Helicobacter Pylori Different Virulence Genes and the Risk of Gastric Cancer in Iranian Patients

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Abstract

Background: Some disease-specific Helicobacter pylori virulence genes can be used for predicting the outcome of diseases. Thus, the current study aimed to explore the frequency of the vacA, cagA, sabA, dupA, babA, oipA, and iceA1 genes in H. pylori isolates, and to determine whether any association exists between the expression of these genes and gastric cancer (GC).

Methods: H. pylori isolates were collected from individuals with digestive diseases. The cagA, vacA, dupA, sabA, babA, oipA, and iceA1 genes were determined by PCR. The qRT-PCR was used for expression analysis of the tested genes.

Results: The presence of the cagA, vacA, dupA, sabA, babA, oipA and iceA1 genes in H. pylori isolates were 41.3%, 95.5%, 31.0%, 93.1%, 55.1%, 82.7%, and 62.0%, respectively. The cagA, dupA, and babA expression in the GC patients was statistically higher than that of the control group (P <0.05).

Conclusions: Our results indicated a high diversity and frequency of H. pylori virulence genes among individuals with gastric diseases in the Iranian population. The cagA, dupA, and babA expression were significantly higher in the GC patients, thus, it may suggest that screening of these genes may help identify peoples at higher risk for GC.

Background

Gastric cancer (GC) is a global concern and remains the third leading cause of mortality from cancer worldwide [1]. Positive serologic tests for Helicobacter pylori is among the main causes of GC, accounting for approximately 89% of GC cases worldwide [1–3]. The relationship of H. pylori infection and several extra-intestinal diseases, such as migraine, Alzheimer's, and mild cognitive impairment, has also been addressed [4–8].

Earlier surveys indicated that those with chronic infection with H. pylori were more likely to develop GC [9–11]. Moreover, the World Health Organization and the International Agency for Research on Cancer (IARC) categorized H. pylori as a Group 1 carcinogen for humans [12].

Among the individuals infected with H. pylori, only 1–3% progress to GC [13]. Some H. pylori virulence genes may play an essential role in the progress of gastric diseases. The strongest candidates include the outer inflammatory protein (oipA), the sialic acid-binding adhesin (SabA), the vacuolating cytotoxin (vacA), the blood group antigen-binding adhesin (babA), the cytotoxin-associated antigen A (cagA), duodenal ulcer promoting gene (dupA), and induced by contact with epithelium gene (iceA) [14–20]. These virulence genes may lower the threshold for neoplastic alteration by dysregulating the host intracellular signaling pathways [13].

In Iran, the rate of H. pylori infection is very high lies between 60% and 90% in different ages and geographic regions [21]. Likewise, the majority of H. pylori-infected subjects are cagA-positive strains that
are related to a greater risk of GC in some Iranian populations [21]. Despite the high prevalence of cagA positive H. pylori in Iran, its contribution to excess cancer risk is unclear. Furthermore, limited studies in Iran have concurrently investigated the relationship to GC of these virulence-related genes. Thus, in the current study, we examined the association of cagA, vacA, dupA, sabA, babA, oipA, and iceA1 genes of H. pylori with GC.

**Methods**

**Study population, samples, and culture**

Gastric biopsies for isolation of H. pylori were taken from subjects with digestive diseases at a teaching hospital in Qom, Iran in the year 2018. Eleven biopsy specimens including four specimens of the greater and lesser curvature of the antrum and four specimens of small and large curvature of the gastric body and two specimens of the duodenum (5 biopsies for histopathological tests and 5 biopsies for culture and molecular tests) and one specimen for testing Rapid urease RUT was taken from each patient.

The culture method was performed as described by Dabiri et al, previously [22]. Briefly, each specimen was placed into brucella Agar with 10% defibrinated sheep blood containing the supplement (5 mg/l amphotericin B, 5 mg/l trimethoprim, 10 mg/l vancomycin). The plates were placed at 37 °C (CO2 10%, N2 85%, O2 5%) and were studied after 5 days of incubation. Urease, catalase, oxidase tests were used for confirmation of H. pylori isolates. The colonies of H. pylori were further used for molecular analysis.

