

Association of *H pylori cagA* and *vacA* genotypes and IL-8 gene polymorphisms with clinical outcome of infection in Iranian patients with gastrointestinal diseases

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10.47; $P = 0.005$).

CONCLUSION: The IL-8 -251 A/T polymorphism and the polymorphisms in *H pylori vacA* gene are involved in limiting the infection outcome to gastritis and peptic ulcer or in favoring cancer onset in Iranian patients.

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Key words: Interleukin 8; *H pylori*; Gastric cancer; Peptic ulcer; Polymorphism

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Abstract

AIM: To find out if a functional promoter polymorphism in the IL-8 gene along with *cagA* status and polymorphisms in *vacA* gene influence the type of diseases in Iranian patients infected by *H pylori*.

METHODS: IL-8 -251 A/T polymorphism was genotyped by oligonucleotide allele specific PCR (ASO-PCR) in a sample of 233 patients with *H pylori* infection undergoing upper gastrointestinal endoscopy. The presence of *cagA* gene and polymorphisms in *vacA* gene was also determined by PCR. Association of these genetic polymorphisms with the development of gastritis, peptic ulcers as well as gastric cancer was tested.

RESULTS: When the patients with different clinical manifestations were compared according to the presence of *cagA* gene or various *vacA* genotypes, only the *vacA* genotypes were significantly different among gastritis, peptic ulcer and gastric cancer patients ($\chi^2 = 17.8$; $P = 0.001$). Furthermore, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases ($\chi^2 =$

INTRODUCTION

Infection with *H pylori* has plausible associations with a variety of clinical outcomes, including chronic gastritis, peptic ulcer and gastric cancer^[1-4]. Variation in the clinical outcome of *H pylori* induced pathology is multifactorial, involving a complex interplay between the host immune responses, pathogen virulence factors, and niche characteristics. Many putative virulence factors have been identified in *H pylori* that contribute to its pathogenesis. The 128-kDa cytotoxin-associated gene encoded antigen A (*cagA*)^[5] and vacuolating cytotoxin antigen gene (*vacA*)^[6] are known as the most important ones. *cagA* gene was identified as a strain-specific *H pylori* gene and has been recognized as a marker for strains that confer increased risk for peptic ulcer disease^[7-8] and gastric cancer^[9]. The *cagA* gene is present downstream of a 40-kb cluster of virulence genes known as the *cag* pathogenicity island (*cag*-PAI). These genes encode a type IV secretion system that forms a syringe-like structure to translocate the immunodominant *cagA* protein into the gastric epithelial cells. *cag*-PAI has also been implicated in the induction of IL-8 in cultured gastric cells^[10]. This property contributes to the proinflammatory power of the strains and thus

to their virulence capability. The difference between *H pylori* strains in virulence capability is also dependent on the expression of *vacA* (87 kDa), which is toxic to epithelial cells *in vitro*^[11-13]. Moreover, mice which were administered *vacA* orally developed gastric ulcers^[14]. Unlike the *cagA*, *vacA* gene is conserved among all *H pylori* strains, although significant polymorphism exists in its gene^[15]. *vacA* alleles possess one of two types of signal regions, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations. The *vacA* signal region encodes the signal peptide and the N-terminus of the processed *vacA* toxin: type s1 *vacA* is fully active, but type 2 has a short N-terminus extension that blocks vacuole formation^[16]. *vacA* s2 strains are rarely isolated from patients with peptic ulcers or gastric adenocarcinoma^[15]. The *vacA* mid-region encodes part of the toxin cell binding domain. Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes^[16]. Consequently, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma^[17,18]. The genetic heterogeneity in immune responses among individuals is another important factor which determines the clinical outcome of *H pylori* infection. Support for this consideration is provided by the low frequency of gastric cancer in some developing countries in spite of the paradoxically high prevalence of *H pylori* infection in those countries^[19,20]. Up to now, there are several reports indicating the association of IL-1 β , tumor necrosis factor α (TNF- α), and IL-10 gene polymorphisms with an increased risk of developing gastric atrophy, hypochlorhydria, and non-cardia gastric cancer^[21-23]. Due to the roles which are played by IL-8 in the pathogenesis of *H pylori* infection, the IL-8 gene is one of the most important candidate host genes in determination of the outcome of *H pylori* infection. This cytokine is produced by gastric epithelial cells as an early response to *H pylori* virulence factors, such as *cagA*^[10]. IL-8 is also a major host mediator involved in neutrophil and phagocyte chemotaxis and activation^[24,25], thereby causing mucosal damage by releasing reactive oxygen radicals^[25]. It is therefore tempting to speculate that mucosal IL-8 production due to *H pylori* infection may be an important factor in the immunopathogenesis of peptic ulcer diseases and may also be relevant in gastric carcinogenesis^[26]. Interestingly, previous studies have suggested that the production of IL-8 is genetically determined and neutrophils from individuals who are homozygous for the AA genotype at the -251 position demonstrated a trend toward higher levels of IL-8 production in response to lipopolysaccharides than those without the allele^[27]. More recently, Ohyauchi *et al* reported that IL-8 -251 A/T polymorphism influences the susceptibility of *H pylori* related gastric diseases in the Japanese population^[28]. Furthermore, in an *H pylori* infected Chinese population the risk of gastric cancer was also significantly elevated in patients with the IL-8-251 AA genotype^[29]. Considering the above information, the aim of this study was to look for an association between IL-8 -251 A/T polymorphism, *vacA* genotypes, the presence of *cagA* gene and clinical outcome of *H pylori* infection in Iranian patients.

