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New Pathogenicity Marker Found in the Plasticity Region of the *Helicobacter pylori* Genome

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Comparison of gastric carcinoma and gastritis isolates showed the presence of genes, probably carcinoma associated (*JHP947* and *JHP940*), that are situated in a *Helicobacter pylori* genome region (45 kb in J99 and 68 kb in 26695) called the "plasticity region." This region presents a great variability of DNA sequences. We investigated, by PCR, the presence of the *JHP940* and *JHP947* genes, as well as the presence of a third gene which seems to be associated with gastritis (*HP986*), on *H. pylori* strains isolated from 200 Brazilian patients, 79 of whom had gastric carcinomas and 53 of whom had duodenal ulcers, to confirm this association. Gastritis isolates ($n = 68$) were included as a control. We also evaluated if these genes were related to the virulence-associated *cagA* genotype. The present methodology did not permit definitive conclusions to be reached regarding the association between the *JHP940* gene and gastric carcinoma or between the *HP986* gene and gastritis. However, we showed that the *JHP947* gene might be implicated in the development of both duodenal ulcer and gastric carcinoma. The presence of the *JHP947* gene was associated with the *cagA*-positive genotype. The *JHP947* gene is a novel virulence marker candidate of *H. pylori*.

Helicobacter pylori is a gram-negative, spiral, microaerophilic bacterium that lives for decades in the hostile environment of the human stomach. *H. pylori* infection has a worldwide distribution, and its prevalence ranges from 2% in developed countries to more than 90% in developing areas (17). This bacterium is the major cause of chronic gastritis and plays an important role in the pathogenesis of peptic ulcer disease, gastric carcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (11, 13, 15, 16, 21, 24, 33). However, why only a minority of *H. pylori*-positive patients develop the severe associated diseases remains unclear. Variation in clinical outcomes has been attributed to differences in environmental factors, bacterial strains, and host genetics. Indeed, a family history of gastric cancer per se, but also in combination with *H. pylori* infection, is associated with an increased risk for gastric carcinoma (19). With regard to the bacterial factors, the influence of particular genes on the outcome of the associated disease (*cagA*, *vacA*, *babA*, and *iceA*) has been evaluated in several studies (25, 26, 29, 34). The cytotoxin-associated gene (*cagA*) was the first one found to be differentially present in *H. pylori* isolates and is considered a marker for the presence of the *cag* pathogenicity island (*cag* PAI). This region includes a number of other genes associated with increased virulence and severe clinical outcomes (5, 22).

Comparison of the genomic contents of two *H. pylori* strains (26695 and J99) (1) has revealed, in addition to the *cag* PAI (5), the presence of regions with different G+C contents, which may represent potential PAIs. Among these (there are eight in 26695 and nine in J99) is one so-called "plasticity region" (2), a large region of 45 kb in strain J99 and 68 kb in strain 26695; it encodes 38 genes in J99, of which 33 are absent in 26695.

A recent study conducted by Occhialini et al. (23) on 17 *H. pylori* strains isolated from patients with gastric adenocarcinoma and 26 *H. pylori* strains isolated from patients with chronic gastritis-associated dyspepsia allowed the identification of two new potential pathogenicity markers in the plasticity region. Indeed, these genes were found more frequently in gastric cancer strains than in strains isolated from patients with gastritis only. One of the genes (*JHP940*) was detected only in some strains from patients with gastric carcinoma, and the second gene (*JHP947*) was found more frequently in strains from patients with gastric cancer (64.7%) than in strains from those without (34.6%). Moreover, a third gene (*HP986*), which is specific to strain 26695, was highlighted: it was detected more often in strains isolated from patients with chronic gastritis-associated dyspepsia (38.5%) than in strains isolated from cancer patients (11.8%).

However, the authors did not evaluate the presence of these new putative virulence markers in strains isolated from peptic ulcer patients. Furthermore, the associations that they observed need to be confirmed on a greater number of strains as well as in another geographic region, since geographic differences with regard to virulence genes of *H. pylori* have been demonstrated (3, 4, 7, 9, 12).

