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Diversity of *Helicobacter Pylori cag*A and *vac*A Genes and Its Relationship with Clinical Outcomes in Azerbaijan, Iran

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ABSTRACT

Purpose: The purpose of this research was to analyze *cag*A and *vac*A genotypes status in *H. pylori* isolates and relationship with clinical outcomes. ***Methods:*** Gastric biopsy specimens were cultured for *H. pylori* isolation and *cag*A and *vac*A genes were detected in these isolates. Data were collected and the results were analyzed using $\chi 2$ and Fishers exact tests by SPSS software version. 16. ***Results:*** Of the total 115 *H. pylori* isolates, 79 (68.7 %) were *cag*A positive and 82 (71.3%) of isolates contained the s1 allele which 33 (28.7%) were subtype s2. s1m2 was the most frequent *vac*A allelic combination in the *H. pylori* isolates examined (63 cases), followed by s2m2 (31 cases), s1m1 (19 cases) and s2m1 (2 case). Strains *cag*A positive were more frequent in peptic ulcer diseases patients than non ulcer diseases patients, as 47 (59.5%) and 32 (40.5%), while *cag*A negative were low, as 15 (41.7%) and 21 (58.3%), respectively. ***Conclusion:*** We found that the *cag*A and *vac*A status were not related to clinical outcomes in this area. Overall, in the present study, *vac*A s1/m2, *cag*A-positive strains were predominant irrespective of clinical outcome, but s2/m1 was rare.

Introduction

Helicobacter pylori (*H. pylori*), is a gram negative bacterial species that colonizes the human stomach and has been associated with human for at least tens of thousands of years.[1] This bacteria is permanently colonizes gastric epithelial cells in approximately 25% of the population in developed countries and 70–90% in developing countries, whereas most infected individuals are asymptomatic. Chronic *H. pylori* infection in susceptible individuals is associated with a variable degree of mucosal damage ranging from mild gastritis and ulcer disease to gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma.[2] Colonization with these bacteria is usually without clinical consequences, but increases the risk of developing peptic ulcer disease, gastric adenocarcinoma and lymphoma.[3]

The clinical outcome of *H. pylori* infection has been associated with bacterial virulence factors, host gastric mucosal factors, and the environment.[4] It is estimated that 50% of the world's population is infected with *H. pylori*, but the factors associated with different outcomes, such as non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD) or gastric carcinoma, are unknown.[5] This diverse clinical outcome may be associated with the expression of virulence factors. The cytotoxin-associated gene (*cag*A), which is not present in every *H. pylori* strain, is considered to be a marker for the cag pathogenicity island, and its expression is associated with severe infection.[6,7] In contrast, the vacuolating cytotoxin gene (*vac*A) is present in most *H. pylori* strains, although the VacA toxin may not be expressed in all cases.[8] The *vac*A gene contains a signal region and a middle region, both of which are divided into two allelic types: s1 or s2, and m1 or m2, respectively. These types are divided into the subtypes s1a, s1b or s1c, and m2a or m2b. Both s1/m1 and *cagA*-positive strains have been reported to be associated with PUD and gastric carcinoma.[9] The purpose of this research was to analyze *cag*A and *vac*A genotypes status in *H. pylori* isolates.

Materials and Methods

Patients

A total of 115 *H. pylori* isolates were obtained from gastric biopsies of patients with gastritis, peptic ulcer and gastroesophageal reflux diseases undergoing endoscopy. This study was approved by the ethical committee of regional Medical Research of Tabriz

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University of Medical Sciences and all patients provided written informed consent for this research.

