

## Original Article

# Helicobacter pylori vacA d1/-i1 Genotypes and Geographic Differentiation between High and Low Incidence Areas of Gastric Cancer in Iran

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## Abstract

**Background:** *Helicobacter pylori* (*H. pylori*)-specific genotypes have been closely correlated with an increased risk of gastric cancer (GC). The present study aimed to determine the distribution of *H. pylori* pathogenic genotypes amongst Iranians infected with strains representing European ancestry in areas with different GC incidence.

**Methods:** A total of 138 *H. pylori* isolates from ten districts in Iran were used for genotyping.

**Results:** The following genotypic frequency was observed: *vacA* s1 (94.9%), s2 (5.1%), m1 (24.6%), m2 (75.4%), d1 (39.9%), d2 (60.1%), i1 (40.6%), i2 (59.4%), *iceA1* (76.8%), *iceA2* (52.9%), *iceA1/2* (29.7%), *babA2* (40.6%), and *cagA* (65.9%). Hierarchical analyses of molecular variance (AMOVA) for the *vacA* d1, d2, i1, and i2 alleles and *iceA1* and *iceA1/2* genes found significant levels of genetic differentiation among populations ( $P < 0.05$ ). Prevalence of the *vacA* d1, i1, and *iceA1/2* (but not *iceA1*) genes and *vacA* d1/i1, *vacA* d1/*iceA1*, *vacA* d1/*iceA1/2*, *vacA* d1/*cagA*+, *vacA* i1/*iceA1*, *vacA* i1/*iceA1/2*, and *vacA* i1/*cagA*+ genotypes were significantly higher (>2- or 3-fold) among *H. pylori* isolates from high incidence GC areas that had age-standardized rates (ASRs) of >20/10<sup>5</sup> (max. 51.8/10<sup>5</sup>) when compared with those from low incidence (ASRs <10/10<sup>5</sup>) GC areas ( $P < 0.005$ , for the latter,  $P = 0.016$ ). In contrast, the *vacA* d2/i2, m2/d2, and m2/i2 genotypes were significantly more prevalent in low compared to high incidence GC areas ( $P < 0.005$ ). The results of Mantel's test only showed a low correlation between genetic and geographic distances for the *iceA1* and *iceA1/2* (but not *vacA* alleles, *iceA2*, *babA2*, and *cagA*) genes among ten districts of Iran ( $r = 0.098$  and  $0.074$ , respectively,  $P < 0.05$ ).

**Conclusion:** We propose that the *H. pylori vacA* d1/-i1 genotypes, which are new determinants of GC, have tremendous potential for differentiating *H. pylori* strains from high and low incidence GC areas in Iran.

**Keywords:** *H. pylori*, genotypes, gastric cancer, incidence, Iran

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## Introduction

*Helicobacter pylori* (*H. pylori*) plays an essential role in the development of gastroduodenal diseases, such as chronic atrophic gastritis, peptic ulcers (PUs), MALT-lymphoma, and gastric cancer (GC).<sup>1</sup> GC is the fourth most common cancer and second leading cause of cancer-related deaths worldwide.<sup>2</sup> The bacterium genotypes could determine the outcome of infection when combined with both environmental and host factors.<sup>3</sup> A recent study has shown that *H. pylori* infection, although strongly associated with the risk of GC, does not increase all-cause mortality and may be protective against lung cancer and stroke.<sup>4</sup> Therefore, it is essential to determine which strains of *H. pylori* could increase the risk of GC. One independent *H. pylori* locus that is

correlated with increased risk of disease is vacuolating cytotoxin A (*vacA*) which encodes the secreted toxin VacA.<sup>5</sup> Considerable differences in vacuolating activities are observed between *H. pylori* strains. This variation is attributed to the *vacA* gene polymorphisms within the signal (s), s1 or s2; middle (m), m1 or m2; and the more recently identified intermediate (i), i1 or i2, regions.<sup>2</sup> The i region plays a functional role in vacuolating activity and is strongly associated with gastric adenocarcinoma.<sup>6,7</sup> This association is independent of and larger than the associations of *vacA* s- or m-type or *cag* status with gastric adenocarcinoma. Recently, an 81 bp deletion located between the m and i regions has been identified and termed the deletion (d) region, as d1 (no deletion) or d2 (with deletion). The d1 type of the *vacA* gene is strongly associated with neutrophil infiltration and gastric mucosal atrophy in both the antrum and the corpus. Thus, the d region genotype is proposed to be an important risk locus for GC in Western strains.<sup>8</sup> The cytotoxin-associated gene A, *cagA*, is the other putative virulence factor of the bacterium which is much more likely to be associated with the development of GC.<sup>9,10</sup> The ulcer-associated *H. pylori* gene, *iceA1*, which is induced by contact with the epithelium is also clinically relevant because the strains harboring this genotype are associated with the development of PUs.<sup>11,12</sup> The other virulence gene that presents in some *H. pylori* strains is *babA2* whose product is a blood-group antigen binding adhesion. It mediates adherence of *H.*

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*pylori* to Lewis b (blood group antigen) in the human gastric epithelial cells.<sup>13</sup> A number of studies have shown a significant correlation between the presence of the *babA2* gene and the development of atrophic gastritis or GC.<sup>14,15</sup>

