

RESEARCH ARTICLE

Helicobacter pylori babA2 Positivity Predicts Risk of Gastric Cancer in Ardabil, a Very High-Risk Area in Iran

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Abstract

Background: Ardabil, a Northwestern province of Iran, was found to have the highest rate of gastric cancer (GC) in the country (ASRs = 51.8/100,000 for males and 24.9/100,000 for females) and one of the highest gastric cardia cancer rates in the world. The aim of the present study was to assess the associations of the *cagA* and *babA2* status of *Helicobacter pylori* with GC in the Ardabil population. **Materials and Methods:** A total of 103 patients with non-atrophic gastritis (56) and GC (47), who underwent endoscopy at the Imam Khomeini Hospital in Ardabil, were assessed. The status of *16S rDNA*, *cagA* and *babA2* genes was determined using PCR and histopathological assessment was performed. **Results:** The following genotypic frequency was observed: *cagA+* (50.6%), *cagA-* (49.4%), *babA2+* (26.5%), *babA2-* (73.5%) *cagA+/babA2+* (19.3%), *cagA-/babA2+* (7.2%), *cagA+/babA2-* (31.3%), *cagA-/babA2-* (42.2%). Although the frequency of the *cagA+*, *cagA+/babA2+* and *cagA-/babA2+* genotypes in patients with GC (55.6%, 25.9%, and 14.8%, respectively) was higher than in those with NAG (48.2%, 16.1%, and 3.6%, respectively), the difference did not reach significance. In contrast, the presence of the *babA2* gene (40.7% vs 19.6%) significantly increased the risk of GC; the age-sex-adjusted odds ratio (95% confidence interval) was 5.068 (1.506-17.058; $P=0.009$), by multiple logistic regression. **Conclusions:** It is proposed that the *H. pylori* *babA2* positivity might be considered as an important determinant of GC risk in Ardabil.

Keywords: *H. pylori* - *cagA* - *babA2* - gastric cancer - high-incidence - Ardabil - Iran

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Introduction

Helicobacter pylori is a microaerophilic gram negative spiral bacterium that is able to localize in the stomach mucosa for at least tens of thousands of year. Several epidemiological studies have shown that the *H. pylori* infection causes a variety of gastrointestinal symptoms such as chronic gastritis and adenocarcinoma (Suerbaum and Michetti, 2002). Although the incidence and mortality of gastric cancer (GC) have declined in recent decades, it is the third-ranking cause of cancer which leads to death and the fifth most common malignant disease in the world (Ferlay et al., 2015). Histologically, GC divided into the intestinal- and the diffuse-type carcinomas. Previous studies have suggested that the interaction between host and environmental factors are jointly responsible for development of GC (Leung et al., 2006).

Stomach cancer is very common in Iran (Alireza et al., 2005a; Alireza et al., 2005b). Ardabil, a volcanic and mountainous land in Northwestern province of Iran that is located in Caspian Sea littoral, includes the highest rate of GC within the country (ASR of 51.8/100,000 in men and 24.9/100,000 in women) (Sadjadi et al., 2003; Babaei et al., 2010). In this area, more than one-third of the GC occurs in the cardia site of the stomach which constitutes

only 5-10% of the entire stomach, and the incidence for cardia sub site was 26.4 and 8.6 for males and females, respectively (Derakhshan et al., 2004; Babaei et al., 2009). *H. pylori* infection has been measured as 89% and 69% in Ardabil province and Iran, respectively (Sadjadi et al., 2003; Malekzadeh et al., 2004; Nouraei et al., 2009). We have previously indicated that all the Iranian *H. pylori* strains, including those with Ardabil signature, represent the European ancestry, influenced by extensive genetic exchange with neighboring countries. However, they have well preserved ethnic and geographic signatures within the country (Latifi-Navid et al., 2010). Ardabil isolates showed approximately equal contribution from the two European ancestral sources, Ancestral Europe1 and Ancestral Europe2 (AE1 and AE2) that were found in purer form in Central Asia (AE1) and North East Africa (AE2).

CagA is one of the putative virulence factors of *H. pylori*, which is encoded by the cytotoxin-associated gene A (*cagA*) that is located in the *cag* pathogenicity island (*cag* PAI) and directly translocated into host gastric epithelial cells by a type-IV secretion system (T4SS) after bacterial attachment (Mattar et al., 2007; Ohnishi et al., 2008). It promotes the tumorigenesis of GC by interfering with multiple signaling pathways (Yong et al., 2015).

