RESEARCH ARTICLE

Helicobacter pylori cag Pathogenicity Island *cagL* and *orf17* Genotypes Predict Risk of Peptic Ulcerations but not Gastric Cancer in Iran

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Abstract

Background: Gastric cancer (GC) is the third most common cancer regarding mortality in the world. The *cag* pathogenicity island (PAI) of *Helicobacter pylori* which contains genes associated with a more aggressive phenotype may involve in the pathogenesis of gastrointestinal disease. We here aimed to examine the associations of *cagH*, *cagL*, *orf17*, and *cagG* genotypes of *H. pylori cag* PAI with severe gastrointestinal disease. Materials and Methods: A total of 242 *H. pylori* strains were genotyped. Histopathological examination and classification of subjects were performed. Results: The frequencies of the *cagH*, *cagL*, *cagG*, and *orf17* genotypes were 40/54 (74.1%), 53/54 (98.1%), 38/54 (70.4%), and 43/54 (79.6%), respectively, in patients with peptidic ulceration (PU),while in the control group, the frequencies were 87/147 (59.6%) for *cagH*, 121/146 (82.9%) for *cagL*, 109/146 (74.7%) for *cagG*, and 89/146 (61.0%) for *orf17*. The results of simple logistic regression analysis showed that the *cagL* and *orf17* genotypes were significantly associated with an increased risk of PU not GC; the ORs (95% CI) were 10.950 (1.446-82.935), and 2.504 (1.193-5.253), respectively. No significant association was found between the *cagH* and *cagG* genotypes and the risk of both the PU and the GC in Iran (*P*>0.05). Finally, multiple logistic regression analysis showed that the *cagL* genotype was independently and significantly associated with the age- and sex-adjusted risk for PU; the OR (95% CI) was 9.557 (1.219-17.185). Conclusions: We conclude that the *orf17* and especially *cagL* genotypes of *H. pylori cag* PAI could be factors for risk prediction of PU, but not GC in Iran.

Keywords: *H. pylori* - *cag* PAI - genotype - peptic ulceration - gastric cancer - prediction - Iran

Asian Pac J Cancer Prev, **16 (15)**, 6645-6650

Introduction

Gastric cancer (GC) is the third common cancer that ends up death in the world* with different incidence rate based on geographical areas (Parkin, 2004). The high incidence of GC has geographical distribution in Asia especially in Iran (Kelley and Duggan, 2003; Sadjadi et al., 2003). The GC has high rate in male and is the third cancer after breast and colorectal cancer in female in Iran (Malekzadeh et al., 2009; Mousavi et al., 2009). Ardabil province is located in Northwestern Iran, is reported as area with the high incidence rate of gastric adenocarcinoma (Sadjadi et al., 2003). The concrete reasons for prevalence of GC, which varies in different geographical areas, remain unknown.

However, several studies have been shown that interaction between host and environmental factors, especially *Helicobacter pylori* (*H. pylori*) infection may play a remarkable role (Covacci et al., 1993; Atherton et al., 1995; Peek and Blaser, 2002; Yamaoka, 2010). This bacterium is found in more than 50 % of the world population and it is a well-known human pathogen. *H. pylori* is the major cause of acute or chronic gastritis, peptic ulcerations (PU) disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. In most of cases the infection remains asymptomatic during life, just 20% develops peptic ulcer or gastric carcinoma (Blaser, 1992a; Blaser, 1992b; 1994; Blaser and Parsonnet, 1994; Parsonnet et al., 1997; McColl and El-Omar, 2002; Sepulveda and Graham, 2002; Kapadia, 2003; Matsuhisa et al., 2003). The *cag* pathogenicity island (*cag* PAI) of *H. pylori* is an important virulence factor that involves several genes with different function in the *H. pylori* (Crabtree et al., 1995a; Crabtree et al., 1995b; Sharma et al., 1995; Tummuru et al., 1995; Censini et al., 1996; Figura and Valassina, 1999; Asahi et al., 2000; Stein et al., 2000; Megraud, 2001). Adherent of bacterium to gastric epithelial cells is critical to initiate the gastric inflammatory response with different clinical outcomes in humans (Hessey et al., 1990; Logan, 1996; Segal et al., 1996; Rieder et al., 1997). *CagL* (a VirB5 ortholog) is a bacterial factor that is known as a pilus

