Diagnostic Microbiology and Infectious Disease 83 (2015) 89–92

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Bacteriology

Detection of *Helicobacter pylori* CagA EPIYA in gastric biopsy specimens and its relation to gastric diseases

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ARTICLE INFO

Article history:
Received 17 February 2015
Received in revised form 30 April 2015
Accepted 11 May 2015
Available online 10 June 2015

Keywords:
Helicobacter pylori
cagA protein
Amino acid repeat sequence
Gastritis
Peptic ulcer

ABSTRACT

CagA of *Helicobacter pylori* undergoes tyrosine phosphorylation in a region containing differing numbers of repeat sequences (EPIYAs), which can result in a modulation of the inflammatory response. This study investigated whether the presence of CagA EPIYA variations in strains of *H. pylori* that are positive for this region contributes to differing degrees of disease severity in the gastric mucosa. In this study, 157 *H. pylori*–positive patients were included, and of those, 40.8% (64/157) were infected with *cagA*-positive strains, which were assayed for the presence of CagA EPIYA-ABC, EPIYA-ABCC, and EPIYA-ABCCC. Peptic ulcers were significantly more prevalent in patients infected with strains containing CagA EPIYA-ABCC/ABCCC than in those with CagA EPIYA ABC strains ($P = 0.044$). This suggests that the number of repetitions of EPIYA-C influences the development of gastroduodenal lesions, highlighting the importance and usefulness of evaluating the *cagA* gene sequence when making therapeutic intervention decisions in patients infected with *H. pylori*.

1. Introduction

For approximately 30 years following the identification of the bacterium *Helicobacter pylori*, a revolution occurred in gastroenterology due to experimental evidence highlighting the relationship between the detection of the bacteria in the gastric mucosa and the development of gastroduodenal diseases (Saxena et al., 2011; Warren and Marshall, 1983). The primary disorder attributed to infection with *H. pylori* is gastritis, a condition that can be observed in almost all *H. pylori*–positive patients. Peptic ulceration, gastric cancer, and lymphoma are all clinical complications resulting from the chronic inflammation caused by this bacterium. The broad clinical spectrum associated with *H. pylori* infection is determined by a complex interaction between bacterial factors, the host and the external environment. The presence of biomarkers identified with bacterial pathogenicity can be useful in distinguishing the various types of lesions in the host (Kusters et al., 2006; Serrano et al., 2007).

The first identified gene associated with pathogenicity in *H. pylori* was *cagA*, which is located at one end of the *cag* pathogenicity island. This island is a region of approximately 40 kb comprising approximately 31 genes that encode components of a type IV secretion system and effector molecules that are injected from bacteria into the host cell (Censini et al., 1996; Covacci et al., 1993). Once injected into gastric epithelial cells, the CagA product is phosphorylated on a tyrosine residue in a 5-amino-acid repeat region containing Glu-Pro-Ile-Tyr-Ala (EPIYA). This phosphorylation event is mediated by a tyrosine kinase (Src) that interacts with the Src2 homology domain. This process results in cytoskeletal reorganization and cell elongation—a phenotype that leads to the dispersal of cells and morphological change associated with the hummingbird phenotype (Higashi et al., 2002).

Examining the structure of the *cagA* gene reveals a 5′ region and a highly conserved 3′ region containing a variable number of repeated sequences that are associated with *H. pylori* pathogenicity (Yamaoka et al., 1998, 1999). *cagA*-positive strains of *H. pylori* found in the Western world typically contain EPIYA-A, EPIYA-B, and EPIYA-C in the C-terminal EPIYA repeat region. In contrast, East Asian *cagA*-positive strains contain EPIYA-A and EPIYA-B but not the EPIYA-C sequence; instead, they contain a unique EPIYA-D sequence (Backert et al., 2010).

In Western countries, studies have shown that the number of EPIYA-C segments found in strains of *H. pylori* influences the degree of pathogenicity and the potential for oncogenicity (Basso et al., 2008; González et al., 2011). Thus, determining the CagA type found in each *H. pylori* infection may confer additional benefit to previously identify populations that have a higher risk of developing peptic ulcer and gastric cancer (Beltrán-Anaya et al., 2014; Huang et al., 2003). Therefore, the aim of

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http://dx.doi.org/10.1016/j.diagmicrobio.2015.05.017
0732-8893/

this study is to investigate whether the presence of CagA EPIYA variations in *H. pylori* is related to differing degrees of gastric mucosal disease in patients from south Brazil.

2. Materials and methods

2.1. Patients and samples

The present study analyzed gastric biopsy specimens obtained from 157 *H. pylori*–positive patients with dyspepsia in Southern Brazil from May 2011 until May 2012. The presence of *H. pylori* infection was assessed by histology, urease testing, and polymerase chain reaction (PCR). Patients were considered *H. pylori* positive when displaying at least 2 positive results. This study was approved by the Ethics Committee in Research of Healthcare (FURG case number: 23116.001044/2011-16). Informed consent was obtained from all patients.

