region of the cagA Gene and Determination of the EPIYA Pattern.

The following primers were used for amplification of the 3-variable region of the cagA gene the primers CAG1: 5- ACCCTAGTGTTAATTTCGTA-3 and CAG2: 5- GTAATTGTCTAGTTTCGC-3 as described by Yamaoka et al. [16] The reaction mixture consisted of 0.5mMof each primer, 1X PCR buffer, 1.5mMMgCl2, sterile water, 0.2M of each deoxynucleotide, 1.25 µL Taq DNA polymerase and 3 µL DNA in a final volume of 25 µL. The amplification conditions were initial denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 95°C for 1min, annealing at 56°C, and extension at 72°C for 1 min, with a final extension at 72°C for 10min. PCR will be carried out in a thermocycler Gene Amp PCR system 9700 (Applied Biosystems). Products of 500 to 850 bp were obtained depending on the type and number of repeats of the EPIYA-C motif in the cagA gene. The PCR products were separated by electrophoresis on 2% Agarose gel and stained with Ethidium bromide and visualized under a UV transilluminator. This procedure allowed for detection of multiple infections.

To rule out the presence of East Asian strain (presence of EPIYA-D), a reaction mixture containing 0.2mM concentrations of each deoxyribonucleoside triphosphate, a 0.4 mM concentration of the forward primer (cagA28F 5- TCTCAGTGTTAATTTCGTA-3), a 0.08 mM Concentration of the reverse primer (cagA-PD (R) 5- TGTTAATTGTCTAGTTTCGC-3), [20] 0.05 U of Taq DNA polymerase/ul, and 3ul of genomic DNA in buffer (10}
mM Tris-HCl [pH 8.3], 1.5 mM magnesium chloride, 50 mM potassium chloride) was incubated at 95°C for 90 s, followed by 35 cycles at 95°C for 30 s, 57°C for 60 s, and 72°C for 30 s and a final extension at 72°C for 5 min. PCR products were separated on a 1.5% (wt/vol) agarose gel.

Statistical analysis
Data analysis was done using SPSS version 17 (SPSS inc., Chicago, IL) program for windows. The association of the specific virulence-associated bacterial genotype and EPIYA motif with the clinical outcome of *Helicobacter pylori* infection was assessed using Chi-square (χ²) or Fisher's exact tests to compare proportions for categorical variables. A value of p < 0.05 was considered statistically significant. Odds ratio was estimated and corresponding 95% confidence intervals were estimated while non parametric correlations between the genotypes and severity of disease were assessed by use Kendall’s tau b test.

RESULTS

Demographic Characteristics

<p>| Table 1: Distribution of cagA, EPIYA motifs among the patients with different clinical outcomes |
|---------------------------------|-------------------------------|-----------------|-----------------|-------------------|-------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>cagA Positive n=39 (%)</th>
<th>Gastritis n=55 (%)</th>
<th>Duodenal ulcer n=5 (%)</th>
<th>Gastric ulcer n=6, (%)</th>
<th>Intestinal Metaplasia n=23 (%)</th>
<th>Gastric cancer n=10 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA</td>
<td>-</td>
<td>26 (47.3)</td>
<td>3 (60)</td>
<td>3 (50)</td>
<td>6 (54.5)</td>
<td>11 (47.8)</td>
</tr>
<tr>
<td>AB</td>
<td>11 (28.21)</td>
<td>9 (23.1)</td>
<td>1 (20)</td>
<td>0 (0)</td>
<td>1 (9.1)</td>
<td>1 (4.3)</td>
</tr>
<tr>
<td>ABC</td>
<td>22 (56.41)</td>
<td>13 (33)</td>
<td>2 (40)</td>
<td>2 (33.3)</td>
<td>4 (36.4)</td>
<td>10 (43.5)</td>
</tr>
<tr>
<td>One C or None</td>
<td>33</td>
<td>22 (56.4)</td>
<td>3 (60)</td>
<td>2 (33.3)</td>
<td>5 (45.5)</td>
<td>11 (47.8)</td>
</tr>
<tr>
<td>ABCC</td>
<td>17 (43.59)</td>
<td>2 (5.1)</td>
<td>1 (20)</td>
<td>2 (33.3)</td>
<td>3 (27.3)</td>
<td>9 (39.1)</td>
</tr>
<tr>
<td>ABCCC</td>
<td>2 (5.13)</td>
<td>14 (35.9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Two C and above</td>
<td>19</td>
<td>14 (35)</td>
<td>1 (20)</td>
<td>2 (33.3)</td>
<td>3 (27.3)</td>
<td>9 (39.1)</td>
</tr>
<tr>
<td>ABD</td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Prevalence of *H. pylori* by age and sex in the enrolled patients

Patients aged 40 and above years had highest prevalence (62.5%); followed by those aged 20-39 Years (64); and lowest those under 20 years (50%). Male patients had a lower prevalence at 59.01% against female patients at 67.21%. However, neither was there any significant association between *H. pylori* infection and age nor sex; p>0.0849041 and p< 0.343563, respectively.