H. pylori Culture and extraction of Genomic DNA

Briefly gastric biopsy samples were homogenized and cultured onto Brucella agar containing 5% sheep blood and antibiotics supplements. Culture plates were incubated at microaerophilic condition at 37 ºC and high humidity for 5-7 days. Organisms were identified as *H. pylori* based on colony morphology, gram staining and positive oxidase, catalase and urease tests. Genomic DNA of total *H. pylori* strains was extracted by using CTAB[10] and stored at – 20 ºC. Briefly, the loop full of bacteria was added to 1.5 ml sterile distilled water, vortexed well and was centrifuged in 1000 g for 10 min. The supernatant was discarded and 270 µl T/E buffer plus 30 µl SDS 10% plus 5 µl proteinase K was added to microtube and then incubated at 50 ºC overnight. One hundred µl of 5 M NaCl solution was added to microtube and mixed well. Eighty µl of prewarmed CTAB/NaCl (65 ºC) solution was added to microtube and vortexed well. Then the microtube was incubated at 65 ºC for 10 minutes. Seven hundred µl of chloroform-isoamylalcohol (24:1) solution was added to the microtube and vortexed for 20 second. The suspension was centrifuged at 12000 g for 5-10 minute at 10 ^{o}C and aqueous phase was transferred into new microtube. Then, 200-300 µl isopropanol was added to each microtube and mixed gently, and incubated at -20 ºC for 30 minute, finally centrifuged at 12000 g for 10 min. The supernatant was discarded and pellet was resuspended in 1 ml of 70% cold ethanol, and then centrifuged at 12000g for 5 min at 10 ºC. The supernatant was discarded and after air drying, the DNA pellet was dissolved in 50 µl T/E (10:1) buffer and incubated at 37 ºC for 30 min, then stored at 4 ºC overnight.

Detection of *cagA* and *vacA* mosaicism distribution

In this study PCR was used to detect the *H. pylori* specific *ure*C gene for confirmation of *H. pylori* isolates, the virulence-associated *vac*A mosaic structure and the presence of *cag*A gene. All primer sets were selected from the published literatures (Table 1).[11,12] PCR reactions were performed in a volume of 50µL containing10mmol/L Tris-HCl, 1.5mmol/L $MgCl_2$, 0.2mmol/L of each deoxynucleotide, 25 pmol of each primer and 2.5 units of Taq polymerase (Geneone, Germany). PCR amplification conditions for *cag*A and *glm*M genes, involved 3 min of pre incubation at 94ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 58 ºC, and 30 s at 72 ºC and 3min at 72°C for final extension. The *vac*A typing was performed with the following conditions: 3 min for pre incubation at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 61 ºC (for m1/m2), 50 ºC (for s1/s2), 44 ºC (for s1a), 52 ºC (for s1b) for annealing, and 3 min at 72°C for final extension. PCR products were visualized by electrophoresis on 1.5% agarose gels with ethidium bromide. DNA from isolates with known genotypes was used as a positive control.

Table 1. Primers for amplification used in this study

DNA region amplified	Primer	Primer sequence	PCR products (bp)
*ure*C (*glm*M)	HP-F	GGATAAGCTTTTAGGGGTGTTAGGGG	294
	HP-R	GCTTACTTTCTAACACTAACGCGC	
*cag*A	cagA-Fm	AGG GAT AAC AGG CAA GCT TTT GA	352
	cagA-Rm	CTG CAA AAG ATT GTT TGG CAG A	
*vac*A-m1	ml -Fm	GGT CAA AAT GCG GTC ATG G	290
	ml -Rm	CCA TTG GTA CCT GTA GAA AC	
*vac*A-m2	m2-Fm	GGA GCC CCA GGA AAC ATT G	352
	m2-Rm	CAT AAC TAG CGC CTT GCA C	
*vac*A-s1 or s2	VA1-F	ATGGAAATACAACAAACACAC	259 or 286
	VA1-R	CTGCTTGAATGCGCCAAAC	
*vac*A-s1a	S1a-Fm	GTC AGC ATC ACA CCG CAA C	190
	S1a-Rm	CTG CTT GAA TGC GCC AAA C	
*vac*A-s1b	S1b-Fm	AGC GCC ATA CCG CAA GAG	187
	S1b-Rm	CTG CTT GAA TGC GCC AAA C	

Statistics analysis

Data were analyzed by SPSS version 16. The Pearson X^2 test was used to evaluate the relationship between individual genotypes and a variety of diseases. Logistic regression analysis was used to relate the different combinations of *vacA* and *cagA* genotypes of *H. pylori* to the presence of peptic ulcers.

Results

Fifty-three of our 115 patients were classified as non-ulcer diseases and, sixty-two patients had proven peptic ulcer disease based on observation during gastroscopy. There was no significant difference between the mean age of patients with and without ulcers. By using primers HP-F and HP-R to amplify the *ure*C gene, the expected PCR product of 294-bp was obtained in all strain isolates. Simultaneously using specific primers, *cag*A gene was detected in 79 (68.7 %) isolates.

In our study, strains carrying the *cag*A gene (*cag*A-positive) were more frequent in PUD patients than NUD patients, as 47 (59.5%) and 32 (40.5%), while strains lacking *cag*A gene (*cag*A-negative) were low, as 15 (41.7%) and 21 (58.3%), respectively (Table 2 and 3).