**Commercial Rapid Urease Test**

The RUT was done as described by the instructions of the manufacturer (HelicotecUT® Plus, Taiwan). The tests were recorded and observed within 1 hour. The color changed from yellow to pink indicated a positive RUT.

**Molecular Identification Of H. Pylori**

DNA was extracted using a commercial kit (Bio Basic, Canada). For molecular confirmation of H. pylori isolates, PCR for 16SrRNA and glmM genes using specific primers was performed based on published papers [23].

**PCR assays for virulence genes (cagA, vacA, dupA, sabA, babA, oipA, and iceA1)**

PCR was carried out according to the study of Dabiri and colleagues [22]. The primers sequences used for vacA, cagA, dupA, sabA, babA, oipA, and iceA1, and the expected product lengths are shown in Table 1. Negative control (Double distilled water) and positive control (H. pylori DNA 26695 and J99) reactions were used in the PCR run.
Table 1
Primer sequences of the tested genes Rerfvers.

| Genes  | Primers (5’ − 3’) | Product (bp) |
|--------|-------------------|--------------|
| glmM   | Forward - AAGCTTTTAGGGTGTTAGGGGTTT  
         | Reverse - AAGCTTACTTTCTAACACTAACGC | 294  |
| 16S rRNA | Forward - CTGGAGAGACTAAGCCCTCC  
         | Reverse - ATTACTGACGCTGATTGTGC | 110  |
| cagA   | Forward - TTGACCAACAACCACAAACCGAAG  
         | Reverse - CTTCCCTTAATTGCGAGATTCC | 183  |
| vacA   | Forward - ATGGAAATACAACAAACAC  
         | Reverse - CAACAAATGGCTGGAATGAT | 136/163 |
| iceA1  | Forward - GTGTTTTTAACCAAAGTATC  
         | Reverse - CTATAGCCASTYTCTTTGCA | 247  |
| babA   | Forward - CACGATCAGTTCAAAAG  
         | Reverse - TTRATGAGCGTGCTGCTTGCG | 138  |
| dupA   | Forward - CAAAGGAACACAACAAACCT  
         | Reverse - CGATATGCGCAAACATTATAGATG | 84  |
| oipA   | Forward - GTTTTTATCGATGGGATTT  
         | Reverse - GTGCATCTCTTATGGCTTT | 401  |
| sabA   | Forward - CCAACAACATTTGAGCTGGTC  
         | Reverse - TTGCAARATRGGTATCATCG | 593  |

Rna Extraction And Qrt-pcr

RNA was extracted using an RNA extraction kit (BioFACT™ Total RNA Prep Kit, Korea) according to the manufacturer’s instructions.

The qRT-PCR was used for expression analysis of *H. pylori* pathogenicity genes. The PCR conditions and primer sequences for the 16srRNA, cagA, vacA, dupA, sabA, BabA, oipA, and iceA1 genes have been described previously [23–30]. A cDNA synthesis kit (BioFACT™ 2-Step 2X RT-PCR Pre-Mix (Taq) Kit, Korea) was used for cDNA synthesis. Amplification was done using 40 cycles, each cycle consisting of 94 °C for 15 s, 55 °C (cagA and dupA), 56 °C (16srRNA and SabA), 53 °C (babA), 52 °C (oipA), 51 °C (vacA) or 49 °C
(iceA1) for 30 s, and 72 °C for 25 s. The reactions were carried out using an ABI step ONE Plus thermal cycler. A 2% agarose gel was used for the analysis of products.

**Statistical Analysis**

Data analysis was carried out using Statistical Package for the Social Sciences software (SPSS version 23.0) and Graph Pad Prism software version 8.0.2. A Chi-square test was used for the analysis of categorical data. ANOVA statistical test and Tukey post hoc test were applied for quantitative analysis. P values of less than 0.05 were considered statistically significant.