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Approximately, the 50 to 60% of *H. pylori* strains harbor *cagA* (Abasiyanik et al., 2002; Basso et al., 2008), whose expression induces the production of IL-8 and mucosal inflammation in cultured gastric cells (Chang et al., 2015; Ferreira et al., 2015). Several studies have been reported that the individuals harboring *cagA*-positive strains suffer from a high risk for gastric mucosal atrophy and GC, compared to those with strains that lack the *cag* PAI (Sozzi et al., 2005; Tan et al., 2006). *orf17* and particularly *cagL*, which are other genotypes of *cag* PAI, significantly increased the risk of peptic ulceration (PU), but not GC. No significant association was found between the *cagH* and *cagG* genotypes and the risk of both the PU and the GC (Raei et al., 2015). *H. pylori* strains may be classified into two types: type I and type II, based on possessing the *cag* PAI (Bagheri et al., 2013). The East Asian subtype of the *cagA* gene (A, B, D) strongly linked to severe gastritis and GC, in comparison with western subtype (A, B, C) (Argent et al., 2008).

Another important virulence factor is *babA2* (blood group antigen binding adhesion) that encoded by the *babA2* gene. BabA is a 78-KDa outer membrane protein (OMP) that binds to lewis b antigens and ABO antigen on the surfaces of gastric epithelial cells (Gerhard et al., 1999). Two *babA* allelic types identified (*babA1* and *babA2*); however, only the *babA2* gene is functionally active. The presence of the 10-bp insertion is the cause of the difference between *babA1* and *babA2* (Ilver et al., 1998). Expression of the *babA2* gene has also been associated with the risk of GC (Lee et al., 2006).

Since the diagnosis of GC in the earlier stages of infection is difficult; therefore, eradication of the *H. pylori* infection might be the main way of cancer conservation. Therefore, it is necessary to determine which strains of *H. pylori* lead to GC. *H. pylori*-specific genotypes could determine the clinical consequences and contribute to understanding of the cause of high tendency for GC (Figueiredo et al., 2002; Peek et al., 2010). The aim of the present study was therefore to determine the *H. pylori* *cagA* and *babA2* status and their associations with GC in Ardabil population.

Materials and Methods

Participants of the study

From February 2012 to January 2014, a total number of

103 consecutive adult patients who underwent endoscopy at the Imam Khomeini Hospital in Ardabil, Northwestern Iran, participated in this study. All patients were born and grew up in the rural or urban areas of Ardabil province. Fresh gastric mucosal biopsy specimens were obtained from each patient and classified by endoscopic and histopathological examinations. The study population consisted of patients with none-atrophic gastritis (NAG) and GC. None of the patients had received none steroidal anti-inflammatory drugs or antibiotics within the previous three months, and all the participants signed a written informed consent.

Histopathological assessment

The biopsy samples were fixed in 10% formalin and embedded in paraffin, and were stained by hematoxylin-eosin, Alcian blue-periodic acid Schiff (pH 2.5) and Giemsa for light microscopy, and histopathological evaluations were also applied according to the updated Sydney classification system (Dixon et al., 1996).

Diagnosis of *H. pylori* infection

During endoscopy, of each patient four biopsies (two from the antrum and two from the corpus) were taken for histological examination and DNA extraction. The Biopsy specimens were frozen at -80°C until processing. Detection of *H. pylori* was performed by using a specific primer set (HP1/HP2) of *H. pylori* 16S rDNA, selected from some previous published works that produced the PCR fragments of 519-bp (Table 1).

DNA extraction and genotyping of *H. pylori*

DNA was extracted from the biopsy specimens by using Genomic DNA purification kit (DNGTM-Plus, CinnaGen Co., Iran) according to the manufacturer's recommendations, and stored at -20°C. After DNA extraction, 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). Amplification was also performed under the following conditions: for *cagA*: 35 cycles of 40 sec at 94°C, 1 min at 50°C (C-terminus), 55°C (middle region), or 56°C (N-terminus) and 1 min at 72°C, and for *babA2*: 35 cycles