In our study the presence of the *vac*A gene also was investigated in all of the isolates by PCR. Complete *vac*A s- and m-region genotypes were obtained in all samples. The majority of them (82 of 115; 71.3%) contained the s1 allele; most of them (80 of 82; 97.5%) were subtype s1a, and 2 of 82 (2.4%) were subtype s1b, However, 33 of 115 (28.7%) were subtype s2 (Figure 1). In this study, we did not find s1c. With regard to the middle region of 115 strains, 21(18%) samples were positive for the middle regions of the *vac*A genes (m1) and 94 (81.7%) were positive for the middle region (m2) by PCR. Meanwhile, PCR product size was 290 bp and 352 bp for m1 and m2, respectively.

Table 2. Distribution of *vac*A genotypes among 115 *cag*A-positive and *cag*A-negative *H. pylori* strains

Genotype	Number (%) of strains		Total (115)	*Pv*
	***cag*A-positive (n=79)**	***cag*A-negative (n=36)**		
s1/m1	19(100%)	0(0%)	19(16.5%)	0.005
s1/m2	53(84.1%)	10(15.9%)	63(54.8%)	0.001
s2/m1	0(0%)	2(100%)	2(1.7%)	0.005
s2/m2	7(22.6%)	24(77.4%)	31(26.9%)	0.001

Table 3. Relationship between clinical outcome and status of *cag*A and *vac*A genotypes by logistic regression analysis

Genotypes	Number (%) of isolates		Total (n=115)	*Pv*
	NUD (n=53)	**PUD (n=62)**		
*vac*As1	34(29.6%)	48 (41.7%)	82 (71.3%)	0.5
*vac*As1a	32 (27.8%)	47 (40.9%)	79 (68.7%)	1
*vac*As1b	1(0.9%)	1 (0.9%)	2 (1.8%)	1
*vac*As2	19 (16.5%)	14 (12.2%)	33 (28.7%)	0.1
*vac*Am1	7 (6.1%)	14 (12.2%)	21 (18.3%)	0.3
*vac*Am2	46 (40%)	48 (41.7%)	94 (81.7%)	0.1
*vac*As1m1	6 (5.2%)	13 (11.3%)	19 (16.5%)	0.1
*vac*As1m2	28 (24.3%)	35 (30.4%)	63 (54.8%)	0.1
*vac*As2m1	1 (0.9%)	1 (0.9%)	2 (1.8%)	0.1
*vac*As2m2	18 (15.7%)	13 (11.3%)	31 (27%)	0.1
*cag*A	32 (27.8%)	47 (40.9%)	79 (68.7%)	0.07

Discussion

The *cag*A gene is part of a 40 kb DNA insertion that is considered to have the typical features of a bacterial pathogenicity island (PAI) and may have originated from a non- helicobacter source. In the present study, 68.7% of the patients were infected with *cag*A-positive strains, similar to another Iranian study.[13] However, this is different from studies from East to South Asian countries where more than 90% of the strains carry the *cag*A gene regardless of clinical outcomes.[14-16] Our result is consistent with studies reported from Europe and the USA where the prevalence of *cag*A-positive strains is between 60-70%.[9,17]

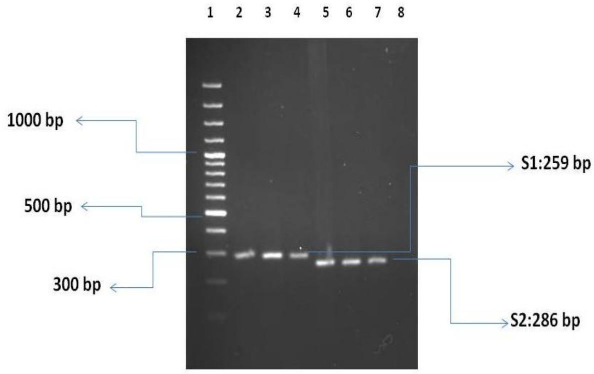

Figure 1. Amplified products of signal region alleles (S1 and S2) by PCR

Lane 1: 100-bp DNA ladder, Lane 2 and 3: S1 genotype *H. pylori*, Lane 4: S1 positive control of *H. pylori*, Lane 5 and 6: S2 genotype *H. pylori*, Lane 7: S2 positive control of *H. pylori*, Lane 8: negative control.