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protein (Backert et al., 2008; Backert and Selbach, 2008; Backert et al., 2011), Integrin α5β1 is a receptor on gastric epithelial cell that *CagL* binds to; by its arginine-glycine-aspartate (RGD) motif, this interaction activates α5β1 receptor and facilitates the delivery of CagA oncoprotein through type 4 secretion system (T4SS) into the epithelial cell (Parsonnet et al., 1997; Kwok et al., 2007; Backert and Selbach, 2008; Backert et al., 2011; Shaffer et al., 2011). *CagL* is associated with two other *cag* PAI proteins; CagI and CagH. All the three factors are essential for the injection of CagA into the epithelial cell and T4SS pili formation, studies have also suggested that pilus dimension might be regulated by CagH. In addition, *cagH* is important for induction of IL-8 secretion by epithelial cells which mediates increased risk of developing GC (Kwok et al., 2007; Shaffer et al., 2011). *cagG* is another factor that is located upstream of the *cagA* gene. The *cagG* has also been shown some homology to the adherence-related or motility-related genes of other bacteria in bioinformatics study (Censini et al., 1996; Weel et al., 1996; Perez-Perez et al., 1999; Audibert et al., 2001; Li et al., 2001). The *orf17* of *H. pylori* shows some homology to a gene from Dickeya zeae that 36% identity is reported in the BLASTp studies. Dickeya zeae, is the aerobic/anaerobic phytopathogen bacterium that causes soft rot disease in a broad range plants species, especially many crops; this bacterium causes bacterial foot rot and it is reported in many Asian countries particularly China which the disease mostly begins at the ligulus (Samson et al., 2005; Zhou et al., 2011). The purpose of this study was to find out the distribution of four target genes of *cag* pathogenicity island (*cagH*, *cagL*, *cagG*, and *orf17*) in *H. pylori* genome and their relationship with gastrointestinal disease in Iran.

Materials and Methods

Collection of tissue specimens

The tissue specimens were collected over seven years from 2007 to 2014 in Iran; from patients with various gastrointestinal diseases as mentioned in Table 1.

H. pylori isolation and culture

Antrum and body biopsies were used for *H. pylori* culture. Biopsies were cultured on selective Brucella agar (Merck, Germany) involving 5% sheep blood, 10 mg/L of vancomycin (Zakaria, Iran), 5 mg/L trimethoprim (MP Biomedicals, France), 2.5 IU/L polymyxin B (MP Biomedicals, France), and 8 mg/L amphotericin B (Bristol-Myers Squibb, USA). Cultures were incubated at 37°C under microaerophilic condition for 3-7 days. Bacterial isolates were identified as *H. pylori* on the basis of Gram-stained morphology and positive urease, catalase, and oxidase tests. Bacterial isolates were harvested in brain heart infusion broth (Merck) enriched with 20% glycerol and 10% inactivated horse serum and stored at -70°C.

Histological assessment and classification

All biopsies were taken from the gastric body (corpus) and antrum of patients with different gastrointestinal disease, and were used for histopathological examination. The biopsy specimens were initially formalin-fixed and paraffin-embedded. Sections of 4μm were obtained and stained with hematoxylin-eosin, Giemsa, and Alcian blue-periodic acid Shiff (pH 2.5). By the use of Sydney system, GC was classified and graded (Table 1) (Dixon et al., 1996).

DNA extraction

Genomic DNA was extracted from the *H. pylori* culture positive gastric biopsy specimens, using DNGTM plus kit (CinnaGen, Tehran, Iran) according to the manufacturer's instructions. Extracted DNA was stored at -20°C until used for polymerase chain reaction (PCR) amplification.