Prevalence of *H. pylori* in different disease conditions

All the patients diagnosed with duodenal ulcer were positive for *H. pylori* infection while it was positive in 74.4% patients with gastric ulcer. Though association was not significant (P < 0.0870), 69.1% of patients with gastritis were *H. pylori* positive. Significantly, (P< 0.0015) GERD was associated with *H. pylori* infection while 60% of patients diagnosed with gastric cancer were *H. pylori* positive.

Prevalence of cagA and distribution of EPIYA motifs pattern

Of the eighty (80) samples positive for *H. pylori* infection thirty nine (39) were cagA positive; a prevalence of 48.75%. PCR amplified products from all the cagA positive strains showed varied patterns in the variable region of the CagA. The observation of no EPIYA ABD pattern indicated there was no presence of East Asian *H. pylori* strain.

The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The ABCCC pattern was rare (5.13%). Five (5) out of the thirty nine (39) cagA positive samples had different EPIYA patterns indicating a 12.8% mixed infection. Because of little representation no significant statistical analysis was deduced from these samples. However, the two samples with both EPIYA ABC and ABCC had Intestinal Metaplasia. None of these samples had gastric cancer.

Association between the EPIYA Motif patterns and gastric pathologies

The presence of cagA was insignificantly associated with peptic ulcer and gastric cancer. Increase in the number of EPIYA C repeats was insignificantly associated with gastric ulcer and duodenal ulcer. EPIYA pattern with more than one

C was significantly associated with Intestinal Metaplasia (p=0.045, OR 3.021, 95% CI 1.026-8.900), and gastric cancer (p=0.008, OR=6.577, 95% CI 1.620-26.704) as shown in Table 2.

### Table 2: Association between the EPIYA Motif patterns and gastric pathologies

<table>
<thead>
<tr>
<th>Gastritis n=55</th>
<th>Duodenal Ulcer n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EPIYA Motif patterns</strong></td>
<td><strong>P value</strong></td>
</tr>
<tr>
<td>Cag A</td>
<td>0.695</td>
</tr>
<tr>
<td>EPIYA AB</td>
<td>0.324</td>
</tr>
<tr>
<td>ABC</td>
<td>0.287</td>
</tr>
<tr>
<td>EPIYA Less than 2 C or none</td>
<td>0.736</td>
</tr>
<tr>
<td>EPIYA more than 1C</td>
<td>0.596</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gastric Ulcer n=6</th>
<th>Peptic Ulcer n=11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P value</strong></td>
<td><strong>OR</strong></td>
</tr>
<tr>
<td>Cag A(26)</td>
<td>0.949</td>
</tr>
<tr>
<td>EPIYA AB(9)</td>
<td>0.309</td>
</tr>
<tr>
<td>ABC</td>
<td>0.665</td>
</tr>
<tr>
<td>EPIYA Less than 2 C or none</td>
<td>0.682</td>
</tr>
<tr>
<td>EPIYA more than 1C</td>
<td>0.570</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intestinal Metaplasia n=23</th>
<th>Gastric Cancer n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P value</strong></td>
<td><strong>OR</strong></td>
</tr>
<tr>
<td>Cag A</td>
<td>0.916</td>
</tr>
<tr>
<td>EPIYA AB</td>
<td>0.153</td>
</tr>
<tr>
<td>ABC</td>
<td>0.042*</td>
</tr>
<tr>
<td>EPIYA Less than 2 C or none</td>
<td>0.449</td>
</tr>
<tr>
<td>EPIYA more than 1C</td>
<td>0.045*</td>
</tr>
</tbody>
</table>

*Significant association

### Correlation between the EPIYA Motif variants with severity of various pathologies

We further evaluated whether each of the pattern correlated with the degree of various pathologies. EPIYA ABC significantly correlated with increased degree of intestinal metaplasia (p=0.039). There was also significant correlation between the EPIYA patterns with more than one C repeat segment with increased degree of intestinal metaplasia (p=0.021). Because of the fewer EPIYA ABCC (2) positive-samples no correlation was made with various pathologies. The degree of chronic inflammatory infiltrate in the gastric mucosa significantly correlated with EPIYA patterns with more than one C repeat segment (p=0.021) as opposed the EPIYA patterns with one or no C repeat segment (p=0.095) as shown in Table 3. Surprisingly, neither of the EPIYA pattern significantly correlated with degree of inflammatory activity nor the degree of gastritis in this study.
DISCUSSION

