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Analysis of 3'-end variable region of the *cagA* gene in *Helicobacter pylori* isolated from Iranian population

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Abstract

Background and Aims—The 3' region of the *cagA* gene, the most well-known virulence factor of *Helicobacter pylori*, contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs. Four segments flanking the EPIYA motifs, EPIYA-A, -B, -C, or -D, were reported to play important roles in *H. pylori*-related gastroduodenal pathogenesis. The aim was to determine the roles of EPIYA segments in gastroduodenal pathogenesis in an Iranian population.

Methods—A total of 92 *cagA*-positive Iranian strains isolated from dyspepsia patients with non-ulcer dyspepsia ($n = 77$), peptic ulcer ($n = 11$) and gastric cancer ($n = 4$) were studied. The EPIYA motif genotyping was determined by polymerase chain reaction and sequencing.

Results—A total of 86 (93.5%) strains had three copies of EPIYA (ABC type), three (3.3%) had four copies (ABCC type) and three (3.3%) had two copies (AB type). The alignment of the deduced protein sequences confirmed that there were no East Asian type EPIYA-D sequences (EPIYATIDFDEANQAG) in Iranian strains. When the prevalence of strains with multiple EPIYA-C segments in Iran was compared with previously published data, it was much lower than that in Colombia and Italy, but was higher than that of Iraq, and the patterns were parallel to the incidence of gastric cancer in these countries.

Conclusion—The structure of the 3' region of the *cagA* gene in Iranian strains was Western type. Although we could not find differences between EPIYA types and clinical outcomes, low prevalence of strains with multiple EPIYA-C segments might be reasons for low incidence of gastric cancer in Iran.

Keywords

cagA; *Helicobacter pylori*; pathogenesis; repeat region; virulence factors

Introduction

Helicobacter pylori infection is recognized epidemiologically as the major causal agent for chronic gastritis, peptic ulcer disease, mucosa-associated lymphoid tissues lymphoma and is also considered to be a risk factor for the development of gastric cancer.^{1,2} *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 to predicting the risk of developing various diseases.³⁻⁵

The *cagA* gene localizes at one end of a 40-kb DNA fragment known as the *cag* pathogenicity island (PAI),⁶ which encodes a type IV secretion system that delivers the CagA protein into host gastric epithelial cells.⁷⁻¹¹ After the delivery, CagA protein is rapidly tyrosine-phosphorylated on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs and interacts with various target molecules, the best studied of which is the cytoplasmic Src homology 2 (SH2) domain of Src homology 2 phosphatase (SHP-2).¹² Mutations of SHP-2 have been found in various human malignancies and altered SHP-2 signaling culminates in the development of gastric adenocarcinoma in genetically engineered mice,^{13,14} indicating that SHP-2 is involved in the development of gastric cancer. Therefore, the EPIYA motifs in the *cagA* gene are thought to play important roles in CagA-SHP-2-related signaling of host gastric epithelial cells.

There are variations in the 3' region of the *cagA* gene, and its structures differed between *H. pylori* strains from Western and from East Asian countries.^{4,5,15,16} Both Western and East Asian strains had two repeat region types: the same 57-bp first repeat region-specific, and 102-bp and 162-bp second repeat-specific, respectively.⁴ Each first and second repeat regions contain EPIYA motifs. Recently, another classification was made according to the four different sequences surrounding EPIYA motifs: EPIYA-A, -B, -C and -D.^{17,18} EPIYA-A and -B segments are conserved in both Western and East Asian strains and correspond to the first repeat region; whereas EPIYA-C is specific for Western strains and EPIYA-D is specific for East Asian strains. The CagA sequence was reported to be assigned a sequence type consisting of the names of the EPIYA segments in its sequence. Depending on the number of EPIYA segments, they are termed as A_nB_nC_n or A_nB_nD_n, where 'n' is the repeating motif and does not have to be equal for A, B, C, and D types (e.g. ABCC).

The East Asian type CagA is reported to exhibit stronger binding activity for SHP-2 and greater ability to induce the morphological changes in gastric epithelial cells compared with the Western type based on the structural differences between EPIYA-C and -D.¹⁹ In addition, the number of EPIYA motifs/segments, especially that in the second repeat region (i.e. EPIYA-C) is reported to be involved in the CagA-SHP2 binding activity and CagA phosphorylation ability.^{20,21} The increased number of EPIYA motifs/segments is also reported to increase the risk of the development of gastric cancer both in Western and East Asian countries.^{4,5,20,22,23}

Iran is located in the Middle East between Europe and East Asian countries, and has close economic and cultural relationships with both countries. Although the patterns of the prevalence of *vacA* genotypes in Iran is similar to those in European countries,²⁴⁻²⁷ it is unclear whether *cagA* EPIYA sequences in Iranian strains is similar to the Western type or not, and whether the number and/or kind of EPIYA motifs/segments in Iranian strains was associated with gastroduodenal diseases, due to few previous information about the patterns of EPIYA motifs in the Iranian population.^{28,29} Currently, only two studies have focused on EPIYA motifs in Iranian strains; however, none of the studies performed sequencing analyses of the repeat regions and one did not take into account the differences between East Asian and Western strains.²⁹ Therefore, the present study was evaluated for 3'-end variable

region of *cagA* in *H. pylori*-related diseases in Iran using polymerase chain reaction (PCR)-based typing and sequencing analyses.