2.2. Clinical sample

Ten biopsies from the gastric antrum and the gastric body were obtained from each patient. Of these, 4 were used for histological analysis. The other 6 biopsies were subjected to urease testing and PCR (Coelho and Zaterka, 2005; Kisa et al., 2002). The samples were maintained in brain–heart infusion broth containing 20% glycerol and were stored at −70 °C until DNA extraction.

2.3. Endoscopic and histologic diagnosis

Endoscopic and histological classifications of the gastric mucosa were established according to the Sydney System (Price, 1991; Tytgat, 1991). The gastric biopsies sent for histological analysis were fixed in 10% formalin and stained with hematoxylin–eosin and Giemsa for the detection of *H. pylori*.

2.4. In-house urease test

For the urease test, 2 biopsies from each patient were incubated immediately after collection in 1.0 mL of urea broth (Isofar, Rio de Janeiro, Brazil), prepared according to manufacturer's instructions, and then stored at 4 °C until use.

2.5. DNA extraction

The biopsies to be assessed by PCR were placed in 1.0 mL of brain–heart infusion broth (Acumedia®, Lansing, MI) supplemented with 20% glycerol and were stored at −70 °C until DNA extraction, which was accomplished using DNAzol® Reagent (Invitrogen®, Grand Island, NY) with 10 μg/μL of Proteinase K (Promega, Madison, WI).

2.6. Detection of the ureA and glmM genes

The presence of the *ureA* and *glmM* genes were analyzed by PCR using the primers described previously, and the PCR was performed as described by Rota et al. (2001) and Espinoza et al. (2011), respectively.

2.7. Detection of β-globin gene

The integrity of DNA present in the samples was analyzed by PCR amplification of a 110-bp fragment from the β-globin human gene. The primers were used and the PCR was performed as described by Saiki et al. (1985).

2.8. Detection of CagA EPIYA

The *cagA* gene in gastric biopsy specimens from *H. pylori*–positive patients was investigated by amplification of a variable region in the 3′ portion of the coding region. The primers used were described previously (Yamaoka et al., 1998). The *cagA* PCR was performed by Batista et al. (2011). The reactions yielded products of 500–850 bp as follows: EPIYA AB, 500 bp; EPIYA-ABC, 640 bp; EPIYA-ABCC, 740 bp; and EPIYA-ABCCC, 850 bp (Queiroz et al., 2012).

2.9. DNA sequencing analyses of the 3′ variable region of cagA

A significant subset of samples was randomly selected. All PCR amplicons were then purified and quantified spectrophotometrically. PCR products were purified with the Polyethyleneglycol (Sigma Aldrich, St. Louis, MO) according to the manufacturer's recommendations. Purified products were sequenced by Macrogen in Seoul, South Korea, in automatic sequencer. Sequence comparisons were subsequently carried out using BLAST Search in the National Center of Biotechnology Information. Nucleotide sequences were transformed into amino acid sequences using the Blastx program (available from: http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared to sequences deposited into the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/).

2.10. Statistical analysis

The association between CagA EPIYA and endoscopic diagnoses was evaluated using the chi-square test. $P \leq 0.05$ was considered statistically significant. All analyses were performed using Stata version 9.2.

3. Results

Of the 157 *H. pylori*–positive patients included in this study, 79 were women and 78 were men, with a mean age of 52.6 years old (range, 20–86 years). Based on the endoscopic diagnoses, 15.9% (25/157) of patients had normal gastric mucosa, 31.2% (49/157) presented with enanthematous gastritis, 23.6% (37/157) had erosive gastritis, 26.8% (42/157) had peptic ulceration, and 2.5% (4/157) presented with gastric cancer.

3.1. Detection of the CagA EPIYA pattern

Of all patients analyzed, 40.8% (64/157) were *cagA* positive. *cagA*-positive strains contained different patterns in the 3′ variable region of the gene: 71.9% (46/64) EPIYA-ABC and 28.1% (18/64) EPIYA-ABCC/EPIYA-ABCCC. No *cagA*-positive strains showed the pattern EPIYA-AB. The relationship between CagA EPIYA-ABC and EPIYA-ABCC/EPIYA-ABCCC and the endoscopic diagnosis are presented in Table 1. The results were confirmed by sequencing of the 3′ variable region of *cagA* in 18/64 (28%) randomly selected PCR products.

Table 1
Association of *H. pylori cagA* and EPIYA with endoscopic diagnoses.

