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Helicobacter Pylori VacA C1 Genotype Is a Benefit Biomarker for Prediction of Gastric Cancer Risk in Ardabil

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ABSTRACT

Background: Gastric cancer (GC) is the fifth common malignant disease and the third leading cause of cancer-related mortality in the world. Ardabil, a Northwestern province of Iran, includes the highest rate of GC within the country. *Helicobacter pylori (H. pylori) vacA* gene plays a major role in generating and maintaining the gastric inflammatory response, which alters the enteric nervous system in various combinations and may contribute to the development of GC. The aim of the current study was to investigate the relationship of the *vacA* c-region genotypes of *H. pylori* with GC among Ardabil population.

Methods: A total of 197 from 259 patients with non-atrophic gastritis (NAG) and GC, who were *H. pylori* positive, were selected and genotyped.

Results: The frequency of *vacA* c1 was 53.7% and c2 42.3%. There was a significant difference between the frequencies of *vacA* c1 in isolates from GC than those from NAG (p<0.05). Though the GC was considered as a dependent factor by the multiple logistic regression analysis, the *vacA* c1 genotype was significantly associated with age- and sex-adjusted risk for GC (p=0.003, odds ratio [OR] = 5.48; 95% confidence interval [CI] =1.80–16.63).

Conclusion: It was proposed that the *H. pylori vacA* c1 genotype could be considered as an important determinant for prediction of risk of GC in Ardabil. It is suggested that interaction between *H. pylori vacA* c-region genotypes and gastric nervous system may contribute to the development of GC.

Keywords: Helicobacter Pylori; VacA C; Nervous System; Gastric Cancer; Ardabil

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INTRODUCTION

Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of cancer leading to death [1]. GC incidence rate is high in Asia (Almost two-thirds) [2]. Iran has the fourth -ranking of GC in Asia, after China, Japan and Korea respectively[3-7]. The incidence of GC in the Northern districts of Iran, particularly Ardabil Province located in northwestern Iran, where the incidence of GC is high, has been reported to be considerably high (ASR of 51.8/100,000 men and 24.9/100,000 women) [8,9]. There is a close relationship between increased risk of GC in high risk regions and *Helicobacter pylori* (*H. pylori*) infection [10]. The 63% of all GC are related to *H. pylori* infection[11]; however, less than 3% of infected individuals develop GC[11, 12]. The prevalence of *H. pylori* infection is 69% in Iran; and the highest frequency (89%) is reported in Ardabil[13]. Strains isolated from Iranian populations, including those with Ardabil signature, are likely to be of European ancestry[14].

One of the pathogenic mechanisms of *H. pylori* changes in the various components of the gastric nervous system, including structural anomalies, sensitivity and motor

function impairment and changes in neurotransmitter is released. Interaction of *H. pylori* with gastric nervous system results in neurons' dysfunction that may play a potential role in development of GC [15].

H. pylori virulence factors could determine the outcome of infection when combined with both environmental and host factors [16]. One independent *H. pylori* locus that is correlated with increased risk of disease is vacuolating cytotoxin A (*vacA*). Suppressing T cell activity, VacA causes a change in function of the immune response and plays an important role in generating and sustaining the inflammatory response plays stomach [17]. Mucosal inflammatory response associated to *H. pylori* infection leads to afferent nerve remodeling and may contribute to the development and processing of various types of diseases including GC.

Genetic studies have shown that the presence of *vacA* is associated with the development of peptic ulcer, severe gastritis, both premalignant and malignant lesions [18] and comprises several polymorphic regions: the signal (s), encoding a part of the N terminus of the mature protein; middle (m), encoding a part of the 55-kDa C-terminal subunit; intermediate (i), located between s and m regions possessing a functional role in vacuole-creating activities, and deletion (d) region. Recently, a 15 bp deletion located at the 3′-end region of *H. pylori vacA* has been identified and termed the c-region, as c1 (with deletion) and c2 (no deletion), which associated with different gastroduodenal diseases [19]. We aimed to determine the impact of *H. pylori vacA* c-region genotypes, age and gender on the development of GC among Ardabil population.

