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Associations between the Plasticity Region Genes of *Helicobacter pylori* and Gastroduodenal Diseases in a High-Prevalence Area

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Background/Aims: Genes associated with the *Helicobacter pylori* (*H. pylori*) plasticity region may play a role in the pathogenesis of *H. pylori*. We compared the genes *jhp0940, jhp0947,* and *jhp0986* in *H. pylori* isolates from patients with different gastroduodenal diseases and in different age groups. **Methods:** The *H. pylori* hyperplasticity region genes *jhp0940, jhp0947*, and *jhp0986* were studied by PCR. We also evaluated whether these genes were related to the cytotoxin-associated gene (*cagA*) and histology findings. **Results:** Of the patient cohort, 71 (62%) were positive for *jhp0940*, 67 (59%) for *jhp0947*, 12 (10%) for *jhp0986*, and 69 (60%) for *cagA*. *jhp0940* (n=18, 67%) and *jhp0947* (n=23, 85%) were found more frequently in duodenal ulcer (DU) patients than in gastritis patients (n=14, 39%; p=0.029 and p$<$0.001, respectively). Gastric ulcer (GU) was more frequently associated with *jhp0940* (17 patients, 77%; p=0.003) than with gastritis (14 patients, 39%). Gastric carcinoma (GC) was more strongly associated with both *jhp0940* (22 patients, 76%; p=0.003) and *jhp0947* (22 patients, 76%; p=0.003) than was gastritis (14 patients, 39%). *jhp0947* was more frequently associated with chronic active inflammation (58 patients, 87%; p=0.009) than with chronic inflammation (9 patients, 13%). Multivariate analysis demonstrated that *jhp0947* was associated with DU (odds ratio, 6.1; 95% confidence interval, 1.87-20). **Conclusions:** The genes *jhp0947* and *jhp0940* were identified in *H. pylori* isolates from patients with GC and DU, while *jhp0940* was also isolated from patients with GU. *jhp0947* was independently associated with DU. **(Gut Liver 2010; 4:345-350)**

Key Words: *Helicobacter pylori*; Hyperplasticity region; Gastric carcinoma; Duodenal ulcer; Gastric ulcer

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative, spiral, microaerophilic bacterium whose infection has a worldwide distribution. Its prevalence varies from 2% in developed countries to more than 90% in developing world.[1] *H. pylori* is the major cause of chronic gastritis and plays an important role in the pathogenesis of peptic ulcer disease, gastric carcinoma (GC), and gastric mucosa-associated lymphoid tissue lymphoma.[2,3] However, why only a minority of *H. pylori*-positive patients develop the severe associated diseases remain unclear. Variation in clinical outcomes has been attributed to differences in environmental factors, bacterial strains, and host genetics. *H. pylori* virulence marker *cagA, vacA, babA*, and *iceA* influence the outcome of the associated diseases that has been evaluated in several studies.[4-7] The cytotoxin-associated gene (*cagA*) present in *H. pylori* isolates is considered a marker for the presence of the *cag* pathogenicity island (*cag* PAI). This region includes a number of other genes associated with an increased virulence and severe clinical outcomes.[8,9] Comparison of the genome of two *H. pylori* strains 26695 and J99 revealed, in addition to the *cag* PAI, the presence of potential PAIs regions with different G+C content so-called "plasticity region."[10] Occhialini *et al*.[11] identified in *H. pylori* strains isolated from patients with gastric adenocarcinoma and in patients with chronic gastritis-associated dyspepsia two new potential pathogenicity markers in the plasticity region. The *jhp0947* was found frequently in strains from patients with gastric can-

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cer (65%) than in strains from those without (35%) while the *jhp0940* was detected only in some strains from patients with GC. The third gene *jhp0986*, which is specific to strain 26695, was detected more often in strains isolated from patients with chronic gastritis-associated dyspepsia (39%) than in strains isolated from cancer patients (12%). The aim of the study was to evaluate the association between the hyperplasticity area genes *jhp0940, jhp0947* and *jhp0986* genes and *H. pylori* infection associated gastritis, peptic ulcer disease and GC in different age groups of the patients.

