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Full Length Research Paper

Evaluation of *cagA* tyrosine phosphorylation DNA motifs in *Helicobacter pylori* isolates from gastric disorder patients in West of Iran

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***Cag* Pathogenicity Island (*cag-PAI*)-containing *Helicobacter pylori* strains induces signal transduction pathways resulting in tyrosine phosphorylation of proteins adjacent to the site of bacterial adhesion on host gastric epithelial cells. Polymerase chain reaction (PCR) assays were designed to test the presence of three nucleotide sequences corresponding to tyrosine phosphorylation motifs (TPMs) A, B, and C in *H. pylori* isolates from gastroduodenal patients in Iran. This cross-sectional descriptive study was performed on 164 antral gastric biopsy specimens which were obtained from patients undergoing upper gastrointestinal tract endoscopy. Initially, *H. pylori* strains were identified by rapid urease test (RUT) and *ureC* gene and then analyzed for the presence of *cagA* gene and TPM subtypes (A, B and C) by PCR with established specific primers. Statistical analyses were performed to find their association with gastroduodenal diseases. The PCR assays demonstrated that motif A was common (13.24% of the isolates), whereas motifs B and C were found in 6.62 and 5.30% of the isolates respectively. TPM-AB, TPM-BC and TPM-AC were found in 21.19, 10.60 and 3.97% of the isolates respectively. 16.56% of the isolates had all kinds of TPMs. Strains lacking a TPM were found in 22.52% of the isolates. The presence of TPM-C alone or in combination with other motifs has a direct diagnostic value but the higher proportion of these strains in gastric ulcer patients merits further investigation.**

Key words: *Helicobacter pylori*, *cagA*, tyrosine phosphorylation motifs, gastroduodenal diseases.

INTRODUCTION

Helicobacter pylori is a major cause of chronic gastritis and involved in the pathogenesis of several diseases such as gastric and duodenal ulcer, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Kargar, 2010, 2011a, b). *H. pylori* is genetically more diverse than the other bacterial species and the genetic diversity of its several virulence factors such as *cagA* and *vacA* can be used as a tool to predict the risk of developing various disease which may be a cause (Shokrzadeh, 2010; Amador, 2009). A commonly used molecular marker of *H. pylori* virulence is the *cagA-PAI* which encodes a type IV secretion system. The presence of the *cagA* gene and/or *cagA* special type (for example, East Asian type) is associated with more severe clinical outcomes than the absence of the gene (Dabiri, 2009; Khayat, 2007). The CagA cytotoxin is directly injected into epithelial cells by a type IV secretion system encoded by genes located in the *cag-PAI*. In the host cell, CagA embeds in the plasma membrane and undergoes phosphorylation on specific tyrosine residues within repeating penta amino acid Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present at the C-terminus of the protein. The C-terminal part which contains the EPIYA motifs has been shown to be highly variable as opposed to the highly conserved N-terminal part (Monstein et al., 2010).

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Table 1. Primers for identification of *cagA* gene and *cagA* TPM types in *H. pylori* (10, 19).

Gene	Primer designation	Nucleotide sequence(5-3′)	Amplicon (bp)
cagA	*cagA-U*	GGA ATA CCA AAA ACG CAA AAA CCA	300
	cagA-L	CCC CAC AAT ACA CCA GCA AAA CT	
TPM A	Mot AF	GAT AGG GAT AAC AGG CAA G	254
	Mot AR	CCT GCA AAA GAT TGT TTG GC	
TPM B	Mot BF	AAC CCT AGT CGG TAA TGG	216
	Mot BR	GCA ACT TGA GCG TAA ATG G	
TPM C	Mot CF	CAA GCG GTA TCA GAA GCT A	179
	Mot CR	TTA ATG CGT GTG TGG CTG TT	
ureC	ureC-F	AAGCTTTTAGGGGTGTTAGGGGTTT	249
	ureC-R	AAGCTTATTTCTAACGC	

Three putative nucleotide TPMs in the CagA protein have been predicted and designated as TPM-A, TPM-B, and TPM-C, although additional motifs such as the EPIYA sequences have also been identified in CagA. The precise link among *cagA*, the production of the CagA protein and strain virulence is controversial. About 68% of strains isolated from dyspeptics in England have *cagA* in the genome, also similar or higher frequencies have been reported in many countries (Gutierrez, 2005; Bindayana, 2006; Reyes, 2007). Nevertheless, *cagA* status alone is insufficient to reliably predict either virulence or association with gastric ulcer (Owen, 2003; Hussein, 2008).

