Original Article

Study of the Cytoxin-Associated Gene A (*CagA* Gene) in *Helicobacter Pylori* Using Gastric Biopsies of Iraqi Patients

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ABSTRACT

Background and Aims: The *Helicobacter pylori CagA* gene is a major virulence factor that plays an important role in gastric pathologies. The size variation of *CagA* gene, which is dependent on the 3′ repeat region, contains one or more Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and *CagA* multimerization (CM) motifs. Four segments flanking the EPIYA motifs, EPIYA −A, −B, −C, or −D, were reported to play a crucial role in the pathogenesis of *H. pylori* infection. The aim was to determine the roles of EPIYA segments and CM motifs in gastroduodenal pathogenesis in an Iraqi population. **Patients and Methods:** Gastric biopsies were collected from 210 patients with gastritis, duodenal ulcer (DU), gastric ulcer (GU), and gastric cancer (GC). The EPIYA motif genotyping was determined by polymerase chain reaction and sequencing. The differences in age, gender, and *CagA* EPIYA motifs of *H. pylori* between GC, DU, GU and gastritis patients were analyzed using a χ^2-test. **Results**: A total of 22 (45.8%) strains had three copies of EPIYA (ABC type), 2 (4.16%) had four copies (ABCC type), 6 (12.7%) had five copies (ABCCC type), 13 (27.08%) had two copies (AB type), 3 (6.25%) had the BC, and 2 (4.17%) had AC motif. The alignment of the deduced protein sequences confirmed that there were no East Asian type EPIYA-D sequences in Iraqi strains. A significant association was found between increase in number of EPIYA-C motifs and GU ($P \leq 0.01$) compared with gastritis. **Conclusions:** The structure of the 3′ region of the *CagA* gene in Iraqi strains was Western type with a variable number of EPIYA-C and CM motifs. A significant association was found between increase in number of EPIYA-C motifs and GU compared with gastritis indicating predictive association with the severity of the disease. The GenBank accession numbers for the partial *CagA* nucleotide sequences determined in this study are JX164093-JX164112.

Key Words: *CagA*, EPIYA, gastric cancer, gastric ulcer, multimerization motifs

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Helicobacter pylori, the causal organism of many gastric disorders is considered to be a risk factor for the development of gastric cancer (GC).[1,2] It is predominantly transmitted within families and infection occurs mostly in early childhood, frequently leading to persistent infection lifelong.[3] *H. pylori* is genetically more diverse than most other bacterial species and the genetic diversity of several virulence factors, such as *CagA* and *VacA*, can be used as a tool for predicting the risk of developing various diseases.[4] *CagA* encodes a 120- to 145-kDa *CagA* protein, and is a marker for the *cag* pathogenicity island, this protein plays an important role in the etiology of *H. pylori*-induced gastric pathologies.[5] The severity of disease outcome could be attributed to possession of the *Cag* pathogenicity island, which encodes a type IV secretion system that facilitates translocation of the *CagA* protein.[6] After the delivery, some *CagA* protein are rapidly tyrosine-phosphorylated on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and interacts with various target molecules, such as phosphorylated, *CagA* binds to a cytoplasmic Src Homology 2 (SH2) domain of Src Homology 2 phosphatase (SHP-2).[2] Because *CagA*–SHP-2 complexes disrupt signal transduction pathways of the cell, the complexes may be involved in the development of atrophic gastritis and the transition from atrophy to intestinal metaplasia.[7] Unphosphorylated *CagA* on these dephosphorylation also contribute to

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the development of *H. pylori* associated gastric diseases, including GC. El-Etr, *et al*,[8] reported that a conserved motif in the C-terminal region of *Cag*A, distinct from the EPIYA motifs designated CRPIA (conserved repeat responsible for phosphorylation-independent activity) or called *Cag*A multimerization (CM) motifs play a vital role in *H. pylori* pathogenesis. The interaction of CM with c-Met subsequently led to the upregulation of β-catenin and nuclear factor κB (NFκB) transcriptional activities, which promoted proliferation and inflammation, respectively.[7]