In this study, the relationship between *cag*A and clinical outcomes was assessed, and although we found that 59.5% of PUD and 40.5% of NUD patients were infected with *cag*A-positive strains, while this findings was not statistically significant (P*v* > 0.05). This finding is in agreement with other reports from Iran,[18-20] but in contrast to many studies from Western countries where *cag*A positive strains are more often isolated from patients with PUD than with NUD.[21] For this difference in the *cag*A status, one possibility which exist is the large genomic variations in the *H. pylori* genomes (e.g., a PCR primer set) that amplifies the *cag*A gene of *H. pylori*.[22] There may be several distinct forms of the *cag*A gene with an uneven geographical distribution and these differences in *cag*A genotypes may provide a marker for differences in virulence among *cag*A-positive *H. pylori* strains and that only some forms of the *cag*A gene are associated with severe gastroduodenal diseases.[23]

All strains of *H. pylori* contain the *vac*A gene, but they vary in terms of their ability to produce cytotoxin.[24] Type s1 and m1 strains demonstrate more toxin activity than s2 and m2 strains.[11,25] The *vacA* genotypes are significantly different in each country. In Western studies, the presence of *vac*As1 and *cag*A has been shown to be significantly associated with peptic ulcers.[26] However, several studies in Asian populations have not confirmed this relationship, indicating that there are important geographic differences.[15] In this research, the frequency results of *vac*A alleles are in agreement with another study from Iran[27] which was reported frequency of s1, s2, m1 and m2 as 69%, 28%, 31% and 61%, respectively.

In our study, we evaluated the combination of *vac*A gene of different alleles in relation to clinical outcomes and no statistically significant correlation was found between these alleles and disease conditions (*pv*>0.05). In this study, predominance of s1 and s1m2 genotypes of *vac*A was observed in all clinical outcomes in patients which is in agreement with other studies from Iran which showed s1 allele is associated with PUD, including DU and GU and also s1/m2 strain is dominant genotype among infected Iranian patients.[18,19,28-30] Similarly, s1/m2 genotype has been found to be predominant in Turkey and in Western countries.[31] However, the *vac*A s1/m1 genotype is more predominant from Afghanistan and India.[32,33]

In the present study, we examined the diversity of the *vac*A gene and the relationship between *vac*A genotypes and *cag*A status with clinical outcomes. The *vac*A s1/m1 genotype was the most virulent genotype, although the prevalence was even higher in PUD than in NUD patients (13 versus 6), but the differences were not statistically significant (*Pv* > 0.05). The prevalence of the s2/m2 genotype, which is reported to be less virulent, was even lower in PUD than in NUD patients (13 versus 18), but again the difference was not statistically significant (*Pv* > 0.05). We also analyzed the signal region and middle region separately, however, no significant relation was found between *vac*A s and m genotypes and clinical outcomes. There are many reports, that s1/m1 genotypes were associated with clinical outcomes such as PUD, whereas s2/m2 genotypes were associated with NUD.[34-36] However, we could not find any relationship between *vac*A genotypes and clinical outcomes. We found that s1/m2 was the most prevalent genotype irrespective of the clinical outcomes. Several studies have been published about the relationship between clinical outcomes and *vac*A and *cag*A status in Iranian populations,[29,30,37,38] where it has been concluded that the *vac*A genotypes are not a good marker for predicting clinical outcomes. In contrast, a study from Shiraz was reported that *vac*A genotypes were significantly different among gastritis, PUD and GC patients.[30] In addition, another study from Shiraz reported that *vac*A genotypes were more frequently found in PUD patients than in NUD patients;[37] since it is well known that almost all strains should possess the *vac*A gene, there finding are questionable.[37] The clinical relevance of the considered virulence- associated genes of *H. pylori* and geographical area is still a subject of controversy. The discrepancy between these reports may have several causes. First, patient selection is extremely important, and the study group should be sufficiently large and diverse with respect to genotypes and clinical symptoms. Second, the PCR assay and typing methods used should be adequate to determine the *vac*A and *cag*A genotypes.

Conclusion

In the present study relationship between *cag*A and *vac*A genotypes and clinical status was not found, which suggest that these genes are not helpful for the universal prediction of specific disease risk. Overall, we found that *vac*As1/m2, *cag*A-positive strains are predominant in our isolates irrespective of clinical outcome.

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Conflict of interest

The authors report no conflicts of interest.

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