Table 1. Oligonucleotide Primers Used for PCR

Genes	Primers	Sequences (5'→3')	Size of PCR products (bp)	Optimized annealing temperature (°C)
<i>16S rDNA</i>	HP1	GCAATCAGCGTCAGTAATGTTC	519	56 (Lu et al., 2002)
	HP2	GCTAAGAGATCAGCCTATGTCC		
<i>cagA</i>	N-terminus	<i>CagA</i> /N-F	CCATTTTAAGCAACTCCATAAACC	413 (Bakhti et al., 2015)
		<i>CagA</i> /N-R	CTGCAAAAAGATTGTTTGGCagA	
	Middle region	<i>CagA</i> /M-F	GGCAATGGTGGTCTGGAGCTAGGC	243 (Bakhti et al., 2015)
		<i>CagA</i> /M-R	GGAAATCTTTAATCTCAGTTCGG	
C-terminus	CAG1	ACC CTAGTC GGT AAT GGG TTA	591-856	50 (Sicinschi et al., 2010)
	CAG2	GTA ATT GTC TAG TTT CGC		
<i>babA2</i>	<i>BabA2</i> F	AATCCAAAAAGGAGAAAAAGTATGAAA	852	55 (Gerhard et al., 1999)
	<i>BabA2</i> R	TGTTAGTGATTTCGGTGTAGGACA		

of 40 sec at 94°C, 1 min at 55°C and 1 min at 72°C. PCR products were visualized by electrophoresis in 1% agarose gel and examined under UV illumination. Primers used for PCR assays of *16S rDNA*, *cagA* and *babA2* genes are also listed in Table 1. As controls, amplified fragments of each gene from seven isolates were purified and sequenced with both forward and reverse primers by 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.

Data analysis

Chi-square (χ^2) or Fisher's exact tests were used to assess the associations between the *cagA* and *babA2* status and GC, whether independently or in combination. Patients with NAG were considered as controls. Odds ratios (ORs) and the corresponding 95% confidence intervals (CIs) were also calculated. The Forward Stepwise LR (Likelihood Ratio) multiple logistic regression analysis was used for each gene individually, with adjustment for the potential confounders, including a threshold age of 40 years and sex. All the P-values were two-sided and $P < 0.05$ was considered statistically significant. Data were collected and analyzed by using SPSS software (SPSS Science, Chicago, IL) version 19.0.

Results

Patients characteristics

The study population consisted of 103 patients, among these, 56 (54.37%) were diagnosed as gastritis and 47 (45.63%) had suffered from GC. Occurrence of *H. pylori* infection was 80.58% (83/103). Among them, the results of *16S rDNA* PCR for 57.44% (27/47) of patients with GC and 100% (56/56) of those with NAG were positive. Generally, the total number of 83 *H. pylori* positive patients, 49 (59%) males and 34 (41%) females, with an average age of 48.7 years (the age range varied between

20 and 90 years), were enrolled in the final analysis. In GC group, 70.4% (19/27) and 29.6% (8/27) were males and females, respectively. Characteristics of patients according to age and sex were shown in Table 2. Results of statistical analysis showed a significant association between age (OR = 11.027, 95% CI, 1.376-88.370; $P = 0.008$), but not sex (OR = 2.058, 95% CI, 0.773-5.878; $P = 0.162$), and GC.

In this study, the prevalence of GC patients according to the anatomical site of the tumor origin was as follows: 53.19% (25/47) with gastric cardia cancer (GCC), 44.68% (21/47) with non-cardia GC (NCGC) and 2.13% (1/47) with both the GCC and the NCGC. Of the 47 GC patients, 27 were infected with *H. pylori*; of them 12 (44.44%) were with GCC, 14 (51.85%) with NCGC and 1 (3.70%) with both the GCC and the NCGC. The prevalence of the intestinal- and the diffuse-type of adenocarcinoma and the squamous-cell carcinoma and the mucinous carcinoma of the stomach was 65.22% (30/46), 30.43% (14/46), 2.17% (1/46) and 2.17% (1/46), respectively. This information was not available for one patients. Among of *H. pylori* infected patients, 53.85% (14/26) and 46.15% (12/26) had the intestinal- and the diffuse-type of adenocarcinoma, respectively. None of the patients had a mixed type of adenocarcinoma.