*Cag*A proteins isolated from different *H. pylori* strains exhibit sequence polymorphisms and duplications, especially in their C-terminal regions containing EPIYA motifs.[9] These motifs are defined as EPIYA-A, EPIYA-B, EPIYA-C, or EPIYA-D according to different flanking amino acids, EPIYA-A and EPIYA-B motifs are common in strains of Western and East Asian origin, EPIYA-C motif, typically present in one to three copies, is characteristic of *Cag*A proteins from strains isolated in European countries, the Americas and Australia.[10] EPIYA-D motif in East Asian has a higher affinity for SHP-2 than does the Western EPIYA-C motif and seems to induce more severe cellular changes, triggers abnormal cellular signals leading to deregulation of cell growth, cell to cell contact and cell migration, elongation of epithelial cells, cytoskeletal alterations known as the "hummingbird phenotype" and increase of epithelial cell turnover, which enhance the risk of damaged cells to acquire precancerous genetic changes.[11,12] Therefore, EPIYA motif diversity may provide a useful tool for prediction of *H. pylori* pathogenic activity, and the accurate determination of the type and number of EPIYA motifs in clinical *H. pylori* isolates can become a useful prognostic tool.[13]

Currently, only one study has focused on EPIYA motifs in Iraq (Kurdistan region) and not performed sequencing analyses of the repeat regions.[14] Therefore, the present study was evaluated for 3′ end variable region of *Cag*A in *H. pylori* related diseases in Iraq using polymerase chain reaction (PCR) based typing and sequencing analyses.

The objectives of this study were to determine type of EPIYA motifs within the *Cag*A 3′ variable region among *H. pylori* isolates using multiple reverse primers by PCR and determine whether there is an association between these motifs and peptic diseases.

PATIENTS AND METHODS

Patient-derived samples

A total of 210 Iraqi patients (79 men and 113 women), mean age, 44.4 ± 1.6 years with dyspeptic symptoms, who underwent endoscopy in Al-Kadhimiya teaching hospital and Al-yarmook hospital at Baghdad, Iraq between June 2010 and August 2011 were included. The endoscopic diagnosis was grouped into three categories: Peptic ulcer [gastric ulcer (GU) and duodenal ulcer (DU)], GC and non-nuclear dyspepsia (NUD). NUD patients were defined as patients who had no endoscopic lesions of ulcers and/or malignancies. After endoscopic examination, the gastric biopsy specimens from the antrum were examined for the presence of *H. pylori* by rapid urease test and PCR. The study protocol was approved by the Ethics and Research Committees of the hospital, and all patients gave informed consent to the study.

Processing of the samples for PCR assay

One antral biopsy specimen from each person was kept in normal saline (0.9% sodium chloride) and preserved immediately at −80°C for molecular analysis.

Each frozen biopsy specimen was thawed, crushed and then genomic DNA was extracted directly from gastric biopsy samples using the QIAamp tissue DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA then quantified through measurement of its OD260 by ND-2000 spectrophotometer (Thermo Scientific Inc., USA). The extracted DNA was eluted in 200 μL of 1XTE buffer (10 mM Tris–HCl, 1 mM EDTA [pH 8.0]) and stored at −20°C until use.

PCR amplification analysis

For confirming the presence of *H. pylori* DNA in biopsy, *ure*C (*glmM*) gene was identified by PCR using primer pairs: *ure*CF and *ure*CR with a 296-bp size product, as described previously.[15] For detecting the presence of the *Cag*A gene, PCR amplification of *Cag*A used previously described primers (*Cag*AF1) and (*Cag*AR1)[16] that amplified the 5′ conserved region of the *Cag*A gene with a 349-bp size product.

Characterization of the C-terminal variable region by EPIYA motif

The forward primer *Cag*A28F and reverse primers *Cag*A-P1C, [*Cag*A-P2GC and *Cag*A-P2TA] (equimolar mixture), *Cag*A-P3E, as described previously[10] and *Cag*A-PD[17] were used to amplify the EPIYA motif encoding sequences A, B, C, and D, respectively. PCR amplification of *Cag*A (The entire 3′ variable region) used previously described primers cag2 and cag4.[18]

All PCR was performed in a volume of 25 μL using GoTaq®Green Master Mix (Promega) containing 10 × PCR buffer, 3 mmol/L $MgCl_2$; 400 μM each deoxyribonucleotide triphosphate (dNTP), 1.5 U Taq DNA polymerase, 10 pmol of each primer, and 200 ng DNA sample. PCR was performed in a thermocycler (MJ MINI, Bio-Rad, France) under the following conditions: initial denaturation for 5 min at 94°C

was followed by 35 cycles of 93°C for 1 min, 55°C for 30 s, and 72°C for 1 min. After a final extension at 72°C for 10 min, the PCR products were examined by electrophoresis on 1.5% agarose gels for 1 h at 60V. *H. pylori* 26695 were used as control strain.