PCR amplification

The final identification of *H. pylori* was confirmed by using *16S rDNA* gene PCR by species-specific primers as shown in Table 2 (Lu et al., 2002). The second PCR was done in order to detect the target genes which were *cagH*, *cagL*, *cagG*, and *orf17* (Table 2). The PCR reaction was carried out in a 30-μl reaction mixture involving MgCl2=1 μl, PCR buffer=3 μl, dNTP mix=0.5 μl, primers (reverse and forward mixture)=1 μl, enzyme=0.2 μl, template DNA=5 μl, and distilled water=19.3 μl (total volume=30 μl). The genes amplification conditions were as follows: predenaturation was carried out at 95°C for 45 s, followed by 30 cycles of 94°C for 45s, 72°C for 45s, and a final extension at 72°C for 7 min. The PCR products were developed under ultraviolet (UV) light after electrophoresis on 1% (w/v) agarose gel. *H. pylori* IRANL15 strain and a no-template reaction used as positive and negative controls in each PCR experiment, respectively.

Amplified fragments of each gene from six 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.

Table 1. Total Frequencies Age and Sex Classes and Disease Types

Characteristics	Total frequency N (%)
Age	
<55	150/241 (62.2)
>=55	91/241 (37.8)
Sex	
Female = 0	98/242 (40.5)
Male = 1	144/242 (59.5)
Non-atrophic gastritis	146/242 (60.3)
Peptic ulcer	54/242 (22.3)
Duodenal ulcer	28/54 (51.9)
Gastric ulcer	20/54 (37.0)
Gastric cancer	42/242 (17.4)
Cardia cancer	18/42 (42.9)
Non-cardia cancer	24/42 (57.1)
Intestinal-type adenocarcinoma	24/42 (57.1)
Diffuse-type adenocarcinoma	16/42 (38.1)
Invasive squamous cell-type carcinoma	1/42 (2.4)
Mucin producing-type adenocarcinoma	1/42 (2.4)

Statistical analysis

For statistical analysis, the SPSS 18.0 software was used (SPSS Inc, Chicago, Illinois, USA). Chi square and Fischer's exact tests were used to analyze the of *H. pylori* genotypes with histopathological and clinical outcomes. A *P-value* of<0.05 indicated significance. A simple and multiple logistic regression were used to calculate the odds ratio (OR) and 95% confidence interval (CI) of the clinical variables. The OR and CI were used to estimate the risk of PU and GC in the current study.

Results

Patients' characteristics

A total of 242 *H. pylori* strains were isolated from Iranian patients (59.5% male and 40.5% female; 37.8% age≥55 and 62.2% age<55) with different ethnic and geographic origins. In the present study, based on histopathological findings, 146 (60.3%) patients presented NAG, 54 (22.3%) had PU, and 42 (17.4%) had GC (Table 1). The total frequencies of *cagH*, *cagL*, *cagG*, and *orf17* genotypes were 61.2%, 86.4%, 71.5%, and 64%, respectively.

Association between the cag PAI genotypes and PU in Iran

The frequency of the *cagH*, *cagL*, *cagG*, and *orf17* genotypes was 74.1%, 98.1%, 70.4%, and 79.6%, respectively in PU group; 28/54 (51.8%) for duodenal ulcer and 20/54 (37.0%) for gastric ulcer, while in the control group (NAG) the following frequencies were reported: 59.6% for *cagH*, 82.9% for *cagL*, 74.7% for *cagG*, and 61.0% for *orf17*. The simple logistic regression analysis demonstrated that the *cagL* and *orf17* genotypes were remarkably associated with an increased risk of PU; the OR (95%CI) was 10.95 (1.446-82.935) and 2.504 (1.193-5.235) respectively (*P*=0.021 for *cagL* and *P*=0.015 for *orf17*). No significant relationship was found between the *cagH* and *cagG* genotypes and the risk of PU (*P*>0.05). Analysis for combined genotypes showed that *cagH*/*cagL* and *cagH*/*orf17* were associated with an increased risk of PU; the OR (95% CI) was 9.756 (1.264-75.303) for *cagH*/*cagL* and 2.861 (1.167-7.013) for *cagH*/*orf17* (*P*=0.029 and 0.02, respectively). No significant correlation was found between the *cagH*/*cagG*,