This study was aimed to determine the *cagA* gene diversity with regard to tyrosine phosphorylation motifs in patients who have gastric disorders in Chaharmahal and Bakhtiari province as well as to evaluate any general associations with gastroduodenal diseases.

MATERIALS AND METHODS

Totally, 200 consecutive patients with dyspeptic symptoms attending the endoscopy suite of gastroenterology section of Hospital of Shahrekord University of Medical Sciences were enrolled. Endoscopic findings of pathologist were recorded at the time of the consultation by the pathologist help. All patients provided written informed consent prior to endoscopy. By using disinfected endoscope, two biopsy specimens were taken from the antrum of each patient. For detection of *H. Pylori,* one piece of each specimen was examined by rapid urease test (RUT). Rapid urease test was performed with a gastro urease kit (Bahar-afshan, Iran), and the second piece from RUT-positive patients was placed in 0.1 ml of sterile phosphate buffer saline solution and sent to biotechnology research centre of Islamic Azad University, Shahrekord Branch (Kargar et al., 2011d). DNA was isolated from biopsy specimens using Genomic DNA purification kit (DNP™, CinnaGen, Iran) according to manufacture's instructions. Primers used for PCR assays of *cag*A tyrosine phosphorylation motifs in *cagA* positive samples and detection of *ureC* gene are listed in Table 1 (Owen, 2003; Kargar, 2011c). DNA extracted from *H. pylori* (D0008, Genekam, Germany) was used as positive control of *cag*A gene and *cagA* tyrosine phosphorylation motifs and sterile distilled water was used as negative control. PCR was done in 20 µl (for *H. pylori*) and 25 µl (for *cag*A tyrosine phosphorylation motifs) of total reaction volume containing 1.5 mM $MgCl_2$ (2.0 mM for *cagA*), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM dNTPs each (fermentas), 0.4 µM primers, 1 U of Taq DNA polymerase (fermentas) and 2 µl (40 to 260 ng/µl) of DNA (Wang et al., 2002). PCR was performed in a DNA thermal cycler (Eppendrof Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany), with 40 cycles for ET2 primer and 35 cycles for *cag*A tyrosine phosphorylation motif's primers. Each cycle consisted of denaturation at 95℃/45 s; annealing at 59℃/30 s fo r ET2, 58℃/45 s for *cagA*, 58℃/45 s for *cagA* MotC and 54℃/45 s for cagA MotA and B; and extension at 72℃/45 s. There was another longer extension of 6 min at 72℃.

PCR products were visualized by electrophoresis on 1% agarose gel, stained with ethidium bromide and examined under ultraviolet illumination. Data were analyzed by using SPSS software (Version 17.*SPSS* Inc, USA) and *p* value was calculated using Chi-square and Fisher's exact tests to find any significant relationship. *P* value less than 0.05 was considered statistically significant.