Sequencing of the 3′ variable region of *CagA*

PCR products for the entire 3′ variable region of the *CagA* gene were purified using the QIA Quick Gel Extraction kit (QIAGEN) according to the manufacturer's recommendations. Purified products were sent to Eton Bioscience Inc., USA for sequencing in both directions. 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. The previously published *CagA* gene sequence of strains *H. pylori* 26695 (AE000511) in GenBank was also included in the analysis.

Statistical analysis

The differences in age, gender, and *CagA* EPIYA motifs of *H. pylori* between GC, DU, GU and gastritis patients were analyzed using a χ^2-test. Statistical significance was set at $P \leq 0.01$.

RESULTS

Out of 210 gastric biopsy specimens, 102 (48.57%) were confirmed to have gastric *H. pylori* infection by RUT and *ureC* gene [Figure 1]. Of them, 58 (56.86%) patients were male and 44 (43.13%) were female with a mean age of 43.10 years (range 15–72 years). Out of 102 *H. pylori*-positive samples, 40 (39.22%) were *CagA*-positive gene [Figure 2] including 6/34 (17.6%) from gastritis patients, 10/23 (43.4%) from GU patients, 21/45 (46.7%) from DU patients and all 3 GC patients (100%). A significant association was found between *CagA* status and clinical outcome for patients ($P \leq 0.01$).

The entire 3′ variable region of the *CagA* gene was amplified with Cag 2, and Cag 4 primers determined the molecular weight resulting fragments for *CagA* regions varied between 450 and 850 bp. There was single-band PCR product in 32 cases and double-band product in 8 cases [Figure 3].

These eight strains were thought to have at least two kinds of strains within the same patient. We successfully separated those subclones by eluted and purified by using QIA Quick DNA Gel Extraction kit (QIAGEN, Hilden, Germany). The purification process was successfully conducted on the samples in which only one fragment was found and the purified DNA of these eight cases were classified with regards to EPIYA types [Table 1].

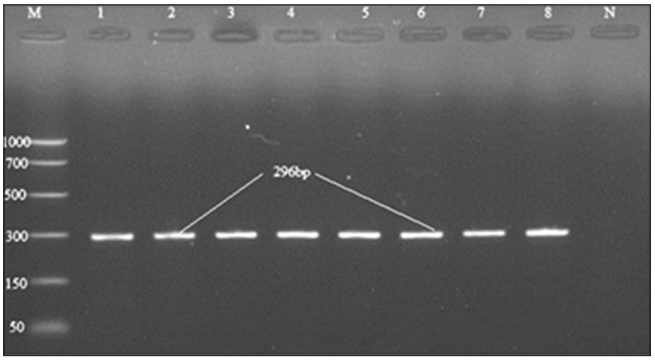

Figure 1: PCR Amplification products of *ureC* (*glmM*) gene 296 bp of *H. pylori*. (M: ladder marker, N: Control negative, Lane 1 to 8 positive results)

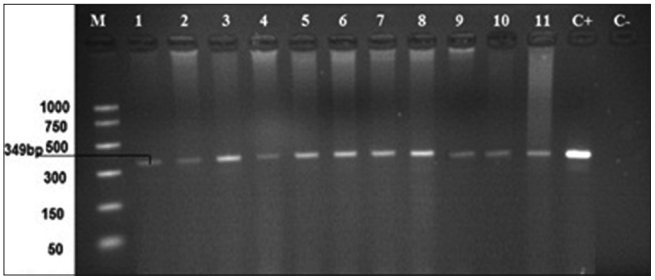

Figure 2: PCR Amplification product of *CagA* gene (5′ conserved region) 349 bp of *H. pylori*. (Lane M: Ladder marker, Lane 1 to11 biopsy samples; C + positive control, C- negative control)