Genotyping of *cagA* and *babA2*

The distribution of the *cagA* or *babA2* positive and negative strains in patients was shown in Table 3. The *cagA* and *babA2* genes were detected in 50.6% (42/83) and 26.5% (22/83) of *H. pylori* strains. In this study, the total prevalence of the *cagA*-, *babA2*-, *cagA*+/*babA2*+, *cagA*-/*babA2*+, *cagA*+/*babA2*- and *cagA*-/*babA2*- were as follows: 49.4% (41/83), 73.5% (61/83), 19.3% (16/83), 7.2% (6/83), 31.3% (26/83) and 42.2% (35/83), respectively. The prevalence of the *cagA*+ and *babA2*- strains was higher than *cagA*- and *babA2*+ strains. In our study, strains carrying the *cagA* and *babA2* genes were also more frequent in GC patients (55.6% and 40.7%, respectively) than in the control group (NAG) (48.2% and 19.6%, respectively). However, no significant association was found between the *cagA*+ strains and the risk of GC ($P > 0.05$). Although, the prevalence of the *cagA*+/*babA2*+ and *cagA*-/*babA2*+ genotype combinations in patients with GC (25.9% and 14.8%, respectively) was higher than in those with NAG (16.1% and 3.6%, respectively), no significant association with GC was found. Furthermore, the *cagA*+/*babA2*- and *cagA*-/*babA2*- genotype combinations did not show any significant association with GC ($P > 0.05$). The presence of the *babA2* gene significantly

Table 2. Distribution of Study Patients According to Age and Sex

Disease	Age			Sex		
	< 40 N (%)	≥ 40 N (%)	Total	Females N (%)	Males N (%)	Total
NAG	17 (31.5)	37 (68.5)	54	26 (46.4)	30 (53.6)	56
GC	1 (4)	24 (96)	25	8 (29.6)	19 (70.4)	27
Total	18 (22.8)	61 (77.2)	79	34 (41)	49 (59)	83

Abbreviations: NAG, non-atrophic gastritis; GC, gastric cancer

Table 3. Association between the *H. pylori cagA* and *babA2* Genes and GC Risk

Genotypes	NAGN (%)	GCN (%)	TotalN (%)	OR (95% CI)	2-tailed P-value
<i>cagA</i> +	27 (48.2)	15 (55.6)	42 (50.6)	1.343 (0.435-3.773)	0.531
<i>cagA</i> -	29 (51.8)	12 (44.4)	41 (49.4)		
<i>babA2</i> +	11 (19.6)	11 (40.7)	22 (26.5)	2.813 (1.022-7.737)	0.041
<i>babA2</i> -	45 (80.4)	16 (59.3)	61 (73.5)		
<i>cagA</i> +/ <i>babA2</i> +	9 (16.1)	7 (25.9)	16 (19.3)	1.828 (0.895-5.985)	0.286
<i>cagA</i> -/ <i>babA2</i> +	2 (3.6)	4 (14.8)	6 (7.2)	4.696 (0.803-27.461)	0.064
<i>cagA</i> +/ <i>babA2</i> -	18 (32.1)	8 (29.6)	26 (31.3)	0.889 (0.328-2.412)	0.817
<i>cagA</i> -/ <i>babA2</i> -	27 (48.2)	8 (29.6)	35 (42.2)	0.452 (0.170-1.203)	0.108

Abbreviations: NAG, non-atrophic gastritis; GC, gastric cancer; OR, odds ratio; CI, confidence interval

increased the risk of GC in the multiple logistic regression with adjustment for age and sex (OR = 5.068, 95% CI, 1.506-17.058; $P=0.009$).