Table 2. Primer Sequence and Conditions of PCR Applied in this Study

Gene and Primer	Sequences (5´ - 3´)	Size of PCR product (bp)	Annealing temperature (°C)
16S rDNA			
HP1	5´-GCAATCAGCGTCAGTAATGTTC-3´	1500	56
HP2	5´-GCTAAGAGATCAGCCTATGTCC-3´		
cagH			
CagH-F	5´-ATGGCAGGTACACAAGCTAT-3´	1113	52
CagH-R	5´-TCACTTCACGATTATTTTAG-3´		
cagL			
CagL-15	5´-AAAACACTCGTGAAAAATACCATATC-3´	263	54
CagL-16	5´-TCGCTTCAAAATTGGCTTTC-3´		
cagG			
CagG-F	5´-TTATAAAATTAAATTACTATTTGC-3´	398	50
CagG-R	5´-GTGGTAAAAACGATGAATCTG-3´		
orf17			
Orf17-F	5´-CTTGATTGATGAAAATTTGGTTG-3´	546	50
Orf17-R	5´-TTAGTGATATATTCATAATTTTCC-3´		

Table 3. Association between cag PAI Genotypes and Peptic Ulceration

cag PAI genotypes	Disease type		*P-value**	OR	CI	Total strains
	PU N (%)	NAG N (%)				
cagH	40/54 (74.1)	87/146 (59.6)	0.061	1.938	0.969-3.873	127
cagL	53/54 (98.1)	121/146 (82.9)	0.021a	10.950	1.446-82.935	174
cagG	38/54 (70.4)	109/146 (74.7)	0.542	0.806	0.403-1.612	147
orf17	43/54 (79.6)	89/146 (61.0)	0.015	2.504	1.193-5.253	132
cagH/*cagL*	40/41 (97.7)	82/102 80.4)	0.029	9.756	1.264-75.303	122
cagH/*cagG*	32/40 (80.0)	70/90 (77.8)	0.776	1.143	0.455-2.869	102
cagH/*orf17*	34/41 (82.9)	73/116 (62.9)	0.022	2.861	1.167-7.013	107
cagL/*cagG*	36/36 (100.0)	96/108 (88.9)	-	-	-	132
cagL/*orf17*	41/43 (95.3)	81/97 (83.5)	0.071	4.049	0.888-18.463	122
cagG/*orf17*	31/35 (88.6)	70/89 (78.7)	0.208	2.104	0.661-6.698	101
cagH/*cagG*/*cagL*	31/31 (100.0)	67/77 (87.0)	-	-	-	98
cagH/*cagG*/*orf17*	28/32 (87.5)	61/75 (81.3)	0.438	1.607	0.485-5.323	89
cagH/*cagL*/*orf17*	34/35 (97.1)	70/84 (83.3)	0.069	6.800	0.858-53.873	104
cagL/*cagG*/*orf17*	31/31 (100.0)	67/75 (89.3)	-	-	-	98

*Peptic ulceration (PU), Non-atrophic gastritis (NAG), OR = Odd Ratio, and CI = 95% Confidence Interval. **P*-value = *P*-value is calculated by comparing the *cag* PAI genotypes positivity with respect to histopathological parameters. aBold face data indicate statistically significant results