Table 1: *CagA* diversity with regard to the number and type of EPIYA motifs in Single and mixed isolates from the same host

Strain group and No. of EPIYA motifs	Type of EPIYA motif	*N*=40	(%)	Age (mean±S.E.) years
Single strain				
2	AB	8	20	42.62±2.41
3	ABC	15	37.5	43.73±1.8
4	ABCC	2	5	50.33±4.5
5	ABCCC	3	7.5	53.81±1.96
3	BC	2	5	40.61±1.37
2	AC	2	5	45.52±2.7
Mixed strain				
2 and 3	AB and ABC	5	12.5	44.9±4.2
3 and 5	ABC and ABCCC	2	5	53
2 and 5	BC and ABCCC	1	2.5	55

Table 2: Association between the numbers of EPIYAC segments and diseases

EPIYA motif	AB (%)	ABC (%)	ABCC (%)	ABCCC (%)
Gastritis	2 (7.5)	4 (12.5)	0 (0)	0 (0)
GU	6 (60)	4 (40)	2 (20)	3 (30)
DU	4 (19.04)	13 (61.9)	0	0
GC	1 (33.33)	1 (33.33)	0	2 (66.66)
Total	13 (27.08)	22 (45.8)	2 (4.16)	6 (12.7)

Among 48 cagA-positive strains, EPIYA typing PCR showed that 22 (45.8%) strains had three copies of EPIYA (ABC type), 2 (4.16%) had four copies (ABCC type), 6 (12.7%) had five copies (ABCCC type), and 13 (27.08%) had two copies (AB type), while the remaining 3 (6.25%) had the BC, 2 (4.17%) had AC motif [Figure 4]. There were no East Asian type strains with EPIYA-D segments in the Iraqi population [Figure 5]. Statistical analysis was carried out by comparing the number of EPIYA-C motifs of strains isolated from GU, DU and GC patients with those of gastritis patients [Table 2]. We found a significant association between the increase in the number of EPIYA-C motifs (≥2) and GU and GC ($P \leq 0.01$) compared with gastritis. Otherwise, the number of EPIYA-C motifs did not associate with DU, indicating predictive association with the severity of the disease.

Sequencing of the 3′ variable region of *CagA*

For comparing and achievement of more details of the 3′ end variable region of the *CagA* gene in the Iraqi population, Nuclolide sequencing of the *CagA* variable regions was performed for 20 randomly selected strains (IRQ1-IRQ20) including 6 from Gastritis, 5 from GU, 7 from DU, and 2 from GC patients. Sequence analyses confirmed that three types of EPIYA motifs were observed: EPIYA-A for EPIY (A/T) KVNKKK (A/T) GQ; EPIYA-B for EPIY (A/T) QVAKKVNAKI; and EPIYA-C for EPIYATIDDLGGPFPL, it has been found no strains possessed the East Asian type of EPIYA-D (EPIYATIDFDEANQAG). We confirmed that PCR methods correctly classified the EPIYA motif types (i.e., strain IRQ8-17 possessed AC type, strain IRQ20-85 possessed ABCCC type, and other strains sequenced ABC type by PCR; the data are identical to data by sequencing). We also found that all cases in EPIYA-A and EPIYA-C were exact EPIYA sequences; however, 5 of 20 cases in EPIYA-A and EPIYA-B were EPIYT, but not EPIYA. The CM motif located within the EPIYA-C segment had the peptide sequence found in strains from Western countries FPLKRHDKVDDLSKVG. We also found that CM motif located upstream of the EPIYA-C segment varied at positions 16 in strain IRQ7-101, FPLKRHDKVDDLSKVR and varied at positions 5 in strain IRQ17-71, FPLKKHDKVDDLSKVG and CM motif located upstream the last EPIYA-C motif in strain IRQ20-85 FPLKRHDKVDDLSKVE and downstream of same EPIYA FPLKRHDKVDDLSKVD [Figure 6].