GC incidence is high in Iran, with age-standardized rates (ASRs) of  $26.1/10^5$  for males and  $11.1/10^5$  for females. It is the first cause of cancer-related deaths in males and the third cancer after breast and colorectal cancers in females.<sup>16,17</sup> There is strong evidence of increased risk of GC in populations with higher rates of *H. pylori* infection.<sup>18</sup> Iran is a country with a high prevalence of *H. pylori* infection (69%).<sup>19</sup> The highest frequency (89%) is reported from Ardabil, a Northwestern province of Iran, where greater than 90% of adults aged 40 or over suffer from chronic gastritis related to *H. pylori* infection; GC is the most common malignancy (31%) with ASRs of  $51.8/10^5$  for males and  $24.9/10^5$  for females.<sup>20-22</sup> The incidence of GC in the Northern districts of Iran, particularly Guilan, Mazandaran, and Golestan Provinces located in Caspian Sea littoral has been reported to be considerably high (ASRs  $>20/10^5$ ), whereas it is low in the Southern regions of Iran, such as Khuzestan, Kerman, and Yazd provinces (ASRs  $<10/10^5$ ).<sup>16,23-25</sup> However, the actual role of bacterial/host factors and geographic or ethnic differences in the incidence of GC in Iran, especially in the high incidence Northern latitude is not clear. In a recent study, we have indicated that the *H. pylori* status in Iran is strongly influenced by genetic exchange with neighboring countries and the ethnic and geographic differentiation has been well persevered within the country.<sup>26</sup> Iranian *H. pylori* strains are similar to others isolated from Western Eurasia and can be placed in the previously described *HpEurope* population. It has been shown that the geographic differences in the cancer risk deriving from *H. pylori* infection could be explained by the phylogeographic origin of *H. pylori* strains. The strains with *European* origin are strongly predictive of increased premalignant histological lesions and epithelial DNA damage.<sup>27</sup> Although Iranian *H. pylori* strains represent *European*

phylogeographic origin, the 2- to 10-fold difference in the incidence of GC between the Northwestern-Northern and Southern regions of Iran is controversial. One hypothesis is the geographic difference in the allelic profiles of *H. pylori* virulence genes in Iran, which show rapid evolution and are largely influenced by selective pressures.

The aim of the present study was to determine: i) Is there any difference in the allelic profiles of *H. pylori* virulence genes between Iranian populations from different geographic locations of Iran, when comparing isolates from high and low incidence areas of GC? ii) Is there a north-south cline in the allelic frequency of *H. pylori* virulence genes in Iran?

## Materials and Methods

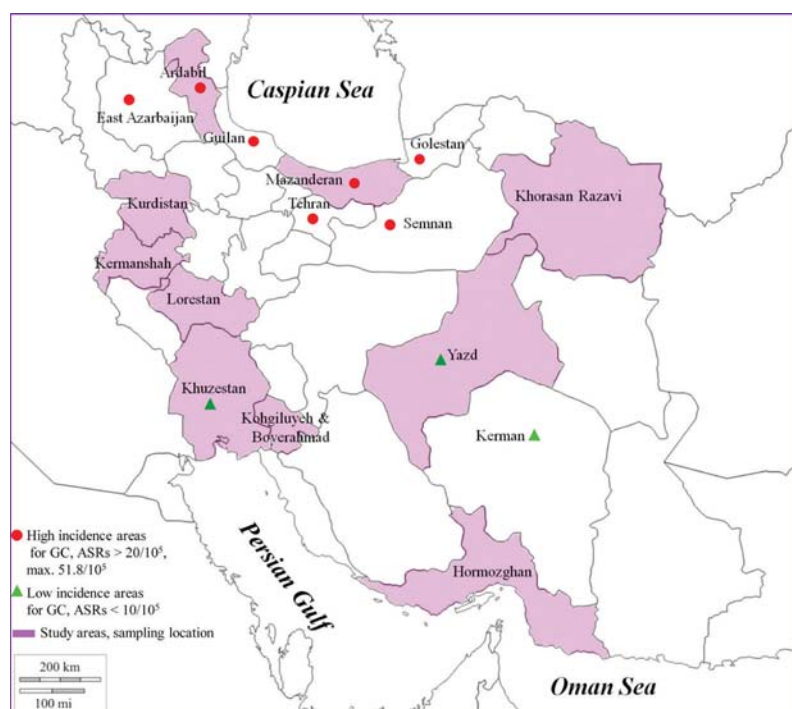
### Patients

A total of 280 patients participated in the study from 2007–2009. Patients referred to the reference endoscopy units in ten districts of Iran that included the high and low incidence areas for GC (Figure 1). Biopsies were taken from patients who were of Iranian nationality, had the same place of birth and residency, and who gave the same ethnic/linguistic origin for both of their parents and for all four of their grandparents.

The study was approved by the Ethics Committee of the Digestive Diseases Research Institute, Shariati Hospital, Tehran University of Medical Sciences based on the ethical principles of human research and experimentation expressed in the Declaration of Helsinki. All subjects underwent endoscopies as part of their treatment process. Informed consent for participation in the study was given by each subject in writing. The structured ethnic/linguistic questionnaire was completed for each subject by direct interview.

### Bacterial isolates and cultivation

A total of 138 *H. pylori* isolates were obtained from gastric bi-



**Figure 1.** Detailed map showing highlighted geographical locations where *H. pylori* isolates were obtained. The high and low incidence areas for gastric cancer (GC) are shown.