Therefore, the aim of the present study was to evaluate the association between new putative virulence genes and two se-

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TABLE 1. Characteristics of the patients

Disease	No. of patients			Mean age (yr) ± SD (range)
	Male	Female	Total	
Chronic gastritis	24	44	68	48.85 ± 15.40 (16–88)
Duodenal ulcer	21	32	53	41.53 ± 15.62 (15–74)
Gastric carcinoma	53	26	79	61.15 ± 13.42 (32–87)
Total	98	102	200	51.77 ± 16.73 (15–88)

vere diseases with different outcomes associated with *H. pylori* infection (duodenal ulcer and gastric carcinoma), controlling for confounding factors such as the age and gender of the patients. The pattern of gastritis associated with the infections caused by strains with or without these genes was also studied.

MATERIALS AND METHODS

Clinical samples, bacterial strains, and culture. This study was approved by the Ethics Committee of the University Hospital, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, and consent to participation was obtained from all patients. *H. pylori* strains were isolated from the gastric biopsy mucosae of 200 patients who underwent endoscopy for evaluation of symptoms related to the upper gastrointestinal tract (duodenal ulcer [$n = 53$] or gastritis associated with non-ulcer dyspepsia [$n = 68$]) or who underwent gastric surgery to remove a gastric carcinoma ($n = 79$) at the University Hospital, UFMG, or at the Luxemburgo Hospital of Oncology, Belo Horizonte, Brazil. Characteristics of the patients are presented in Table 1.

Tissue samples for culture were ground separately in a tissue homogenizer (Kontes, Vineland, N.J.) and plated onto petri dishes containing freshly prepared Belo Horizonte medium (28). Incubation was performed at 37°C under a microaerobic atmosphere obtained by using a gas generation system (Anaerocult C; Merck, Darmstadt, Germany). Plates were evaluated after 3, 6, 9, and 12 days of incubation. *H. pylori* was identified by macroscopic and microscopic morphology, a positive urease test, positive oxidase and catalase reactions, and PCR amplification of the *ureA* gene.

The two *H. pylori* strains whose genomes were sequenced previously (2, 31) were included as controls: strain J99, isolated from a patient with duodenal ulcer, and strain 26695 (NTCC 12455), isolated from a dyspeptic patient with gastritis.

For extraction of total DNA, the *H. pylori* strains were thawed, plated onto brain heart infusion agar plates supplemented with 10% sheep blood, and incubated at 37°C under microaerobic conditions for 3 days. Cells were harvested in 500 μl of sterile distilled water and centrifuged at 6,000 × *g* for 5 min, and the pellets were stored at −80°C until processing.

DNA extraction from bacterial cultures. *H. pylori* DNA was extracted with the QIAamp tissue kit (QIAGEN Inc., Santa Clarita, Calif.) according to the manufacturer's recommendations, with minor modifications (20). Briefly, the bacterial pellets, stored at −80°C, were suspended in 180 μl of lysis buffer (buffer ATL) and homogenized by vortexing. Twenty microliters of a proteinase K solution (20 mg/ml) was then added, followed by overnight incubation at 56°C. A second lysis buffer (buffer AL) provided in the kit was added, and the sample was incubated at 70°C for 10 min. Next, 200 μl of ethanol was added, and this mixture was then loaded onto the QIAamp spin column and centrifuged at 6,000 × *g* for 1 min. The QIAamp spin column was placed in a 2-ml collection microtube, and the contained filtrate was discarded. The column material was washed twice (250 μl each time) with the first washing buffer (buffer AW1) and twice (250 μl each time) with the second washing buffer (buffer AW2) provided in the kit. Finally, the DNA was eluted with 100 μl of distilled water preheated to 70°C (two elutions, each with 50 μl). The DNA concentration was determined by measuring the absorbance at 260 nm.

