DNA sequence analysis of \textit{cagA} 3′ motifs of \textit{Helicobacter pylori} strains from patients with peptic ulcer diseases

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The \textit{Helicobacter pylori} \textit{cagA} gene is a major virulence factor that plays an important role in gastric pathologies. DNA sequence data for the \textit{cagA} 3′ region of Western isolates differ markedly in their EPIYA motifs from those of East Asian isolates. An increase in the number of these motifs is known to be associated with gastric cancer. Whether such an association is also the case for peptic ulceration was investigated in this study. Gastric biopsies were collected from 96 patients with duodenal ulcer (DU), gastric ulcer (GU) and gastritis. The types of EPIYA motif detected by PCR among 28 DU strains were 13 ABC, eight ABCC, six ABCCC, and in one patient both ABC and ABCCCCC; among nine GU strains were two ABC, five ABCC and two ABCCC; and among 40 gastritis strains were 35 ABC and five ABCC. DNA sequencing was carried out to confirm the detection of the EPIYA motif types and to analyse their peptide sequences. A significant association was found between the number of the EPIYA-C motifs (≥2) and peptic ulceration (\(P=0.00001\)) compared with gastritis. In conclusion, this study shows that our patients harboured \textit{cagA}-positive \textit{H. pylori} strains with EPIYA motifs of the Western type and that the increase in the number of EPIYA-C motifs was significantly associated with DU and GU but not with gastritis, indicating predictive association with the severity of the disease.

INTRODUCTION

\textit{Helicobacter pylori} is the causative agent of gastritis and peptic ulcers and plays an important role in the development of gastric cancer. The severity of disease outcome has been attributed to possession of the \textit{cag} pathogenicity island, which encodes a type IV secretory system that facilitates translocation of the CagA protein (Backert \textit{et al.}, 2004). This protein plays an important role in the aetiology of \textit{H. pylori}-induced gastric pathologies. When CagA is secreted into gastric epithelial cells, some CagA molecules are tyrosine-phosphorylated through their EPIYA motifs, whilst other CagA molecules remain unphosphorylated (Poppe \textit{et al.}, 2007; Selbach \textit{et al.}, 2003; Stein \textit{et al.}, 2002). Phosphorylated CagA causes dysregulation of the epithelial structure through its effect on the signal system of the host cell (Brandt \textit{et al.}, 2005), whilst unphosphorylated CagA also contributes to the development of \textit{H. pylori}-associated gastric diseases, including gastric cancer (El-Etr \textit{et al.}, 2004; Hirata \textit{et al.}, 2002). Recently, Suzuki \textit{et al.} (2009) reported that a conserved motif in the C-terminal region of CagA, distinct from the EPIYA motifs and designated CRPIA (conserved repeat responsible for phosphorylation-independent activity), plays a pivotal role in \textit{H. pylori} pathogenesis. The size variation of the CagA protein has been shown to be related to the presence of the repeat sequences containing the EPIYA motifs within the 3′ variable ends (Argent \textit{et al.}, 2005). \textit{H. pylori} strains isolated in Western countries contain the EPIYA-A, EPIYA-B and Western \textit{cagA}-specific EPIYA-C segments. The latter motif varies in number among distinct Western CagA proteins, mostly ranging from one to three (Ren \textit{et al.}, 2006). The EPIYA repeats of strains isolated in East Asian countries contain the EPIYA-A, EPIYA-B and East Asian CagA-specific EPIYA-D segments. The objectives of this study were to determine the number and type of EPIYA motifs within the \textit{cagA} 3′ variable region among \textit{H. pylori} isolates using multiple reverse primers by PCR and DNA sequence analysis and to determine whether there is an association between these motifs and peptic ulcer diseases.

METHODS

Patients. A total of 96 patients was included in this study: 29 duodenal ulcer (DU), 10 gastric ulcer (GU) and 57 gastritis patients. Patients attended the endoscopy unit at the Istanbul Teaching Hospital, Istanbul, Turkey, and were selected on a consecutive basis. 

Abbreviations: DU, duodenal ulcer; GU, gastric ulcer.

The GenBank/EMBL/DDBJ accession numbers for the partial \textit{cagA} nucleotide sequences determined in this study are GQ899170–GQ899174, GU132848–GU132860, GU143415 and GU143416.
Those who had received antibiotics, non-steroidal anti-inflammatory drugs or proton pump inhibitors 4 weeks before endoscopy were excluded. Two antral biopsies were obtained from each patient. Of these, 84 patients (29 DU, nine GU and 46 gastritis) aged 20–65 years (44 females) who were H. pylori-positive by culture were analysed further. Informed written consent was obtained from all patients. The study was approved by the ethical committees of Fatih University and the Istanbul Teaching Hospital.