MATERIALS AND METHODS

1. *H. pylori* strains

H. pylori strains were isolated from the gastric biopsy of 114 patients who underwent endoscopy for evaluation of symptoms related to the upper gastrointestinal tract with gastritis associated with non-ulcer dyspepsia in 36 (32%), gastric ulcer (GU) in 22 (19%), GC in 29 (25%) and duodenal ulcer (DU) in 27 (24%) (Table 1). They attended the gastroenterology outpatient and endoscopy suite from June 2004 to December 2008. The study was approved by the Ethics Review Committee of the Aga Khan University. All patients gave an informed consent for endoscopy and participation in the study. None of the patients had received antibiotics, acid reducing drugs such as H_2-receptor antagonists, acid pump inhibitors, non-steroidal anti-inflammatory drugs or bismuth compounds in the last 4 weeks.

Table 1. Details of Patients from Which *Helicobacter pylori* Isolates Were Obtained

Total no.	114
Age, yr	
Mean±SD	47±16
Range	18-82
Gender	
Male	80 (70)
Female	34 (30)
Symptoms	
Abdominal pain	77 (67)
Weight loss	16 (14)
Melena	11 (10)
Hematemesis	10 (9)
Diagnosis	
Gastritis	36 (32)
Gastric ulcer	22 (19)
Gastric carcinoma	29 (25)
Duodenal ulcer	27 (24)
Histology	
Chronic active inflammation	89 (78)
Chronic inflammation	25 (22)

Values are presented as number (%).

2. Culture and identification of *H. pylori*

The specimens were transported immediately in sterile normal saline to isolate *H. pylori*. Thus, within three hours of collection each specimen was homogenized in sterile eppendorf tubes with electric homogenizer. The resulting suspension was inoculated onto Columbia Blood Agar (Oxoid) medium and Dents supplement (containing vancomycin, trimethoprim, and polymyxin) and incubated at 37°C under microaerophilic conditions for 4-6 days. Plates were then examined for bacterial growth and typical colonies were selected for identification. The identity of *H. pylori* was confirmed by Gram stain and by the production of urease and catalase. One half of the homogenate was used for culture, and the other half was kept at −80°C. *H. pylori* isolates were defined as gram-negative spiral-shaped bacilli that were catalase positive and rapidly (less than 1 hour) urease positive. *H. pylori* NCTC 11637 (type strain) was used as a positive control for the culture conditions and identification tests.

3. Histological analysis

Formalin-fixed and paraffin-embedded gastric biopsy specimens were routinely processed. All biopsy specimens for histological examination were fixed in 10% formalin, embedded in paraffin wax on the oriented edge, and cut into 5 μm thick sequential sections. All tissue sections were stained with hematoxylin and eosin for histological examination. The degree of acute and chronic inflammation, as well as the *H. pylori* density was scored according to the updated Sydney system.[12] The bacterial density was graded from 0 to 3 (0, absent; 1 to 3, from few and isolated bacteria to colonies). The infiltration of gastric mucosa by mononuclear cells and polymorphonuclear leucocytes, atrophy, and intestinal metaplasia were graded as follows: 0, none; 1, mild; 2, moderate; 3, marked. Chronic inflammation was defined according to an increase in lymphocytes and plasma cells in the lamina propria graded into mild, moderate or marked increase in density. Chronic active gastritis indicated chronic inflammation with neutrophilic polymorph infiltration of the lamina propria, pits or surface epithelium graded as 0, nil; mild, $<1/3$ of pits and surface infiltrated; moderate, 1/3-2/3; and marked, $>2/3$. Antrum and corpus gastritis were scored by total sum of grade of gastritis (mild, 1; moderate, 2; marked, 3 infiltration with lymphocytes and plasma cells) and activity of gastritis (mild, 1; moderate, 2; marked, 3 infiltration with neutrophilic granulocytes) either in the antrum or in the corpus, a maximum of a sum of 6 points for each individual patient.

Atrophy was defined as the loss of inherent glandular tissue, with or without replacement by intestinal-type epithelium. For optimal histological evaluation, all gastric biopsy specimens included surface epithelium and muscularis mucosae. Lymphoid aggregates were defined as accumulations of lymphocytes and plasma cells without a germinal centre.