MATERIALS AND METHODS

A total of 259 patients affected with non-atrophic gastritis (NAG) and GC; who referred to the Endoscopy unit at the Imam Khomeini Hospital in Ardabil participated in the study. Gastric biopsy specimens were taken from the antrum and/or the corpus, and classified by endoscopic and histopathological examinations.

DNA extraction and diagnosis of H. pylori infection and genotyping of H. pylori

H. pylori infection was diagnosed by rapid urease test and PCR amplification of *H. pylori 16S rDNA*. DNA was extracted from the biopsy specimens with the Genomic DNA Purification kit (DNGTM-Plus, CinnaGen Co., Iran) according to the manufacturer's protocol and stored at -20°C. Polymerase chain reaction (PCR) was performed in a volume of 30 µL reaction volume containing 50 ng of genomic DNA, 3 µL of 10X PCR buffer (CinnaGen, Iran), 1 mM MgCl2, 200 µM of each dNTP (CinnaGen Co., Iran), 0.5 µM of each specific primer and 2 U of Taq DNA polymerase (CinnaGen Co., Iran). The PCR conditions were 96°C for 3 min and then 35 cycles of 96°C for 40 seconds, optimized annealing temperature for each allele (Table 1) for 40 seconds, 72°C for 40 seconds, and a final incubation at 72°C for 7 min. PCR products were electrophoresed and visualized by a UV trans illuminator (Figure 1). As controls, amplified fragments of each gene/allele from ten isolates were purified and sequenced with both forward and reverse primers using a 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.

Data analysis

Data collected and analyzed using binary logistic regression model and the Chi-square (χ^{2}) or Fisher's exact tests in SPSS software (version 19); P value of < 0.05 indicated statistically significant level. Odds ratios (ORs) and the corresponding 95% confidence intervals (CIs) were also computed. For determine the effect of each allele in GC, using simple logistic regression analysis by the Enter method. The Forward Stepwise LR (Likelihood Ratio) multiple logistic regression analysis was used for each allele, with adjustment for the potential confounders, including a threshold age of ≥55 years and sex.

Table 1. Oligonucleotide Primers Used for PCR

Genes	Primers	Sequences (5′→3′)	Size of PCR products (bp)	Optimized annealing temperature (ºC)
16 S rDNA	HP1	GCA ATC AGC GTC AGT AAT GTT C	519	56
	HP2	GCT AAG AGA TCA GCC TAT GTC C		
vacA				
c1/-c2	c1-F	ATC ATY SGT TAT GRH AAT GTT TCT	c1: 600-700	55
	R-nd	TTA TGC TCT AAA CTG GCT A		
	c2-F	ATT ATA ATT TAG TAG GAG TGC AAG G	c2: 600-700	55
	R-nd	TTA TGC TCT AAA CTG GCT A		