**Culture.** The two antral biopsies were homogenized together in 100 μl saline solution and then handled as follows: (i) 50 μl was inoculated onto Columbia agar plates containing 5 % horse blood and incubated under microaerophilic conditions in a CO₂ incubator at 37 °C for 5–7 days; (ii) 20 μl was used for a rapid urease test; and (iii) the remainder was stored at −80 °C. The growth cultures of H. pylori were identified by colony morphology, Gram staining and positive reactions to oxidase, catalase and urease activities. Cultures were harvested and stored in Brucella broth containing 20 % glycerol at −80 °C. From each isolated bacterial culture, six single colonies from separate spots on the plates were subcultured for three passages. A total of 588 isolates that included a sweep of the entire colonies and the six single colonies of each isolate were studied by PCR and the amplified products were analysed by DNA sequencing.

**Extraction of genomic DNA.** Single clones and subclones were harvested and DNA was extracted using a QIAamp DNA Mini kit (Qiagen).

**PCR.** Amplification of the cagA 3′ variable region was performed using primers cag2 (5′-GGAAACCTTAGCTGGTTAATG-3′) and cag4 (5′-ATCTTTCGAGCTTGCTATCG-3′), as described previously (Argent et al., 2005). The forward primer cag4F (5′-TTCTCAAAGGAGCAATTGGC-3′) and reverse primers cag1-P1C (5′-GTCCCTGTCTTCTTTTTATTAACTTGAGCTTGTCTATCG-3′), cagA-P2CG (5′-TTTAGGCAACTT-TTACGTATAATTGGG-3′), and cagA-P3E (5′-ATCAATTGTAGCCTTAAATGCGG-3′) were used to amplify the EPIYA motif encoding sequences A, B, C, and D, respectively (Argent et al., 2005).

**DNA sequence analysis.** DNA sequencing of the cagA 3′ region was performed using a BigDye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), according to the manufacturer’s recommendations. Oligonucleotide screening by BLAST analysis was used to identify H. pylori CagA peptide sequences. Alignment of partial CagA peptide sequences showing the EPIYA motifs of H. pylori isolates from patients with DU, GU and gastritis including the reference strain 26695 are shown in Fig. 2. Statistical analysis was carried out by comparing the number of EPIYA-C motifs of strains isolated from DU+GU patients with those of gastritis patients (Table 1). We found a significant association between the increase in the number of EPIYA-C motifs (≥2) and peptic ulceration (P=0.00001) compared with gastritis. We also detected the highly conserved CRPIA repeat motifs (FPLKRHDKVIDILSKV) among the cagA genes in all strains isolated. These were located in the C-terminal region and, regardless of the number of C motifs, there was always one CRPIA motif located before and one after such motifs (Fig. 2). No statistical significance was found between the age, gender and the severity of the disease.

The prevalence of H. pylori and possession of the cagA gene in strains isolated from dyspeptic patients in Turkey are known to be very high (70%), as reported previously by us and others (Bulent et al., 2003; Erzin et al., 2006; Salih et al., 2007). More recently, Umit et al. (2009) also demonstrated 77 % cagA positivity in dyspeptic patients. A similar finding was found in this study. The H. pylori cagA gene encodes the CagA protein, a major virulence factor strains. The type and number of EPIYA motif repeats in our strains were as follows: among the 28 DU strains, there were 13 ABC, eight ABCC, six ABCCC, and in one patient both ABC and ABCCCCG; among the nine GU strains, there were two ABC, five ABCC and two ABCCC; and among the 40 gastritis strains, there were 35 ABC and five ABCC (Table 1). All strains that gave an EPIYA-positive PCR product were further confirmed by DNA sequencing. DNA from only one of the six colonies of each strain that gave similar EPIYA motifs by PCR was sequenced. The consensus sequences of the EPIYA-A (KVNKKKQTGQ), EPIYA-B (QVAKKVNAKI) and EPIYA-C (TIDILQ-GPEPL) motifs were detected in strains from patients with different peptic ulcer diseases. The cagA 3′ variable region of different sizes due to the variable number of EPIYA motifs was detected in strains from these patients. DNA sequencing further confirmed our results obtained by PCR amplification. In Fig. 1, patient 1407 had EPIYA motif repeats of ABC (detected in five of the isolated colonies) and ABCCCCC (detected in the sixth colony). The alignment of partial CagA peptide sequences showing the EPIYA motifs of H. pylori isolates from patients with DU, GU and gastritis is shown in Fig. 2. Statistical analysis was carried out by comparing the number of EPIYA-C motifs of strains isolated from DU+GU patients with those of gastritis patients (Table 1). We found a significant association between the increase in the number of EPIYA-C motifs (≥2) and peptic ulceration (P=0.00001) compared with gastritis. We also detected the highly conserved CRPIA repeat motifs (FPLKRHDKVIDILSKV) among the cagA genes in all strains isolated. These were located in the C-terminal region and, regardless of the number of C motifs, there was always one CRPIA motif located before and one after such motifs (Fig. 2). No statistical significance was found between the age, gender and the severity of the disease.

