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Evaluation of Helicobacter pylori *vacA* and *cagA* Genotypes and Correlation With Clinical Outcome in Patients With Dyspepsia in Hamadan Province, Iran

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Background: *Helicobacter pylori* is known to be a causative agent of chronic active gastritis, peptic ulcer and gastric cancer in human. Diverse genotypes of *H. pylori* strains have different virulence potency and geographic distribution.

Objectives: The purpose of this study was to investigate the association between the cytotoxin-associated gene (*cagA*), and the various vacuolating cytotoxin (*vacA*) genotypes of *H. pylori* strains and clinical outcomes in patients referred to Shahid-Beheshti Hospital in Hamadan, Iran.

Patients and Methods: In this cross-sectional study, biopsy samples were collected consecutively from 153 patients with gastric cancer (GC), peptic ulcer dyspepsia (PUD) and non-ulcer dyspepsia (NUD) in the gastroenterology department of Shahid-Beheshti Hospital in Hamadan province, the west of Iran. *H. pylori* infection was confirmed in 83 patients (3 with GC, 27 with PUD, and 53 with NUD) by histology, rapid urease test (RUT) and culture. Genomic DNA was extracted from the bacterial isolate and was further confirmed with 16S rRNA gene sequencing as *H. pylori*, and characterized based on *cagA* and *vacA* genotyping using the polymerase chain reaction (PCR) method.

Results: In this study, *vacA* genotypes s1/m2, s1/m1, s2/m2 and s2/m1 were determined in 43.4%, 19.3%, 13.2% and 6% of the isolated *H. pylori*, respectively. The *vacA*s1 genotype was detected in 52 (62.6%) isolates, of which the *vacA*s1a genotype was detected in 45.2, 40.7, and 66.6% of the isolates from patients with NUD, PUD, and GC, respectively. The *cagA*-positive genotype was determined in 73 (87.9%) isolates and 10 (12.1%) were negative. The frequency rates of *cagA* gene were 84.9, 92.6 and 100% in isolates of patients with NUD, PUD, and GC, respectively. The *cagA*-positive genotype is strongly associated with s1a/m2 and s1a/m1 *vacA* genotypes.

Conclusions: The most predominant *VacA* genotypes in our areas were s1/m2 and s1/m1, which regard as the genotypes with more virulence intensity. The *H. pylori vacA*s1a, *cagA* genotypes have a significant relationship with the presence of PUD and GC in Iranian patients with dyspepsia.

*Keywords:*Helicobacter Pylori; *VacA*; *cagA*; Genotyping

1. Background

Helicobacter pylori can colonize the stomach of about fifty percent of the world's population. The bacteria are primarily observed on the mucosa of the gastric antrum (1). Colonization with *H. pylori* itself is not a disease, but H. pylori is an etiologic agent of the acute or chronic gastritis, and a predisposing condition to the peptic ulcer disease, gastric carcinoma, and B-cell mucosa associated lymphoid tissue (MALT) lymphoma (2-4). The clinical outcome of *H. pylori* infection depends on a diversity of factors, which are related to the host (e.g. blood group antigens and polymorphisms in interleukin 1 gene cluster), the bacterium (e.g. strain-specific genes, differential gene expression, phase variation and allelic variation), and the environment (e.g. age and smoking) (5). The genes of *H. pylori* (e.g. *CagA* and *VacA*) have been identified as being virulence-associated. These virulence and pathogenicity factors are well-implicated in studies for their role in the progress of diseases leading to gastric ulcer and gastric cancer (6-8). The vacuolating cytotoxin (*vacA*) gene is present in virtually all *H. pylori* strains and contains at least two variable regions, the signal (s) region, which encodes the signal peptide, and the middle (m) region. There are two major s-region types, s1 and s2, and three known s1-region subtypes (s1a, s1b, s1c). The m region has been divided into two allelic type, m1 and m2. Among type m1 strains, subtypes m1a and m1b have been identified (9). Although all strains of *H. pylori* contain the *vacA* gene, they vary in terms of their ability to produce cytotoxin. The amount of cytotoxin produced is highest with the s1/m1 allele, followed by the s1/m2 allele, while no

cytotoxin activity is found when s2/m2 is present. Specific *vacA* genotypes are associated with the level of toxin production and clinical diseases like PUD and GCin different parts of the world (10-12). The cytotoxin-associated gene (*cagA*) is a marker of 40 kb genomic pathogenicity island. A significant association between the presence of ulcers or gastric carcinoma and *vacA* type s1 and *cagA* genes has been reported (13). *H. pylori* eradication leads to the healing of gastritis and peptic ulcer disease, and probably also has a beneficial effect on regression of atrophic gastritis and prevention of distal gastric cancer (14, 15).

