RESEARCH ARTICLE

Role of Plasticity Region Genes and cagE gene of cagPAI of Helicobacter pylori in Development of Gastrointestinal (GI) Diseases

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Abstract

Background: Helicobacter pylori is a Gram-negative, micro aerophilic bacterium in the human stomach that is associated with the development of gastrointestinal ailments such as peptic ulcer (PU) and gastric cancer (GC). In the present study, plasticity region genes (jhp0940, jhp0945 and jhp0947) and cagE gene of cagPAI were assessed independently and in combination for their ability to predict clinical consequences. Materials and Methods: A total of 211 strains which were isolated from patients with different gastrointestinal diseases (114 with non-atrophic gastritis, 59 with PU, and 38 with GC) were genotyped by PCR and sequencing. Data were collected and analyzed using SPSS software version 19. Logistic regression models were applied to determine relationships between the plasticity region genes and cagE of H. pylori and clinical status. Results: The cagE gene (71.1%) had the highest frequency and jhp0945 (13.7%) was the least abundant among the genes examined. The jhp0940 gene was significantly associated with GC (P = 0.0007), but not PU. On multiple logistic regression analysis, adjusted for both age and sex, the jhp0940 genotype was significantly associated with GC (odds ratio, OR = 2.8, 95%CI = 1.1–7.0; P = 0.027). The jhp0940+/jhp0945+/jhp0947+ genotype was also linked to an increased risk of GC (OR = 50.4, 95%CI = 5.1–500.0; P = 0.0008) while no genotype correlation was found with PU in Iran (P > 0.05). Conclusions: Given the high frequency of cagE, this gene could be a suitable marker for the presence of cagPAI in Iranian strains. The jhp0940 genotype could also be a strong predictor of GC in Iran.

Keywords: Helicobacter pylori- plasticity region genes- cagE- gastrointestinal diseases- Iran

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Introduction

Gastric cancer (GC) is the third cancer-related mortality in the world (Ferlay et al., 2015), so that each year more than one million people are diagnosed with the disease and almost 700,000 of them succumb to it (Parkin et al., 2005). GC is a multifactorial multi-stage disease and Helicobacter pylori-specific genotypes, host factors, and environmental co-factors play a remarkable role in its development (Zabaleta 2012).

H. pylori infection increases the risk of GC by approximately 10% (Choi et al., 2007). It has been reported that peptic ulcer (PU) was developed in approximately 3-10% of H. pylori-infected patients, compared to none of the uninfected patients (Kusters et al., 2006). The studies have shown that variability in virulence factors of H. pylori plays a role in bacterial pathogenesis (Figueiredo et al., 2002).

The cag pathogenicity island (cag PAI) of H. pylori is an important virulence factor which contains 27 to 31 genes (Israel and Peek 2001) which encode components of a bacterial type IV secretion system and inject the CagA protein in the host gastric epithelial cells. It has been shown that strains lacking the cag PAI are less virulent compared to strains carrying it (Proenca Modena et al., 2007). The cagA (cytotoxin-associated gene A) is one of the virulence genes that is located in cag PAI and encodes cancer-causing CagA protein (Nguyen et al., 2008; Hatakeyama 2011; Wroblewski et al., 2010). The H. pylori CagA protein is a 120- to 140-kDa protein that is correlated with H. pylori pathogenesis (Douragh et al., 2009b).

Another member of cag PAI is called cagE (cytotoxin associated gene E) (Censini et al., 1996; Sozzi et al., 2005), and is linked to an increased induction of IL-8 secretion in the gastric epithelial cells (Lima and Rabenhorst 2009). The cagE gene along with the cagA was introduced as a more accurate biomarker for determining the presence cag PAI (Douragh et al., 2009a). The presence of cagE has essential role in the risk to develop sever gastritis, peptic ulcer, and gastric cancer (Chomvarin et al., 2008; Ali et al., 2005; Tan et al., 2005).

