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Prevalence of EPIYA Motifs in *Helicobacter pylori* Strains Isolated from Patients with Dyspeptic Disorders in Northern Iran

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Abstract

Background: Cytotoxin-associated gene A (*CagA*)-positive strains of *Helicobacter pylori* are associated with gastroduodenal diseases. Evidences have suggested that the type of *H. pylori CagA* EPIYA motifs may be associated with recurrent dyspepsia (i.e. gastritis, peptic ulcer, or gastric cancer). We investigated the prevalence of different EPIYA motifs (A, B, C, or D) in *H. pylori* strains isolated from patients with recurrent dyspepsia who underwent upper gastrointestinal (GI) endoscopy.

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Materials and Method: *H. pylori* strains were isolated from biopsy specimens of 220 patients with recurrent dyspepsia. The presence of *glm*M gene, as a housekeeping gene, *Cag*A gene, and pattern of *Cag*A EPIYA motifs were determined using polymerase chain reaction (PCR) method. The association between the type of motifs and disease state was determined by the Chi-square test, Fisher's exact test, and logistic regression.

Results: *Cag*A-positive *H. pylori* strains were identified in 125 (57%) of patients, including 36 (28.6%) gastritis, 31 (24.6%) duodenal ulcer, and 58 (46.4%) gastric cancer. The frequency of pattern of *Cag*A EPIYA motifs were detected as 39 (31.2%) AB motifs, 54 (43.2%) ABC motifs, 32 (25.6%) ABCC motifs,and no D motifs. The risk of gastric cancer occurrence was estimated to be 2.57 times higher in patients infected by strains with ABCC motif when compared with gastritis and duodenal ulcer patients (p=0.03). Moreover, patients with C-containing motifs were 2.27 times more likely to be afflicted with gastric cancer than with duodenal ulcer. AB motif was more associated with gastritis and duodenal ulcer than ABC and ABCC motifs.

Conclusion: The results suggested that *Cag*A-EPIYA ABCC might be associated with gastric cancer, while EPIYA-AB might be associated with duodenal ulcer.

Keywords: *Helicobacter pylori*; Cytotoxin-associated gene A; Dyspeptic disorders

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Introduction

Helicobacter pylori colonize in the stomach of more than 50% of the world's population and play an essential role in the development of various gastro-duodenal diseases (such as gastritis, peptic ulcer, and gastric cancer) (1-3). Several potential virulence factors such as *CagA*, VacA, OipA, and HomB have been suggested to play a role in the pathogenesis of organism (4-5). *CagA* gene is one of the members of

a cluster of genes referred to as Pathogenicity Island (PAI) (6). Epidemiological studies have revealed that *CagA*-positive strains of *H. pylori* are much more likely to be associated with the development of gastric cancer (7-8). *CagA*-positive strains produce a type IV secretion apparatus that is used to directly inject *CagA* into host gastric epithelial cells (9). *CagA* is phosphorylated by host cell kinases, forms a complex with SHP-2 (Src homology region 2 containing phosphate 2), and alters multiple host signaling pathways. Dysregulation in this pathway is responsible for increased cell proliferation, cell to cell contact, cell migration, elongation of epithelial cell, and increase in epithelial cell turnover which leads to morphologic changes, inflammation, and atrophy of the infected cells (6, 8, 10).

CagA is a polymorphic gene containing a highly conserved 5′ end and a variable 3′ end (4). It contains an EPIYA motif, a repeat region classified in four types, A, B, C, and D. Different strains of *H. pylori* have been detected according to the presence of this repeat region (11). EPIYA-C and -D serve as the primary *CagA* phosphorylation sites and are required for binding to SHP-2 (12). Two different combinations of these motifs have been found in two distinct geographical locations, Western *CagA* and Eastern *CagA* (13). EPIYA-A and B are conserved in both two combinations (Western and Eastern), while EPIYA-C (up to 3 repeats) is specific for Western combination, and EPIYA-D is specific for Eastern strains (14). Among Western isolates, molecular epidemiological studies have indicated a correlation between an increased number of EPIYA-C motifs and the type of the consequent disease (9, 12, 14). Indeed, Western strains isolated from gastric cancer samples, frequently show multiple EPIYA-C motifs (15, 16). This increase may be due to elevated morphological transformation because of increased *CagA* phosphorylation and SHP-2 binding. Eastern *CagA* containing the EPIYA-D motif demonstrates higher affinity for SHP-2 than Western *CagA*. This leads to greater morphological changes in infected cells (17) as well as higher levels of inflammation and atrophy (18). These findings along with the fact that Eastern strains predominate in countries with the highest rates of gastric cancer, suggest that Eastern *CagA* may have the potential to induce more severe forms of gastric disease (19, 20). In a study in Tehran, the capital of Iran, *CagA* motifs isolated from gastric biopsy specimens were demonstrated to be of Western type (21). Since gastric cancer is highly prevalent in northern parts of Iran similar to that of eastern Asia, we proposed that *CagA* motif in *H. pylori* isolated from patients in northern Iran might be different from other parts of Iran. Therefore, we investigated the prevalence of different EPIYA motifs in *H. pylori* isolates from the gastric biopsy specimens from the north of Iran.