**Table 1.** Oligonucleotide primers used for PCR.

Genes	Primers	Sequences (5'→3')	Size of PCR products (bp)	Optimized annealing temperature (°C)	References	
<i>16S rDNA</i>	HP1	GCAATCAGCGTCAGTAATGTTTC	519	56	28	
	HP2	GCTAAGAGATCAGCCTATGTCC				
<i>vacA</i>	s1/-s2	s1a-F	GTC AGC ATC ACA CCG CAA C	s1a: 190	54	46
		s1a-R	CTG CTT GAA TGC GCC AAA C			
		s1b-F	AGC GCC ATA CCG CAA GAG	s1b: 187	54	
		s1b-R	CTG CTT GAA TGC GCC AAA C			
		s2-F	GCT AAC ACG CCA AAT GAT CC	s2: 199	54	
		s2-R	CTG CTT GAA TGC GCC AAA C			
	m1/-m2	VAG-F	CAA TCT GTC CAA TCA AGC GAG GCG	m1: 570	56	47
		VAG-R	GCG TCT AAA TAA TTC CAA GG			
	i1/-i2	VAC F1	GTTGGGATTGGGGGAATGCCG	i1: 426	53	6
		C1R	TTAATTTAACGCTGTTTGAAG			
		VACF2	GTTGGGATTGGGGGAATGCCG			
		C2R	GATCAACGCTCTGATTGA	i2: 432	53	
	d1/-d2	VAS-5 F	ACTAATATTGGCACACTGGATTG	d1: 367–379	45	8
VAGF-R		CTCGCTTGATTGGACAGATTG				
	mD1-F	AGGTYATTAACCCACCCAA	d1: 223	45	This study	
	md1-R	CTCGCTTGATTGGACAGATTG				
<i>cagA</i>	N-terminus	cagAN-F	CCATTTTAAGCAACTCCATAAACC	413	56	48
		cagAN-R	CTGCAAAAAGATTGTTGGCAGA			
	Central region	cagAC-F	GGCAATGGTGGTCTCGGAGCTAGGC	243	55	48
		cagAC-R	GGAAATCTTTAATCTCAGTTCGG			
	C-terminus	CAG1	ACC CTAGTC GGT AAT GGG TTA	591–856	50	49
		CAG2	GTA ATT GTC TAG TTT CGC			
<i>babA</i>	<i>babA2</i>	BABA2F	AATCCAAAAAGGAGAAAAAGTATGAAA	832 or 601	55	42, 46
		BABA2R	TGTTAGTGATTTCGGGTAGGACA			
		BABA2R607	CTTTGAGCGCGGGTAAGC			
<i>iceA</i>	<i>iceA1</i>	iceA1-F	GTGTTTTTAACCAAAGTATC	247	48	50
		iceA1-R	CTATAGCCASTYTCTTTGCA			
	<i>iceA2</i>	iceA2-F	GTTGGGTATATACAATTTAT	229 or 334	48	
		IceA2-R	TTRCCCTATTTCTAGTAGGT			

**Table 2.** Frequency of the *vacA* alleles and *iceA1*, *iceA2*, *babA2*, and *cagA* genes in *H. pylori* isolates from different geographic locations in Iran.

Provinces	Strains (N)	Allele frequency (%)												
		<i>vacA</i> s1	<i>vacA</i> s2	<i>vacA</i> m1	<i>vacA</i> m2	<i>vacA</i> i1	<i>vacA</i> i2	<i>vacA</i> d1	<i>vacA</i> d2	<i>cagA</i> +	<i>babA2</i>	<i>iceA1</i>	<i>iceA2</i>	<i>iceA1/2</i>
Ardabil	28	27 (96.4)	1 (3.6)	8 (28.6)	20 (71.4)	16 (57.1)	12 (42.9)	15 (53.6)	13 (46.4)	20 (68.9)	9 (32.1)	24 (85.7)	19 (67.8)	15 (53.5)
Mazanderan	13	12 (92.3)	1 (7.7)	7 (53.8)	6 (46.2)	9 (69.2)	4 (30.8)	9 (69.2)	4 (30.8)	11 (84.6)	5 (34.4)	10 (76.9)	6 (46.1)	3 (23.0)
Kurdistan	10	9 (90.0)	1 (10.0)	2 (20.0)	8 (80.0)	4 (40.0)	6 (60.0)	3 (30.0)	7 (70.0)	4 (40.0)	5 (50.0)	3 (30.0)	8 (80.0)	1 (20.0)
Lorestan	10	9 (90.0)	1 (10.0)	2 (20.0)	8 (80.0)	5 (50.0)	5 (50.0)	5 (50.0)	5 (50.0)	7 (70.0)	3 (30.0)	10 (100)	4 (40.0)	4 (40.0)
Kermanshah	11	10 (90.9)	1 (9.1)	1 (9.1)	10 (90.9)	4 (36.4)	7 (63.6)	3 (27.3)	8 (72.7)	8 (72.7)	3 (27.3)	8 (72.7)	9 (81.8)	6 (54.5)
Khorasan Razavi	15	15 (100)	0 (0.0)	1 (6.7)	14 (93.3)	3 (20.0)	12 (80.0)	3 (20.0)	12 (80.0)	9 (60.0)	9 (60.0)	13 (86.6)	5 (33.3)	3 (20.0)
Kohgiluyeh & Boyerahmad	7	7 (100)	0 (0.0)	3 (42.9)	4 (57.1)	2 (28.6)	5 (71.4)	3 (42.9)	4 (57.1)	4 (57.1)	4 (57.1)	6 (85.7)	3 (42.8)	2 (28.5)
Khuzestan	22	22 (100)	0 (0.0)	5 (22.7)	17 (77.3)	6 (27.3)	16 (72.7)	5 (22.7)	17 (77.3)	14 (63.6)	10 (45.4)	17 (72.3)	7 (31.8)	2 (28.5)
Yazd	14	13 (92.9)	1 (7.1)	2 (14.3)	12 (85.7)	3 (21.4)	11 (78.6)	4 (28.6)	10 (71.4)	9 (68.3)	6 (42.8)	9 (64.3)	8 (57.1)	3 (21.4)
Hormozgan	8	7 (87.5)	1 (12.5)	3 (37.5)	5 (62.5)	4 (50.0)	4 (50.0)	5 (62.5)	3 (37.5)	5 (62.5)	2 (25.0)	6 (75.0)	4 (50.0)	2 (25.0)
<b>Total</b>	<b>138</b>	<b>131 (94.9)</b>	<b>7 (5.1)</b>	<b>34 (24.6)</b>	<b>104 (75.4)</b>	<b>56 (40.6)</b>	<b>82 (59.4)</b>	<b>55 (39.9)</b>	<b>83 (60.1)</b>	<b>91 (65.9)</b>	<b>56 (40.6)</b>	<b>106 (76.8)</b>	<b>73 (52.9)</b>	<b>41 (29.7)</b>