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Role of Helicobacter pylori Plasticity Region Genes and of cagPAI (cagE) in Gastric Cancer and Peptic Ulcer Diseases
A number of *H. pylori* virulence genes are located outside the *cag* PAI, within the plasticity region that is a large chromosomal segment including strain-specific genes transferred from other species (Romo-Gonzalez et al., 2009). Variability in plasticity region genes may be responsible for differences in *H. pylori* pathogenesis (Alm et al., 1999; Alm and Trust 1999). It has been reported that *jhp0940*, *jhp0945*, *jhp0947*, and *jhp0949* in the *H. pylori* strains in Western countries are associated with an increased in IL-8, IL-12, and tumor necrosis factor alpha (TNF-α) (Romo-Gonzalez et al., 2009; de Jonge et al., 2004; Occhialini et al., 2000; Lehours et al., 2004; Rizwanet al., 2008; Santos et al., 2003; Proenca Modena et al., 2007). The presence, absence, and activity of plasticity region genes may be related to the severity of gastric mucosal injury and increased risk of development of different gastroduodenal diseases. The aim of present study was to examine the relationship between *cagE*, *jhp0940*, *jhp0945*, and *jhp0947* genes and gastroduodenal diseases in Iran.

**Materials and Methods**

**Gastric biopsies**

Gastric biopsies were obtained from patients with different gastroduodenal diseases referred to the Endoscopy unite in Iran from 2007 to 2014. The total final study population consisted of patients with non-atrophic gastritis (NAG), gastric cancer (GC), and peptic ulcers (PU) (gastric ulcers (GU), duodenal ulcers (DU)).

**Histological examination and classification**

Gastric biopsy specimens were taken from the antrum and the corpus and then biopsies were formalin-fixed and embedded in paraffin. For histopathological examination, biopsies were stained with Hematoxylin- eosin, and Giemsa, and Alcian blue-periodic acid Shiff (pH 2.5). By use of Sydney classification system, histopathological evaluations were performed and tumors were classified into intestinal or diffuse adenocarcinoma (Kersulyte et al., 2000).

**H. pylori isolation and cultivation**

All the tissue specimens taken from both antrum and corpus were cultured and identified on selective Brucella agar plates (Merck, Germany) containing 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), under microaerobic conditions. The Cultures were incubated at 37°C for a maximum of 5–7 days. Bacterial isolates were identified as *H. pylori* according to negative Gram staining, morphology, and positive catalase, oxidase, and urease tests, as well as PCR amplification of *H. pylori* 16SrDNA (Lu et al., 2002).

**DNA extraction and PCR amplification**

Genomic DNA was extracted from *H. pylori* isolates using the Genomic DNA purification kit (Fermentas, UK) according to the manufacturer’s instructions. Extracted DNA was stored at -20°C. Genotyping of *cagE*, *jhp0940*, *jhp0945*, and *jhp0947* genes were determined by PCR methods and using species-specific primers as shown in Table 1. Negative controls included *Escherichia* DH5α and deionized water. PCR was performed in a total volume of 30μL that contained 3μL of 10X PCR buffer (Cinna Gen, Iran), 1μL of MgCl2 200mM, 2 U of Taq DNA polymerase (Cinna Gen, Iran), 0.5μMof of each primer, and 25ng of bacterial DNA. The PCR amplification conditions were 96°C for 180 s; then 35 cycles of 96°C for 40 s (denaturation), optimized annealing temperature for each gene (Table 1) for 40 s, and 72°C for 40 s (extension); and finally, 72°C for 7 min (final extension). PCR products were electrophoresed on 1% (w/v) agarose gel and visualized by a UV transilluminator (Figure 1). The band sizes according to gene and allele are listed in Table 1. To confirm, the amplified fragments of each gene from five isolates were purified and sequenced with both 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**

For statistical analysis, the SPSS version 19 was used. Simple logistic regression analysis by the *Enter* method was used to determine the effect of each pathogenic gene and genotype combinations in gastroduodenal diseases. We used the multiple logistic regression analysis by the Forward Stepwise Likelihood Ratio (LR) method to determine which factor(s) has/have a relative influence on GC and other gastric diseases, after controlling for age and sex variables. In all comparative analysis, NAG patients were considered as the control group. A P value of < 0.05 was indicated as statistically significant.