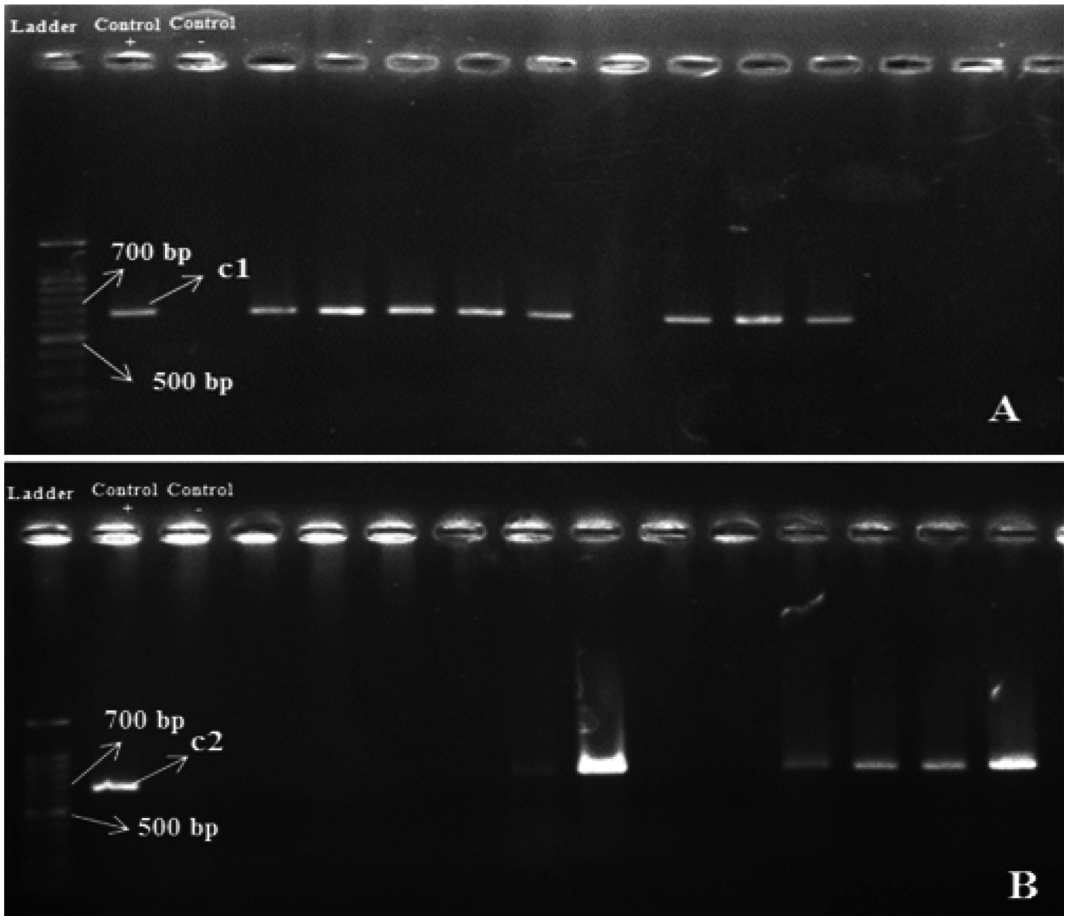

Figure 1. PCR products of *c* region of the *vacA* gene.

Histopathologic Examination and Classification of Subjects

Gastric biopsy specimens were taken from pyloric gland area (the antrum) and fundic gland area (the corpus). One biopsy from the antrum and/or fundus was fixed in 10% formalin and embedded in paraffin. The tissue sections for histopathologic examinations were provided, and then the histo-morphologic architecture of the tumors was expressed according to Lauren's classification as intestinal or diffuse carcinomas. Biopsies were stained with hematoxylin–eosin, Alcian blue-periodic acid Shiff (pH 2.5) and Giemsa for light microscopy, and histopathological evaluations were also applied according to the updated Sydney classification system[20].

RESULTS

Classification of patients and presence of H. pylori in gastric biopsy specimens

A total of 259 patients from rural and urban areas of Ardabil with non-atrophic gastritis (186) and GC (73) participated in the current study. Histopathological evaluations were performed for samples**.** Patients were classified into 2 groups; females: 45.9% and males: 54.1%. Patients were also classified into 2 age groups; age <55 (54.4%) and age ≥55 (45.9%). The age information was not available for one patient. Of 73 patients with GC, 86.3% had age ≥55 and 72.6% were male. GC was more common among patients with age ≥55 and male group. In NAG group, 29.0% of patients were age ≥55, and 46.8% were male. Characteristics of patients according to age and sex were shown in Table 2. The current study, statistical analysis showed that both the age ≥55 and male sex were significantly associated with GC [OR (95% CI) = 3.01 (1.67-5.43) and 17.11 (7.94-36.83), respectively; $P = 0.00$] (Table 3). 197 (76.1%) patients were infected by *H. pylori*. Electrophoresis of the PCR products of *16S rDNA* for 46.19% (91/197) of patients revealed bands with the size of 519 bp which confirmed the presence of *H. pylori*.