Amplification of DNA by PCR. Oligonucleotide primers were designed on the basis of published sequences of *H. pylori ureA* (6), *cagA* (two sets of synthetic oligonucleotide primers were used, and strains were considered to be *cagA* positive when at least one of the reactions was positive) (32), and specific open reading frames (ORFs) (*JHP940*, *JHP947*, and *HP986*) from the plasticity region (23) (Table 2). Amplification of *H. pylori* genomic DNA sequences was carried out in a total volume of 50 μl containing 5 μl of 10× PCR buffer (Eurobio, Les Ulis, France), 1.5 mM $MgCl_2$, 200 μM each deoxynucleotide (Eurobio), 2 U of Eurobiotaq DNA polymerase (Eurobio), 0.5 μM each primer, and 10 ng of *H. pylori* DNA. Each reaction mixture was amplified for 35 cycles (except for the *cagA* gene [40 cycles]) as follows: 1 min at 94°C, 1 min of annealing at either 60°C (for *cagA* and *JHP947*), 58°C (for *HP986*), 56°C (for *JHP940*), or 45°C (for *ureA*), and 1 min at 72°C. After the last cycle, extension was continued for another 7 min. Amplicons were analyzed on a 1% agarose gel stained with ethidium bromide.

Histological study. Samples of the antral and oxyntic gastric mucosae were fixed in 10% formalin and embedded in paraffin wax by routine methods. Five-micrometer-thick histological sections stained with hematoxylin and eosin were obtained for histological analysis. The mucosae were analyzed in terms of the degree of inflammatory reaction and activity, which were scored as follows, according to the revised Sydney system (8): 0, none; 1, mild; 2, moderate; 3, marked. For patients with gastric carcinoma, multiple biopsy specimens were obtained from the tumor lesion to confirm the diagnosis.

Statistical analysis. Data were analyzed with the SPSS statistical software package (version 10.0; SPSS Inc., Chicago, Ill.).

Variables such as gender, mean age, *JHP947* gene status (negative or positive), *cagA* status (negative or positive), and the intensity and activity of antral and oxyntic gastritis (scored as defined above) were evaluated.

Data were examined by logistic regression analysis for association of *JHP947* gene status (the dependent variable) with disease (gastritis versus duodenal ulcer versus gastric carcinoma), with adjustments made for potential confounding factors such as age and gender.

Logistic regression analysis was also performed to analyze the relation be-

TABLE 2. Oligonucleotide primers used for PCR

Gene	Nucleotide positions	Sequence (5′→3′)	Size of PCR product (bp)	Reference
ureA	304–321	GCCAATGGTAAATTAGTT	411	6
	697–714	CTCCTTAATTGTTTTTAC		
cagA	157–181	GATAACAGGCAAGCTTTTGAGGGA	394	32
	527–550	CCATGAATTTTTGATCCGTTCGG		
	910–935	ATGGGGAGTCATGATGGCATAGAACC	717	32
	1602–1626	ATTAGGCAAATTAAAGACAGCCACC		
JHP940	139–158	GAAATGTCCTATACCAATGG	591	23
	711–729	CCTAAGTAGTGCATCAAGG		
JHP947	91–109	GATAATCCTACGCAGAACG	611	23
	682–701	GCTAAAGTCATTTGGCTGTC		
HP986	21–39	GCATGTCCCAAATCGTAGG	566	23
	568–586	TGCATTTCGCATTGGCTCC		

TABLE 3. Variables associated with gastric carcinoma and duodenal ulcer diseases in the multivariate analysis

Variable	Finding for:					
	Gastric carcinoma			Duodenal ulcer		
	OR	95% CI	*P*	OR	95% CI	*P*
Old age	1.07	1.04–1.10	0.000	0.98	0.95–1.00	0.059
Male gender	0.32	0.14–0.74	0.008			
cagA-positive status	4.67	1.56–13.97	0.006	4.51	1.56–13.06	0.005
JHP947 gene-positive status	4.14	1.47–11.66	0.007	2.75	1.08–6.96	0.033

tween the presence of a duodenal ulcer or gastric carcinoma and the *JHP947* and *cagA* status, with adjustments made for age and gender.

For patients without a duodenal ulcer or gastric carcinoma, data were evaluated by logistic regression analysis for association between the severity of antral and oxyntic gastritis and *JHP947* gene status, with adjustments made for *cagA* status.