**Results**

In this study, 190 patients with digestive disorders were enrolled. Among them, 13 were excluded from the study due to the unavailability of demographic information. Based on histological and endoscopic findings, patients were classified into three groups: 91 people without ulcer (NUD = non-ulcer dyspepsia), 55 with peptic ulcer disease (PUD), and 31 with GC. According to the RUT, 51 (28.8%) tissue samples were positive for *H. pylori* and 126 (71.2%) specimens were negative. In the histology method, 40 (22.6%) biopsy specimens were positive and 137 (77.4%) specimens were negative. Likewise, in the culture method, *H. pylori*-positive and -negative samples were 42 (23.7%) and 135 (76.3%) respectively. Among the specimens with a positive culture, 13 samples were excluded from the study due to a low number of bacteria and insufficient amounts of DNA and RNA for molecular tests. Finally, 29 isolates of *H. pylori* remained for further analysis (Fig. 1).

The patients with positive culture were assessed for the relation of gender, age, ethnic group, history of smoking, and alcohol intake with the severity of illness (Table 2). No statistically significant difference was observed between these variables and gastroduodenal diseases. However, the age groups of > 60 years had an increased rate of GC disease.
Table 2
Distribution of 29 patients with different clinical outcomes, according to age, gender, ethnic group, history of smoking, and alcohol intake.

| Parameters          | Non-ulcer (N = 13) | Peptic ulcer (N = 8) | GC (N = 8) | P-value |
|---------------------|--------------------|----------------------|------------|---------|
| Gender              |                    |                      |            |         |
| Male                | 6 (46%)            | 4 (50%)              | 7 (87.5%)  | 0.15    |
| Female              | 7 (54%)            | 4 (50%)              | 1 (12.5%)  |         |
| Age                 |                    |                      |            |         |
| ≤ 20                | 0                  | 0                    | 0          | 0.07    |
| 40–60               | 13 (100%)          | 8 (100%)             | 8 (100%)   |         |
| > 60                | 0                  | 0                    | 0          |         |
| Ethnic group        |                    |                      |            |         |
| Iranian             | 12 (92.3%)         | 7 (87.5%)            | 7 (87.5%)  | 0.90    |
| Afghan              | 1 (7.7%)           | 1 (12.5%)            | 1 (12.5%)  |         |
| History of smoking  |                    |                      |            |         |
| Yes                 | 1 (7.7%)           | 2 (25%)              | 1 (12.5%)  | 0.45    |
| No                  | 12 (92.3%)         | 6 (75%)              | 7 (87.5%)  |         |
| Alcohol intake      |                    |                      |            |         |
| Yes                 | 0                  | 0                    | 0          | 0.90    |
| No                  | 13 (100%)          | 8 (100%)             | 8 (100%)   |         |

Pcr Results Of The Tested Genes

The presence of the cagA, vacA, dupA, sabA, babA, oipA, and iceA1 genes was investigated in patients with a positive culture for the H. pylori. There was no statistical relationship between these genes and clinical Consequences (Table 3). The cagA and vacA genes were detected in 41.3% (12/29) and 96.5% (28/29) of the patients, respectively. Similarly, to vacA, sabA, and oipA were found more commonly in patients with H. pylori. Of the 29 H. pylori-infected samples, dupA, babA and iceA1 were detected in 31.0% (9/29), 55.1% (16/29), and 62.0% (18/29), respectively. As shown in Table 3, cagA, dupA, and babA were most commonly found in the GC patients, whereas sabA, oipA, and iceA1 were most commonly found in the Non-ulcer patients (p > 0.05). Although, the vacA gene was frequently found in all patient groups; there was no significant difference among the investigated patient (p > 0.05) (Table 3).
Table 3
The cagA, vacA, DupA, SabA, BabA, OipA, and iceA1 status of H. pylori isolates obtained from 29 patients with different clinical outcomes.