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<table>
<thead>
<tr>
<th>EPIYA motif</th>
<th>DU</th>
<th>GU</th>
<th>DU + GU</th>
<th>Gastritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>13</td>
<td>2</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>ABCC</td>
<td>8</td>
<td>5</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>ABCCC</td>
<td>6</td>
<td>2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>ABCCCCG</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>9</td>
<td>37</td>
<td>40</td>
</tr>
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</table>
This protein varies in size due to the possession of repeat units of the EPIYA-AB and Western-type C motifs within the 3’ variable end. Isolates from East Asian countries possess the EPIYA-AB and East Asian EPIYA-D motifs. It is well known that all strains found at different geographical locations around the world have the EPIYA-A and/or -B motifs (which play a role in downregulation of Src kinase activity). However, the C motifs are only found in Western strains and the D motif only in East Asian strains (Argent et al., 2008). The strains analysed in this study were of the Western type and generally possessed between one and three C motifs. However, in one strain isolated from a DU patient, up to five C motifs were detected. This might be due either to the acquisition of these motifs during the infection period through microevolution or to infection with two different strains. Recently, Argent et al. (2008) indicated that analysis of different cagA data from GenBank and the literature showed that Western cagA is significantly more likely to undergo duplication of the C motif than the East Asian D motif. The EPIYA motifs present in the EPIYA-C and EPIYA-D segments are major sites of CagA tyrosine phosphorylation (Ren et al., 2006). It has been shown that CagA proteins with a larger number of EPIYA-C motifs in Western strains lead to increased phosphorylation of the protein, increased formation of the hummingbird phenotype in vitro, and are more likely to be associated with the development of gastric cancer (Argent et al., 2005). Azuma et al. (2004) reported previously that aspects other than the CagA level of tyrosine phosphorylation may have an impact on its cellular effects. CagA from East Asian strains with a consensus sequence motif not found in Western strains led to greater activation of the

![Fig. 1. The cagA 3’ end EPIYA motif repeats of the isolate from patient 1407 showing the ABC (1) and ABCCCCC (2) motifs. Forward primer cagA28F and reverse primers cagA-P1C, cagA-P2CG and cagA-P2TA were used for amplification of the EPIYA-A, -B and -C motifs, respectively. M, Molecular size marker.](image1)

![Fig. 2. Alignment of partial CagA peptide sequences (showing the EPIYA and CRPIA motifs) of H. pylori strains isolated from patients with peptic ulcer diseases including the H. pylori reference strain 26695. The deduced amino acid sequence of the EPIYA-A (KVNKKKTGQ), EPIYA-B (QVAKKVNAA) and EPIYA-C (TIDDLGGPFPL) motifs is shown. Asterisks indicate the CRPIA motifs. The numbers on the right indicate the final amino acid in each line.](image2)
phosphatase. However, they indicated that, despite the absence of this consensus sequence in Western strains, they could still induce cellular damage. Most of our strains of *H. pylori* isolated from patients with gastritis possessed ABC-type EPIYA motifs, with a few with minor variations in these motifs. Whilst less than half of the strains from DU and even less from GU patients were of the ABC type, the rest had variable numbers of multiple repeats of the C motif that was significantly associated with these diseases. This indicates that such multiple repeats play an important role in the development of peptic ulceration, in addition to other possible factors, as some patients were still infected with *H. pylori* strains possessing the ABC motifs. It should also be emphasized that the carcinogenic potential of *H. pylori* in patients with GU will have a greater impact on the progression of the disease than those from DU patients. Very few of our gastritis patients had atrophy and none had intestinal metaplasia, a fact that makes the correlation of these factors with the severity of the disease negligible. El-Etr et al. (2004) reported previously that, following translocation of the CagA protein into gastric epithelial cells, some CagA molecules undergo tyrosine phosphorylation at EPIYA motifs, whilst others remain unphosphorylated or become dephosphorylated. In one-third of the epithelial cells, gene expression was altered by the CagA protein; however, the changes were not affected by the phosphorylation state of CagA (Suzuki et al., 2009). Recently, Suzuki et al. (2009) provided evidence that the unphosphorylated form of CagA induces a variety of host cell proliferative and immune responses in gastric epithelial cells. They concluded that the unphosphorylated CagA activity also plays a pivotal role in promoting the survival, multiplication and dissemination of gastric epithelial cells, leading to *H. pylori* long-term colonization. Although Suzuki et al. (2009) recently showed that the CRPIA motif in unphosphorylated CagA is involved in interaction with and activation of the signalling system that promotes epithelial proliferation and pro-inflammatory responses, their role seems to be in concert with other triggering mechanisms, as this motif is present in all strains possessing cagA. In this study, we detected these CRPIA motifs, which might be one of the factors important in pathogenesis. However, correlation of their exact role in peptic ulceration requires further investigation.

In conclusion, an increase in the number of EPIYA-C motifs seems to have a significant association with peptic ulceration. This study showed that patients with DU and GU harboured cagA-positive *H. pylori* strains with EPIYA motifs of the Western type with a variable number of EPIYA-C motifs, indicating a predictive association with the severity of the disease.

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**REFERENCES**