Many studies have shown infection with cagA –positive *Helicobacter pylori* strain to be closely associated with gastric cancer and peptic ulcers.[3,4] Nonetheless, the majority of the *Helicobacter pylori* infected patients do not develop serious diseases. In the present study, 62.99% of dyspeptic patients in Kenya were found to be infected with *H. pylori* which were lower than reported.[21] On average 48.75% of the infecting strains harbored the cagA gene which is within the global prevalence of cagA –positive *H. pylori*, which range from 43% to 90%.[22-24] The differences in the prevalence of *H. pylori* can be explained by differences in the diagnostic method used, age of patients, geographic area and the environmental health conditions in which people live or that the rate of infection is decreasing.[24]

In this study we observed no EPIYA ABD pattern indicating the absence East Asian *H. pylori* strain in Kenya as was found elsewhere in Africa. The most occurring EPIYA pattern was the ABC (56.41%) followed by ABCC at 43% and AB at 28.21%. The predominance of ABC pattern mirrors the study by Chomvarin et al.,[25] among the dyspeptic patients in Northern Thailand. The presumed violent ABCC pattern was rare (5.13%) as observed elsewhere. [26] This shows that the Kenyan *H. pylori* are of Western type. Analysis of motifs have shown that males are more likely to have ABC pattern than females (OR=3.036, 95% CI 1.075–8.575, p=0.047) a link that has been not documented elsewhere. There was no other pattern associated with gender. Though it may be speculated that the number of EPIYA C repeats increases with age we found no association between age with increase in C repeats or any of the EPIYA patterns as opposed to what was found by Breurec et al.[24] in Senegal.

Surprisingly, none of the gastric pathologies including peptic ulcer and gastric cancer was associated with the presence of cagA gene alone. This differs from many other studies that associated cagA gene with peptic ulcer and gastric cancer.[27-29] The lack of association correlates with another recent study in Kenya by Kimang’a et al.[30] that found no significant association of cagA gene with gastric pathologies. However, 60% and 54.5% of patients with gastric cancer and peptic ulcer, respectively had cagA-positive *H. pylori* strain indicating possible risk. Other pathologies had their distributions below a 50% mark.

In this study none of the EPIYA patterns was significantly associated with gastritis, duodenal ulcer or gastric ulcer. EPIYA ABC was significantly associated with intestinal metaplasia (OR=2.885, 95% CI 1.018–8.175, p=0.042) and not gastric cancer. Patients with more than one C repeat had an odds ratio (OR, 3.021) 2-fold of having intestinal metaplasia than those with one or no C segment (OR, 1.458). Our analysis of gastric histopathology found a significant correlation between the EPIYA patterns with more than one C repeat segment with increased degree of Intestinal metaplasia (p=0.021) as had been reported elsewhere.[31] Gastric cancer was significantly associated with samples with more than one C repeat (OR=6.577 95% CI 1.620-26.704, p=0.008). It has been documented that the number of EPIYA-C motifs influence the degree of virulence and oncogenic potential of cagA –positive *H. pylori*.[31] Studies have shown that an increase in the number of phosphorylation sites in the C-terminus of CagA is associated with the carcinogenic potential of *H. pylori*.[32-34]

Therefore, it is more likely that those patients with chronic gastritis infected with a *H. pylori* strain with cagA gene that encodes two or more EPIYA-C motifs (25.45%) are at higher risk of developing intestinal metaplasia and gastric cancer. Thus, determining the EPIYA motifs in CagA, rather than detecting cagA alone, would be a better marker for assessing the risk of serious gastric pathology. [35] Gastric histopathology analysis showed a significant correlation between the density of *H. pylori* colonization with the degree of inflammation, severity of gastritis and epithelial damage at p=0.001 and degree of mucosa infiltration at p=0.01 which was in support of previous studies.[36,37] The density of *H. pylori* colonization was not significantly correlated with Intestinal metaplasia and gastric cancer leading to an assumption that there are virulent factors that cause dangerous pathologies. We also evaluated the distribution of mixed infections and found no association with gastric cancer or any other gastric pathology as opposed to the study by Batista et al. 2011.[38]
CONCLUSION
In summary, this study showed that cagA-positive *H. pylori* infection is averagely prevalent in dyspeptic patients in Kenya and all the cagA EPIYA patterns were of Western type. We found that infection by *H. pylori* cagA strains with multiple EPIYA C repeats to be associated with gastric cancer but not peptic ulcer in Kenya; but the low prevalence of these strains might contribute to the low incidence of gastric cancer in this country. This shows that determining the EPIYA motifs in CagA, rather than detecting cagA gene alone, would be a better marker for assessing the risk of serious gastric pathology.

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