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vacA Genotypes of *Helicobacter pylori* in Relation to *cagA* Status and Clinical Outcomes in Iranian Populations

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SUMMARY

Mosaicism in *vacA* alleles with two distinct families of *vacA* signal sequences (s1 and s2) and two distinct families of middle region alleles (m1 and m2) has been reported. Research suggests that the *vacA* s1 genotype is closely associated with duodenal ulcer disease and with high cytotoxin production. The aims of this study were to evaluate the role of *vacA* genotyping with respect to gastric inflammation and injury, and clinical presentation in Iranian populations. Genomic DNA of biopsy specimens from patients with gastritis, peptic ulcer disease (PUD), or gastric cancer (GC) were characterized based on *ureC* (*glmM*), *cagA*, and *vacA* genotyping by using polymerase chain reaction. Of 167 patients including 33 with PUDs, 129 with non-ulcer dyspepsia (NUD), and 5 with GC, 96 (57.5%) cases were infected by *Helicobacter pylori*. Among these patients, *H. pylori* were isolated from 19 (57.7%) PUD patients, 74 (68.7%) NUD patients, and 3 (60%) GC patients. The *cagA* was detected in 76% of *H. pylori*-positive cases. The *vacA* s1-m2 genotype was the most prevalent in 7/19 PUD (37%) and 30/74 NUD (40.5%) patients with *H. pylori* infection. The prevalence of *vacA* s2-m1 (8%) was high in Iranian isolates. A significant association was not found between *H. pylori* genotypes and clinical outcomes. The *vacA* genotypes and *cagA* status were not useful markers for gastroduodenal diseases in Tehran, Iran.

INTRODUCTION

Although *Helicobacter pylori* have been accepted as a major cause of gastroduodenal diseases, it is still unknown what factors are responsible for the different outcomes such as asymptomatic gastritis, peptic ulcer disease (PUD), or gastric cancer (GC). The rapid changes in the epidemiology of these different manifestations of *H. pylori* infection suggest an environmental factor, an interaction between an environmental factor and the host, or a change in prevalence of strains differing in virulence. Two phenotypic characteristics among *H. pylori* strains, the vacuolating cytotoxin encoded by the *vacA* gene (VacA) and a high-molecular-weight protein encoded by the cytotoxic associated gene A (CagA), are considered possible candidates for the identification of strains with enhanced virulence. For example, gastric mucosa from patients infected with *H. pylori* containing a 40-kb region that includes the *cagA* gene (*cag* pathogenicity island) typically exhibits more severe inflammation than that of gastric mucosa infected with *cagA*-negative strains (1). The *vacA* gene is present in most *H. pylori*, but the *vacA* product may not be expressed in all cases.

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There has been an attempt to characterize and classify differences in the *vacA* gene and to associate specific genotypes with different *H. pylori*-associated diseases. Atherton et al. divided the middle region of the gene into two types (m1 and m2) and the cleaved signal sequence into two distinct families (s1 and s2) (2). The combination of the two signal sequence families and two distinct families of middle region alleles resulted in four possible combinations (subtypes) of signal sequence and middle region alleles, and Atherton et al. identified three of the four possible subtypes (s2-m1 was not found). Later the presence of the s2-m1 genotypes was reported but with very rare prevalence (0 to 3%) (3). The current consensus is that s1-m1 strains showed a high level of vacuolating cytotoxin activity, whereas s2-m2 strains did not exhibit vacuolating cytotoxin activity. It remains unclear whether the *vacA* genotypes were useful markers for clinical outcomes. Initial reports indicated that the s1 genotype would be found in close association with clinical outcomes in Western countries (2); however the prevalence of this genotype was extremely high (almost 100%) in East Asian countries irrespective of the clinical outcomes (4). The prevalence of *H. pylori* infection is about 50% of the world's population and has been reported to be 60 to 90% in Iran (5–8). However, there is little information about the virulence factors of *H. pylori* in Iran in relation to gastroduodenal diseases (3,6–8). The purpose of this study was to determine the prevalence of *H. pylori* isolates in patients with a full spectrum of clinical manifestations of *H. pylori* infection to evaluate whether the postulated associations with *vacA* genotypes and *cagA* gene would be applicable to patients with *H. pylori* infection in Iranian populations.