Material and Methods

Patients

Patients with recurrent dyspeptic symptoms who received upper gastrointestinal (GI) endoscopy at Tooba Outpatients Clinic of Mazandaran University of Medical Sciences (MAZUMS), Sari Iran, were enrolled in the study. Three gastric biopsy specimens were obtained from each patient and used for the rapid urease test, *H. pylori* culture, and pathological examinations. All procedures were performed between January 2010 and July 2011. Patients with a history of previous gastric surgery, those who had received *H. pylori* eradication treatment, or those who had recently used H2 receptor blockers, proton pump inhibitors, and non-steroidal anti-inflammatory drugs (NSAIDs) were excluded. Based on the endoscopic and histopathological assessments, samples were divided into three groups of gastritis, duodenal ulcer, and gastric cancer. The Medical Research Ethic Committee of Mazandaran University of Medical Sciences approved the study protocol and written informed consents were taken from all patients.

Culture of H. pylori

Biopsy specimens were placed in sterile thioglycolate broth media (Merck, Germany) and quickly transferred to the microbiology lab at 4°C for further processing. After dissection and homogenization of the specimens, 100 µl of each homogenized specimen was plated on Columbia agar (Mast, UK) containing 7% fetal calf serum (Gibco, USA), 10% defibrinated sheep blood (Jihad Daneshgahi, Iran), and *H. pylori* selective antibiotic tablets (Mast, UK). Plates were incubated at 37 °C under microaerophilic conditions for 7 days and subcultured from single colonies. *H. pylori* colonies were confirmed via morphology, Gram staining, and positive oxidase, catalase, and urease tests. The presence of *H. pylori* colonies was further confirmed by PCR amplified of glmM housekeeping gene, as previously reported (22).

Amplification of the CagA gene

Genomic DNA was extracted from a single bacterial colony using a commercial kit (Bioneer, South Korea) based on manufacturer's instructions. The presence of *CagA* in isolated bacteria was detected by a 298-bp amplicon size as the gene was amplified with primers D008 5'-ATAATCGTAAATTAGACA ACTTGAGCGA-3' and R008 5'-TT AGAATAATCA ACAAACATCACGCCAT-3' using the conditions described previously (5).

Amplification of EPIYA motifs

For the PCR amplification of the 3' variable region of the *CagA* gene containing different EPIYA motifs, we used the two primers designed by Effrosini et al. for EPIYA-A, -B, and -C (23) and the two primers designed by Kathleen et al. for EPIYA-D (3). PCR was performed in a volume of 25 µl containing 10X PCR buffer (KCl 50 mM, Tris-HCl 10 mM, and 1.5% (vol/vol) triton x-100), 1.5 mM MgCl2, 100 µM of each deoxynuclotides triphosphate (dNTP), 1.5 µ of taq DNA polymerase (Fermentase), 10 pmol of each primer, and 100 ng DNA sample. The reaction took place in an Eppendorf thermo cycler (Hamburg, Germany) under the following conditions: a first denaturation step at 94 °C for 5 minute following by 30 cycle of 93 °C for 1 minute, 50 °C for 45 seconds, and 72 °C for 1 minute, as well as a final extension at 72 °C for 5 minutes. PCR products were examined by 1.5% agarose gel electrophoresis.

Statistical analysis

The association between the type of motifs and disease state was determined by the Chi-square test, Fisher's exact test, and logistic regression. The logistic model goodness of fit was evaluated with the Hosmer-Lemeshow test. Also association among the mean age with type of motifs, sex and disease states were done by t-test and Univariate Analysis of Variance (ANOVA). Gamma correlation was employed to detect association between two ordinal variables.