**Results**

**Characteristics of patients and genotyping**

A total of 214 *H. pylori* strains from patients were obtained and genotyped. In the present study, based on histopathological findings: 114 patients had NAG, 59 had...
PU (29/59 with gastric ulcer and 30/59 with duodenal ulcer), and 38 had GC (cardia cancer: 14/38, non cardia cancer 23/38 and cardia and non cardia gastric cancer: 1/38; and intestinal type: 20/38 and diffuse type: 18/38); and three patients were excluded from analyses because in their histopathological evaluations no tumor tissue and lymphoma were recognized. As shown in Table 2, patients were classified into 2 groups; females: 87/211 and males: 124/211. Also Patients were classified into 2 age groups; including patients with age <55: 133/211 and those with age >=55: 77/211 (Table 2). The age information for one Patient was not available. The frequency of the jhp0940+ genotype one of the most important risk factor(s) related to GC. When the GC was considered as a dependent factor by the multiple logistic regression analysis, only the jhp0940+ genotype was remarkably associated with the age-adjusted risk for GC; the odds ratio (OR) was 3.858 (95% confidence interval, CI, 1.762-8.447; p = 0.0007, q= 0.0029), whileno significant relationship was found between, cagE+, jhp0940+, jhp0945+ and jhp0947+ genotypes and the risk of GC (q=0.05).The major and novel finding in our study was that the jhp0940+ genotype one of the most important factors of H.pylori associated with gastric cancer in Iran. We used multiple logistic regression analysis to find the most important risk factor(s) related to GC. When the GC was considered as a dependent factor by the multiple logistic regression analysis, only the jhp0940+ genotype was remarkably associated with the age and sex-adjusted risk for GC. The OR was 2.810 (95% CI, 1.126–7.012; p = 0.027). The interesting thing to note is that the simultaneous presence of the jhp0940+ gene with cagE, jhp0947, and jhp0945 genes increased virulence strains than the strains that had only jhp0947 gene. So that simultaneous presence of three genes jhp0940/jhp0945/jhp0947 of H.pylori plasticity region showed very strong relationship with GC (OR= 50.4). According to Table 4, Analysis for genotype combinations with GC risk showed that jhp0940+/cagE+, jhp0940+/jhp0947+, jhp0940+/jhp0947/cagE+, jhp0940+/jhp0947/jhp0945+cagE+, and jhp0940+/jhp0945+cagE+ were associated with an increased risk of GC. The OR was 8.1 (95% CI, 2.3-29.2; p = 0.001), 6.000 (95% CI, 2.1-16.7; p = 0.0006), 4.4 (95% CI, 1.1-16.6; p = 0.029), 4.8 (95% CI, 1.6-14.9; p = 0.006), 50.4 (95% CI, 5.1-500.0; p = 0.0008), 9.4 (95% CI, 1.4-

Table 1. Primer Sequence and Conditions of PCR Applied in This Study

| Genes | Primers | Sequences (5’→3’) | Size of PCR products (bp) | Annealing temperature (°C) |
|-------|---------|-------------------|--------------------------|--------------------------|
| 16 S rDNA | HP1 | GCA ATC AGC GTC AGT AAT GTT C | 519 | 56 |
| | HP2 | GCT AAG AGA TCA GCC TAT GT C | | |
| cagE | Forward | TTGAAAACCTCAAGGATAGGATAGGC | 508 | 54 |
| | Reverse | GCCTAGCGTAATATCACCATTACCC | | |
| jhp0940 | Forward | GAAATGTCCTATACCAATGG | 381 | 48 |
| | Reverse | CCTAAGTAGTGATCAAGGG | | |
| jhp0945 | Forward | ACTCCAGCCAGTTGTTAAA | 380-400 | 48 |
| | Reverse | TTCTTGCGAGTTAGGATTTGG | | |
| jhp0947 | Forward | GATAATCTACGCAGAAGCC | 368 | 48 |
| | Reverse | GCTAAAGTCTATGCGCTTGGC | | |

