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ORIGINAL ARTICLE

# *Helicobacter pylori vacA i* region polymorphism but not *babA2* status associated to gastric cancer risk in northwestern Iran

**Batool Mottaghi · Reza Safaralizadeh · Morteza Bonyadi · Saeid Latifi-Navid · Mohammad Hossein Somi**

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**Abstract** *Helicobacter pylori*-specific genotypes have been strongly associated with an increased risk of gastric cancer (GC). The aim of the present work was to study the associations of *H. pylori* virulence factors, *vacA i* region polymorphisms and *babA2* status with GC risk in Azerbaijan patients. The DNA extracted from gastric biopsy specimens was used to access the *babA2* and *vacA* genotypes. Overall, *babA2* was present in 85.39 % (76/89) of *H. pylori* strains: 19 out of 24 (79.16 %) strains from GC, 16 out of 17 (94.14 %) strains from peptic ulcer disease (PUD) and 41 out of 48 (85.14 %) strains from chronic gastritis. No significant association was found between *babA2* genotype and clinical outcomes ($P > 0.05$). *i1 vacA* polymorphism was detected in 46/89 (51.68 %) strains: in 21/24 (87.5 %), 6/17 (35.29 %) and 19/48 (39.58 %) patients with GC, PUD and chronic gastritis, respectively. *i2* allele was detected in 43 (48.31 %) out of all 89 strains examined: 3 (14.28 %) of 24 strains from GC, 11 (64.71 %) of 17 from PUD, and 29 (60.42 %) of 48 strains from chronic gastritis. In this study, multiple linear regression analysis confirmed the strong association of *i1* allele with GC (partial regression correlation $0.455 \pm 0.101$; $P = 0$). Results of multiple logistic regression analysis showed that *vacA i1* genotype was significantly associated with GC compared with a control group (gastritis) (odds ratio 13.142, 95 % CI 3.116–55.430; $P = 0$). Findings from the measurement of *H. pylori babA2* and *vacA* genotypes indicate a strong correlation between the *vacA i1* allele and GC risk in the Azerbaijan area of Iran.

**Keywords** *Helicobacter pylori* · *babA2* · *vacA i1* · Gastric cancer

B. Mottaghi · R. Safaralizadeh (✉) · M. Bonyadi
Department of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran
e-mail: safaralizadeh@tabrizu.ac.ir

S. Latifi-Navid
Department of Biology, Faculty of Sciences, University of Mohaghegh Ardabili, Ardabil, Iran

M. H. Somi
Liver and Gastrointestinal Diseases Research Center, (LGDRC), Tabriz University of Medical Sciences, Tabriz, Iran

## Introduction

*Helicobacter pylori* is a spiral shaped, flagellated, microaerophilic, gram-negative bacterium, which efficiently colonizes the stomach mucosa of more than 50 % of the human population [1, 2]. It is documented to play a causative role in the pathogenesis of various gastroduodenal diseases including chronic gastritis, peptic ulcer disease (PUD), gastric cancer (GC) and mucosa-associated lymphoid tissue (MALT) lymphoma [3, 4]. However, most infected individuals show no clinical symptoms and only a minority of patients develops serious clinical outcomes [5, 6], indicating that the clinical outcome is affected by differences in genetic susceptibility of the host, variability of bacterial virulence, and environmental factors [7].

The major bacterial virulence factors include adhesions (BabA, SabA, OipA, AlpA, AlpB and HopZ), the products of the *cag* pathogenicity island (*cag*PAI) and the vacuolating cytotoxin (VacA) [8, 9]. Adherence of *H. pylori* to the gastric epithelial cells is the initial stage of colonization, which leads to persistence of infection and delivery of virulence factors to host epithelial cells [8–10]. The blood-group antigen-binding adhesin (BabA), a 78-kDa outer

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membrane protein, encoded by the *babA2* gene, has been shown to mediate the adherence of *H. pylori* to human Lewis (α-1, 3/4 difucosylated) blood-group antigens on gastric epithelium [11, 12]. Although two distinct *babA* alleles (*babA1* and *babA2*) and one highly homologous gene *babB* have been identified, only the *babA2* allele is functionally active [12, 13]. Analyses of binding characteristics of *H. pylori* strains worldwide suggest that the BabA adhesin has changed in response to host mucosal glycosylation patterns to allow *H. pylori* to acclimatize to its host and to maintain persistent colonization. The usefulness of the BabA adhesion in predicting clinical outcomes probably depends on the geographical origin of the strains [9].