2. Objectives

There are no data regarding the prevalence and molecular characteristics of *H. pylori* in patients with gastrointestinal discomfort in Hamadan province. This study aimed to evaluate the *H. pylori vacA*, cagA genotypes and their correlation with clinical outcomes in patients with dyspepsia in Hamadan, the west of Iran.

3. Materials and Methods

3.1. Clinical Specimen

The study population consisted of 153 adult patients (89 men and 64 women) aged 16-88 (mean age, 53) years. This descriptive cross- sectional study was performed on 153 adult patients [4 gastric cancer (GC), 42 peptic ulcer dyspepsia (PUD) and 107 non-ulcer dyspepsia (NUD)] underwent an upper gastroduodenal endoscopy for the diagnosis and treatment purposes in the gastroenterology department of Shahid-Beheshti Hospital (it's a governmental and referral hospital) in Hamadan province from May 2010 to February 2011. The diagnosis of gastroduodenal diseases was based on the clinical, endoscopic and histopathological examinations. The clinical variables were extracted from medical records of the patients by a specialist physician. The ethics committee of the Hamadan University of Medical Sciences approved the study protocol with the number 546788 and the written consent was obtained from all the patients. Subjects who had received anti-microbial therapy, H2 receptor blockers, and proton pump inhibitors in the preceding 20 days prior to endoscopy or anti-H. Pylori treatments in the past were excluded from the study. Biopsy specimens of antrum from each patient were collected for the rapid urease test, culture, histopathology and PCR.

3.2. Bacterial Isolation and Culture

During each endoscopy, three antral biopsies were obtained and subjected to the following tests: The first biopsy for rapid urease test (RUT), the second for culture, and the last for histopathology following the standard protocol as described earlier (16). The biopsy specimens for culture were taken into screw capped bottle containing Thioglycolate broth (Merck, Germany) with 0.5% agar and 3% yeast extract and immediately transported to the microbiology lab at Faculty of Medicine, Hamadan University of Medical Sciences. Since *H. pylori* is a fastidious organism, the culture of *H. pylori* has done with some modifications as follows: gastric biopsy specimens were ground with tissue homogenizer and then inoculated onto Brucella Agar (Merck, Germany) media with 10% sheep blood and 10% fetal bovine serum (GIPCO), and Campylobacter Selective Supplement (Merck, Germany), and incubated under microaerophilic (5% O2, 10% CO2, and 85% N2) conditions at 37°C for 3 to 5 days. Organisms were identified as *H. pylori* on the basis of morphology on Gram stain observation and by oxidase, catalase, urease tests and 16s rRNA PCR (Table 1). All the isolated *H. pylori* strains were kept frozen at -70°C in the tryptone soya broth medium containing 15% (vol/vol) glycerol until genotyping was performed.

3.3. PCR of H. Pylori Genes

DNA was extracted from the bacterial growth by boiling in 200 μL of sterile double distilled water for 10 min and cooling it on ice for 5 min. It was then centrifuged at 12000 rpm for 10 min. Fifty Mm NaOH as 1/5 was added to the supernatant and centrifuged at 12000 rpm for 5 min. The supernatant containing DNA was used for PCR. The genus-specific PCR involving 16S rRNA gene (17) and genotyping of *cagA* and *vacA* genes were performed using specific primers (9) (Table 1). PCR was performed in a 25 μL reaction volume containing 5μL of genomic DNA, 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide (Fermentase, Co), 0.5 μM of each specific primer and 1U of Taq DNA polymerase (Fermentase, Co). All the PCR reactions were performed using thermocycler (Eppendorf 5323, Germany). The amplified product was electrophoresed in 1.5% agarose containing 0.5 μg/mL ethidium bromide and examined under a *transilluminator* .