| Genes | Non-ulcer (N = 13) | Peptic ulcer (N = 8) | GC (N = 8) | P-value |
|-------|--------------------|----------------------|-----------|---------|
| cagA  | Positive 3 (23%)   | 4 (50%)              | 5 (62.5%) | 0.17    |
|       | Negative 10 (77%)  | 4 (50%)              | 3 (37.5%) |         |
| vacA  | Positive 13 (100%) | 7 (87.5%)            | 8 (100%)  | 0.25    |
|       | Negative 0         | 1 (12.5%)            | 0         |         |
| DupA  | Positive 3 (23%)   | 1 (12.5%)            | 5 (62.5%) | 0.06    |
|       | Negative 10 (77%)  | 7 (87.5%)            | 3 (37.5%) |         |
| SabA  | Positive 13 (100%) | 8 (100%)             | 6 (75%)   | 0.60    |
|       | Negative 0         | 0                    | 2 (25%)   |         |
| BabA  | Positive 6 (46.2%) | 4 (50%)              | 6 (75%)   | 0.40    |
|       | Negative 7 (53.8%) | 4 (50%)              | 2 (25%)   |         |
| OipA  | Positive 12 (92.3%)| 7 (87.5%)            | 5 (62.5%) | 0.20    |
|       | Negative 1 (7.7%)  | 1 (12.5%)            | 3 (37.5%) |         |
| iceA1 | Positive 9 (69.2%) | 5 (62.5%)            | 4 (50%)   | 0.60    |
|       | Negative 4 (30.8%) | 3 (37.5%)            | 4 (50%)   |         |

H. Pylori Virulence Gene Expression

Expression of the Virulence-associated genes cagA, vacA, dupA, sabA, babA, oipA, and iceA1 was detected using qRT-PCR. Expression was calculated based on the cycle threshold for the gene of the target relative to that for the 16srRNA (control group). As shown in Fig. 2A, cagA expression in the GC group was 2336-fold higher than that of the control group, which was statistical significance (P < 0.0001). Likewise, dupA and babA expressions in the GC group were statistically 378- and 3.4-fold higher than that
of the control group, respectively (p < 0.05) (Fig. 2B, C). On the contrary to the cagA, dupA, and babA, the expression of vacA and sabA genes in the GC group were significantly 1.6- and 4.5 times lower than that of the control group, respectively (p < 0.05). For oipA and iceA1 genes, there was no significant difference between the GC and control groups (p > 0.05).

**Discussion**

In the current study, we indicated the association between virulence genes of *H. pylori* and the GC. *H. pylori* infections are frequently asymptomatic and only a few individuals develop GC [31]. It has been indicated that some *H. pylori* virulence genes may play a significant role in the progress of post-*H. pylori* infection disorders such as GC.

In the previous studies, cagA and vacA of *H. pylori* were shown to have a significant association with peptic ulcers and GC [32–36]. Nevertheless, other studies have not established this association, specifying geographic variation [37]. Based on our analysis, the cagA gene was observed in 41.3% of the *H. pylori*-infected patients. The current reports are in comparison with earlier reports that show the cagA gene was present in about 65% of *H. pylori* isolates obtained from Asian and Western peoples [37–39]. The cagA-positive isolates of *H. pylori* have been also reported to be associated with gastric mucosal atrophy and GC [35, 40]. In a meta-analysis study, it was shown that patients infected with cagA-positive *H. pylori* isolates have an increased risk for GC [32]. Similarly, the subsequent analysis showed that infection by cagA-positive *H. pylori* isolates can increase the risk of GC [35]. Gohardani *et al.* showed that the expression of cagA was higher in Iranian patients with peptic ulcers compared to those with non-ulcer dyspepsia [41]. However, in studies conducted in Korea and Japan, there was no significant association between the cagA gene and the severity of gastroduodenal diseases [42, 43]. According to recent studies, the diversity of cagA types may explain such discrepancy [37].

The expression of babA and dupA genes were also significantly associated with GC in the current study.