4. Extraction of genomic DNA

The bacterial cells on chocolate agar plate was washed twice with phosphate buffer saline (PBS, pH 8.0) then centrifuged at 3,000 rpm for 20 minutes. *H. pylori* DNA was extracted by a phenol/chloroform method similar to the method previously described.[13] Briefly, bacterial pellet was resuspended in Tris-Cl buffer containing ethylenediaminetetraacetate (T.E pH 8.0) and lysozyme and was then incubated at 37°C for 30 minutes. The suspension was treated with sodium dodecyl sulphate (SDS), proteinase K and RNase A. DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated by sodium acetate and ice-cold absolute alcohol, and washed with ice cold alcohol (70%). The pellet of DNA was finally resuspended in TE buffer. DNA content and purity was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer (Beckman DU-600; Beckman Instruments, Fullerton, CA, USA).

5. Polymerase chain reaction

Amplification of *jhp0940, jhp0947, jhp0986* and *cag A* genes by PCR was performed in a volume of 50 μL containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol KCl, 1.5-2.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleoside triphosphates, 2 units Taq DNA polymerase (Promega) and 25 pmol of both forward and reverse primers (Table 2) used before (synthesized by MWG Automatic synthesizer).[11,14] PCR was performed in a Perkin Elmer 9700 thermal cycler. The amplification cycles for different genes are given in Table 1. Positive and negative reagent control reactions were performed with each batch of amplifications. DNA from *H. pylori* strains ATCC 43504 (*cagA* positive), ATCC 51932 (*cagA* negative) was used to define the accuracy of the *cagA*. After PCR, the amplified PCR products were electrophoresed in 2% agarose gels containing 0.5% x Tris/acetate/ethylenediaminetetraacetic acid, stained with ethidium bromide, and visualized under a short wavelength ultraviolet light source.

6. Statistical assessment

The statistical package for social science SPSS (Release 16, standard version; SPSS Inc., Chicago, IL, USA) was used for data analysis. The descriptive analysis was done for demographic and clinical features. Results were presented as mean±standard deviation for quantitative variables and number (percentage) for qualitative variables. Differences in proportion were assessed by using Pearson chi square, Fisher exact or likelihood ratio test where appropriate. Variables such as gender, age, *jhp0947, jhp0940, jhp0986* and *cagA* status (negative or positive), and the intensity and activity of gastritis (scored as defined above) were evaluated. Data were examined by logistic regression analysis for association of *jhp0947, jhp0940* and *jhp0986* gene status (the dependent variable) with disease, e.g., DU, GU, etc with adjustments made for potential confounding factors such as age group, *cagA*, chronic gastritis. A p value of ≤0.20 in the univariate analysis was used as a significant level for inclusion in the full model. In the multivariate analysis, a p value of ≤0.05 was considered significant. The odds ratios (OR) of significant covariates, as well as their 95% confidence intervals (CI), were determined.

Table 2. Oligonucleotide Primers Used for PCR

Gene	Sequence (5'→3')	PCR cycles	Size of PCR product, bp
JHP0940			
F	GAAATGTCCTATACCAATGG	1 cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 56°C	591
R	CCTAAGTAGTGCATCAAGG	for 1 min and 72°C for 1 min, 1 cycle of 72°C for 7 min	
JHP0947			
F	GATAATCCTACGCAGAACG	1 cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 60°C	611
R	GCTAAAGTCATTTGGCTGTC	for 1 min and 72°C for 1 min, 1 cycle of 72°C for 7 min	
JHP0986			
F	GCATGTCCCAAATCGTAGG	1 cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C	566
R	TGCATTTCGCATTGGCTCC	for 1 min and 72°C for 1 min sec, 1 cycle of 72°C for 7 min	
CagA			
F	GGTCAAAATGCGGTCATGG	1 cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 56°C	297
R	TTAGAATAATCAACAAACATCACGCCAT	for 1 min and 72°C for 1 min, 1 cycle of 72°C for 5 min	

RESULTS

1. Distribution of the hyperplasticity region genes

Seventy-one (62%) of the *H. pylori* isolates were positive for *jhp0940*, 67 (59%) for *jhp0947* and 12 (10%) for *jhp0986*. The *jhp0986* was identified in 12 (100%) (p=0.017) of the *H. pylori* strains isolated from only male patients.