RESULTS

Out of 200 gastric biopsy specimens, 164 (82%) were confirmed to have gastric *H. pylori* infection by RUT and *ureC* gene. 79 (48.17%) of the patients were male and 85 (51.83%) were female with a mean age of 47 years old (range 15 to 88 years old). 16 patients (9.76%) had gastric ulcers, 22 (13.41%) had duodenal ulcers, 3 (1.83%) had gastric cancer, 3 (1.83%) had duodenit, 34 (20.73%) had gastric nodularity, 52 patients (31.71%) had gastric erosion and 159 (96.95%) had gastritis. Out of 164 *H. pylori- positive* samples, 151 (92.07%) were *cagA*-positive. There was a significant relationship between *cagA* gene and gastric nodularity disease (*p* =

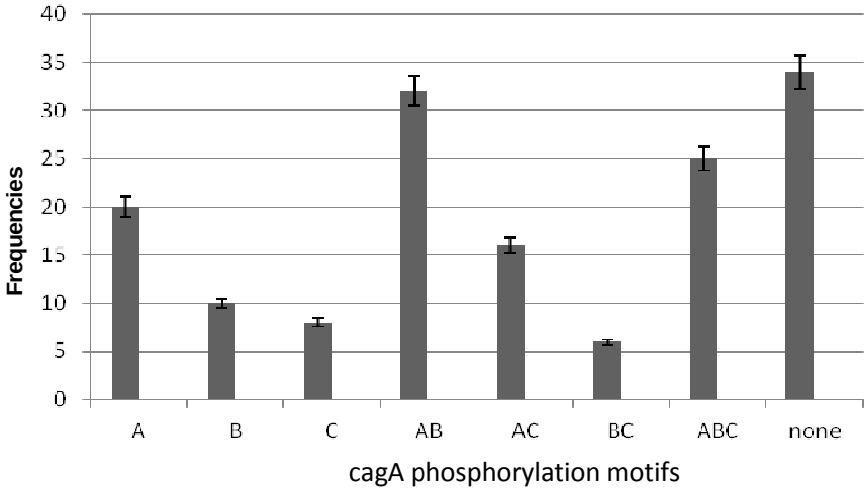

Figure 1. Distribution of *cagA* TPMs in *cagA*-positive, *H. pylori* isolates (n = 151).

Table 2. Distribution of *cagA* TPMs in *H. pylori* isolates in relation to patient diseases.

Motif disease (n)	CagA-positive								CagA-negative
	A	B	C	AB	AC	BC	ABC	No motif	
G.U (16)	0	0	0	2 (12.50)	5 (31.25)	2 (12.5)	3 (18.75)	2 (12.50)	2 (12.50)
D.U (22)	4 (18.18)	2 (9.09)	0	2 (9.09)	2 (9.09)	1 (4.55)	3 (13.64)	4 (18.18)	4 (18.18)
G.C (3)	0	0	0	0	1 (33.34)	0	0	2 (66.66)	0
G.N (34)	1 (2.94)	1 (2.94)	1 (2.94)	7 (20.59)	4 (11.77)	0	5 (14.71)	9 (26.47)	6 (17.64)
Due (3)	0	0	0	0	0	0	1 (33.34)	2 (66.66)	0
Gas (159)	20 (12.58)	10 (6.29)	8 (5.03)	31 (19.50)	15 (9.43)	5 (3.14)	25 (15.72)	32 (20.13)	13 (8.18)
G.E (52)	2 (3.85)	1 (1.92)	4 (7.70)	15 (28.85)	7 (13.46)	1 (1.92)	8 (15.38)	8 (15.38)	6 (11.54)

G.U, gastric ulcer; D.U, duodenal ulcer; G.C, gastric cancer; G.N, gastric nodularity; Due, duodenit; Gas, gastritis and G.E, gastric erosion.

0.01). Among 151 *cagA*-positive strains, TPM typing PCR showed that the *cagA* genes with no kind of TPM (22.52%) and the combination of A and B TPMs (21.19%) were predominant strains and the frequency of TPM-C (5.29%) and the combination of C and B (3.97%) were the lowest of all (Figure 1). TPM-A and TPM-B were widely distributed among the isolates regardless of disease symptoms while TPM-C was rare as a single motif. There is a significant association between some diseases and *cagA* tyrosine phosphorylation motifs; for instance gastric ulcer and the combination of A and C motifs (p = 0.01), gastric nodularity and TPM-A (p = 0.03), gastric erosion and TPM-A (p = 0.02) also combination of A and B motifs (p = 0.03). Also, despite the low frequency of TPM-C, it is related with more severe gastric disorders (Table 2).