Table 4. Association between *cag* PAI Genotypes and Gastric Cancer

cag PAI genotypes	Disease type		*P-value**	OR	CI	Total strains
	PU N (%)	NAG N (%)				
cagH	21/42 (50.0)	87/146 (59.6)	0.269	0.678	0.340-1.351	108
cagL	35/42 (83.3)	121/146 (82.9)	0.945	1.033	0.412-2.589	156
cagG	26/42 (61.9)	109/146 (74.7)	0.108	0.552	0267-1.140	136
orf17	23/42 (54.8)	89/146 (61.0)	0.471	0.775	0.388-1.550	112
cagH/cagL	21/28 (75.0)	82/102 (80.4)	0.534	0.732	0.273-1.960	103
cagH/cagG	19/33 (57.6)	70/90 (77.8)	0.029a	0.388	0.166-0.908	89
cagH/orf17	20/38 (52.3)	73/116 (62.9)	0.261	0.654	0.312-1.372	93
cagL/cagG	24/29 (82.8)	96/108 (88.9)	0.378	0.600	0.193-1.867	120
cagL/orf17	22/28 (78.6)	81/97 (83.5)	0.547	0.724	0.253-2.070	103
cagG/orf17	19/31 (61.3)	70/89 (78.7)	0.061	0.430	0.178-1.039	89
cagH/cagG/cagL	19/24 (79.2)	67/77 (87.0)	0.350	0.567	0.173-1.861	86
cagH/cagG/orf17	19/30 (63.3)	61/75 (81.3)	0.054	0.396	0.154-1.018	80
cagH/cagL/orf17	20/26 (76.9)	70/84 (83.3)	0.461	0.667	0.227-1.959	90
cagL/cagG/orf17	19/23 (82.6)	67/75 (89.3)	0.394	0.567	0.154-2.089	86

*Gastric cancer (GC), Non-atrophic gastritis (NAG), OR = Odd Ratio, and CI = 95% Confidence Interval. **P*-value = *P*-value is calculated by comparing the *cag* PAI genotypes positivity with respect to histopathological parameters. aBold face data indicate statistically significant results

cagL/cagG, *cagL/orf17*, *cagG/orf17*, *cagH/cagG/cagL*, *cagH/cagG/orf17*, *cagH/cagL/orf17*, and *cagL/cagG/orf17* combined genotypes and the risk of PU ($P>0.05$). Multiple logistic regression analysis revealed that the *cagL* genotype was independently and significantly correlated with the age and sex-adjusted risk for PU; the OR (95% CI) was 9.557 (1.219-7.185) ($P=0.032$) (Table 3).

Association between the cag PAI genotypes and GC in Iran

Among the 42 GC patients, 18/42 (42.9%) were with cardia cancer (CC), 24/42 (57.1%) with non-cardia cancer (NCC), 16/42 (38.1%) with diffuse-type adenocarcinoma, 24/42 (57.1%) with intestinal-type adenocarcinoma, 1/42 (2.4%) with mucin producing-type adenocarcinoma, and 1/42 (2.4%) with invasive squamous cell-type carcinoma. Chi-square test demonstrated no correlation between the *cagH*, *cagL*, *cagG*, and *orf17* genotypes and risk of GC ($P>0.05$). Statistical analysis for combined genotypes illustrated that *cagH/cagG* reduced the risk of GC; the OR (95% CI) was 0.388 (0.166-0.908) ($P=0.029$). However, no. remarkable relationship was found between the *cagH/cagL*, *cagH/orf17*, *cagL/cagG*, *cagL/orf17*, *cagG/orf17*, *cagH/cagG/cagL*, *cagH/cagG/orf17*, *cagH/cagL/orf17*, and *cagL/cagG/orf17* combined genotypes and the risk of GC ($P>0.05$) (Table 4).