Methods

Patients

A total of 190 Iranian patients (81 men and 109 women; mean age, 45.4 ± 1.6 years) with dyspeptic symptoms underwent endoscopy in Taleghani Hospital, Tehran, Iran. All endoscopies were performed by one experienced endoscopist. The endoscopic diagnosis was grouped into three categories: peptic ulcer, gastric cancer and non-ulcer 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 culture and PCR. This study was granted Institutional Review Board approval by Shahid Beheshti University M.C. and received ethical approval from the ethics committee. All subjects were provided a copy of the written consent and assured of their anonymity and confidentiality of data obtained.

H. pylori culture and preparation of DNA for PCR amplification

Two antral biopsy specimens from each person were kept in transport medium consisting of thyoglycolate with 1.3 g/L agar (Merck, Homburg, Germany) and 3% yeast extract (Oxoid, Basingstoke, UK). Gastric biopsies were cultured on brain heart infusion (BHI) agar with 10% (v/v) sheep blood and *Campylobacter*-selective supplement (Merck). The cultured plates were incubated at 37°C for 3–5 days in a microaerobic atmosphere (5% O₂, 10% CO₂, 85% N₂) in a CO₂ incubator (Innova-Co 170; New Brunswick Scientific, Edison, NJ, USA). The organisms were identified as *H. pylori* by Gram staining, colony morphology, and positive oxidase, catalase and urease reactions. The bacteria were harvested, and genomic DNA was extracted by using the QIAamp tissue DNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

PCR amplification analysis

For confirming the presence of *H. pylori* DNA in biopsy and culture, the *glmM* gene was identified by PCR using primer pairs: forward 5'-GGATAAGCTTTTAGGGGTGTTAGGGG-3' and reverse 5'-GCTTACTTTCTAACACTAACGCGC-3' with a 296-bp size product.³⁰ For detecting the presence of the *cagA* gene, we also used PCR using primer pairs: forward (*cagA*F1) 5'-AACAGGACAAGTAGCTAGCC-3' and reverse (*cagA*R1) 5'-TATTAATCGTGTGTGGCTG-3' with a 349-bp size product.³¹

To determine of number and type of EPIYA motifs, we initially used the PCR to identify the individual EPIYA motifs by using a single forward primer (28F) and multiple reverse primers (*cagA*-P1C to amplify the EPIYA(Q/K)VNKKK(T/A)G repeat, *cagA*-P2CG and *cagA*-P2TA for the EPIY(A/T)QVAK repeat, and *cagA*-P3E to amplify the EPIYATID repeat.³² The entire 3' variable region was amplified with *cag2* as a forward primer (5'-GGAACCCTAGTCGGTAATG-3') and *cag4* as a reverse (5'-ATCTTTGAGCTTGTCTATCG-3'). All PCR in this study was performed in a volume of 25 µL containing 10 × PCR buffer, 500 nM of each primer, 2 mmol/L MgCl₂; 200 µM each deoxyribonucleotide triphosphate (dNTP), 1.5 U Taq DNA polymerase, and 200 ng DNA sample. PCR was performed in a thermocycler (AG 22331; Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation for 5 min at 94°C was followed by 30 cycles of 93°C for 1 min, 58°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 1.2% agarose contained gels according to standard procedures. *H. pylori* 26695 were used as control strain.

DNA sequence analysis

Polymerase chain reaction products for the entire 3'-variable region of the *cagA* gene were purified using the QIA Quick Gel Extraction kit (QIAGEN). The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was employed for sequencing. The ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) was utilized according to the manufacturer's instruction for electrophoresis and data collection. Nucleotide sequences were aligned and analyzed by use of BioEdit ver. 7.0.9 and Lasergene ver. 6.0 software (DNASTAR, Madison, WI, USA). The previously published *cagA* gene sequence of strains *H. pylori* 26695 in GenBank was also included in the analysis.

Statistical analysis

Data were analyzed by using SPSS ver. 6.1.3 software (SPSS, Chicago, IL, USA). Pearson's χ^2 -test and Fisher's exact tests were used to assess relationships between categorical variables. The Mann-Whitney *U*-test was used to compare quantitative variables. Significance was defined as $P < 0.05$. All figures including percentage in table and article were rounded down if they were $P < 0.05$ and were presented as whole numbers if they were $P > 0.05$.