DISCUSSION

In this study, we examined *CagA* C-terminal variations in *H. pylori* strains from Iraqi patients. We found that the C-terminal region varied considerably in size, from 450 to 850 bp. Variation in the size of *CagA* is due to the presence of a variable number of repeat sequences including EPIYA motifs and CM motifs located in the 3′ region of the gene. Encoded in their cagA 3' end, Iraqi *H. pylori* strains had one to three EPIYA-C motifs in an ABC, ABCC, and ABCCC *CagA* pattern. In the present study, we found that there were no strains with EPIYA-D segments, in agreement with a study examining Iraqi (Kurdistan region) strains.[14] The absence of ABD type *CagA* might be the reason for lower incidence of GC in Iraq. The incidence of GC in Iraq is unusually rare (age-standardized rate [ASR] for men 5/10^5), which is

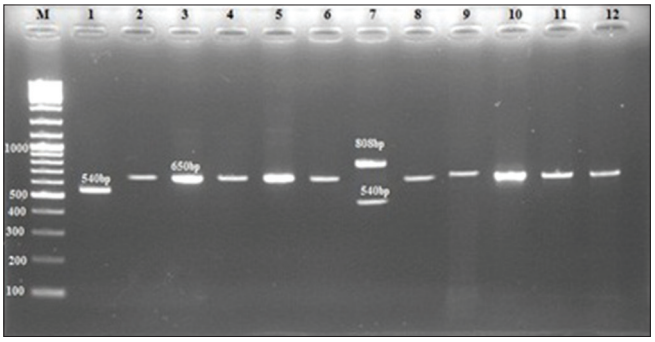

Figure 3: PCR products of the *cagA* 3' end variable region using primers Cag2-Cag4. Lanes: M, 100-bp DNA marker; 1 to 6 and 8 to12, Single *H. pylori* strains, 7 mixed isolates

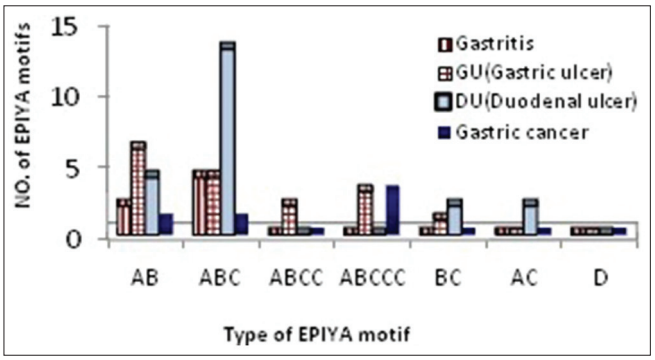

Figure 4: Association between number and type of *CagA* EPIYA motifs and *H. pylori*-associated diseases

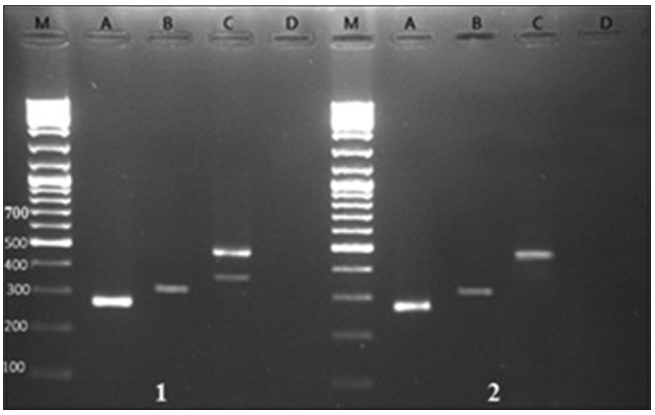

Figure 5: PCR amplification of *CagA* 3' end EPIYA motif from *H. pylori* strains. Using the forward primer *CagA* 28F and the reverse primers *CagA*-P1C (EPIYA -A), *CagA*-P2CG and *CagA*-P2TA (equimolar mixture; EPIYA- B), *CagA*-P3E (EPIYA -C) and *CagA* –PD (EPIYA -D). (1) ABCC motifs, and (2) ABC motifs. M: 100-bp DNA marker