Table 2. Characteristics of Patients Enrolled in This Study

| Characteristics | Frequency (N) | Percent (%) |
|----------------|---------------|-------------|
| Sex            |               |             |
| Male           | 124/211       | 58.8        |
| Female         | 87/211        | 41.2        |
| Age            |               |             |
| >=55           | 77/211        | 36.5        |
| <55            | 133/211       | 63.0        |
| No data        | 1/211         | 0.5         |
| Diseases       |               |             |
| NAG            | 114/211       | 54.0        |
| PU             | 59/211        | 28.0        |
| Duodenal ulcer | 29/59         | 49.2        |
| GC             | 50/59         | 50.8        |
| Cardia gastric cancer | 14/38 | 36.8 |
| Non cardia gastric cancer | 23/38 | 60.5 |
| Cardia&Non cardia gastric cancer | 2.6 |
| Intestinal-type adenocarcinoma | 20/38 | 52.6 |
| Diffuse-type adenocarcinoma | 18/38 | 47.4 |

NAG, nonatrophic gastritis; PU, peptic ulcer; GU, gastric ulcer; DU, duodenal ulcer; GC, gastric cancer.
The Correlation of the H. pylori cagE, jhp0940, jhp0945, and jhp0947 Genotypes with the Risk of PU

The frequency of cagE+, jhp0940+, jhp0945+, and jhp0947+ genotypes was 61.0%, 47.5%, 5.1%, and 42.4%, respectively in PU patients (Table 3). The results of simple logistic regression analysis demonstrated that no genotype was significantly associated with risk of PU (P>0.05). Also, no significant relationship was found between genotype combinations and PU risk (P>0.05) (Table 4).

Discussion

H. pylori is a major determinant of different gastrointestinal disease progression. Strain-specific H. pylori genes and their different genotypic combinations could determine the clinical outcomes (Ramis et al., 2013). cag PAI and plasticity regions are among the low GC

| Genotypes | Frequency N (%) |
|-----------|-----------------|
| jhp0940+vs.jhp0940- | 41/114 (36.0) 26/38 (68.4) 28/59 (47.5) 95/211 (45.0) |
| jhp0945+vs.jhp0945- | 10/38 (26.3) 5/13 (38.5) 1/16 (6.3) 16/67 (24.0) |
| jhp0947+vs.jhp0947- | 18/54 (33.3) 21/28 (75.0) 9/25 (36.0) 48/107 (44.9) |
| cagE+vs.cagE- | 7/12 (58.3) 0/5 (0.0) 0/18 (0.0) 7/39 (17.9) |

Table 3. The Total Frequency of H. Pylori Genotypes in Dyspeptic Patients

CI, confidence interval; a Bold face data indicate statistically significant results; bFalse discovery rate-adjusted p-value.
The cag PAI of *H. pylori* is one of the virulence factors that involves several genes with different functions (Israel and Peek, 2001). The *cagA* and *cagE* genes are the two main virulence factors of cag PAI which can be employed as markers of the presence of cag PAI (Ramis et al., 2013; Douraghi et al., 2009a). In the present study, 150 (71.1%) isolates out of 211 contained cagE. The prevalence of cagE in isolates of Iran was higher than that of Turkey (59.3%), Malaysia (59%) and England (62%) but lower than that of Brazil (89.3%) and India (85.4%)(Douraghi et al., 2009a; Erzin et al., 2006; Tiwari et al., 2007). Accordingly, it is suggested that cagE would be a more proper marker of the presence of cag PAI in the Iranian isolates as it is in isolates of Japan (Maeda et al., 1999), France (Audibert et al., 2001) and Brazil (Ribeiro et al., 2003). Several studies have shown that the cagE gene is a more accurate marker of the persistent presence of cag PAI compared to other genes (Ikenoue et al., 2001; Sozzi et al., 2005). In the present study, the presence of cagE gene in GC and PU isolates showed no meaningful relationship with the mentioned diseases. This finding is in line with Proenca Modena et al. (2007) study in which no meaningful relationship between cagE and gastrointestinal diseases were found (Proenca Modena et al., 2007).

More than 50% of *H. pylori*-specific genes are located in the plasticity region (Romo-Gonzalez et al., 2009). Many of them are significantly associated with the risk of *H. pylori*-associated diseases (Sugimoto et al., 2012; de Jonge et al., 2004; Occhialini et al., 2000). Recently jhp0940, jhp0945, jhp0947, and jhp0949 genes of the plasticity region from Western countries have been reported to be associated with an increased risk of gastroduodenal disease (Romo-Gonzalez et al., 2009; de Jonge et al., 2004; Occhialini et al., 2000; Lehours et al., 2004; Rizwan et al., 2008; Santos et al., 2003; Proenca Modena et al., 2007).