MATERIALS AND METHODS

Patients and Bacterial strains

In the present study 298 patients were classified at the time of endoscopy into those having gastritis ($n = 199$), gastric ulcer ($n = 12$), duodenal ulcer or duodenitis ($n = 67$) and non-cardia gastric carcinoma ($n = 20$). This classification was also confirmed by histological examinations. These patients have been referred for upper gastrointestinal endoscopy to the Gastroenterology Section of the University Hospitals (Namazi and Shahid Faghihi) of Shiraz University of Medical Sciences between 2002 and 2005. Patients who had received non-steroidal anti-inflammatory drugs were excluded. *H pylori* strains were successfully isolated from the gastric biopsies of 286 patients (150 males, 136 females; median age 45.3 ± 16.6 years). The present study was approved by the local ethics committee.

Bacterial culture and histological examination

Biopsy specimens were taken from the antrum and corpus of the stomach. These specimens were used for the rapid urease test, bacterial culture, and histological assessment. After 5 d of culture on selective agar plates, the organisms were identified as *H pylori* by Gram staining, colony morphology, and positive oxidase, catalase and urease reactions.

Preparation of patients and H pylori genomic DNA

After 3-5 d of culture, *H pylori* colonies were pooled from the plates and washed using phosphate-buffered saline. *H pylori* genomic DNA was prepared after bacterial cell lysis using SDS and proteinase K solution and phenol-chloroform extraction. Patients genomic DNA was extracted from EDTA anticoagulated peripheral blood leucocytes using a salting out method. The DNA samples were maintained at -70°C until use in polymerase chain reaction (PCR).

Analysis of IL-8 and bacterial vacA and cagA genotypes

All primer sets used were selected from the published literature and are shown in Table 1. An allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was used to detect the polymorphism at position -251 of the IL-8 gene^[30]. As an internal control, the β -globin specific primers were included in the ASO-PCR (Table 1). For IL-8 genotyping, 10 μL of PCR reaction mixture consisting of 250 ng of genomic DNA, 200 $\mu\text{mol/L}$ dNTPs, 2.25 mmol/L MgCl_2 , 1 \times Taq DNA polymerase buffer, 2 units of Taq DNA polymerase (Boehringer Mannheim, Germany), 10 pmol of each test primer and 5 pmol of internal control primers were employed. Then, a touch-down procedure was followed that consisted of 25 s at 95°C , annealing for 45 s at temperatures decreasing from 68°C (four cycles) to 61°C (20 cycles), and an extension step at 72°C for 40 s. The annealing temperature for the remaining 5 cycles was 58°C for 40 s. For *cagA* and *vacA* genes the PCR was performed using a thermal cycler (Master Cycler; Eppendorf, Germany) under the following conditions: an initial denaturation for 5 min at 94°C , 35 cycles of 60 s at 94°C , 60 s at 57°C and 60 s at