DISCUSSION

cagA is a highly polymorphic gene with diversity in its 3´ end region which is important for the biological activity of the protein on gastric epithelial cells. This region has the tyrosine phosphorylation site and the SHP-2 phosphatase binding site. The interaction of SHP-2 with these proteins activates a series of signaling pathways, causing an increase in proliferation and abnormal cell motility. It is therefore important to extend studies on polymorphism in this 3´ of *cagA* among populations and to correlate this diversity with different kinds of gastric disorders (Reyes et al., 2007). *H. pylori* strains having c*agA* gene have been considered more virulent than *cagA* negative strains (Jafari et al., 2008). The prevalence of *cagA*-positive *H. pylori* varies from one geographic region to another, for example, 97% in Korea, 94% in Malaysia, 90% in China, 78% in Turkey and 53% in Kuwait (Nahaei et al., 2008). In the present study, *cagA* gene was found in 92.07% of the *H. pylori*-positive isolates. Because of high positivity in all *H. pylori* isolates, this finding does not substantiate the role of *cagA* gene alone as an important marker for increased virulence. This finding is also in agreement with studies from East Asia. In this study, PCR assays

were applied to assess the presence of nucleotide sequence motifs corresponding to deduced CagA amino acid TPMs (A, B and C) in *H. pylori*; because these motifs are associated with tyrosine phosphorylation of CagA protein, they were investigated to determine if they might provide markers for more severe disease outcome as a result of increased *H. pylori*–host epithelial cell interactions. Polymorphisms within *cagA* may affect the biological function of the protein and might explain the lack of a consistent correlation between *cagA* and disease severity (Owen et al., 2003).

According to the results obtained, the frequency of TPM-A was higher than the other TPMs. This motif was highly associated with non ulcer dyspepsia (NUD) diseases like gastric nodularities and gastric erosions. Our data are in agreement with those of Owen et al. (2003) which suggested that TPM-A was a common feature (71%) of *H. pylori* strains isolated from patients in England irrespective of associations with chronic clinical disease. Other data on TPM frequencies are indicated that *H. pylori* have been isolated from 15 gastritis and 18 gastric cancer patients in Costa Rica (Occhialini et al., 2001). Nucleotide sequencing and deduced amino acid sequences showed relative frequencies that were significantly higher than ours with 100% for TPM-A and 58% for TPM-B, whereas TPM-C was not detected in any strain. The reasons of such differences from our results are unclear, but they could be due to the geographic conditions. Our data suggest that a higher proportion of gastric ulcers-associated strains had TPM-C which demonstrates that this motif plays a role in the pathogenesis of *H. pylori* unlike TPM-A and TPM-B which are more frequently associated with slight diseases. These observations are in agreement with other reports showing that TPM-C and not TPM-A are required for phosphorylation and the lack of TPM-C results in lower phosphorylation activity in strains containing either TPM-A alone or both TPM-A and TPM-B (Odenbreit, 2001; Puls, 2002). These findings are contradicted by other reports showing that TPM-C does not play any role in the pathogenesis of the bacterium (Owen et al., 2003). Our results indicated that there is a relationship between the combination of TPM-A and C with severe gastric disorders. Also, there is an association between the combination of TPM-A and B and slight diseases so we can conclude that TPM-B may not play an important role in the pathogenesis of the bacterium. Although the frequency of the cagA genes with no phosphorylation motifs are high; but they do not play an important role in the formation of severe gastric disorders.

Conclusion

In conclusion, the present study showed that while TPM-A and B were common in the west of Iran, there was a direct association between the presence of TPM-C and the occurrence of peptic ulcer diseases. The presence of these TPMs and the EPIYA motif repeats and their associations with disease of the need of investigation in a wider selection of trains in relation to the degree of inflammation in the host gastric tissue as well as the level of tyrosine phosphorylation of CagA protein in infected epithelial cells.

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