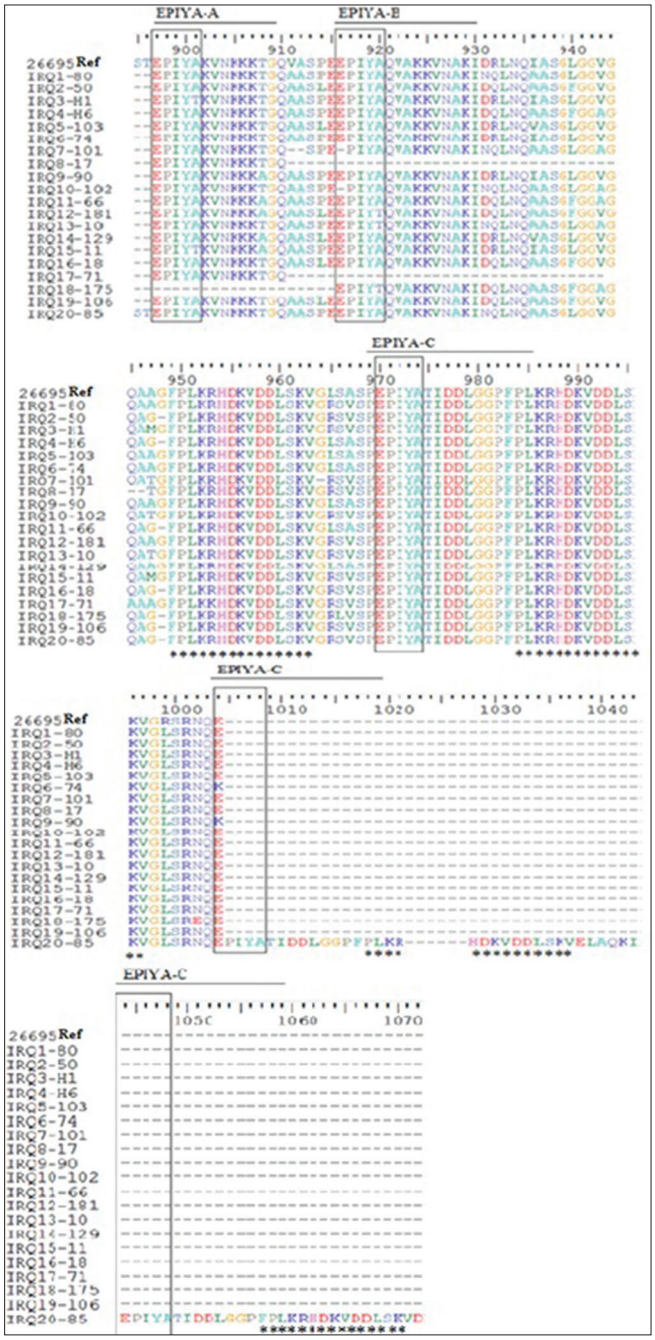

Figure 6: Alignment of partial *CagA* peptide sequences (showing the EPIYA and CM motifs) from 20 Iraqi *H. pylori* strains including the *H. pylori* reference strain 26695 (CM marked with*)

much lower than that in East Asian countries (e.g., Japan 69.2/10^5 and South Korea 70.02/10^5),[19-21] despite the country's geographical proximity to Turkey and Iran, where the incidence of GC differs hugely among these countries, being 8.9-14.1/10^5 and 38-69/10^5, respectively.[22,23]

The prevalence of strains with more than one repeat region (i.e., more than one EPIYA-C; e.g., ABCC) was 51.1% in Colombia and 33.3% in Italy,[24] which was much higher than in the current study (16.75%) and in one Iraqi study no strain was found to have more than three phosphorylation motifs (0%). It might be simply due to small number of severe diseases in the study by Hussein *et al*,[14] (e.g., 20 cases with peptic ulcer disease (PUD) and 29 cases with non-PUD). Low prevalence of strains with multiple EPIYA-C segments might be reasons for low incidence of GC in Iraq. Therefore, EPIYA motif diversity may prove useful in the prediction of *H. pylori* pathogenic activity, and the accurate determination of the type and number of EPIYA motifs in clinical *H. pylori* isolates can become a useful prognostic tool.[24]

Somewhat interestingly, the mean age of patients infected with ABCC type and ABCCC type strains were higher than those with other types. As suggested by Yamaoka *et al*,[25] the level of acid production increases in advanced age and it is possible that strains with higher number of EPIYA C segments may be less resistant to the acid.