Table 1 Primer sequences used for detection of *cagA* gene status and IL-8 or *vacA* gene polymorphisms

Locus	Primers	Size (bp)
IL-8 -251	Common primer, 5'-tgc ccc ttc act ctg tta ac-3' A allele, 5'-cca caa ttt ggt gaa tta tca at-3' T allele, 5'-cca caa ttt ggt gaa tta tca aa-3'	336
β -globin	5'-aca caa ctg tgt tca cta gc-3' 5'-caa ctt cat cca cgt tca cc-3'	100
<i>cagA</i>	5'-aat aca cca acg cct cca ag-3' 5'-ttg ttg ccg ctt ttg ctc tc-3'	400
<i>vacA</i>		
S1 and s2	5'-ctg ctt gaa tgc gcc aaa c-3' 5'-atg gaa ata caa caa cac-3'	s1 = 259 s2 = 286
m1 and m2	5'-gcg tct aaa taa ttc cca gg-3' 5'-caa tct gtc caa tca agc gag-3'	m1 = 570 m2 = 645
<i>glmM</i>	5'-aag ctt tta ggg gtg tta ggg gtt t-3' 5'-aag ctt act ttc taa cac taa cgc-3'	294

72°C, with a final extension step at 72°C for 5 min. The PCR system for *cagA* contained 10 × PCR buffer, 2.5 μ L; MgCl₂, 1.2 mmol/L; dNTP, 200 μ mol/L; *cagA* specific primers, 10 pmol; Taq DNA polymerase, 1.0 U; and the DNA template, 50 ng. To confirm the identification of the bacteria as *H. pylori*, 10 pmol of *glmM* specific primers were included in each PCR reaction for *cagA* gene (*glmM* is a conserved gene formerly known as *ureC*). For detection of *vacA* polymorphisms a multiplex PCR was performed using the specific primers for amplification of s and m genes (Table 1). The PCR system for *vacA* genotyping was similar to *cagA* genotyping. PCR products were examined by 2% agarose gel electrophoresis and photographed using an ultraviolet reflection analyzer.

Statistical analysis

Fisher's exact test and the χ^2 test were used to analyze the data from different disease groups. All tests were performed two tailed with a confidence interval (CI) of 95%. A *P*-value of less than 0.05 was accepted as statistically significant. The Statistical Package for the Social Sciences (SPSS) version 11.5 was used for statistical analysis.

RESULTS

Prevalence of the *cagA* and *vacA* gene in different disease groups

Table 2 shows the distribution of the 286 strains according to their *cagA* and *vacA* types in different patient groups. To detect the *cagA* subtype of *H. pylori*, *cagA* specific PCR was performed on extracted bacterial DNA from patients with gastric diseases. Amplified *cagA* DNA fragments were detected in 219/286 (76.6%) of our *H. pylori*. Sixty seven patients (23.4%) were *cagA*-, although they were *glmM*+ or *vacA*+. When the patients with different clinical manifestations were compared according to the presence of the *cagA* gene (Table 2), insignificant differences in *cagA* status were found among patients with peptic ulcer, gastritis and gastric cancer ($\chi^2 = 1.9$; *P* = 0.38). Also, when the frequency of *cagA*+ and *cagA*- subtypes were compared among patients with gastritis and gastric ulcer

Table 2 IL-8 -251 A/T polymorphism, *cagA* status and *vacA* gene polymorphism in patients with gastric diseases

Locus	Genotype	Patients Gastritis <i>n</i> (%)	Peptic Ulcer <i>n</i> (%)	Gastric Cancer <i>n</i> (%)	<i>P</i> value
IL-8 -251	AA	22 (14.4)	14 (23.0)	9.0 (47.4)	0.013
	AT	74 (48.4)	28 (45.9)	6.0 (31.6)	
	TT	57 (37.3)	19 (31.1)	4.0 (21.1)	
<i>cagA</i>	+	148 (74.4)	54 (80.6)	17 (85.0)	0.39
	-	51 (25.6)	13 (19.4)	3.0 (15.0)	
<i>vacA</i>	s1m1	48 (26.5)	21 (32.3)	12.0 (66.7)	0.001
	s1m2	74 (40.9)	33 (50.8)	3.0 (16.7)	
	s2m2	59 (32.6)	11 (16.9)	3.0 (16.7)	