Discussion

Ardabil, a Northwestern province of Iran, is one of the areas with the highest GC incidence rate in the world. In the present study, we therefore investigated the association of the *cagA* and *babA2* status with the risk of GC in Ardabil population. Through analyzing the data by means of logistic regression, the results showed no significant association between the *cagA* status of *H. pylori* and GC risk; however, the importance of *CagA* EPIYA polymorphisms has not been confirmed in Ardabil population yet; therefore, determining the associations of the *CagA* EPIYA polymorphisms with GC is important in this high-risk area. Recent studies have shown that the multiple repeats of *H. pylori CagA* EPIYA-C phosphorylation sites predict risk of gastric ulcer (GU) in Iran (Honarmand-Jahromy et al., 2015). The GU and GC have etiologic factors in common, which are likely to be caused by the *H. pylori*-induced atrophic gastritis (Hansson et al., 1996). We found that the *cagA* gene is highly prevalent in Ardabil patients (50.6%), but it could not be considered as an important risk factor for GC. However, in agreement with other studies (Dabiri et al., 2009; Joobar et al., 2012), a high frequency of the *cagA* gene was also observed in GC patients in our study (55.6%). Several studies on Tehran population, of Iran, indicated no significant association between the presence of the *cagA* gene and GC risk (Jafari et al., 2008; Dabiri et al., 2010; Bagheri et al., 2013). In contrast, study conducted by Cittelly et al. in Colombians showed that the frequency of the *cagA* gene was significantly higher in GC patients (80%) than in control groups (51.4%) ($P<0.01$). They proposed that the presence of the *cagA* gene significantly increased the risk of GC (Cittelly et al., 2002). Also, study performed by Cavalcant et al. from a high-risk area of GC in Brazil demonstrated that the presence of the *cagA* gene particularly associated with the risk of GC (OR=10.36) (Cavalcante et al., 2012). A recent follow-up study from a high-risk area of GC in Spain showed that infection with *cagA+vacAs1/m1* strains was related to the progression of gastric precancerous lesions (OR = 4.80) compared with those infected with strains that harbored the *cagA-/-vacAs2/m2* genotype (González et al., 2011). In other study which was performed in a low-risk area of Okinawa of Japan, a strong relationship was found between *cagA* gene and GC risk in comparison with control groups, in a multiple logistic regression (OR = 6.68) (Matsunari et al., 2012). The prevalence of the *cagA* gene in Western and Eastern countries was reported to be 60% and 95%, respectively, however, only in Western countries, a significant association with GC was found (Kim et al., 2001; González et al., 2011). A meta-analysis conducted by Zhao et al. also showed that the *H. pylori CagA* antibody of the serum could not be useful for detecting GC in East Asian countries, with the highest incidence rates of GC (Zhao et al., 2014). This is in agreement with the results of the present study

which was conducted in a genotype level and restricted to Ardabil, with the highest GC incidence rate in Iran and different ethnic group characteristics, Azeri with an Altaic language family. Interestingly, it has also been shown that the *H. pylori CagA*-negative non-virulent isolates may be a potential risk factor of pancreatic cancer (Chen et al., 2015).

Our findings demonstrated that the prevalence of the subjects harboring *babA2-* strains was significantly higher than the subjects with *babA2+* strains. The *babA2+* *H. pylori* isolates were present in approximately 26.5% of the study population. The patients infected with the *H. pylori* strains which carry *babA2* gene showed a higher risk of developing GC which was about 5.068 fold, by the multiple logistic regression; therefore, it could be considered an important virulence factor in Ardabil population. We have previously shown no significant difference in the distribution of the *babA2* and *cagA* genes among *H. pylori* isolates from high (34.2% and 75.6%, respectively) and low (44.4% and 63.9%, respectively) incidence GC areas ($P>0.05$) (Latifi-Navid et al., 2013). In Iran, the frequency of *babA2* genotype was 40.6% (Abadi et al., 2013; Latifi-Navid et al., 2013), which was strongly associated with GC in comparison with the control groups ($P=0.0004$) (Abadi et al., 2013). Similarly, study conducted by Gerhard et al. showed that frequency of the *babA2* gene in GC patients (78%) was higher than those with gastritis (51%), with significant difference (Gerhard et al., 1999). Erzin et al. showed that the frequency of the *babA2* gene in GC patients (87.9%) was higher than those with NAG (23.3%) ($P=0$) (Erzin et al., 2006). Furthermore, in Germany, Turkey, or northern Portugal, *babA2* expression was closely associated with the severity of gastrointestinal disease (Gerhard et al., 1999; Azevedo et al., 2008; Erzin et al., 2008), while in Portuguese and Thai populations, the presence of the *babA2* gene was not associated with GC risk (Gatti et al., 2006; Chomvarin et al., 2008). These results reflect the fact that the associations of *H. pylori* virulence genes/alleles with GC risk have been widely varied according to geographic variation/ or ethnic origin.

We proposed that the *H. pylori babA2* positivity might be considered as an important determinant of GC risk in Ardabil. Hence, this study provided us with valuable information which might be helpful to reduce the incidence of GC in high-risk areas, particularly Ardabil. We propose that more investigations with a sufficient number of samples are necessary in order to better depicting the bacterial genotype diversity and the role of environmental and host factors and their associations with GC in this the very high-incidence area.

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