3.4. Statistical Analysis

Descriptive statistics, the Chi-square and Fisher's exact tests were used to analyze the data. P Value of < 0.05 was considered statistically significant.

4. Results

Patients consisted of 64 females and 89 males (mean age 53 years, range 16-88 years) (Table 2). From 153 biopsy samples, 137 (89.5%) samples were positive for H. pylori using the histology, 76 (49.7%) by the rapid urease test, 83 (54.2%) by culture [3 gastric cancer (GC), 27 peptic ulcer dyspepsia (PUD) and 53 non- ulcer dyspepsia (NUD)] and confirmed by 16s rRNA gene PCR. PCR amplification of H. pylori 16s rRNA gene gave band size of 446 bp.

4.1. PCR of cagA and vacA Genes

Out of 83 culture positive samples, 73 (87.9%) samples yielded the 508bp product representing *cagA* positive,

in which 25 (92.6%) isolates found in 27 patients with PUD, 3 (100%) isolates in 3 patients with GC, and 45 (84.9 %) isolates in 53 patients with NUD. Sixty-eight (81.9 %) isolates of *H. pylori* were found to be *vacA*-positive using the specific primers for s1 52 (62.7 %) and s2 16 (19.3 %) genes (Table 2). Thirty-seven (44.6%) of 83 strains yielded the 190 bp product representing *vacA* signal sequence genotype s1a, 2 (2.4%) yielded the 187 bp product representing genotype s1b and 13 (15.7%) yielded the 213bp product representing genotype s1c. Sixteen (19.3%) yielded the 199 bp product representing genotype s2. Fourteen (16.9%) isolates were classified as type m1a and 7 (8.4%) were classified as type m1b. Forty-seven (56.6%) isolates were classified as type m2 (Table 2). The relationship between the particular *vacA* genotypes and the occurrence of different gastrointestinal diseases was also determined (Table 2). Combinations of *vacA* homologue containing signal sequence and middle region (s1a/m1, s1a/m2, s1b/m1, s1b/m2, s1c/m1, s1c/m2, s2/m1 and s2/m2) were estimated. The s1a/m1 and s1a/m2 combinations were found in 10 (12%) and 27 (32.5%) patients, respectively. The combination of s1b/m1 was found in 2 (2.4%) patient and s1b/m2 in 2 (2.4%) isolates. The s1c/m1 and s1c/m2 were found in 6 (7.2%) patients. The s2m2 combination was identified in 11 (13.3%) and s2m1 in 5 (6 %) out of 83 patients (Table 3). Distribution of the combination of *vacA* homologue containing signal sequence and middle region with the occurrence of different gastrointestinal diseases was also determined (Table 3). S1/m2 (36 patients) was the predominant combination found in 10 (37%) out of 27 patients with PUD, 24 (45.3%) out of 53 patients with NUD, 2 (66.6%) with GC, respectively. All 27 (100%) s1a/m2 and 10 (100%) s1a/m1 *vacA* genotypes were associated with the *cagA*-positive genotype.

Table 1. Oligonucleotide Primers Used for *cagA* and *vacA* Typing

Primer Sequence Annealing Size of Designation (5′ to 3′) 0C Product (bp)
cagA ACCCCTAGTCGGTAATGGG 49 508
GCTTTAGCTTCTGAYACYGC
vacA s1 ATGGAAATACAACAAACACAC 48 259
CTGCTTGAATGCGCCAAAC
vacA s1a GTCAGCATCACACCGCAAC 54 190
CTGCTTGAATGCGCCAAAC
vacA s1b AGCGCCATACCGCAAGAG 55 187
CTGCTTGAATGCGCCAAAC
vacA s1c CTCTCGCTTTAGTGGGGYT 52 213
CTGCTTGAATGCGCCAAAC
vacA s2 CCTAACACGCCAAATGATCC 54 199
CTGCTTGAATGCGCCAAAC
vacA m1a GGTCAAAATGCGGTCATGG 48 290
CCATTGGTACCTGTAGAAAC
vacA m1b GGCCCCAATGCAGTCATGGAT 56 291
CTGTTAGTGCCTAAAGAAGCAT
vacA m2 GGAGCCCCAGGAAACATTG 50 352
CATAACTAGCGCCTTGCAC
16s rRNA CTGGAGAGACTAAGCCCTCC 49 446
AGGATCAAGGTTTAAGGATT