Results

According to microbial culture, *H. pylori* was isolated from 220 biopsy specimens from patients with recurrent dyspeptic symptoms and confirmed by biochemical tests and the presence of *glmM* housekeeping gene. PCR amplification of *CagA* gene revealed 125 (56.81%) *CagA*-positive and 95 *CagA* negative isolates. Of 125 *CagA*-positive H. pylori isolates, 36 (28.6%) were from patients gastritis, 31 (24.8%) from duodenal ulcers, and 58 (46.4%) from gastric cancer (Table 1). The number of detected strains with AB motif was 39 (31.2%), with ABC motifs were 54 (43.2%), and with ABCC motifs were 32 (25.5%). No EPIYA-D motif was detected. Logistic regression analysis after adjusting the age and sex, demonstrated that cancer risk was estimated to be 2.57 times higher in patients infected by strains with ABCC motif when compared with gastritis and duodenal ulcer patients (p= 0.03). For duodenal ulcer and gastritis, there was a reverse correlation between the ABCC motif and both diseases (Table 3) which confirmed that AB motif was more associated with gastritis and duodenal ulcer than C-containing motifs. Further analyses with Gamma correlation revealed that fewer number of C motif (AB or ABC) was correlated with gastritis and duodenal ulcer, while higher number of C motif was correlated with gastric cancer (p= 0.068). Table 2 presents the frequency of *CagA* genotypes (EPIYA motifs) in patients with different dyspeptic disorder. EPIYA motifs which contained C segment (i.e., ABC and ABCC motifs) were showed that patients with these motifs were 2.27 times more likely to be afflicted with gastric cancer than duodenal ulcer (p= 0.068). Furthermore, these patients were 1.57 times more likely to be afflicted with gastric cancer than gastritis (p= 0.231), and 0.692 times more likely to be afflicted with gastritis than duodenal ulcer (p= 0.318).

Table1. Age, sex and disease state of 125 patients that *CagA* positive H.pylori isolated.

Sex	Gastritis		Duodenal ulcer		Gastric cancer		Total	
	N (%)	Age (mean±SD)	N (%)	Age (mean±SD)	N (%)	Age (mean±SD)	N (%)	Age (mean±SD)
Male	16 (44.4%)	49.50±14	12 (38.7%)	38.75 ±16.91	30 (51.7%)	46.13 ±15.7	58 (46.4%)	45.53 ±15.7
Female	20 (55.6%)	43.10 ±17.31	19 (61.3%)	36.26 ±14.55	28 (48.3%)	39.11 ±14.50	67 (53.6%)	39.4 ±15.16
All patients	36 (28.8%)	45.94 ±16.03	31 (24.8%)	36.26 ±14.55	58 (46.4%)	42.74 ±15.42	125(100%)	42.06 ±15.7

N= frequency

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Table 2. Frequency of pattern of cag-A EPIYA motifs in *H pylori*

	Gastritis (N=36)	Duodenal ulcer (N=31)	Gastric cancer (N=58)	Total (N=125)
EPIYA-AB	12(33.3%)	13(41.4%)	14(24.1%)	39(31.2%)
EPIYA-ABC	16(44.4%)	15(48.4%)	23(39.7%)	54(43.2%)
EPIYA-ABCC	8(22.2%)	3(9.7%)	21(36.2%)	32(25.6%)

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Table 3. Association between *H pylori*- associated diseases and all variables.

	EPIYA motif	N (%)	OR(95%CI)	P value
Gastritis				
	EPIYA-ABb	12 (33.3%)	1	0.18
	EPIYA-ABC	16 (44.4%)	0.58(0.27-1.28)	0.10
	EPIYA-ABCC	8 (22.2%)	0.46(0.18-1.17)	0.24
Gastric Cancer				
	EPIYA-ABb	14 (24.1%)	1	0.08
	EPIYA-ABC	23 (39.7%)	1.01(.48-2.16)	0.98
	EPIYA-ABCC	21(36.2%)	2.5(1.06-6.28)	0.03
Duodenal ulcer				
	EPIYA-ABb	13 (41.9%)	1	0.09
	EPIYA-ABC	15 (48.4%)	0.96 (0.41-2.22)	0.91
	EPIYA-ABCC	3 (9.7%)	0.24 (.07-0.89)	0.03

a. Adjusted for age and gender

Discussion

This study showed the strain of *H. pylori* isolated from patients in north of Iran is similar to *H. pylori* types isolated from other regions of Iran: the Western type. No strain with EPIYA-D segment (Eastern strain type) was isolated (21, 24). Thus, it seems that although the north of Iran is an endemic region for gastric cancer like East Asian countries (Japan, China, and Korea), the H.pylori strains in the north of Iran are different from those countries. Several in vivo and in vitro studies have reported that East-Asian types of *CagA* are associated with higher prevalence of gastric cancer compared to that of Western types; however, there is not enough evidence to explain the role of geographical region in clinical outcome of infection with *CagA* positive-*H. pylori* strains (4). Our data revealed that *CagA*-positive *H. pylori* with ABCC motifs are associated with the risk of gastric cancer as 2.57 times of the risk of *H. pylori* with AB motifs. Furthermore, duodenal ulcer was associated with *CagA*-positive *H. pylori* containing AB motifs. This finding is in agreement with reports of Baso et al. (9) and Batista et al. (14). In contrast, Shokrzadeh et al. found no difference between EPIYA-C types and clinical outcomes in Tehran, Iran (21). This difference may be due to small number of samples in this study (i.e. 11 cases with peptic ulcer disease and 4 cases with gastric cancer in the study by Shokrzadeh et al.). Another possibility is the geographical difference, since north of Iran is a high risk area for developing gastric