MATERIALS AND METHODS

Population studied

A total of 167 patients who received upper-endoscopy treatments during February to January 2006 in Tehran, Iran, were enrolled in this study. None of the patients had received nonsteroidal anti-inflammatory drugs or antibiotics within the previous 3 months. Informed consent was obtained from all patients, and the protocol was approved by the ethical committee of the Research Center for Gastroenterology and Liver Diseases in Shaheed Beheshti University of Medical Science.

Isolation and identification

Three biopsy specimens were taken from the greater curve of the antrum; two were used for histological examination and one for *H. pylori* culture. Gastric biopsy specimens for culturing were kept in transport medium consisting of thyoglycolate with 1.3 g/L agar (Merck Co., Humbuerg, Germany) with 3% yeast extract (Oxoid Ltd., Basingstoke, UK) and brought to the laboratory on the day of endoscopy. In each case, the gastric biopsy specimens were cultured on Brucella agar with 7% sheep blood and supplements with different antibiotics incubated (Merck) under microaerophilic conditions at 37°C for 3 – 10 days.

Preparation of genomic DNA and polymerase chain reaction (PCR)

DNA from each *H. pylori* isolate was extracted from the fresh gastric biopsy specimens using the QIAamp tissue method (Qiagen, Hilden, Germany). The genotypes of *vacA* single sequences (s1 or s2) and middle regions (m1 or m2), and the presence of *cagA* and *glmM* (*ureC*) were determined by PCR. The primer sequences are listed in Table 1. All PCR mixtures were prepared in a volume of 25 μl containing 1 × PCR buffer, 500 nM of each primer, 1.5 mM $MgCl_2$; 200 μM each dNTP, 1.5U Taq DNA polymerase, and 300 ng DNA sample. The mixtures were placed in a thermocycler (Eppendorf AG 22331; Eppendorf, Hamburg, Germany), and then PCR products were visualized by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and examined under UV illumination.

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Data analysis

The chi square and Fisher's exact tests were used for analysis of categorical data. The Mann-Whitney rank sum test was used for assessing differences between ordered categories such as histological grade or cytotoxin production. The association between the diversity of *cagA* and *vacA* genes and clinical outcomes was analyzed with the chi square test. Analyses were done using Sigma Stat for Windows V2.03 (SPSS, Chicago, 1ll., USA). A *P* value of <0.05 was accepted as statistically significant.

RESULTS

The study population consisted of 167 patients (88 men and 79 women) with a mean age of 44 years (range, 10 to 81 years). The patients were classified at the time of endoscopy as having PUD (n =33), GC (n = 5), or no evidence of mucosal ulceration but with chronic gastritis as diagnosed by histological examination (NUD) (n = 129). Based on PCR for *ureC* (*glmM*) using DNA from biopsy specimens, 96 (57%) patients were positive for *H. pylori* infection. The prevalence of *H. pylori* was 57.5% (19 of 33) among patients with PUD, 60% (3 of 5) among patients with GC, and 57% (74 of 129) among patients with NUD. The distribution of patients (total number, number of infected patients, and number of PUD) according to age is shown in Table 2.

vacA genotypes

Possible combinations of *vacA* s and m regions were determined in the Iranian population (Table 3). Among 96 samples positive for *ureC* (*glmM*), only three samples were not amplified both by *vacA* s and m regions (two from NUD and one from PUD). In four samples *vacA* s regions (s1) were amplified, but *vacA* m regions were not detected (three from NUD and one from PUD). Overall, 22 samples were classified as *vacA* s1-m1, 40 as s1 -m2, 19 as s2-m2, and 8 as s2-m1 genotypes. Likewise, 4 *H. pylori* isolates were s1-m0 genotype. Out of 66 *vacA* s1 strains, 63 samples were successfully sub-typed using s1a and s1b specific primers. Among them, 27 (43%) were s1a positive and 36 (57%) were s1b positive. In the case of m1 sub-typing, the distribution of m1a and m1b was 77 and 23%, respectively. We did not find any relationship between *vacA* genotypes and clinical outcomes (Table 3).

cagA status and its relation with *vacA* genotypes

Among 96 samples positive for *ureC* (*glmM*), the *cagA* gene was detected in 73 samples (76%). Out of 93-*vacA*-positive strains, 71 (76%) isolates were *cagA*-positive. Fifty-eight (88%) strains with *vacA* s1 genotype were *cagA*-positive, whereas only 13 (48%) strains with *vacA* s2 genotype were *cagA*-positive, showing that the presence of the *cagA* gene was highly significantly associated with the *vacA* s1 genotype ($P < 0.001$), in agreement with previous studies (2). In particular, most samples (96%) with the *vacA* s1-m1 genotype were *cagA* positive. As with *vacA* genotypes, the prevalence of the *cagA* gene was not related to clinical outcomes (79% o f PUD patients and 74% of NUD patients were positive for *cagA*) (Table 4).