Table 2. Distribution Between *vacA* Subtypes, *Cag A* and Clinical Outcomes [a,b,c]

Genotypes	PUD (n = 27)	NUD (n = 53)	Cancer (n = 3)	Total (n = 83)	X^2	P Value
***vacA*s1**	16 (59.3)	33 (62.3)	3 (100)	52 (62.7)	1.925	0.382
***vacA*s2**	5 (18.5)	11 (20.8)	0	16 (19.3)	0.801	0.670
***vacA*m1**	8 (29.6)	12 (22.6)	1 (33.3)	21 (25.3)	0.568	0.853
***vacA*m2**	13 (48.1)	32 (60.4)	2 (66.7)	47 (56.6)	1.217	0.544
***vacA*s1/m1**	6 (22.2)	9 (17)	1 (33.3)	16 (19.3)	0.711	0.701
***vacA*s1/m2**	10 (37)	24 (45.3)	2 (66.7)	36 (43.4)	1.183	0.554
***vacA*s2/m1**	2 (7.4)	3 (5.7)	0	5 (6.0)	0.296	0.862
***vacA*s2/m2**	3 (11.1)	8 (15.1)	0	11 (13.3)	0.722	0.697
CagA	25 (92.6)	45 (84.9)	3 (100)	73 (88)	1.424	0.491

[a] Patients distribution consisted of 153 adult patients (89 men and 64 women) aged 16-88 years (mean age, 53).
[b] Data are presented as No.(%).
[c] Abbreviations: PUD; peptic ulcer dyspepsia, NUD; non-ulcer dyspepsia.

Table 3. Distribution Between Allelic Variants of *vacA* and Clinical Outcomes [a,b]

Allelic variants of *vacA*	PUD (n = 27)	NUD (n = 53)	Cancer (n = 3)	Total (n = 83)	X^2	P Value
s1						
s1a	11 (40.7)	24 (45.3)	2 (66.1)	37 (44.6)	0.764	0.682
s1b	0	2 (3.8)	0	2 (2.4)	1.16	0.560
s1c	5 (18.5)	7 (13.2)	1 (33.3)	13 (15.7)	1.118	0.572
s2	5 (18.5)	11 (20.8)	0	16 (19.3)	0.801	0.610
m1						
m1a	6 (22.2)	7 (13.2)	1 (33.3)	14 (16.9)	1.638	0.441
m1b	2 (7.4)	5 (9.4)	0	7 (8.4)	0.382	0.826
m2						
-	13 (48.1)	32 (60.4)	2 (66.7)	47 (56.6)	1.217	0.544
s1a/m1a	3 (11.1)	4 (7.5)	1 (33.3)	8 (9.6)	2.267	0.322
s1a/m1b	1 (3.7)	1 (1.9)	0	2 (2.4)	0.328	0.849
s1b/m1a	0	0	0	0	-	-
s1b/m1b	0	0	0	0	-	-
s1c/m1a	1 (3.7)	2 (3.8)	0	3 (3.6)	0.117	0.943
s1c/m1b	1 (3.7)	2 (3.8)	0	3 (3.6)	0.117	0.943
s1a/m2	7 (25.9)	18 (34)	2 (66.7)	27 (32.5)	2.179	0.336
s1b/m2	0	2 (3.8)	0	2 (2.4)	1.160	0.560
s1c/m2	4 (14.8)	3 (5.7)	0	7 (8.4)	2.228	0.328
s2/m1a	2 (7.4)	1 (1.9)	0	3 (3.6)	1.682	0.431
s2/m1b	0	2 (3.8)	0	2 (2.4)	1.160	0.560
s2/m2	3 (11.1)	8 (15.1)	0	11 (13.3)	0.722	0.697

[a] Data are presented as No.(%).
[b] Abbreviations: PUD; peptic ulcer dyspepsia, NUD; non-ulcer dyspepsia.