Results

Of 190 patients, 141 (74.2%) were proven to be infected with *H. pylori*. In 141 *H. pylori* obtained, 92 (73.6%) were positive for the *cagA* gene including 77 strains isolated from 121 NUD patients (55.4%), 11 from 16 peptic ulcer patients (68.8%) and all four gastric cancer patients. There were no significant differences in *cagA* positive rate, age and sex among different diseases (Table 1).

Among 92 *cagA*-positive strains, EPIYA typing PCR showed that 86 (93.5%) strains had three copies of EPIYA (ABC type), three (3.3%) had four copies (ABCC type) and three (3.3%) had two copies (AB type) (Table 1). There were no East Asian type strains with EPIYA-D segments in the Iranian population. Somewhat interestingly, the mean age of patients infected with ABCC type strains were higher than those with other types.

The entire 3'-variable region of the *cagA* gene was amplified in 96.7% ($n = 89$) of *cagA*-positive isolates (Fig. 1). Among 89 detected cases we observed a single-band PCR product in 86 cases and double-band product in three cases. These three strains were thought to have at least two kinds of strains within the same patient. These three cases with double bands were classified as ABCC (two cases) or AB (one case) in the EPIYA typing described above. When we compared the size of the double-band PCR products with that of single band with known type, we found that the cases with double-band product could be classified as AB/ABCC (Fig. 1).

For comparing and achievement of more details of the 3'-end variable region of the *cagA* gene in the Iranian population, we performed nucleotide sequencing of the *cagA* variable regions from 14 randomly selected strains including five from NUD, five from ulcer and four from gastric cancer patients. Sequence analyses confirmed that three types of EPIYA motifs were observed: EPIYA-A for EPIYAKVNKKK(A/T/V/S)GQ; EPIYA-B for EPIY(A/T)(Q/K)VAKKVNAKI; and EPIYA-C for EPIYATID-DLG (Fig. 2). We found no strains possessing the East Asian type of EPIYA-D (EPIYATIDFDEANQAG).^{17,18} We confirmed that PCR methods correctly classified the EPIYA motif types (i.e. strain H.PI10 possessed AB type, strain H.PI11 possessed ABCC 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, four of 14 cases in

EPIYA-B were EPIYT, but not EPIYA. Nucleotide sequence data reported are available in GenBank accession number FJ849779-FJ849792.

Discussion

CagA is a highly immunogenic protein with a molecular weight of between 120 000 and 140 000.³³⁻³⁴ Variation in the size of CagA is due to the presence of a variable number of repeat sequences including EPIYA motifs located in the 3' region of the gene.⁴⁻⁵⁻¹⁵⁻³³ The two Csk binding sites are designated as segments EPIYA-A and -B, and the SHP-2 binding sites in Western and East Asian CagA are designated as segments EPIYA-C and -D, respectively.¹⁷⁻²¹⁻³⁵⁻³⁶ The EPIYA-D segment has perfect matches with the high affinity binding sequence for SH2 domains of SHP-2 (pY-[V/T/A/I/S]-X-[L/I/V]-X-[F/W]).³⁵ On the other hand, the sequences of EPIYA-C differ from SHP-2 binding sequence by a single amino acid located in the pY+5th position. Therefore, the East Asian type CagA was reported to have stronger activity to bind to SHP-2 than the Western type CagA.³⁵ In the present study, we found that there were no strains with EPIYA-D segments, in agreement with a recent study examining Iranian strains.²⁸ Our data is not surprising because the patterns of the prevalence of *vacA* s and m genotypes in Iran is also similar to those in European countries.²⁴⁻²⁷ The incidence of gastric cancer in Iran (age-standardized rate [ASR] for men 13.95/100 000) is much lower than that in East Asian countries (e.g. Japan 69.2/100 000 and South Korea 70.02/100 000) (www-dep.iarc.fr/dataava/infodata.htm). The absence of ABD type CagA might be the reason for lower incidence of gastric cancer in Iran.