opsy cultures of the patients. Gastric biopsies were cultured on selective Brucella agar (Merck, Germany) that contained 10% blood, vancomycin (10 mg/mL; Zakaria, Iran), trimethoprim (5 mg/mL; MP Biomedicals, France), and amphotericin B (4 mg/mL; Bristol-Myers Squibb, USA). Plates were incubated at 37°C under microaerobic conditions and examined for visible bacterial colonies within 3–5 days. Bacterial isolates were identified as *H.*

*pylori* according to Gram's stain, as Gram-negative spiral forms, in addition to positive urease, oxidase and catalase tests, as well as PCR amplification of *H. pylori 16S rDNA*.<sup>28</sup> We performed single colony isolation in order to ensure that each strain consisted of only a single genotype. Bacterial isolates were harvested in brain heart infusion broth (Merck, Germany) enriched with 20% glycerol and 10% inactivated horse serum and stored at -70°C.



### DNA extraction and genotyping

DNA was extracted from *H. pylori* isolates with the Genomic DNA Purification kit (Fermentas, UK) according to the manufacturer's instructions. The bacterial genotypes; the *vacA* signal sequences, s1 (s1a or s1b) or s2; the middle (m1 or m2), intermediate (i1 or i2), and deletion regions (d1 or d2); and the *iceA1*, *iceA2*, *babA2*, and *cagA* genes were determined by PCR. Negative controls included *Escherichia coli* DH5 $\alpha$  and deionized water. The recruited primers are described in Table 1. PCR was performed in a total volume of 30  $\mu$ L that contained 3  $\mu$ L of 10X PCR buffer (CinnaGen, Iran), 200  $\mu$ M of each dNTP (CinnaGen, Iran), 1 mM MgCl $_2$ , 2 U of *Taq* DNA polymerase (CinnaGen, Iran), 0.5  $\mu$ M of each primer, and 25 ng of bacterial DNA. The amplification was performed over a total of 30 cycles. Each cycle consisted of the following steps: denaturation at 96°C for 40 s, optimized annealing temperature for each allele (Table 1) for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 7 min. PCR products were electrophoresed and visualized by a UV transilluminator. Comparison of the PCR products of the *vacA* alleles and the *iceA1*, *iceA2*, *babA2*, and *cagA* genes with the molecular ladder revealed the bands' sizes. The band sizes according to gene and allele are listed in Table 1.

The amplified fragments of the *vacA* alleles and the *iceA1*, *iceA2*, *babA2*, and *cagA* genes from seven isolates were purified, cloned into the compatible site of the pTG19-T PCR cloning vector, and sequenced with both M13 forward and reverse primers using BigDye technology on an ABI3700XL DNA sequencer (Applied Biosystems). The BLAST program (<http://www.ncbi.nlm.nih.gov>) was used to match the nucleotide sequences with the published sequences in GenBank.

### Statistical analysis

We used the hierarchical analyses of molecular variance (AMOVA) in GenAlEx 6.5<sup>29</sup> to determine whether the means of genetic variation in the allelic frequencies of bacterial virulence genes were all equal between the Iranian populations either from the entire populations or those from the high and low incidence areas of GC. This model of analysis separates the total genetic variance into components attributable to different sources of variation. The significance was tested using 999 random permutations. Pearson chi-square ( $\chi^2$ ) and Fisher's exact tests were used to determine whether the frequency of each gene/allele and their combinations differed between the Northwestern-Northern and Southern geographic regions of Iran. The analysis was performed using SPSS v.14. A *P* value of < 0.05 indicated significance. We used the Mantel's test<sup>30</sup> in GenAlEx 6.5 to evaluate the relationship between genetic and geographic distance matrices. This test takes into account that the set of all pair-wise distances, genetic or spatial, is not independent. This test was performed when considering distance matrices between populations from ten districts of Iran. The significance was tested using 999 random permutations.

## Results

We obtained a total of 138 *H. pylori* isolates from dyspeptic patients aged 17–90 years (mean age: 44.47 years). Of these, 69 were female.

Electrophoresis of the *I6S rDNA* PCR products from the 138 *H. pylori* isolates revealed bands of 519 bp which confirmed the

identity of isolates as *H. pylori*. Most isolates with the *iceA2* allele could be divided into two types according to the presence of repeated sequences of 105 nucleotides and whether PCR products were 229 bp (*iceA2*-1) or 334 bp (*iceA2*-2) long. Of 73 *iceA2*-positive isolates, 48 showed PCR products that were 229 bp whereas 28 showed the PCR products of 334 bp. Only three of the isolates had both PCR products (229 bp and 334 bp), however none of these differences were statistically significant. The negative control *E. coli* DH5 $\alpha$  did not produce any PCR product with the *H. pylori*-specific primers.