DISCUSSION

The geographic distribution of distinct *H. pylori* genotypes and the prevalence of virulent bacterial genotypes in several regions, particularly in Iran, remain unknown. This study included 167 unselected patients, of whom 96 were infected with *H. pylori*. In the present study, we examined the diversity of the *vacA* gene and the relationship between *vacA* genotypes and *cagA* status. Although the *vacA* s1-m1 genotype is reported to be the most virulent genotype, the prevalence was even higher in NUD than in PUD patients (27 versus

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11%), although the differences were not statistically significant. The prevalence of the s2-m2 genotype, which is reported to be less virulent, was even lower in NUD than in PUD patients (18 versus 32%), but again the differences were not statistically significant. Overall, *vacA* s1 -m2 was the most prevalent genotype irrespective of the clinical outcomes.

When we analyzed the signal region and middle region separately, we found no relationship between *vacA* s and m genotypes and clinical outcomes (Table 3). Previous reports showed that s1-m1 genotypes were the most virulent, whereas s2-m2 genotypes were avirulent based on the activity of in vitro cytotoxin activities (2,7,10). Accordingly, there are many reports, especially from European countries, that s1-m1 genotypes were associated with clinical outcomes such as PUD and GC, whereas s2-m2 genotypes were associated with NUD (11–13). Surprisingly, however, we could not find any relationship between *vacA* genotypes and clinical outcomes. In our Iranian population, the prevalence of s1-m1 was even higher in NUD cases (27%) compared with PUD cases (11%), and that of s2-m2 was lower in NUD cases (18%) compared with PUD cases (32%). In the Iranian population, we found that s1-m2 was the most prevalent genotype irrespective of the clinical outcomes. Interestingly, we also found the high prevalence of *vacA* s2-m1 (8%) in the Iranian population, which was a much higher prevalence than that noted in other reports (3).

To date, five studies have been published about the relationship between clinical outcomes and *vacA* and/or *cagA* status in Iranian populations (3,6–8,10). Mohammadi et al. reported that the *vacA* s1 genotype was detected in 79 and 68% of patients with PUD and NUD, respectively, in Tehran (3). Similar to our data, they reported that s1-m2 genotypes were the predominant genotypes in Iran. The authors of another study from Tehran also reported that the *vacA* s1 genotype was detected in 79 and 77% in patients with PUD and NUD, respectively (6). Overall, we confirmed that the *vacA* genotypes should not be a good marker for predicting clinical outcomes. In contrast, Kamali-Sarvestani et al. from Shiraz, a southern city of Iran, reported that the *vacA* genotypes were significantly different among gastritis, PUD, and GC patients (7). In addition, another study from Shiraz reported that *vacA*-positive strains (56.92%) were more frequently found in PUD patients than in NUD patients (8); however, since it is well known that almost all strains should possess the *vacA* gene, the accuracy of their study is questionable (8).

Strains from Western countries predominantly possessed *vacA* s1a or s1b/m1a, or *vacA* m2a genotypes, whereas in strains from South Asia *vacA* s1a/m1c genotypes and in those from East Asia *vacA* s1c/m1b, or m2b genotypes are predominant (14–16). In Iran, *H. pylori* with positive *cagA* and *vacA* s1b/m1a strains are found in the majority of all patients with different clinical status. Thus the Iranian strains genotypes are closer to Uruapan strains than to Asian. Overall, it is clear that the importance of the *vacA* genotype is different in the different geographic regions even within Iranian populations. We also found that the *cagA* status was not related to clinical outcomes in Iranian populations. Interestingly, this is in agreement with other studies in Iran, including studies in both Tehran (6) and Shiraz (7,8). The *cagA* status was reported to be related to clinical outcomes such as PUD and GC, especially in European and North American populations (17–19), whereas the prevalence of *cagA* was almost 90% in East Asian countries irrespective of the diseases (20). Our study showed that the prevalence of *cagA*-positive strains was 76%, which was similar to many reports from European and North American populations (17,21). In contrast, one study from Tehran reported that the prevalence of the *cagA* gene was only 44% (6). The reason for this discrepancy is unclear; however, the study authors used different primer pairs for the *cagA*, and sequence diversity might exist in different geographic locations. In agreement with previous consensus, we confirmed that the prevalence of the *cagA* gene was closely linked with the *vacA* s1 genotypes; therefore the linkages between the *cagA* and *vacA* genes should be consistent irrespective of the geographic locations. Overall, in the present study, we