The presence of *babA2* is associated with increased risk of duodenal ulcer disease and adenocarcinoma, and when found in conjunction with *cagA* and *vacA* s1 alleles, it leads to an even greater risk of developing more severe disease [14].

The VacA induces cytoplasmic vacuolation and apoptosis processes in the human epithelial cells [5, 15]. The gene encoding this (*vacA*) is present in the majority of *H. pylori* strains. However, considerable differences in the vacuolating activities of *vacA* products for cell lines are observed between strains, and this is primarily due to variations in the *vacA* gene structure [16]. The variable regions are located in the signal region (*s1* or *s2*) and the mid region (*m1* or *m2)*. The *vacA s1/m1* chimeric strains have greater vacuolation than *s1/m2* strains, and there is typically no vacuolating activity in *s2/m2* strains [2, 5, 16, 17]. Recently, Rhead et al. identified a new polymorphic region in *vacA* located between the *s* and *m* regions, which they termed the intermediate (*i*) region. The *i* region encodes part of the p33 VacA subunit, and 2 allelic variants of this region, *i1* (vacuolating) and *i2* (non-vacuolating), were described. *i1*-type strains, but not *i2*-type strains, were associated with gastric carcinoma in an Iranian population [18, 19] and were an independent predictor of PUD disease in an Italian population [20]. Recently, the *i* region has been shown to be a better predictor of disease severity than any of the *s* or *m* region, though the *i* region seems to overlap with the *s* and m regions. This means that the more toxic *i1* region is often associated with *s1*–*m1* [21].

The incidence rate of GC is high in East Azerbaijan province located in northwestern Iran, where GC is the leading cause of cancer-related deaths in males (ASRs = $26.0/10^5$) and the fourth type of cancer after breast, skin and esophagus cancers in females (ASRs = $11.6/10^5$) [22]. The main aim of the present work was to study the associations of *H. pylori* virulence factors (*vacA*, *i* region polymorphisms, *babA2*) with GC and PUD in Azerbaijan patients.

## Methods

### Patients

In total, 89 strains from 89 patients [patients with endoscopic findings and pathology tests for PUD (17 subjects), GC (24 subjects) and non-atrophic chronic gastritis (48 subjects)] seeking treatment at Imam Reza and Shahid Madani hospitals in Tabriz, Iran, were tested. The biopsy specimens were obtained during a one-year period, from 2012 to 2013. Forty-five patients were women (50.56 %), and 44 were men (49.44 %). *H. pylori* infection was diagnosed by rapid urease test and PCR. Three biopsy specimens were obtained from the antrum of the stomach of each patient during endoscopy. One was inserted into a urease test tube; one was placed in a sterile tube and frozen at −70 °C for further polymerase chain reaction (PCR) analysis; and the other was used for histological examination.

### Histological examination

The biopsy specimens were first fixed in 10 % formalin and then embedded in paraffin before the tissue sections were prepared for histopathological examinations. GC was classified and graded using the Sydney system [23].

### DNA extraction and PCR

Using protocol DNG™ Plus kit (CinnaGen, Tehran, Iran), DNA was extracted from the second gastric biopsy specimens of those patients that tested positive on the rapid urease tests. Before using the kit, the tissue was completely broken by scalpel. Extracted DNA was stored at −20 °C until polymerase chain reaction (PCR) amplification was performed.