Endoscopic diagnosis	*cagA*		*P* value
	Negative	Positive	
Normal mucosa (n = 25)	64.0% (16/25)	36.0% (9/25)	
Enanthematous gastritis (n = 49)	53.1% (26/49)	46.9% (23/49)	0.368
Erosive gastritis (n = 37)	59.5% (22/37)	40.5% (15/37)	0.718
Peptic ulcer (n = 42)	59.5% (25/42)	40.5% (17/42)	0.716
Gastric cancer (n = 4)	100.0% (4/4)	0% (0/4)	0.203
Total	93	64	
	Genotype *cagA* EPIYA		
	ABC	ABCC/ABCCC	
Normal mucosa (n = 9)	88.9% (8/9)	11.1% (1/9)	
Enanthematous gastritis (n = 23)	82.6% (19/23)	17.4% (4/23)	0.562
Erosive gastritis (n = 15)	73.3% (11/15)	26.7% (4/15)	0.359
Peptic ulcer (n = 17)	48.0% (8/17)	52.0% (9/17)	0.044
Total	46	18	

3.2. Association between the numbers of EPIYA C segments and gastric diseases

Peptic ulcers were significantly more prevalent in patients infected with strains containing CagA EPIYA-ABCC/ABCCC than in those with CagA EPIYA ABC strains ($P = 0.044$) (Table 1).

4. Discussion

The *cagA* gene is considered to be an important factor in the bacterial pathogenicity associated with gastric adenocarcinoma and peptic ulcers. This fact highlights the importance of its detection in patients infected with *H. pylori* (Abdullah et al., 2012; Hatakeyama, 2004; Serrano et al., 2007). Of all *H. pylori*–positive patients analyzed in this study, 40.8% (64/157) were *cagA* positive, which is similar to the prevalence previously detected in Brazil (Leite et al., 2005; Ramis et al., 2010).

CagA displays a variable number of repeat sequences (EPIYA) in its 3′ region (Yamaoka et al., 1998, 1999). Once injected into a gastric epithelial cell, this region undergoes tyrosine phosphorylation and interacts with the phosphatase SHP-2, which has binding affinity for tyrosine-phosphorylated EPIYA C. This interaction leads to cellular changes that are thought in gastric carcinoma. The greater the number of EPIYA-C segments, the higher the affinity for SHP-2 (Ando et al., 2006). In this study, all *cagA*-positive patients harbored EPIYA A, B, and C, which confirms that the geographically Western strains have EPIYA C in the C-terminal repeat region. Additionally, the number of EPIYA-C segments seemed to influence the pathogenicity of the strain, which agrees with previous studies (Basso et al., 2008; González et al., 2011).

In the present study, 52.0% of the patients *H. pylori* positive with peptic ulcer disease presented CagA EPIYA-ABCC/EPIYA-ABCCC segments ($P = 0.044$). A significant association similar between peptic ulcer (54.5%) and CagA variants carrying 2 or more EPIYA C also was detected in Mexico, recently (Beltrán-Anaya et al., 2014). These data are consistent with other studies, and the presence of a larger number of CagA EPIYA-C segments is considered a risk factor for gastroduodenal ulceration (Panayotopoulou et al., 2010; Salih et al., 2010).

Batista et al. (2011), in a study conducted in Brazil, showed an association between the presence of CagA EPIYA and the development of gastric cancer. In this work, none of the patients with gastric cancer had the *cagA* gene. This absence may be due to the small number (2.5%) of gastric cancer cases, as larger numbers would be needed for a meaningful investigation of the presence of this gene. Still, it is worth noting that, in atrophic gastritis and intestinal metaplasia, disorders that precede dysplasia, and the development of gastric cancer, there is a marked decrease in the colonization of the gastric mucosa by *H. pylori*, which may lead to false-negative results in infected patients who develop gastric cancer (Forman, 1998).

Although that most strains of *H. pylori* possess the 3 standards of EPIYA A, B, and C, we cannot exclude the hypothesis that some strains may possess alternative sequences that would not be recognized by the primers used. However, the primer pair used for amplification of the 3′ region of *cagA* in this study has been used in other studies showing high confidence in determining the presence of *cagA* region (Batista et al., 2011; Queiroz et al., 2012).

On the other hand, it is important to note that not only *cagA* contributes to heterogeneity and correlation of gastric disease manifestation but also other factors like *vacA*, *babA*, and *iceA* (Atherton et al., 1995; Gerhard et al., 1999; Peek et al., 1998). Several studies have showed a strong indication that the association of the virulence factors may increase the risk of the developing gastric malignant tumors Therefore, the studies of these genes in each region are important (González et al., 2011; Ramis et al., 2010; Vaziri et al., 2013).

In conclusion, it was observed that infection by *cagA*-positive *H. pylori* strains harboring multiple EPIYA-C repeats is associated with the presence of peptic ulcer disease, while infection with strains containing only 1 EPIYA-C copy was correlated with the development of mild lesions in the gastric mucosa. The analysis of the diversity of clinical manifestations associated with differences in the number of repeats within the *cagA* gene may be an important diagnostic and prognostic tool for patients infected by *H. pylori*.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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