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found that *vacA* s1-m2, *cagA*-positive strains are predominant in strains isolated from Tehran, Iran, irrespective of clinical outcomes. We also found that the s2-m1 genotype, which is reported to be rare, is not rare in Iranian populations.

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Table 1

Oligonucleotide primers used for *cagA, ureC* (*glmM*) and *vacA* alleles

Gene	Primer designation	Sequence	PCR product size (bp)	Reference
cagA	CagA F1	AACAGGACAAGTAGCTAGCC	349	9
	CagA R1	TATTAATGCGTGTGTGGCTG		
vacA s1s2	VAIF	ATGAAAAAAACCCTTTTAC	259 (s1)	9
	VAIXR	CGAATTGCAAGTGATGGT	286 (s2)	
glmM	GlmM1-R	GCTTACTTTCTAACACTAACGCGC		9
	GlmM2-F	GGATAAGCTTTTAGGGGTGTTAGGGG	296	
vacA m1a	VA3-F	GGTCAAAATGCGGTCATGG	300 (m1a)	9
	VA3-R	CCATTGGTACCTGTAGAAAC		
vacA m1b	VAm-F3	GGCCCCAATGCAGTCATGGAT	300 (m1b)	9
	VAm-R3	GCTGTTAGTGCCTAAAGAAGCAT		
vacA m2	VA4-F	CATAACTAGCGCCTTGCAC	400 (m2)	9
	VA4-R	GGAGCCCCAGGAAACATTG		
s1a	VA1-R	CTGCTTGAATGCGCCAAAC	190	2
	SS1-F	GTCAGCATCACCGCAAC		
s1b	VA1-R	CTGCTTGAATGCGCCAAAC	187	2
	SS3-F	AGCGCCATACCGCAAGAG		

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Table 2

Distribution of patients according to age

Disease	Age of patients (y)						
	10–20	20–30	30–40	40–50	50–60	Upper 60	Total
PUD	1[1)] (1)[2)]	6 (2)	5 (1)	8 (7)	6 (3)	7 (5)	33 (19)
GC	0 (0)	0 (0)	0 (0)	2 (1)	0 (0)	3 (2)	5 (3)
NUD	2 (0)	9 (4)	17 (9)	28 (15)	30 (13)	43 (33)	129 (74)

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

[1)] Number of patients.

[2)] Number of patients with *H. pylori* infection.

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Table 3

Correlation between *vacA* subtypes and clinical outcomes

***vacA* genotype**	**PUD *n* = 19 no. (%)**	**NUD *n* = 74 no. (%)**	**GC *n* = 3 no. (%)**	**Total *n* = 96 no. (%)**
s1	10 (53)	53 (72)	3 (100)	66 (69)
s2	8 (42)	19 (27)	0	27 (28)
m1	4 (21)	26 (35)	0	30 (31)
m2	13 (68)	43 (58)	3 (100)	59 (61)
s1/m1	2 (11)	20 (27)	0	22 (23)
s1/m0	1 (5)	3 (4)	0	4 (4)
s1/m2	7 (37)	30 (41)	3 (100)	40 (42)
s2/m1	2 (11)	6 (8)	0	8 (8)
s2/m2	6 (32)	13 (18)	0	19 (20)
Untypable	1 (5)	2 (3)	0	3 (3)

Abbreviations are in Table 2.

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Table 4

Correlations between *cagA* status and clinical outcomes

Patient group	**No. of patients *n* = 167 no.**	***H. pylori* positive *n* = 96 no. (%)**	***cagA* negative *n* = 23 no. (%)**	***cagA* positive *n* =73 no. (%)**
PUD	33	19 (57.5)	4 (21)	15 (79)
GC	5	3 (60)	0 (0.0)	3 (100)
NUD	129	74 (57)	19 (25.7)	55 (74.3)

Abbreviations are in Table 2.