($\chi^2 = 1$; *P* = 0.3), gastritis and peptic ulcer ($\chi^2 = 0.75$; *P* = 0.38), gastritis and cancer ($\chi^2 = 0.61$; *P* = 0.42) or between peptic ulcer and gastric cancer ($\chi^2 = 0.01$; *P* = 0.75), the difference always remained insignificant. In the case of *vacA* gene, three of the four possible combinations of signal sequence and middle-region types were identified. The s1/m1 type was found in 81/264 (30.7%) of the isolates, the s1/m2 type in 110/264 (41.7%) of the isolates, and the s2/m2 type in 73/264 (27.7%) of the isolates. The distribution of *vacA* genotypes (s1/m1, s1/m2 or s2/m2) were significantly different among peptic ulcer, gastritis and gastric cancer patients ($\chi^2 = 17.8$; *P* = 0.001). Interestingly, the difference in distribution of three different *vacA* genotypes between patients with gastric cancer and gastritis ($\chi^2 = 12.57$; *P* = 0.0018) was more significant than the difference in distribution of *vacA* genotypes between patients with gastric cancer and peptic ulcer ($\chi^2 = 7.97$; *P* = 0.018). In fact, the frequency of s1m1 genotype was notably higher in gastric cancer patients (66.7%) compared to those with gastritis or peptic ulcer (26.5% and 32.3%, respectively). Furthermore, similar to previous reports^[15], there is a strong statistical linkage between the s1 genotype of *vacA* and the presence of the *cag* island ($\chi^2 = 27.95$; *P* = 0.0000001; OR = 4.99, 95% CI = 2.61-9.58). Similarly, the s2 genotype is associated with the lack of the *cag* island. In fact, strains that are *cag*+ are more likely to possess the *vacA* s1 allele than *cag*- strains.

Prevalence of the IL-8 -251 A/T polymorphism in different disease groups

The allelic frequencies of IL-8 -251 A/T polymorphism and genotype distributions are given in table 2. The IL-8 -251 A/T polymorphism showed no evidence of deviation from the Hardy-Weinberg equilibrium, with a non-significant χ^2 value ($\chi^2 = 0.05$, *P* = 0.4). Interestingly, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and those with benign diseases ($\chi^2 = 10.47$; *P* = 0.005). Moreover, when the patients were categorized to high producer (AA) and intermediate + low producer (AT + TT) genotypes, a more meaningful difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases was noticeable (OR = 4.45, 95% CI = 1.53-12.94; $\chi^2 = 8.58$, *P* = 0.003). In fact, the prevalence of AA genotype in gastric cancer

patients was 47.4% compared to 16.8% in benign diseases. While comparing different patient groups, no significant differences were demonstrated in frequencies of IL-8 -251 A/T genotypes between patients with peptic ulcer and gastritis ($\chi^2 = 2.4$; $P = 0.25$) or gastric cancer ($\chi^2 = 4.22$; $P = 0.12$). However, IL-8 -251 A/T polymorphism showed a significant difference between patients with gastric cancer and gastritis ($\chi^2 = 12.5$; $P = 0.001$).

DISCUSSION

After exposure to *H pylori*, the clinical manifestations are variable and depend on host and pathogen factors. There is no information on the prevalence and role of *H pylori* genotypes and/or the role of IL-8 -251 A/T genotypes in the disease outcome of Iranian patients. Therefore, in the present study, we determined the presence of the *cagA* gene (as a marker of *cag* pathogenicity island) and the genotypes of *vacA* gene in the infecting strains along with the distribution of host IL-8 genotypes in relation to the occurrence of different clinical manifestations in Iranian patients with *H pylori* infection. It has been shown that exposure of gastric epithelial cells to *cag+* *H pylori* strains can activate the proto-oncogenes *c-fos* and *c-jun*, a crucial step in the development of *H pylori*-related neoplasia^[31]. The presence of *cagA* has been statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer^[7-9], although some studies deny this association^[32-34]. Audibert and her colleagues reported that *cagA* status is not sufficient to predict the IL-8 induction ability of *H pylori* and is not correlated with the presence or absence of ulcer^[35]. In a series of patients from Taiwan, the presence of *cagA* gene in the PAI also showed no relationship to the type of disease and/or the histological features of the patients^[36]. The results of the present study also shows that the prevalence of *cagA* is not significantly different among different disease groups ($P > 0.05$), which is in accordance with the results of other Asian countries. Therefore, the *cag*-PAI may not be the principal virulence factor, as suggested by the absence or sporadic distribution of the *cag*-PAI genes among strains from varied clinical outcomes. However, considering the high prevalence of *cag+* strains in Iranian patients (76.6%), the relationship of *cag* status with disease type is more difficult to establish in our population. Therefore, larger sample sizes are recommended for such studies.