Gerhard *et al.*, and Hocker *et al.* revealed that *H. pylori* strain to carry babA was associated with the highest risk of the ulcer [44, 45]. In our results, however, there was not any association between babA- and dupA-positive *H. pylori* isolates and the development of GC, even though the expression of babA and dupA genes were also significantly associated with GC.

Our findings were inconsistent with the dupA gene being a marker for intestinal metaplasia and GC. In this survey, there was not any association between the presence of dupA and GC. Hong Lu *et al.* indicated that dupA was associated with a reduced risk for GC and an increased risk for duodenal ulcers [46]. A study in Brazilian adults also described that the existence of dupA was statistically lower in patients with GC [47]. However, Argent *et al.*, reported that the dupA-positive *H. pylori* strains were more frequent in GC patients than duodenal ulcers (71% vs. 50%) [48]. The difference between these studies may be related to differences in the descriptions of geographic variations of circulation strains or patient groups.
In the current study, the *vacA* gene was present in 96.5% of *H. pylori* isolates, but no significant association between this gene and GC was observed. Likewise, in Asian studies, the existence of *vacA* was not statistically associated with GC [42, 43].

The *iceA1* gene was not associated with GC in this study. However, some reports have proposed a reverse correlation [37, 38]. Two distinct allelic variants of *iceA1* exist, in which *iceA1* has been suggested to be associated with gastric disease [37].

The *oipA* gene was existing in 82% of *H. pylori* isolated in the current which was following the previous studies [49, 50]. Kudo et al. and Yamaoka et al. also identified the *oipA* gene from 30% and 45.9% of *H. pylori* isolates, respectively [51, 52]. Similar to our result, Shao *et al.*, declared that no relationship exists between the *oipA* gene and gastrointestinal diseases [53]. Overall, the association between the *oipA* gene and GC needs further investigation.

In our study, the expression of some virulence genes of *H. pylori* was significantly higher in GC patients. Thus, it has been revealed that the eradication of *H. pylori* in patients with early GC would prevent the progress of new cancer [54].

**Conclusion**

Our finding indicated a high diversity and frequency of *H. pylori* virulence genes in GC patients in the Iranian population. Although there was not a significant association between different virulence genes with the clinical outcomes, the *cagA, dupA, and babA* expression in the GC group was significantly higher than that of the control group. Thus, it may suggest that screening of these genes may help identify populations at higher risk for GC.

**Abbreviations**

*Helicobacter pylori* (*H. pylori*)

Outer inflammatory protein (*oipA*)

Sialic acid-binding adhesin (*SabA*)

Vacuolating cytotoxin (*vacA*)

Blood group antigen-binding adhesin (*babA*)

Cytotoxin-associated antigen A (*cagA*)

Duodenal ulcer promoting gene (*dupA*)

Induced by contact with epithelium gene (*iceA*)
Gastric Cancer (GC)

Polymerase chain reaction (PCR)

Real-time quantitative PCR (qRT-PCR)

International Agency for Research on Cancer (IARC)

Rapid urease test (RUT)

Complementary DNA (cDNA)

Deoxyribonucleic Acid (DNA)

Ribonucleic Acid (RNA)

Non-ulcer dyspepsia (NUD)

Peptic ulcer disease (PUD)

**Declarations**

**Ethics approval & consent to participate**

The study protocol was approved by the ethics committee of the School of Medicine, Tehran Azad University of Medical Sciences (No: IR.IAU.TMU.REC.1397.310). Writing informed consent, obtained from all patients.

**Consent for publication**

Not applicable.

**Availability of data and material**

All data and materials are available from the corresponding author.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**
[MK], writing and original draft preparation. [AH], correspondence(s). [MZ] and [RF], designing, investigation and writing. [RN], Review and revise of the manuscript. All authors have read and approved the manuscript.

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**Figures**
Figure 1

Samples included in the study.
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Figure 2

H. pylori virulence gene (cagA, dupA, and babA) expression.
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H. pylori virulence gene (cagA, dupA, and babA) expression.