2. Hyperplasticity region genes and diseases

Jhp0947 gene was found in 23 (85%) (p<0.001) of the *H. pylori* strains isolated from patients with DU and 22 (76%) (p=0.003) with GC compared to 14 (39%) with NUD gastritis (Table 3). *Jhp0940* was present in 18 (67%) (p=0.029) of the *H. pylori* strains isolated from DU, 17 (77%) (p=0.004) with GU and 22 (76%) (p=0.003) with GC compared to 14 (39%) with NUD gastritis (Table 3). There was a low frequency of *jhp0986* gene in *H. pylori* isolates. It was positive in 4 (11%) isolates from NUD gastritis, 4 (18%) with GU, 2 (7%) with GC and 2 (7%) with DU (Table 3). Multivariate analysis demonstrated *jhp0947* was associated with DU (OR, 6.1; 95% CI, 1.87-20.12) while *jhp0940* was associated with GU (OR, 2.8; 95% CI, 0.92-9) (Table 4).

3. Association of *cagA* and hyperplasticity region genes with diseases

CagA was positive in 69 (60%) isolates. It was significantly associated with GC in 21 (72%) (p=0.04) compared to 17 (47%) from gastritis (Table 5). In *cagA* positive strains, *jhp0940* gene was associated with GU in 11 (85%) (p=0.010) and GC in 18 (86%) (p=0.002) compared to NUD gastritis 6 (35%) while *jhp0947* was associated with GC in 17 (81%) (p=0.011) and DU 16 (89%) (p=0.003) compared to 7 (41%) in NUD gastritis.

4. Association of *cagA* and hyperplasticity area genes with histology

H. pylori isolates were associated with chronic active inflammation in 89 (78%) and with chronic inflammation in 25 (22%) patients (Table 1). *Jhp0947* was significantly associated with chronic active inflammation in 58 (65%) (p=0.009) compared to chronic inflammation in 9 (36%) (Table 3). *Jhp0986* was significantly associated with chronic inflammation in 6 (24%) (p=0.023) compared to chronic active inflammation in 6 (7%) (Table 3). *Jhp0940* was associated with both chronic active inflammation 57 (64%) and chronic inflammation in 14 (56%) (p=0.463) (Table 3). However, in the presence of *cagA*, *jhp0947* was

Table 3. Association of Hyperplasticity Region Genes with Diseases

Diagnosis	*JHP0947*, n (%)		p-value	*JHP0940*, n (%)		p-value	*JHP0986*, n (%)		p-value
	Positive	Negative		Positive	Negative		Positive	Negative	
Gastritis (GS)	14 (39)	22 (61)		14 (39)	22 (61)		4 (11)	32 (89)	
Gastric ulcer (GU)	8 (36)	14 (64)	0.847	17 (77)	5 (23)	0.004	4 (18)	18 (82)	0.462
Gastric carcinoma (GC)	22 (76)	7 (24)	0.003	22 (76)	7 (24)	0.003	2 (7)	27 (93)	0.684
Duodenal ulcer (DU)	23 (85)	4 (15)	<0.001	18 (67)	9 (33)	0.029	2 (7)	25 (93)	0.693
Histology									
Chronic active inflammation	58 (65)	31 (65)	0.009	57 (64)	32 (36)	0.463	6 (7)	83 (93)	0.023
Chronic inflammation	9 (36)	16 (64)		14 (56)	11 (44)		6 (24)	19 (76)	

GU, GC and DU were compared against GS. Differences in proportion were assessed by using Pearson chi square, Fisher exact or likelihood ratio test where appropriate. A p-value <0.05 was considered significant.

Table 4. Factors Associated with Duodenal and Gastric Ulcer in the Multivariate Analysis

Variable	Duodenal ulcer			Gastric ulcer		
	AOR	95% CI	p-value	AOR	95% CI	p-value
CagA	1.20	0.45-3.21	0.70	1.15	0.41-3.18	0.80
Chronic gastritis	0.90	0.25-3.27	0.89	2.0	0.68-6.0	0.21
Age group	0.48	0.19-1.24	0.13	1.0	0.35-2.8	0.97
JHP0947	6.1	1.87-20.12	0.003	0.37	0.13-1.0	0.049

A p value of ≤0.20 in the univariate analysis was used as a significant level for inclusion in the full model. In the multivariate analysis, a p value of ≤0.05 was considered significant. The adjusted odds ratios (AOR) of significant covariates, as well as their 95% confidence intervals (95% CI), were determined.