The *vacA* s1 allele was the most prevalent gene (94.9%, 131/138) compared with the *vacA* s2 allele (5.1%, 7/138). Of 131 *vacA* s1 alleles, 127 (96.95%) were s1a and 4 (3.05%) were s1b. In the 138 strains tested, *vacA* genotypes were present in the following percentages: m1 (24.6%, 34), m2 (75.4%, 104), d1 (39.9%, 55), d2 (60.1%, 83), i1 (40.6%, 56), and i2 (59.4%, 82). The *iceA1* gene was present in 76.8% (106/138) of strains, whereas the *iceA2* gene was present in 52.9% (73/138). A number of strains showed both *iceA1* and *iceA2* genes, namely the *iceA1/2* gene. The frequencies of the *iceA1/2* and *babA2* genes were 29.7% (41/138) and 40.6% (56/138), respectively. The *cagA* gene was the fourth most prevalent gene after *vacA* s1 (94.9%), *iceA1* (76.8%), and *vacA* m2 (75.4%), with a frequency of 65.9% (91/138). Table 2 shows the frequency of the virulence genes and alleles in different loci of the *H. pylori* isolates from different geographic locations in Iran.

The individual AMOVA for the *vacA* d1, d2, i1, and i2, alleles as well as the *iceA1* and *iceA1/2* genes for 138 *H. pylori* isolates from the ten geographic regions of Iran found significant levels of genetic differentiation among populations (*P* < 0.05). The test showed that a considerable portion of the total genetic variation resulted from differences between individual *H. pylori* isolates within-populations, 91% for *vacA* i1 and *iceA1*, 90% for *vacA* i2, 88% for *vacA* d1, and 93% for *vacA* d2 and *iceA1/2*, while the between-populations genetic variation accounted for the remaining 9% (*vacA* i1 and *iceA1*), 10% (*vacA* i2), 12% (*vacA* d1), and 7% (*vacA* d2 and *iceA1/2*). When the analysis was restricted to the Northwestern-Northern (Ardabil and Mazandaran) and Southern (Khuzestan and Yazd) regions of Iran, AMOVA for the *vacA* d1, d2, i1, and i2 alleles and *iceA1/2* gene (but not *iceA1*) revealed a significant genetic difference among populations (*P* < 0.05). AMOVA statistics showed the distribution of genetic variance as 88% and 83% within- and 12% and 17% between-populations for the *vacA* alleles (d1, d2, i1, and i2) and *iceA1/2* gene, respectively.

The results of  $\chi^2$  and Fisher's exact tests showed a significantly higher prevalence of the *vacA* d1 and i1 genotypes and *iceA1/2* gene among *H. pylori* isolates from the high incidence GC areas compared with those from low incidence areas in Iran [24/41 (58.5%) vs. 9/36 (25.0%), 25/41 (61.0%) vs. 9/36 (25.0%), and *iceA1/2*: 18/41 (43.9%) vs. 5/36 (13.8%), respectively, *P* < 0.005; Table 3]. The results of analysis showed no significant difference in the distribution of the *vacA* s1, s2, m1, and m2 genotypes and *iceA1*, *iceA2*, *babA2*, and *cagA* genes among *H. pylori* isolates from high and low incidence GC areas (*P* > 0.05).

The frequency of genotype combinations of the *vacA* d1/i1, *vacA* d1/*iceA1*, *vacA* d1/*iceA1/2*, *vacA* d1/*cagA*+, *vacA* i1/*iceA1*, *vacA* i1/*iceA1/2*, and *vacA* i1/*cagA*+ were significantly higher among *H. pylori* isolates from the high incidence areas of GC compared with those from the low incidence areas in Iran (*P* < 0.005; for the latter, *P* = 0.016). The genotype combinations of the *vacA* d2/i2, m2/d2, and m2/i2 were also significantly more prevalent among *H. pylori*

**Table 3.** Frequency of *vacA* alleles and *iceA1*, *iceA2*, *babA2*, and *cagA* genes in *H. pylori* isolates from Northwestern-Northern (NW-N) and Southern (S) geographic regions of Iran representing the high and low incidence of gastric cancer, respectively.

		Allele frequency (%)														
	GC¶ incidence	Strains (N)	<i>vacA</i> s1	<i>vacA</i> s2	<i>vacA</i> m1	<i>vacA</i> m2	<i>vacA</i> i1	<i>vacA</i> i2	<i>vacA</i> d1	<i>vacA</i> d2	<i>cagA</i> +	<i>cagA</i> -	<i>babA2</i>	<i>iceA1</i>	<i>iceA2</i>	<i>iceA1/2</i>
NW-N Iran	High†	41	39 (95.1)	2 (4.9)	15 (36.6)	26 (63.4)	25 (61.0)	16 (39.0)	24 (58.5)	17 (41.5)	31 (75.6)	10 (24.4)	14 (34.2)	34 (82.9)	25 (61.0)	18 (43.9)
S Iran	Low‡	36	35 (97.2)	1 (2.8)	7 (19.4)	29 (80.6)	9 (25.0)	27 (75.0)	9 (25.0)	27 (75.0)	23 (63.9)	13 (36.1)	16 (44.4)	26 (72.2)	15 (41.7)	5 (13.9)
	<b>Total</b>	<b>77</b>	<b>74 (96.1)</b>	<b>3 (3.9)</b>	<b>22 (28.6)</b>	<b>55 (71.4)</b>	<b>34 (44.2)</b>	<b>43 (55.8)</b>	<b>33 (42.9)</b>	<b>44 (57.1)</b>	<b>54 (70.1)</b>	<b>23 (29.9)</b>	<b>30 (39.0)</b>	<b>60 (77.9)</b>	<b>40 (51.9)</b>	<b>23 (29.9)</b>

¶GC= gastric cancer; †Ardabil (NW Iran) and Mazandaran (N Iran), (Age-standardized rates: ASRs = 51.8/10<sup>5</sup> and 26.78/10<sup>5</sup>, respectively); ‡Khuzestan and Yazd (S Iran), (ASRs < 10/10<sup>5</sup>)

**Table 4.** Frequency of combinations of the *vacA* m1, i1, and d1 alleles and *iceA1*, *babA2*, and *cagA* genes in *H. pylori* isolates from Northwestern-Northern (NW-N) and Southern (S) geographic regions of Iran representing the high- and low incidence of gastric cancer, respectively.