Zhang *et al*., recently found that increased numbers of EPIYA motifs in *H. pylori* infection alone is not the only independent factor for the development of GC.[1] *H. pylori* virulence factors, smoking, gender, host genetic, environmental, and dietary factors play a major role in determining whether or not a person infected with *H. pylori* will develop GC.[1] Moreover, another possibility as reported by Acosta, *et al*,[26] using site-specific mutagenesis, suggests that tyrosine phosphorylation at EPIYA-C is sufficient, but not exclusive, to activate translocated *CagA*, suggesting that other motifs besides EPIYA-C are used for phosphorylation of *CagA* proteins as well. For this reason it is not only important to study the tyrosine phosphorylation motif, EPIYA, but also another important amino acid sequence, Multimerization motif (CM motif), which surrounds the EPIYA-C motif and EPIYA-D in Western and East Asian *CagA* species.[1] The most common Western *CagA*-ABC, possesses two identical CM sequences (FPLKRHDKVDDLSKVG, referred to as W-CM sequence). In contrast, East Asian *CagA*-ABD carries a single CM sequence (FPLRRSAAVNDLSKVG, referred to as E-CM sequence) located immediately after the EPIYA-D segment.[8,27]

The type and number of CM motifs may influence the potential of individual *CagA* proteins to multimerize in host cells, and this may affect the ability of *CagA* to disturb host cell function via SHP-2 deregulation.[27] Some studies have reported important proinflammatory and prooncogenic activities of *CagA* that are independent of the EPIYA motifs, but which may be as important for disease incidence.[24,27]

In the present report, we have shown that in 85% of *CagA*-positive strains, the CM motifs had the peptide sequence FPLKRHDKVDDLSKVG, typically found in

H. pylori strains from Western countries and we found different types of CM motifs in 3 (15%) strains, which vary from the sequence supposed for W-CM. The two modified W-CM motifs of *H. pylori* in strain IRQ20-85 are not registered in database, isolated from patient suffering from GC. These findings indicate that there is explicit positive correlation between the number and type of CM motifs and various gastroduodenal diseases associated to *H. pylori* infection.

In conclusion, the structure of the 3′ region of the *CagA* gene in Iraqi strains was Western type with a variable number of EPIYA-C and CM motifs. A significant association was found between increase in number of EPIYA-C motifs and GU, GC compared with gastritis, indicating predictive association with the severity of the disease. Since sequencing analyses provide us much more information than PCR-based typing alone, further studies with a larger number of Iraqi strains using sequencing analyses will be necessary to establish the roles of EPIYA and CM regions in gastroduodenal pathogenesis.