Table 5. Association between *cagA* and Hyperplasticity Region Genes and Diseases

	CagA, n (%)		p-value
	Positive	Negative	
Diagnosis			
Gastritis	17 (47)	19 (53)	
Gastric ulcer	13 (59)	9 (41)	0.38
Gastric carcinoma	21 (72)	8 (28)	0.04*
Duodenal ulcer	18 (67)	9 (33)	0.12
Histology			
Chronic active inflammation	58 (65)	31 (35)	0.05*
Chronic inflammation	11 (44)	14 (56)	
Gene			
JHP 0947			
Positive	43 (64)	24 (36)	0.341
Negative	26 (55)	21 (45)	
JHP 0940			
Positive	47 (66)	24 (34)	0.111
Negative	22 (51)	21 (49)	
JHP 0986			
Positive	7 (58)	5 (42)	0.869
Negative	62 (61)	40 (39)	

*p-value <0.05 was considered significant.

not significantly associated with chronic active inflammation in 39 (67%) (p=0.087) and neither *jhp0986* 3 (27%) (p=0.075) with chronic inflammation.

DISCUSSION

The study showed that the hyperplasticity region genes *jhp0947* and *jhp0940* were present in 60% of *H. pylori* isolates while *jhp0986* was infrequently demonstrated. The *jhp0940* was demonstrated in strains isolated from patients in comparatively older age group though they did not show any gender distribution. The *H. pylori* strains with *jhp0947* and *jhp0940* were significantly associated with GC and DU while *jhp0940* was also associated with GU (Table 3). In these *H. pylori* strains, *cagA* was only significantly associated with GC but not with DU and GU (Table 5). However, when these *cagA* positive strains were positive for *jhp0947* it was significantly associated with both GC and DU while *jhp0940* with GC and GU. *H. pylori* strains with *jhp0947* were significantly associated with chronic active inflammation in 65% (Table 3). However, histological inflammation was not augmented when they were concurrently *cagA* status positive.

In a previous study, Occhialini *et al*.[11] found that the *jhp0947* gene was present more frequently in strains isolated from patients with GC (64.7%; 11 of 17) than in those isolated from patients with gastritis alone (34.6%; 9 of 26). Also, Santos *et al*.[15] demonstrated that *jhp0947* gene was present in Brazilian strains from patients with GC and DU and appeared to be implicated in the development of both DU and GC. In our study, there was an independent association between the presence of *jhp0947*-positive *H. pylori* strains and DU. This is consistent with the previous studies.[11,15] However, in contrast we also noted that there was an association not only between the *jhp0940* gene and GC and DU but also between *jhp940* and GU in our *H. pylori* strains.[11,15] The *jhp0986* gene was demonstrated in only few of our *H. pylori* isolates. As, we did not use different modality such as hybridization to confirm this finding, failure with PCR does not necessarily mean that *jhp0986* gene was absent in our *H. pylori* isolates. In this study, we have demonstrated an association between the presence of the *jhp0940* gene and GU. The *jhp0947* and *jhp0940* genes, as well as the *cagA* gene, which is a marker of the *cag* PAI, may be implicated in the development of not only DU and GC but also GU in patients with *H. pylori* infection. This is also the first study in Pakistan that has suggested a relation between positive *cagA* and *jhp0947* or *jhp0940* with GC, DU and GU. We noted that the hyperplasticity genes were not associated with the *cagA*. In the presence of *cagA* positivity, no increase in severity of histological gastritis was demonstrated. In conclusion, this study demonstrated that the new hyperplasticity region genes *jhp0947* and *jhp0940* as virulence marker of *H. pylori* that are associated with GC and peptic ulcer disease. The functional analysis of these genes in a larger number of *H. pylori* isolates will provide further insight into its role in the virulence of *H. pylori* and the evolution of the infection.

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