DNA sequence analysis of *cagA* 3' motifs of *Helicobacter pylori* strains from patients with peptic ulcer diseases

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The *Helicobacter pylori* *cagA* gene is a major virulence factor that plays an important role in gastric pathologies. DNA sequence data for the *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 ABCCCC; 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 *cagA*-positive *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.

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INTRODUCTION

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 *cag* pathogenicity island, which encodes a type IV secretory system that facilitates translocation of the CagA protein (Backert *et al.*, 2004). This protein plays an important role in the aetiology of *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 *et al.*, 2007; Selbach *et al.*, 2003; Stein *et al.*, 2002). Phosphorylated CagA causes dysregulation of the epithelial structure through its effect on the signal system of the host cell (Brandt *et al.*, 2005), whilst unphosphorylated CagA also contributes to the development of *H. pylori*-associated gastric diseases, including gastric cancer (El-Etr *et al.*, 2004; Hirata *et al.*, 2002). Recently, Suzuki *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 *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 *et al.*, 2005). *H. pylori* strains isolated in Western countries contain the EPIYA-A, EPIYA-B and Western *cagA*-specific EPIYA-C segments. The latter motif varies in number among distinct Western CagA proteins, mostly ranging from one to three (Ren *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 *cagA* 3' variable region among *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 *cagA* nucleotide sequences determined in this study are GQ899170–GQ899174, GU132848–GU132850, 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'-GGAACCTAGTCGGTAATG-3') and *cag4* (5'-ATCTTTGAGCTTGCTATCG-3'), as described previously (Argent *et al.*, 2005). The forward primer *cagA28F* (5'-TTCTCAAA-GGAGCAATTGGC-3') and reverse primers *cagA-P1C* (5'-GTCCT-GCTTCTTTTATTAACTTKAGC-3'), *cagA-P2CG* (5'-TTTAGC-AACTTGAGCGTAAATG GG-3'), *cagA-P2TA* (5'-TTTAGCAACTT-GAGTATAAATGGG-3') and *cagA-P3E* (5'-ATCAATTGTAGCGT-AAATGGG-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' 500–850 bp product 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 was carried out using CLUSTAL W.

Statistical analysis. The differences in age, gender and *cagA* EPIYA motifs of *H. pylori* between DU, GU and gastritis patients were analysed using a χ^2 test. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

H. pylori was found in 84 (88 %) of the 96 patients enrolled in this study, as determined by culture and a rapid urease test. We detected the *cagA* 3' variable region of *H. pylori* by PCR in strains isolated from 77 of the 84 patients (92 %): 28/29 from DU (97 %), 9/9 from GU (100 %) and 40/46 from gastritis (87 %) patients. The type of EPIYA motif in these strains was also determined by PCR and we found that our strains possessed the Western-type EPIYA motifs A, B and C. The PCR amplicons of the EPIYA motifs ranged in size from 550 to 850 bp depending on the number of the C motif repeats possessed by the different

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 ABCCCC; 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 (KVNKKKTGQ), EPIYA-B (QVAKKVNNAKI) and EPIYA-C (TIDDLG-GPFPL) 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 ABCCCC (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 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 (FPLKRHDKVDLSKVG) 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

Table 1. *H. pylori cagA* 3' end EPIYA motifs of strains from patients with peptic ulcer diseases

EPIYA motif	DU	GU	DU + GU	Gastritis
ABC	13	2	15	35
ABCC	8	5	13	5
ABCCC	6	2	8	0
ABCCCC	1	0	1	0
Total	28	9	37	40

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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