### PCR amplification

PCR was used to detect the *H. pylori*-specific *16S rDNA* gene [24], *vacA* gene (*i* region polymorphisms) [19] and the presence of *babA2* gene [25]. PCR amplification was carried out in a 25-µL reaction mixture containing 12.5 µL Master Mix (CinnaGen PCR Master Kit, Tehran, Iran), 1 µM concentrations of each primer, 0.4 µM concentration of each inner control primer (*16S rDNA* gene) and 5 µL volume of template DNA. PCR primers and conditions of each PCR are listed in Table 1. The PCR negative control consisted of the reaction performed without bacterial DNA (i.e., DNA extracted from gastric biopsies, which emerged *H. pylori*-negative on the basis of the URT test and PCR). Positive controls were obtained from *H. pylori* previously cultured from strains obtained from Guilan and

**Table 1** Primer sequence and conditions of PCR applied in this study

| Gene and primer | Sequences | Size of PCR product | PCR cycles |
|---|---|---|---|
| *babA2* | | | |
| Bab7-F | 5′-CCAAACGAAACAAAAAGCGT-3′ | 271-bp | 1 cycles of 95 °C for 30 s; 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s; 1 cycle of 72 °C for 10 min |
| Bab7-R | 5′-GCTTGTGTAAAAGCCGTCGT-3′ | | |
| *vacA* i region | | | |
| *i1* | | | |
| Vac-F1 | 5′-GTTGGGATTGGGGGAATGCCG-3′ | 426-bp | 1 cycle of 95 °C for 90 s; 35 cycles of 95 °C for 30 s, 53 °C for 60 s and 72 °C for 30 s; 1 cycle of 72 °C for 5 min |
| C1R | 5′-TTAATTTAACGCTGTTTGAAG'-3′ | | |
| *i2* | | | |
| Vac-F1 | 5′-GTTGGGATTGGGGGAATGCCG-3′ | 432-bp | |
| C2R | 5′-GATCAACGCTCTGATTTGA-3′ | | |
| *16S rDNA* | | | |
| HP1 | 5′-GCAATCAGCGTCAGTAATGTTC-3′ | 519-bp | |
| HP2 | 5′-GCTAAGAGATCAGCCTATGTCC-3′ | | |

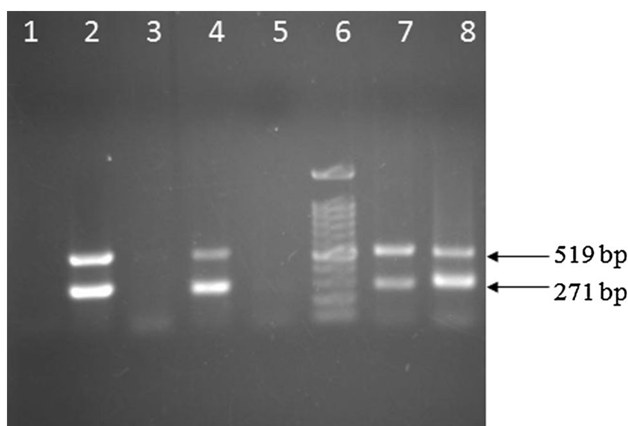


**Fig. 1** PCR amplification, performed with *babA2* primers (*lane 1* negative control without DNA; *lane 2* positive control; *lanes 4, 7, 8* clinical positive; *lanes 3, 5* clinical negative; *lane 6* 100–1,000 bp DNA ladder)

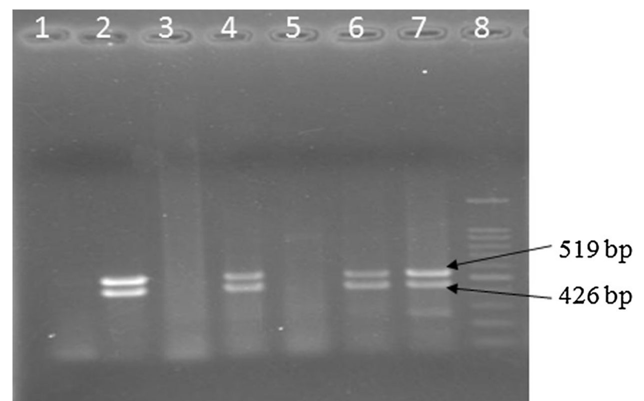


**Fig. 2** PCR amplification, performed with *i1* primers (*lane 1* negative control without DNA; *lane 2* positive control; *lanes 4, 6, 7* clinical positive; *lanes 3, 5* clinical negative; *lane 8* 100–1,000 bp DNA ladder)