In Western countries, there are increasing evidences that the multiple number of EPIYA segments, especially EPIYA-C segments, are involved in the development of gastroduodenal diseases.⁵⁻²⁰⁻²³ However, we could not confirm the data that number of EPIYA-C segments play important roles in gastroduodenal diseases in an Iranian population, in agreement with a recent study in Iran.²⁸ It is important that the incidence of gastric cancer is also variable even among Western countries and the incidence was relatively high in Colombia (ASR for men 33.22/100 000) and Italy (19.9/100 000). We previously reported that the prevalence of strains with more than one second repeat region (i.e. more than one EPIYA-C; e.g. ABCC) was 51.1% in Colombia and 33.3% in Italy,⁵ which was much higher than in the current study (three cases, 3.3%) and one Iranian study (12%).²⁸ In our previous study, the prevalence of strains with multiple second repeat regions isolated from chronic gastritis patients was 12.5% in the USA, and the incidence of gastric cancer was 7.61/100 000 (ASR for men). A recent study also showed that the prevalence of strains with multiple EPIYA-C segments was 0% in Iraq,²⁸ and the incidence of gastric cancer was 4.58/100 000 (ASR for men). Overall, although we could not find any differences between EPIYA types and clinical outcomes, low prevalence of strains with multiple EPIYA-C segments might be reasons for low, but not extremely low incidence of gastric cancer in Iran. We hypothesize that the prevalence of multiple EPIYA-C segments might be good markers for predicting the grovel risk of gastric cancer in each area studied. Somewhat interestingly, the mean age of patients infected with ABCC type strains were higher than those with ABC types. We previously hypothesize that *H. pylori* strains with multiple EPIYA segments are normally present at very low levels in high-acid conditions and they would be 'invisible' by culture or PCR methods until acid levels decrease (e.g. with increasing age), allowing their prevalence to increase sufficiently to become detectable.⁵ Therefore, our current data support our hypothesis.

It is also true that both our current study and a recent study in Iran²⁸ could not confirm the relationship between EPIYA types and clinical outcomes. It might be simply due to small number of severe diseases in both studies (e.g. 17 cases with peptic ulcer disease [PUD] and 42 cases with non-PUD in a study by Hussein *et al.*²⁸). Another possibility is that this is due

to the lack of intact *cag* PAI in many Iranian strains. We recently reported that at least 62.3% of Iranian isolates possessed partially deleted *cag* PAI,³⁷ indicating that CagA might have no function in these strains without type IV secretion system. The prevalence of partially deleted *cag* PAI in Iranian strains is much higher than that in any other regions.³⁸⁻³⁹ Further studies will be necessary to confirm the function of *cagA* repeat regions in relation to type IV secretion system (e.g. whether non-translocated CagA alone without *cag* PAI play some roles in gastroduodenal pathogenesis or not).

Finally, we performed sequencing of the *cagA* repeat region in Iranian strains for the first time. We confirmed that our PCR-based methods well matched the sequence data. In a previous study, the absence of amplicon of the *cagA* variable regions for sequencing could be an indicator of the *cagA* negative strains;⁴⁰ whereas we could not amplify the region in three cases with *cagA*-positive strains. Because the *cagA* variable regions are not conservative regions, we should use multiple primer pairs to detect the regions for typing and sequencing. One advantage for sequencing is that we could detect even one amino acid mutation, which is impossible using PCR-based methods. As mentioned above, the important differences between EPIYA-C and EPIYA-D is only one amino acid exchanges (i.e. EPIYA-C is EPIYATIDD and EPIYA-D is EPIYATIDF); the difference could not be detected by previously reported PCR-based typing systems including that used in the current study. Fortunately, however, we could confirm that there were no cases with EPIYA-D (or EPIYATIDF). Sequence analyses also showed that there were many cases with EPIYT, but not EPIYA in EPIYA-B segments. In contrast, all cases in EPIYA-A and EPIYA-C were exactly EPIYA sequences. As for the SHP-2 binding ability, there seem to be no differences between EPIYA and EPIYT in EPIYA-C segments.³⁵ However, it is unknown whether the function of EPIYA differs to that of EPIYT in EPIYA-B segments. Anyway, sequencing analyses provide us much more information than PCR-based typing alone; therefore, further studies with a larger number of Iranian strains using sequencing analyses will be necessary to establish the roles of EPIYA regions in gastroduodenal pathogenesis.

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Figure 1.

Polymerase chain reaction products of the *cagA* 3'-end variable region. Lane 1, ladder mix; lane 2, negative control; lane 3, EPIYA-AB; lane 4, EPIYA-ABCC; lanes 5 and 6, EPIYA-ABC; lane 7, *Helicobacter pylori* 26695 (EPIYA-ABC); lane 8, EPIYA-AB/ABCC (ABCC in EPIYA typing method); lane 9, EPIYA-ABC; lane 10, EPIYA-AB/ABCC (AB in EPIYA typing method); lane 11, EPIYA-ABCC.

Figure 2.
Alignment of 3'-end variable region sequences of CagA with respect to the number and types of EPIYA motifs. We show data of 14 samples randomly selected.

Table 1

CagA diversity with regard to the EPIYA motifs genotype in *cagA*-positive clinical isolates

EPIYA motif genotype	n	Age	PUD	NUD	GC
AB	3	41.7 ± 2.7	0	3	0
ABC	86	45.3 ± 1.7	10	73	3
ABCC	3	53.3 ± 9.9	1	1	1

GC, gastric cancer; NUD, non-ulcer dyspepsia; PUD, peptic ulcer diseases.