Genotypes	NW-N Iran†		S Iran‡		Total (n = 77) No. (%)
	High incidence areas for GC¶ (n = 41) No. (%)	Low incidence areas for GC (n = 36) No. (%)			
<i>vacA</i> d1/i1	<b>21 (51.2)</b>	<b>6 (16.7)</b>			<b>27 (35.1)</b>
<i>vacA</i> m1/i1	14 (34.1)	6 (16.7)			20 (26.0)
<i>vacA</i> m1/d1	15 (36.6)	7 (19.4)			22 (28.6)
<i>vacA</i> m1/ <i>babA2</i>	6 (14.6)	4 (11.1)			10 (13.0)
<i>vacA</i> m1/ <i>iceA1</i>	12 (29.3)	6 (16.7)			18 (23.4)
<i>vacA</i> m1/ <i>iceA1/2</i>	5 (12.2)	0 (0.0)			5 (6.5)
<i>vacA</i> m1/ <i>cagA</i> +	14 (34.1)	7 (19.4)			21 (27.3)
<i>vacA</i> d1/ <i>babA2</i>	11 (26.8)	6 (16.6)			17 (22.1)
<i>vacA</i> d1/ <i>iceA1</i>	<b>20 (48.8)</b>	<b>7 (19.4)</b>			<b>27 (35.1)</b>
<i>vacA</i> d1/ <i>iceA1/2</i>	<b>8 (19.5)</b>	<b>0 (0.0)</b>			<b>8 (10.4)</b>
<i>vacA</i> d1/ <i>cagA</i> +	<b>23 (56.1)</b>	<b>8 (22.2)</b>			<b>31 (40.3)</b>
<i>vacA</i> i1/ <i>babA2</i>	10 (24.4)	4 (11.1)			14 (18.2)
<i>vacA</i> i1/ <i>iceA1</i>	<b>20 (48.8)</b>	<b>6 (16.7)</b>			<b>26 (33.8)</b>
<i>vacA</i> i1/ <i>iceA1/2</i>	<b>10 (24.4)</b>	<b>0 (0.0)</b>			<b>10 (13)</b>
<i>vacA</i> i1/ <i>cagA</i> +	<b>20 (48.8)</b>	<b>8 (22.2)</b>			<b>28 (36.4)</b>
<i>iceA1/cagA</i> +	25 (61.0)	20 (55.6)			45 (58.4)
<i>iceA1/2/cagA</i> +	9 (22.0)	3 (8.3)			12 (15.6)
<i>iceA1/babA2</i>	11 (26.8)	13 (36.1)			24 (31.2)
<i>iceA1/2/babA2</i>	4 (9.8)	4 (11.1)			8 (10.4)
<i>vacA</i> m1/i2	1 (2.4)	1 (2.8)			2 (2.6)
<i>vacA</i> m2/i1	10 (24.4)	3 (8.3)			13 (16.9)
<i>vacA</i> m2/i2	<b>16 (39.0)</b>	<b>26 (72.2)</b>			<b>42 (54.5)</b>
<i>vacA</i> m2/d1	9 (21.9)	2 (5.5)			11 (14.3)
<i>vacA</i> m2/d2	<b>17 (41.5)</b>	<b>27 (75.0)</b>			<b>44 (57.1)</b>
<i>vacA</i> d1/i2	3 (7.3)	3 (8.3)			6 (7.8)
<i>vacA</i> d2/i1	3 (7.3)	3 (8.3)			6 (7.8)
<i>vacA</i> d2/i2	<b>14 (34.1)</b>	<b>24 (66.7)</b>			<b>38 (49.3)</b>
<i>vacA</i> m1/i1/ <i>cagA</i> +	13 (31.7)	6 (16.7)			19 (24.7)
<i>vacA</i> m1/i1/ <i>babA2</i>	4 (9.8)	3 (8.3)			7 (9.1)
<i>vacA</i> m1/i1/ <i>iceA1</i>	11 (26.8)	5 (13.9)			16 (20.8)
<i>vacA</i> m1/i1/ <i>iceA1/2</i>	5 (12.2)	0 (0.0)			5 (6.5)
<i>vacA</i> m1/d1/ <i>babA2</i>	5 (12.2)	4 (11.1)			9 (11.7)
<i>vacA</i> m1/d1/ <i>cagA</i> +	14 (34.1)	7 (19.4)			21 (27.3)
<i>vacA</i> m1/d1/ <i>iceA1</i>	12 (29.3)	6 (16.7)			18 (23.4)
<i>vacA</i> m1/i1/d1	14 (34.1)	6 (16.7)			20 (26.0)
<i>vacA</i> m1/d1/ <i>iceA1/2</i>	5 (12.2)	0 (0.0)			5 (6.5)
<i>cagA</i> +/ <i>babA2/iceA1</i>	10 (24.4)	12 (33.3)			22 (28.6)
<i>cagA</i> +/ <i>babA2/iceA1/2</i>	3 (7.3)	3 (8.3)			6 (7.8)

†Ardabil (NW Iran) and Mazandaran (N Iran), (Age-standardized rates, ASRs = 51.8/10<sup>5</sup> and 26.78/10<sup>5</sup>, respectively); ‡Khuzestan and Yazd (S Iran), (ASRs < 10/10<sup>5</sup>); ¶GC, Gastric cancer; Boldface data indicate statistically significant results by the Pearson Chi-square (χ<sup>2</sup>) and Fisher's exact tests (P < 0.005; for *vacA* i1/*cagA*+, P = 0.016).

isolates from low incidence areas of GC compared with those from high incidence areas (P < 0.005; Table 4).