Mazanderan provinces. PCR products were analyzed by electrophoresis on 1.2 % agarose gels run in TAE buffer and stained with ethidium bromide. The PCR product was examined in parallel with a molecular size marker of 100-bp DNA ladder (CinnaGen, Iran). Electrophoresis of the *16S rDNA* PCR products from the *H. pylori*-positive samples revealed bands of 519 bp, which confirmed the identity of *H. pylori* (Figs. 1, 2, 3). The BLAST program (http://www.ncbi.nlm.nih.gov) was used to match the nucleotide sequences with the published sequences in GenBank.

## Statistical analysis

The SPSS version 19 software was used for statistical analysis. Fisher's exact and Chi-square tests were applied to establish any statistically significant associations. A $P$ value <0.05 was considered as significant. Multiple regression analysis was used to determine the relative influence of the pathogenic factors of bacteria on GC and other gastric diseases. In all comparison analysis, patients with gastritis were considered as the control group.

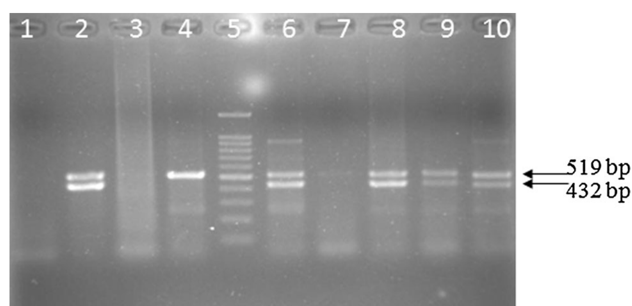


**Fig. 3** PCR amplification, performed with *i2* primers (*lane 1* negative control without DNA; *lane 2* positive control; *lanes 6, 8, 9, 10* clinical positive; *lane 3* clinical negative; *lane 4 H. pylori*—positive without *i2* allele) (*lane 5* 100–1,000 bp DNA ladder)

## Results

### The relationship between age and sex to clinical symptoms

A significant relationship between age and clinical symptoms was observed. Twenty-three out of 24 (95.8 %) patients with GC were 40 years or older, while the prevalence of gastritis was almost the same in patients older than 40 years (45.8 %, 22/48 patients) and those less than 40 years 54.2 % (26/48) of age ($P = 0$). No significant correlation was found between age, sex and prevalence of PUD ($P > 0.05$).

### The correlation of frequencies of *babA2* and *vacA* genotypes with GC and PUD

PCR was used to determine the presence of *babA2* and *vacA* genotypes of *H. pylori* in the DNA extracted from *H. pylori*-positive rapid urease test biopsies. Overall, the presence of *babA2* in *H. pylori* strains was 85.39 % (76/89): 19 (79.16 %) of 24 strains from GC, 16 (94.14 %) of 17 from PUD, and 41 (85.14 %) of 48 strains from chronic gastritis (Table 2). No significant association was noted between the *babA2* genotype and clinical outcomes ($P > 0.05$).

Presence of *vacA* gene in our *H. pylori* strains was 100 % (89/89). *i1 vacA* polymorphism was detected in 46/89 (51.68 %) strains: 21/24 (87.5 %), 6/17 (35.29 %) and 19/48 (39.58 %) patients with GC, PUD and non-atrophic chronic gastritis, respectively, and *i2* allele was detected in 43 (48.31 %) of all 89 strains examined: 3 (14.28 %) of 24 strains from GC, 11 (64.71 %) of 17 from PUD, and 29 (60.42 %) of 48 strains from non-atrophic chronic gastritis (Table 2).