REFERENCES

1. Zhang C, Xu S, Xu D. Risk assessment of gastric cancer caused by *Helicobacter pylori* using *CagA* sequence markers. PLoS ONE 2012;7:e36844-51.
2. Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, *et al.* SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. Science 2002;295:683-6.
3. Konno M, Fujii N, Yokota S, Sato K, Takahashi M, Sato K, *et al*. Five-Year Follow-Up study of mother-to-child transmission of *Helicobacter pylori* infection detected by a random amplified polymorphic DNA fingerprinting method. J Clin Microbiol 2005;43:2246-50.
4. Atherton C, Cao P, Richard M, Tummuru R, Blaser M, Cover T. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori* association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem 1995;270:171-7.
5. Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, *et al*. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc Nat Acad Sci 1993;90:5791-5.
6. Backert S, Selbach M. Role of type IV secretion in *Helicobacter pylori* pathogenesis. Cell Microbiol 2008;10:1573-81.
7. Suzuki M, Mimuro H, Kiga K, Fukumatsu M, Ishijima N, Morikawa H. *Helicobacter pylori CagA* phosphorylation-independent function in epithelial proliferation and inflammation. Cell Host Microbe 2009;5:23-34.
8. El-Etr S, Mueller A, Tompkins L, Falkow S. Merrell S. Phosphorylation-independent effects of *CagA* during interaction between *Helicobacter pylori* and T84 polarized monolayers. J Infect Dis 2004;190:1516-23.
9. Hatakeyama M, Higashi H. *Helicobacter pylori CagA*, a new paradigm for bacterial carcinogenesis. Cancer Sci 2005;96:835-43.
10. Argent RH, Zhang Y, Atherton JC. Simple method for determination of the number of *Helicobacter pylori CagA* variable-region EPIYA tyrosine phosphorylation motifs by PCR. J Clin Microbiol 2005;43:791-5.
11. Hatakeyama M. *Helicobacter pylori CagA* a bacterial intruder conspiring gastric carcinogenesis. Int J Cancer 2006;119:1217-23.
12. Chuang C, Yang H, Sheu S, Hung K, Wu J, Cheng H, *et al*. *Helicobacter pylori* with stronger intensity of *CagA* phosphorylation lead to an increased risk of gastric intestinal metaplasia and cancer. BMC Microbiol 2011;11:121-8.
13. Suerbaum S, Josenhans C. *Helicobacter pylori* evolution and phenotypic diversification in a changing host. Microbiology 2007;5:441-52.
14. Hussein NR, Mohammadi M, Talebkhan Y, Doraghi M, Letley P, Muhammad M, *et al*. Differences in virulence markers between *Helicobacter pylori* strains from Iraq and those from Iran, potential importance of regional differences in *H. pylori*-associated disease. J Clin Microbiol 2008;46:1774-9.
15. Lu J, Perng R. Shyu Y, Chen H, Lou Q, Chong K, *et al*. Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. J Clin Microbiol 1999;37:772-4.
16. Yamaoka Y, Kodama T, Gutierrez O, Kim J, Kashima K, Graham D. Relationship between *Helicobacter pylori* iceA, cagA, and vacA status and clinical outcome, studies in four different countries. J Clin Microbiol 1999;37:2274-9.
17. Jones K, Joo Y, Jang S, Yoo Y, Lee H, Chung I, *et al.* Polymorphism in the *CagA* EPIYA Motif Impacts Development of Gastric Cancer. J Clin Microbiol 2009;47:959-68.
18. Rudi J, Christof K, Maiwald M, Kuck D. Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and *CagA* protein expression, cytotoxin production, and associated diseases. J Clin Microbiol 1998;36:944-8.
19. Argent H, Thomas R, Letley D, Rittig M, Hardie K, Atherton JC. Functional association between the *Helicobacter pylori* virulence factors VacA and *CagA*. J Med Microbiol 2008;57:145-50.
20. Jones K, Whitmire J, Merrell S. A tale of two toxins, *Helicobacter pylori CagA* and VacA modulate host pathways that impact disease. Front Microbiol 2010;1:115.
21. Othman T, Abdulljabar R, Saeed A, Kittani S, Sulaiman M, Mohammed SA, *et al*. Cancer incidence rates in the kurdistan region/iraq from 2007-2009. Asian Pac J Cancer Prev 2011;12:1261-4.
22. Salih B, Bolek B, Arikan S. DNA sequence analysis of *cagA* 3' motifs of *Helicobacter pylori* strains from patients with peptic ulcer diseases. J Med. Microbiol 2010;59:144-8.
23. Shokrzadeh L, Baghaei K, Yamaoka Y, Dabiri H, Jafari F, Sahebekhtiari N, *et al*. Analysis of 3′end variable region of the *CagA* gene in *Helicobacter pylori* isolated from Iranian population. J Gastroenterol Hepatol 2010;25:172-5.
24. Sicinschi L, Corvea P, Peek R, Camargo M, Piazuelo M, Romero-Gallo J. *CagA* C-terminal variations in *Helicobacter pylori* strains from Colombian patients with gastric precancerous lesions. Clin Microbiol Infect 2010;16:369-78.
25. Yamaoka Y, E-Zimaity H, Gutierrez O. Relationship between the *CagA* 3' repeat region of *Helicobacter pylori* gastric histology and susceptibility to low pH. Gastroenterology 1999;117:342-9.
26. Acosta N, Quiroga A, Delgado M, Bravo M, Jaramillo C. *Helicobacter pylori CagA* protein polymorphisms and their lack of association with pathogenesis. World J Gastroenterol 2010;21:3936-43.
27. Ren S, Higashi H, Lu H. Structural basis and functional consequence of *Helicobacter pylori CagA* multimerization in cells. J Biol Chem 2006;281:32344-52.

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