The results of Mantel's test showed low correlation between genetic and geographic distances for the *iceA1* (r = 0.098) and *iceA1/2* (r = 0.074) genes among the ten districts (P < 0.05). No significant association was found between the genetic and geographic distances for the *vacA* alleles (s1, s2, m1, m2, i1, i2, d1, d2) and the *iceA2*, *babA2*, and *cagA* genes in Iran (P > 0.05).

## Discussion

In the present study we determined the distribution of *H. pylori* virulence genes/alleles between the high and low incidence areas for GC in Iran. The most prevalent genes were *vacA* s1a, *iceA1*, *vacA* m2, and *cagA* followed by *vacA* d2 and i2 genotypes and the *iceA2* gene, respectively. The *vacA* m1, d1, and i1 genotypes and *iceA1/2* and *babA2* genes occurred at low and intermediate

frequencies in Iran. The most predominant genotype combinations were *iceA1/cagA+*, *vacA* m2/d2, and *vacA* m2/i2, respectively. BLAST analysis using the sequenced fragments of each gene/allele from seven isolates showed that these sequences exclusively matched the corresponding sequences in *H. pylori* J99, 26695, 60190, and Tx30a.

Ogiwara et al. showed that in Western countries ( $n = 266$ ) the frequencies for the *vacA* alleles were s1 (80.8%), m1 (64.3%), i1 (71.8%), and d1 (74.1%) alleles and the *vacA* s1/m1/i1 (64.0%) and s1/m1/i1/d1 genotypes (64.0%). In Eastern countries ( $n = 244$ ), the frequencies were 100% (s1), 92.6% (m1), 97.5% (i1), 98.0% (d1), 92.6% (*vacA* s1/m1/i1), and 92.6% (*vacA* s1/m1/i1/d1).<sup>8</sup> In Western countries, strains that harbored the *vacA* s1, m1, i1, or d1 genotype had a significantly increased risk for the development of GC (adjusted ORs: 3.17, 10.65, 8.57, and 8.04, respectively). In East Asian countries there was no significant correlation between the *vacA* genotypes, clinical consequences and histopathological changes.<sup>8</sup> A recent follow-up study performed on 321 patients from a high-risk area of GC in Spain has shown that infection with *cagA+/vacA* s1/m1 strains was associated with the progression of gastric precancerous lesions (OR = 4.80) compared with those infected with strains that harbored the *cagA-/vacA* s2/m2 genotype.<sup>31</sup> In Iran, the frequencies of *vacA* s1, s2, m1, and m2 have been previously reported as 69.0%–80.3% (s1), 19.7%–31.0% (s2), 30.7%–49.7% (m1), and 50.3%–69.3% (m2).<sup>6,32–36</sup> The present study also reported higher frequencies of *vacA* s1 and m2, of which the frequency of m2 was more than twice the rate reported in both Western and Eastern countries. The relationship between *H. pylori vacA* genotypes, particularly the s1/m1/i1 alleles, and development of GC in Iran has been shown in several reports. This has reflected the importance of this risk locus in the development of severe gastrointestinal diseases.<sup>6,37</sup> However, Siavoshi et al. have shown no significant correlation between *H. pylori vacA* s and m region alleles to the types of gastritis, non-atrophy, atrophy, or intestinal metaplasia (IM) and severe forms of atrophy or IM in first-degree relatives of GC patients.<sup>36</sup>

The *iceA* gene has two main allelic variants, *iceA1* and *iceA2*. The *iceA1* gene represents sequence homology with the *Neisseria lactamica nlaIIIR* gene encoding a CTAG-specific restriction endonuclease. In contrast, the *iceA2* gene has no homology with the nucleotide sequences of the known genes released in the EMBL/GenBank database and its function remains unclear.<sup>12,38</sup> *H. pylori* strains might acquire these genes by genetic exchange with other bacteria. However, there is no evidence showing that both the *iceA1* and *iceA2* genes have been derived from a common ancestor.<sup>39</sup> Shiota et al. conducted a meta-analysis with a total of 5357 patients in order to determine the prevalence of the *iceA1* gene and its relationship to clinical outcomes. There was a significantly higher overall prevalence of *iceA1* in Asian countries (64.6%, 1791/2771) compared to Western countries (42.1%, 935/2218;  $P < 0.0001$ ). In contrast, the prevalence of *iceA2* was significantly higher in Western countries (45.1%, 844/1871) than Asian countries (25.8%, 651/2522;  $P < 0.0001$ ). The presence of *iceA1* significantly correlated with PUs.<sup>12</sup> In the present study, the prevalence of *iceA1* was 76.8%, whereas *iceA2* was 52.9%. Both were substantially greater than rates reported from Western and Eastern countries. We showed the presence of both the *iceA1* and *iceA2* genes (*iceA1/2*) in the same strains (29.7%, 41/138). In most studies, the presence of both genes in the same strains might be explained by mixed infection, for which strains that harbored the *iceA1/2* genotype were

excluded from the study.<sup>40</sup> However, Gonzalez-Vazquez et al. have recently reported the presence of both the *iceA1* and *iceA2* genes in the same strains.<sup>41</sup> In the present study, single colonies were used and genotyped. Each pair of primers was used for PCR amplification of the genes/alleles from all of the strains. Strains positive for the *vacA* i1 allele were also checked for the presence of the *vacA* i2 allele. Therefore, there was no possibility of any mixed infection; the presence of the *iceA1/2* genotype was confirmed.