In this study, it was observed that the frequency of *i1* allele [87.5 % (21/24)] in *H. pylori* strains isolated from GC patients compared with *i2* allele is much higher than that in patients with gastritis [39.58 % (19/48)].

### Effects of *vacA* alleles in GC and PUD

In this study, multiple linear regression analysis confirmed the strength of the correlation of *i1* allele with GC (partial regression correlation 0.455 ± 0.101; $P = 0$). Results of multiple logistic regression analysis showed that *vacA i1* genotype was significantly associated with GC compared with a control group (gastritis) [odds ratio (OR) 13.142, 95 % confidence interval (CI) 3.116–55.430; $P = 0$].

## Discussion

If specific *H. pylori* virulent markers can be used to predict PUD or GC risk, patients with gastritis alone can be chosen for *H. pylori* eradication. The adhesion protein BabA2 has been suggested as one of those markers. Two corresponding genes encoding BabA have been cloned and named *babA1* and *babA2*. Only the *babA2* gene is functionally active [12, 13]. Many differences in the prevalence of the *babA2* genotype of *H. pylori* and its association with clinical symptoms have been reported. For example, the distribution of the *babA2* genotype in Western countries has been reported to be 36–72 % in different studies and associated with the development of GC [14, 26]. The frequency rate in Eastern countries was 100 % but without a GC association [27]. The present study showed that the frequency of the *babA2* genotype is 85.39 %, occurring in gastritis (85.42 %), peptic ulcer (94.12 %) and GC (79.17 %). In our study, there was no significant association between the *babA2* genotype and gastric symptoms ($P > 0.05$). These results were not in accordance with previous studies in Iran. Eshaghi et al. [28] and Abadi et al. [29] reported the frequency of the *babA2* genotype in Isfahan and Sari as 71.6 and 40.6 %, respectively. In both studies, a strong association was observed between the *babA2* genotype and GC [28, 29]. The results of this study are consistent with those found in Italy and Brazil [25, 26]. The present study was restricted to a Northwestern province of Iran with a high-incidence rate of GC and different ethnic group characteristics (Azeri with an Altaic language family), which might explain why the results are not consistent with the some other reports within the country. In addition, Latifi-Navid et al. showed that the *babA2* genotype could not differentiate the *H. pylori* strains from the high and low incidence GC areas [6, 22]. In European countries such as Finland, Germany and Portugal, *babA2* is a biomarker for PUD [30, 31]. Mizushima et al. [32] demonstrated that the frequency of the *babA2* genotype is higher in Japan than in Western countries and that there is no significant correlation between the *babA2* genotype and clinical outcome in Japan. These results point to the usefulness of *babA2* as a virulence marker in predicting

**Table 2** Prevalence of *babA2*, *i1* and *i2* alleles among different disease groups

| Virulence markers | Diseases groups | | | Total strains |
|---|---|---|---|---|
| | Gastric cancer | Peptic ulcer | Chronic gastritis | |
| *babA2* | 19/24 (79.17) | 16/17 (94.12) | 41/48 (85.42) | 76/89 (85.39) |
| *i1* | 21/24 (87.5) | 6/17 (35.29) | 19/48 (39.58) | 46/89 (51.68) |
| *i2* | 3/24 (14.29) | 11/17 (64.71) | 29/48 (60.42) | 43/89 (48.31) |

Numbers in parenthesis are percentage of those genes

clinical outcome, which is most likely dependent on the geographic origin of the *H. pylori* strains.