The prevalence of H. pylori vacA c genotypes and its correlation with the risk of GC

The *vacA* c-region genotypes could be determined in

Table 2. Characteristics of patients enrolled in this study

Characteristics	Types of gastroduodenal diseases		
	Non-atrophic gastritis	Gastric cancer	Total
Patients NO. (%)	186/259 (71.8)	73/259 (28.2)	259/259 (100.0)
Sex groups NO. (%)			
Male	87/186 (46.8)	53/73 (72.6)	140/259 (54.1)
Female	99/186 (53.2)	20/73 (27.4)	119/259 (45.9)
Age groups NO. (%)			
≥55	54/186 (29.0)	63/73 (86.3)	117/259 (45.2)
<55	132/186 (71.0)	9/73 (12.3)	141/259 (54.4)

Table 3. Associations between the risk of gastric cancer and age, and gender

Characteristic/ confounder	Simple logistic regression		
	P value	OR	95% CI
Gastric cancer			
Sex			
Male	0.00a	3.01	1.67-5.43
Female	1 (ref)	1 (ref)	1 (ref)
Age, y			
≥55	0.00	17.11	7.94-36.83
<55	1 (ref)	1 (ref)	1 (ref)

Abbreviations: OR, odds ratio; CI, confidence interval.
[a]Boldface data indicate statistically significant results

82 *H. pylori*-positive patients. They were not detected in nine specimens, so were excluded from the analysis. Generally, a total of 82 *H. pylori*-positive patients, 43 (52.4%) with GC and 39 (47.6%) with NAG, were enrolled in the final analysis. The size of the PCR products of the *vacA* c1 and *vacA* c2 genotypes was 600-700 bp. The amplified products of the *vacA* c1 and *vacA* c2 genotypes showed more than 96% similarity with those in GenBank. Total prevalence of *vacA* c1 was 57.3% and c2 42.7%. As shown in Table 4, the frequency of the *vacA* c1 genotype in patients with GC was 74.4, and in NAG group was 38.5%. There was a significant difference between the frequencies of *vacA* c1 in isolates from GC than those from NAG ($p<0.05$). The results of simple logistic regression analysis showed that this genotype was significantly associated with the risk of GC; the OR (95% CI) was 4.65 (1.81-11.92; $P = 0.02$). Though the GC was considered as a dependent factor by the multiple logistic regression analysis, the *vacA* c1 genotype was significantly associated with the age- and sex-adjusted risk for GC ($p= 0.003$, OR = 5.48; 95% CI =1.80–16.63).

DISCUSSION

Gastric cancer is very common in Ardabil**,** a Northwestern province of Iran with the highest GC incidence rate in the country. There is a close relationship between *H. pylori*-specific factors and GC. Ardabil is a province with a high prevalence of *H. pylori* infection (89%). Strains with the particular *vacA* genotypes have been found to be benefit biomarkers for GC. 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) [21]. The results of a study from Northeastern of Brazil where there is a high prevalence of gastric cancer has shown that infection with the most virulent *H. pylori* strains, carrying the *cagA* gene and s1m1 *vacA* alleles, predominates and is correlated with more severe *H. pylori*-associated diseases; and infection with *cagA*$^+$ strains was significantly associated with gastric cancer (p = 0.016, OR = 10.36, 95% CI = 1.35-217.31) [22]. A meta-analysis study on 17374 patients demonstrated that individuals carrying strains of *H. pylori vacA* s1 (vs. s2), m1 (vs. m2), s1m1 (vs. s1m2), and s1m1 (vs. s2m2) are at an increased risk of developing GC [OR of 5.32 (95% CI 2.76-10.26), 2.50 (95% CI 1.67-3.750), 2.58 (95% CI 1.24-5.38), and 4.36 (95% CI 2.08-9.10), respectively][23].