Discussion

The presence of *H. pylori* in the gastric mucosa has been known as an essential risk factor of different gastrointestinal disease including; NAG, PU, and GC. *H. pylori* has several virulence factors that might take part in mucosal damage (Mahdavi et al., 2002; Erzin et al., 2006; Kusters et al., 2006; Miernyk et al., 2011; Yeh et al., 2011; Shiota et al., 2012). This study investigated the relevance of various virulence factors (*cagH*, *cagL*, *cagG*, and *orf17*) and their relationship with sever gastrointestinal disease. The *cagH* genotype has not been surveyed on genomics level; however, we found that the *cagH* genotype had no relationship with gastrointestinal disease in Iran ($P>0.05$). Recently, it has been demonstrated that 86.6% of the *H. pylori* isolates from Indian patients were *cagL* positive. Other studies in Malaysia, Singapore, and Taiwan demonstrated that more than 85% of the isolates were *cagL* positive (Schmidt et al., 2010; Yeh et al., 2011). A study on 61 isolates from patients with digestive disease in Iran, showed that 96.7% were *cagL* positive. This report was concordant with the results from Taiwan, where the 98.6% of the patients were *cagL* positive, but no remarkable association was detected between the *cagL* genotype and clinical outcomes ($P>0.05$) (Kwok et al., 2007; Yeh et al., 2011; Wang et al., 2013; Yadegar et al., 2014). These results are consistent with our results in GC group but not in PU group, while the *cagL* genotype is remarkably and independently associated with the risk of PU in Iran ($P=0.021$), but no association was found with the risk of GC ($P>0.05$). Previously studies showed that *cagG* mutants were impaired to induce inflammatory response as well as increase proliferation of epithelial cells, and also determined that lack of *cagG* gene leads to the loss of CagA translocation/phosphorylation (Censini et al., 1996; Tomb et al., 1997; Fischer et al., 2001). Recently reports showed that the loss of *cagG* genotype has been associated with reduced adherence to epithelial cells (Mizushima et al., 2002). An *in vivo* study in China has also demonstrated no significant relationship between *cagG* and clinical outcomes. Several studies have proposed that the lack of *cagG* genotype led to the loss of induction of IL-8 (Censini et al., 1996; Hsu et al., 2002). However, other studies have proposed that the complete deletion of *cagG* gene resulted in no reduction in IL-8 induction (Fischer et al., 2001). According to these studies, it is impossible to discriminate whether or not the lack of inflammation with *cagG* mutant is associated with reduced colonization or loss of T4SS, or both (Figura and Valassina, 1999). A study on 145 *H. pylori* isolates in China showed that *cagG* was detected in 91.7% isolates, and 100% of isolates from patients with PU (duodenal ulcer and gastric ulcer) were *cagG* positive but not statistically significant ($P>0.05$). *cagG* genotype was known as a conserve gene in Chines population and

there was no significant differences in the frequencies of *cagG* gene in isolates from patients with various digestive disease. Hsu et al reported that of the 120 isolates from patients with different gastrointestinal disease in Korea 86.7% (104/120) were *cagG* positive (Hsu et al., 2002). Mizushamia et al used PCR to investigate the distribution of *cagG* gene in 236 clinical *H. pylori* isolates in Japan, 97% of isolates were *cagG* positive. For both isolates from Korea and Japan, *cagG* positive isolates demonstrated no significant association with gastrointestinal disease ($P>0.05$) (Mizushima et al., 2002). In other study, the level of gastric mucosal inflammation was compared in the antrum and the body (corpus) of both the *cagG* positive and negative group. It was illustrated that the level of inflammation was relatively higher in *cagG* positive than *cagG* negative group, both in the antrum and the body ($P>0.05$), but no relationship was detected between *cagG* genotype and the intensity of gastritis (Shimoyama et al., 1998; Yamaoka et al., 1998). Consistent with the previous studies, we showed that the *cagG* genotype had no remarkable association with digestive disease in Iran ($P>0.05$).

The *orf17* genotype has also not been studied on genomics level, we showed that *orf17* gene had no association with GC group ($P>0.05$) but there was a remarkable relationship between the presence of *orf17* gene and an increased risk of PU in Iran ($P=0.015$).

This report is the first one regarding the relevance of the *H. pylori cagL* and *orf17* genotypes to PU in Iran. We have proposed that the *orf17* and particularly *cagL* genotypes of *H. pylori* cag PAI could be as beneficial factors for the risk prediction of PU, but not GC in Iran. In addition, specific *H. pylori* virulent biomarkers could be used to predict PU or GC risk and selected for *H. pylori* eradication in patients with NAG. Based these initial results, our future goals are to evaluate several virulence factors simultaneously, in order to clarify the correlation between the virulence factors and gastrointestinal disease.

Acknowledgements

This work was supported by a grant from University of Mohaghegh Ardabili. The supporter had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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