The frequency of the *babA2* genotype in Western countries has been reported as 36%–72%, which was associated with the development of GC.<sup>42,43</sup> The frequency rate was 100% in Eastern countries, without a GC association.<sup>44</sup> In Iran, the *babA2* genotype, which frequency was 40.6%, strongly correlated with GC compared to non-ulcer and ulcer patients ( $P = 0.0004$ ).<sup>15</sup> In the present study, the frequency rate was 40.6%; however, it could not differentiate the *H. pylori* strains, particularly between the high and low incidence GC areas. The frequency of the *cagA* gene in Western and Eastern countries has been reported as more than 60% and 95% with and without an association to GC, respectively.<sup>31,45</sup> A frequency of 65.9% in the present study is consistent with earlier reports, however its association with GC is still controversial in Iran.<sup>32,36</sup>

We performed an individual AMOVA for the virulence genes/alleles for 138 *H. pylori* isolates that were distributed into ten geographic regions in Iran. The AMOVA for the *vacA* d1, d2, i1, and i2 alleles and *iceA1* and *iceA1/2* genes found significant levels of genetic differentiation among populations. These results have shown that considerable variation in the virulence genes has been well preserved at the population level. When the analysis was restricted to the four districts that represented high (Ardabil and Mazandaran) and low (Khuzestan and Yazd) incidence areas for GC, the AMOVA for *iceA1* did not show any differentiation between populations. The prevalence of the *vacA* d1, i1, and d1/i1 genotypes and *iceA1/2* gene (but not *iceA1*) were significantly higher (>2- or 3-fold) among *H. pylori* isolates from the high (ASRs > 20/10<sup>5</sup>; max. 51.8/10<sup>5</sup>) than low incidence (ASRs < 10/10<sup>5</sup>) areas for GC in Iran ( $P < 0.005$ ). The *vacA* d1/*iceA1/2* and *vacA* i1/*iceA1/2* genotypes and the *cagA* or *iceA1* genes in combinations either with the *vacA* i1 or d1 alleles (*vacA* d1/*iceA1*, *vacA* d1/*cagA+*, *vacA* i1/*iceA1*, and *vacA* i1/*cagA+*) showed significantly higher frequency (>2- or 3-fold) in the high than low incidence GC areas. In contrast, the *vacA* d2/i2, m2/d2, and m2/i2 genotypes were significantly more prevalent (approximately 2-fold) in the low rather than high incidence areas ( $P < 0.005$ ). These results have suggested that the *H. pylori vacA* d1/i1 alleles could be considered risk biomarkers in the high incidence GC areas in Iran. The prevalence of the *vacA* s1(a) allele was very high and mimicked the pattern observed in Eastern countries, while the prevalence of the *vacA* m1, d1, and i1 was low compared with both Western and Eastern countries.<sup>8</sup> In addition, the *vacA* s1(a) allele showed a similar distribution between the high and low incidence areas in Iran therefore it could not differentiate *H. pylori* strains between these areas with different GC incidence. In the high incidence areas, the distribution of the *vacA* m1 (36.6%), m1i1 (34.1%), m1d1 (36.6%), m1/*iceA1* (29.3%), m1/*iceA1/2* (12.2%), and m1/*cagA+* (34.1%) genotypes were high compared to the low incidence areas, which had the following distributions: 19.4% (m1), 16.7% (m1i1), 19.4% (m1d1), 16.7% (m1/*iceA1*), 0.0% (m1/*iceA1/2*), and 19.4% (m1/*cagA+*), however the differences did not reach statistical significance. In all analyses, the *iceA1/2* genotype has shown considerable impact in geographic



differentiation of the strains, particularly between the high and low incidence areas. Therefore, this genotype may be of importance as a new risk marker although its function is not clear. The relatively small sample size might have a negative impact on the final models. However, several comparative analyses have confirmed the consistency and reliability of these results.

We performed Mantel's test to determine whether there was a north-south cline in the allelic frequency of *H. pylori* virulence genes in Iran. The results only showed a low correlation between the genetic and geographic distances for the *iceA1* and *iceA1/2* (but not *vacA* alleles, *iceA2*, *babA2*, and *cagA*) genes among the ten districts ( $r = 0.098$  and  $0.074$ , respectively,  $P < 0.05$ ). The lack of, or weak trend, has indicated that these geographic differences in the distribution of the *H. pylori* virulence genes/alleles might be overwhelmed by the specific regional influences.

In conclusion, the present study showed the determinant role of the *H. pylori vacA* d and i region genotypes and the *iceA1/2* gene rather than the *vacA* s and m region genotypes and *iceA1*, *iceA2*, *babA2*, and *cagA* genes in the geographic differentiation of *H. pylori* strains in Iran. The presence of the *vacA* m2 genotype and *cagA* or *iceA1* genes might not be considered as independent of the *vacA* d and i regions in the differentiation of *H. pylori* strains between high and low incidence GC areas in Iran. The presence of both the *iceA1* and *iceA2* genes (*iceA1/2*) in the same strains might be of importance as a new risk marker, although its function was not clear. There was a weak or no north-south cline in the allelic frequency of *H. pylori* virulence genes/alleles in Iran, which indicated the likely presence of specific regional influences. We have proposed that the *H. pylori vacA* d1/-i1 genotypes, which are new determinants of GC, have great potential to differentiate *H. pylori* strains between the high and low incidence areas of GC in Iran.

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