cancer (25). Gamma correlation test revealed that with increasing the numbers of EPIYA-C motifs, the risk of gastric cancer increased as well. There are many reports about the role of EPIYA motifs of *CagA* in pathogenicity of *H. pylori*. Following injection of *CagA* protein into the gastric and duodenal epithelial cells, the EPIYA motifs are tyrosine phosphorylated by Src and Ab1 family kinases, which result in the impairment of a variety of intracellular signaling pathways (10). The number of present EPIYA motifs is related to the level of *CagA* phosphorylation that occurs in epithelial cells infected by *H. pylori* (4). Patients infected with a *H. pylori* strain with stronger *CagA* phosphorylation have more sever chronic inflammation with an increased risk of gastric cancer. Also it is important to note the due to this fact that tyrosine-phosphorylated EPIYA-C makes as many sites to interact *CagA* protein with SHP-2 domains of host cells. This binding activates SHP-2 and induction of stronger and longer periods of SHP-2 activity which leads to an increased signal transduction, more morphological damage, and finally the development of gastric cancer. Recent studies indicated the prevalence of strains with more than one repeat region (such as ABCC) as 51.1% in Colombia, 33.3% in Italy (20), and 3.3% (21) and 12% (24) in two different parts of Iran, while the present study showed the prevalence of these strains in the north of Iran as 25.6%. As mentioned before, these differences may be due to the high prevalence of gastric cancer and geographical and environmental difference in northern parts of Iran. Overall, the current study confirmed patients with one or more repeats of the C region showed a 2.27-fold increased risk of developing gastric cancer than duodenal ulcer.

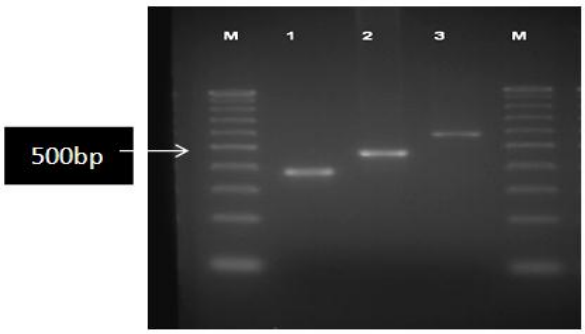

Figure 1. PCR products of the *CagA* 3′ end variable region. M= DNA marker [100bp- 1000bp]; lane 1: AB motifs (379 bp), lane 2: ABC motifs (470 bps), and lane 3: ABCC motifs (570 bps).

Also it is important to note the due to this fact that tyrosine-phosphorylated EPIYA-C makes as many sites to interact *CagA* with SHP-2 domains of host cells. This binding activates SHP-2 and induction of stronger and longer periods of SHP-2 activity, leads to an increased signal transduction, greater morphological damage, and involved in development of gastric cancer. Therefore recent studies indicated prevalence of strains with more than one repeat region (such as ABCC) was 51.1% in Colombia and 33.3% in Italy population (20), also this current study 25.6% but in other recent studies in Iran revealed 3.3% (21) and 12% (24). This discordant as mentioned before, may be due to the high prevalence of gastric cancer and geographical and environmental difference in northern parts of Iran. Overall this current study confirmed patients with one or more repeats of the C region showed a 2.27-fold increased risk of developing gastric cancer than duodenal ulcer. With regard to this result we proposed to exact understanding the role of EPIYA motif in gastroduodenal diseases in population of Iran, Other studies needed to investigate the role of EPIYA motif in gastroduodenal diseases in other provinces with high incidence of gastric cancer such as Ardabil, golestan and gilan to compare with this recent studies.

Conclusion

There was a significant statistical association between the presence of the type of *CagA* EPIYA motifs of *H. pylori* and the development of dyspeptic disorders. EPIYA-CC was significantly associated with gastric cancer, while EPIYA-AB was associated with duodenal ulcer. It should be noted that the presence of an EPIYA-CC does not necessarily lead to a gastric cancer since there was a high percentage of patients with gastritis and duodenal ulcer infected with *H. pylori* containing EPIYA-C or EPIYA-CC motifs. Further studies needed to investigate the role of EPIYA motifs in patients with dyspeptic disorders in other provinces of Iran which are endemic regions of gastric cancer such as Ardabil, Golestan and Gilan provinces.

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