5. Discussion

H. pylori are one of the most genetically diverse bacterial species with geographic genetic variations. Studies in several countries have demonstrated different distribution of *vacA* alleles and the presence of *cagA* gene in *H. pylori* genotypes strains and their association with gastro duodenal diseases (18-20). In Europe and North America, the prevalence of *cagA*-positive *H. pylori* has been reported between 64% and 79% whereas in Asia (e.g. Japan, Korea, China and Turkey), the frequency of *cagA*-positive *H. pylori* strains is usually over 90% (21). Few studies have been done on *cagA* status in Iran. The *CagA* gene was present in 44% of the patients according to an investigation by Siavoshi et al. (22). This number was 87.9% in our study, which was not near to the number reported in the European and North American populations. No significant association between *cagA* status and diseases has been observed in Asian countries; although the majority of *H. pylori* strains are *cagA*-positive, but the previous studies from Iran reported that the *cagA*-positive strains were more frequent among patients with gastric cancer compared to other patients (22). The *vacA* genotypes show considerable geographic variations. In our study, a *cagA*-positive genotype was determined in 87.9% of the *H. pylori* samples and the *vacA* genotypes s1/m2, s1/m1, s2/m2 and s2/m1 were determined in 43.4%, 19.3%, 13.2% and 6%, respectively. This observation is similar to China and Turkey; but in the middle region, results are substantially different from Brazil and Portugal with m1 dominant type. Also, Aydin et al. from Turkey and the large multicenter study of van Doorn et al. reported an equal prevalence of m1 and m2 subtypes (6, 23-26). Martin et al. and Nogueira et al. have been observed that allele s1 was associated with high degrees of gastric tissue inflammation; although this correlation was in companion with *cagA* positivity and m1 allele, which we found no association (21, 27). However, the current study does not rule out an association between the expression of *vacA* or *cagA* protein and the virulence of *H. Pylori* (28-30). In a recent study by Ghotaslou et al. in 2013, 68.7% of the patients were infected with *cagA*-positive strains, similar to another Iranian study (31). However, this is different from studies from East to South Asian countries where more than 90% of the strains carry the *cagA* gene regardless of clinical outcomes. Our result is higher than the reported values from Europe and the USA where the prevalence of *cagA*-positive strains is between 60-70% (32). In the pres-

ent study, out of 83 culture-positive samples, 73 (87.9%) samples yielded the 508 bp product representing *cagA*-positive, in which 25 (92.6%) isolates were found in 27 patients with PUD, 3 (100%) out of 3 patients with GC, and 45 (84.9 %) out of 53 patients with NUD. No correlation of *cagA* could be established with GC and PUD, although *cagA* had been identified as a virulence marker and associated with increased severity of disease in some geographic regions (33, 34). High prevalence of *cagA* (80-90%) independent of the disease status had been reported in some Indian studies. These studies indicate that *cagA* cannot be considered as the sole virulence marker for determination of the disease outcome at least in India as has been reported from other geographic regions (12). The present study suggests that *vacA*s2 region variants are not involved in gastric carcinogenesis and PUD. The s1a/m1 and s1a/m2 combinations were found in 10 (12%) and 27(32.5%) patients, respectively. The combination of s1b/m1 was not found in any patient and s1b/m^2 was found in 2 (2.4 %) isolates. The s1c/m1 was found in 6 (7.2%) and s1c/m2 in 6 (7.2%) patients. The s2m2 combination was identified in 11 (13.3%) out of 83 patients. The s2m1 combination was found in 5 (6 %) patients. We also found a large number of *cagA*-positive *H. Pylori* (87.9%) in our patient population. This is the first study in our area that reports high prevalence of *cagA* gene in the *vacA*-positive strains. These differences may be geographical and because of high frequency of *cagA* positive strains in our population with no significant difference between disease and control populations. Excluding the mixed infection and combining the groups, we observed that vacAs1a/m2 was frequently found in our study population, which is similar to several other findings.

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