Studies on jhp0945 gene are very few and far between. In the present study, we observed that the frequency of the jhp0945-positive isolates in patients with GC, PU, and NAG was 26.3%, 5.1%, and 14.0%, respectively. There was no significant difference between the frequencies of the jhp0945-positive in the isolates from PU or GC and those from NAG (P > 0.05). In contrast, Sugimoto et al indicated that in Western isolates, the presence of jhp0945 was significantly associated with GC (OR = 2.27), DU (OR = 1.86), and GC (OR = 1.92), while in East Asia, the jhp0945-positive isolates significantly increased the risk for GC (OR = 2.58) (Ramis et al. 2013). Although jhp0945 gene had no relationship with PUD or GC on its own, it showed a meaningful relationship with cancer along with the jhp0940 and jhp0947 genes.

The jhp0947 gene, with unknown function has been identified as the most sensitive marker of the plasticity region for *H. pylori* related diseases (Santos et al., 2003; Occhialini et al., 2000) and has been proposed to be associated with severe tissue damage and PU (de Jonge et al. 2004) or development of GC (Santos et al., 2003). The percentage of jhp0947 gene was reported 58% in a study in Iran on 143 first-degree relatives GC patients included 68/143 with pan gastritis, 64/143 with antral-predominant gastritis, and 11/143 with corpus-predominant gastritis, with or without atrophy or intestinal metaplasia, and its distribution in 3 groups of gastritis showed no significant difference (P > 0.05) (Siavoshi et al., 2011). In this study, the percentage of jhp0947 gene was 49.8%. The findings showed that jhp0947 gene had no significant association with gastrointestinal disease (P > 0.05) but there was a significant relationship between the simultaneous presence of jhp0947, and jhp0940 genes and an increased risk of GC in Iran (P = 0.0006; OR = 6.00). The results of our study conform to previous reports from the Western (Colombia and the United States) and East Asian (South Korea and Japan) countries (Sugimoto et al. 2012). In 2000, Occhialini et al. (Occhialini et al., 2000) demonstrated that the frequency of jhp0947 in isolates from GC patients (64.7%) was higher than that of gastritis patients (34.6%). Moreover, a study in Brazil (Santos et al., 2003) reported that, in multivariate analysis, the presence of the jhp0947 was linked to GC (OR = 2.94) and DU (OR = 4.84), but not with gastritis. A study on a Dutch population showed a significant relationship between the presence of jhp0947 and DU, but not with gastritis (de Jonge et al., 2004).

JHP940 is a protein kinase, which leads to indirectly up regulating phosphorylation of NF-κB p65 at Ser276. It seems that JHP940 plays a key role in enhancing the gastric inflammatory and inflammation-related different clinical outcomes such as gastric cancer (Hwang et al., 2002; Furuta et al., 2002; El-Omar et al., 2000). Of 211 *H. pylori* isolates from dyspeptic patients, 45.0% carried the jhp0940 gene. The prevalence of JHP940 gene in this study was similarly distributed among Japanese isolates (40.0%) while the prevalence of JHP940 was in isolates from Costa Rican, Peruvian, and Spanish were 30.0%, 60.0%, and 5.0%, respectively (Rizwan et al., 2008).

In the present study, JHP940 gene had no association with PU group (P > 0.05). Other studies showed no significant correlation between the presence of JHP940 and any gastrointestinal diseases (Santos et al., 2003) or even a preventive effect on GC (Romo-Gonzalez et al., 2009) and PU (Sugimoto et al., 2012). The findings of this study showed that jhp0940 gene had strong relationship with GC group (P = 0.0007); the OR in a simple logistic regression model was 3.858. However, in multiple logistic regression analysis, including age, sex, jhp0940, jhp0945, jhp0947, and cagE status, when the GC was considered as a dependent factor, the jhp0940-positive genotype remained in the final model (P = 0.027, OR = 2.810; 95% CI = 1.126-7.012). We recommend that the jhp0940 gene of *H. pylori* could be as beneficial biomarker for the risk prediction of GC, but not PU in Iran.

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Competing interest

The authors declare that they have no conflict of interest.

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