Recently, two disease-related regions: intermediate (i) region (located between sand m regions) and deletion (d)

Table 4. Associations between the risk of GC and *vacA* c-region genotypes

genotypes	NAG No.(%)	GC No.(%)	Total No.(%)	Simple logistic regression		
				P value	OR	95% CI
c- region						
c1	15/39 (38.5)[a]	32/43 (74.4)	47/82 (57.3)	0.02	4.65	1.81-11.92
c2	24/39 (61.5)	11/43 (25.6)	35/82 (42.7)	1(ref)	1(ref)	1(ref)

Abbreviations: NAG, non-atrophic gastritis; GC, Gastric cancer; OR, odds ratio; CI, confidence interval.
[a]Boldface data indicate statistically significant results

region (located between the i and m regions) have been identified as new factors determining of GC risk [24,25]. The results of a recent meta-analysis study showed that the *vacA* i1 allele of *H. pylori* is associated with an increased risk of GC in the middle Asian ($p < 0.001$, OR = 10.89; 95% CI= 4.11–20.88)[26]. The results of a study by Ferreira et al. [27] on 192 *H. pylori*-infected patients with gastric carcinoma (56), with chronic superficial gastritis (114), and with chronic atrophic gastritis (22) from the North of Portugal showed that the *vacA* i1 genotype was significantly associated with gastric atrophy and for gastric carcinoma, with OR= 8.0; 95% CI=2.3–27.0 and OR= 22.0; 95% CI=7.9–63.0, respectively.

Latifi-Navid et al. (2013) have reported that the *vacA* d1/-i1 alleles from strains with *European* ancestry could probably be considered as determinant biomarkers in the high incidence areas of GC in Iran (Ardabil and Mazanderan) [28]. The results of two recent studies from East Azerbaijan region in Northwestern Iran, where the incidence of GC is high, demonstrated that there are high association of the *vacA* i1 (OR = 13.14) [29] and *vacA* d1genotypes (OR = 8.04) [30] with GC. On the contrary, in a study, Ogiwara et al. showed that in East Asia and Southeast Asia countries where GC incidence is high, there was no correlation between the *vacA* –s, -m1, -i1, and -d1 genotypes and GC. In Western countries, strains with *vacA* s1, m1, i1, or d1 genotypes had a significant relationship with risk of GC [25]. We have recently identified a novel polymorphic site in the 3′-end region of *H. pylori vacA*, denoted by c1/-c2 (c1: with deletion of 15 bp). Despite its biological role is not yet known, the *vacA* c1 type has been proposed as a new determinant of GC risk. We have previously examined 217 isolates recovered from 114 patients with NAG, 57 patients with PUD, and 46 patients with GC, and have shown that the *vacA* m1, i1, d1, c1, and *cagA* genotypes were significantly associated with the risk of GC (OR was 4.29, 6.11, 3.18, 15.13, and 2.59, respectively), and *vacA* i1 and *cagA* genotypes with the risk of PUD (OR was 2.80 and 2.62, respectively). Finally, logistic regression analysis showed that the *vacA c1* genotype was a strong correlation in male GC patients ≥55 than in male NAG patients ≥55 the OR was 36.66 (P= 0.004) [19].

In the present study, statistical analysis showed that the frequency of the *vacA* c1 genotype in patients with GC was significantly higher than in NAG patients; simple logistic regression analysis confirmed the relationship between *vacA* c1 genotype with GC in Ardabil; the OR (95% CI) was 4.65 (1.81-11.92; P = 0.02). Finally, after controlling for age and sex variables, the *vacA* c1 genotype remained in the final model when the GC was considered as a dependent factor by the multiple logistic regression analysis. In fact, the results of multiple logistic regression confirmed the high correlation of the *vacA* c1 genotype with the risk of GC (p = 0.003, OR = 5.48; 95% CI = 1.80–16.63). However, it is still not clear biological role and its impact on the enteric nervous system. Although all of these studies have shown the relationship between *vacA* genotypes of *H. pylori* to gastric cancer; however, none of these genotypes associated with abnormal nerve cells of the stomach is not yet known.

CONCLUSION

Results of the present study revealed that the *vacA* c1 genotype was strongly associated with the incidence of GC in high-risk areas, particularly Ardabil. However, the role of the interaction between *H. pylori vacA c*-region genotypes and gastric nervous system is unknown. It is suggested that neural activity impaired by this genotype may have a potential role in the development of GC, but this hypothesis requires further investigation. It seems that the unfolding relationship between neurological changes caused by inflammation induced by *H. pylori vacA* genotypes could help to identify new therapeutic approaches for GC.

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