Another virulence factor of *H. pylori* is the *vacA* gene. For *vacA*, a new polymorphic region (the *i* region) has been shown to be a major determinant of toxicity. González-Rivera et al. [33] showed that the VacA *i* region is a main determinant of VacA effects on human T cell function. The *vacA* type *i1* strains were shown to be a better marker of disease than *s1* or *m1* genotypes in Western and Iranian strains [19]. In Greece, an *i1* polymorphism was found to be associated with obvious chronic inflammatory infiltration and activity of chronic gastritis in the antrum while no relation was found with the presence of gastric atrophy and intestinal metaplasia [34]. These results were discordant with findings of our study. In our study, it was observed that the frequency of the *i1* allele (87.5 %) in *H. pylori* strains isolated from GC patients compared with that of *i2* allele is much higher than the relative frequency of these alleles (39.58 %) seen in patients with gastritis. Furthermore, the results of multiple logistic regression analysis showed that *vacA i1* genotype was significantly associated with GC compared with a control group. The results were in accordance with the previously reported prevalence of *H. pylori vacA i1* polymorphism in Iran. Recently, Basso et al. [20] showed that *i1* is associated not only with GC but also with DU in Italian isolates. A relationship was described by Yakoob et al. [35] between *i1* and GC, GU (gastric ulcer) and DU, while Hussein et al. [18] failed to find such a relationship between *i1* and GU in Iraq isolates. In a recent study, Lui and et al. [36] reported that most of the strains found were of the *i1* allelic type (102/104, 98.1 %), but with no association between the *vacA i1* allelic type and clinical outcome. In a Portuguese population, patients infected with *vacA i1* strains showed an increased risk of gastric atrophy and gastric carcinoma, with odds ratios of 8.0 (95 % CI 2.3–27) and of 22 (95 % CI 7.9–63), respectively [37]. In a Belgian population, the *vacA s1* and *i1* genotype strains emerged as the best markers of GC and duodenal ulcer [38]. In a recent study, Winter et al. [39] showed that non-i1 strains (*vacA s2/i2* or *s1/i2* strains) can cause metaplasia in mice, and in human gastric biopsy specimens, the *vacA i1* allele was strongly associated with precancerous intestinal metaplasia, which was almost totally absent in subjects infected with the strains harboring the *vacA i2* genotype, even in a *vacA s1, cagA(+)* background. These studies indicate that all three of the bacterial, host and environmental factors may be important in leading to particular clinical and pathological outcomes of the infection. Recently, Latifi-Navid et al. [6] demonstrated that the *vacA d1/-i1* genotypes, the newly recognized determinants of GC, have great potential for differentiating *H. pylori* strains from high and low incidence GC areas in Iran. They showed a very high (96.1 %) prevalence of the *vacA s1(a)* allele in Iran, similar to the pattern observed in Eastern countries. A distribution of more than 95 % was noted in both high and low incidence areas in Iran, implying that the *vacA s1(a)* allele was not capable of differentiating the *H. pylori* strains from these areas with different GC incidence rates. On the other hand, this study showed that the presence of the *vacA m* region genotype and the *cagA* gene might not be considered as independent of the *vacA d* and *i* regions in the differentiation of *H. pylori* strains from the high and low incidence GC areas in Iran. These findings indicate that the *H. pylori vacA i1* and d1 genotypes could probably be considered as determinant biomarkers in the prediction of GC risk, particularly in the high-incidence areas of GC. Given the age-standardized GC incidence rate of more than 20 per 100,000 estimated for East Azerbaijan in Iran [22] as well as our previous finding on the *vacA d1* allele [40], we considered the occurrence of the *vacA i1* and *babA2* genotypes and their association with the GC risk in the present study. Thus, we propose that the *i1 vacA* polymorphism could be an important biomarker for predicting GC risk in the Azerbaijan area in Iran. In addition, we have described the association between age and clinical symptoms. We observed that 95 % of patients with GC were 40 years or older, but the prevalence of gastritis was not significantly different in patients 40 years or older (45.8 %) and in patients less than 40 years (54.2 %) of age ($P = 0$). These findings confirm results reported in other parts of the world [41]. In sum, the *vacA i1* allele is frequently found in *H. pylori* strains from GC disease in Azerbaijan and it can

be an important biomarker for predicting GC risk in the Azerbaijan area in Iran.

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**Conflict of interest** None.

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