Furthermore, the lack of association may be due to the fact that the development of cancer or ulcer disease is a complex process that also involves factors other than the *cag*-PAI, such as *vacA*. Certain *vacA* genotypes causing a high vacuolating activity are correlated with increased disease severity in humans^[15]. Several studies have also shown that the presence of *vacA* is associated with peptic ulcer diseases^[14,17-18]. The *vacA* gene displays a considerable polymorphism, especially in the signal region (genotypes s1 and s2) and in a mid region (genotypes m1 and m2). Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes^[15]. Consequently, *vacA* s1/m1 strains cause more direct epithelial damage and are more frequently associated with

peptic ulceration and gastric carcinoma^[9,17,18]. The results of the present study also show a significant difference in *vacA* genotype distribution between gastric cancer and gastritis ($P = 0.0018$) or peptic ulcer ($P = 0.018$) patients. The marked increase of s1m1 genotype in gastric cancer patients (66.7%) compared to those in patients with gastritis (26.5%) or peptic ulcer (32.3%) confirms the pathogenic role of this virulence determinant in Iranian patients. However, different disease outcomes were encountered in subjects infected with *H pylori* strains sharing the same virulent *vacA* genotype, s1m1. The different outcomes of *H pylori* infection may depend not only on other bacterial factors but also on the different genetic background of the host. Concerning host genetic factors, Thye *et al* performed a genome wide screen analysis to identify the genetic factor(s), which define susceptibility to *H pylori* infection^[37] and suggested the presence of a possible linkage with chromosomes^[4]. Considering the location of the human IL-8 gene on chromosome 4 (4q13-q21), the results of their study may support the hypothesis that the IL-8 gene polymorphism is probably associated with *H pylori* induced gastrointestinal diseases. Interestingly, the inheritance of the IL-8 -251A allele was associated with progression of gastric atrophy in patients with *H pylori* infection and increased the risk of gastric cancer in Japanese and Chinese people^[28,29,38]. Our results also indicate that gastric cancer is significantly associated with the functional polymorphism in the promoter region of the IL-8 gene. Specifically, individuals genetically predisposed to produce more IL-8 are at a higher risk of developing gastric cancer. The finding that there was an increased risk of gastric cancer in high IL-8 producers was in agreement with the concept that IL-8 may influence the risk of developing gastric cancer by altering the quality and vigor of inflammatory responses produced by the host after exposure to *H pylori*. In addition, IL-8 stimulated neutrophils to synthesize active radicals such as nitric oxide^[25]. These radicals by their mutagenic potential^[39] could cause mutations in gastric epithelial cells. In addition, IL-8 by inducing angiogenesis would be one of the important factors in gastric carcinogenesis. In support of this hypothesis, the expression of IL-8 has been associated with increased vascularization and poor prognosis in gastric carcinoma^[40,41]. Thus, inheritance of the high producer allele of IL-8 (carriers of -251 A allele) may induce chronic gastritis, which may then be followed by the development of gastric cancer.

In conclusion, similar to studies performed in China and Japan, the association between *cagA* positivity and virulence of *H pylori* strains was equally frequent among Iranian patients with different disease types. Moreover, the present study provides further evidence that in addition to genetic polymorphism of the *vacA* gene in the pathogen, genetically determined differences in IL-8 production via promoter polymorphisms could contribute to individual susceptibility to gastric cancer development after *H pylori* infection in Iranian patients.

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