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 (95% CI), were determined.

RESULTS

Distribution of the *JHP940*, *JHP947*, and *HP986* ORFs. The *JHP940* gene was found in only 3 of 200 *H. pylori* strains isolated, which were from 1 patient with gastritis, 1 with a duodenal ulcer, and 1 with gastric carcinoma. Low frequency was also observed for *HP986*: this gene was not found in strains isolated from gastritis or duodenal ulcer patients and was present in only 4 of 79 (5.1%) strains isolated from patients with gastric carcinoma. On the other hand, the *JHP947* gene was found in 140 (70.0%) of the *H. pylori* strains analyzed.

Study of the association between *JHP947* and severe diseases. Thirty (44.1%) of the 68 patients with gastritis, 42 (79.2%) of the 53 patients with duodenal ulcer, and 68 (86.1%) of the 79 patients with gastric carcinoma were colonized by a *JHP947*-positive strain.

In the univariate analysis, when patients with gastritis and gastric carcinoma were compared, the presence of the *JHP947* gene was positively associated with gastric carcinoma ($P < 10^{-3}$), old age ($P = 0.14$), and male gender ($P = 0.047$). In the multivariate analysis, the presence of the *JHP947* gene remained associated only with gastric carcinoma ($P < 10^{-3}$; OR, 2.94; 95% CI, 1.86 to 4.64). In the groups of patients with duodenal ulcer and gastritis, the *JHP947* gene was associated only with duodenal ulcer disease ($P < 10^{-3}$; OR, 4.84; 95% CI, 2.13 to 10.96).

Diseases and *JHP947* and *cagA* status. Among the 140 *H. pylori* strains harboring the *JHP947* gene, 127 (90.7%) were also *cagA* positive. Since the presence of the *JHP947* gene was associated with the *cagA*-positive genotype ($P = 0.01$), we investigated whether or not the *cagA* and *JHP947* genes were independently associated with duodenal ulcer and gastric carcinoma by logistic regression, adjusting for confounding factors. The results of the univariate analysis showed that old age, *cagA*$^+$ status, and *JHP947* gene-positive status were associated with gastric carcinoma ($P < 10^{-3}$ for all variants) and duodenal ulcer ($P = 0.01$ for age; $P < 10^{-3}$ for the other variables). With respect to gender, no association was seen with duodenal ulcer ($P = 0.62$), but male gender was associated with gastric carcinoma ($P < 10^{-3}$).

In the multivariate analysis, the *cagA* and *JHP947* genes were independently associated with duodenal ulcer. These genes were also independently associated with gastric carcinoma. Old age and male gender of the patients were also independently associated with gastric carcinoma but not with duodenal ulcer (Table 3).

***cagA* status, *JHP947* status, and gastric mucosa histology.** We also evaluated whether the presence of the *cagA* and *JHP947* genes in the *H. pylori* strains was associated with the severity of gastritis in the subset of patients without duodenal ulcer or gastric carcinoma.

In the univariate analysis, the presence of the *JHP947* gene was associated with *cagA*-positive status ($P < 10^{-3}$), the activity ($P = 0.002$) of oxyntic gastritis, and the activity of antral gastritis ($P = 0.024$). However, in the multivariate analysis, the gene remained associated only with *cagA*-positive status ($P = 0.012$; OR, 5.54; 95% CI, 1.46 to 21.01). On the other hand, the intensity of gastritis in both the antral ($P = 0.02$) and oxyntic ($P = 0.008$) gastric mucosae, as well as the activity of antral ($P < 10^{-3}$) and oxyntic ($P < 10^{-3}$) gastritis, was significantly greater in patients colonized by a *cagA*-positive strain than in patients colonized by a *cagA*-negative strain.

DISCUSSION

H. pylori infection is associated with clinical outcomes as different as distal gastric carcinoma and duodenal ulcer. Among the factors that may be linked to infection outcome, bacterial virulence factors such as cytotoxin and the *cag* PAI have been extensively investigated. Although these known bacterial markers undoubtedly contribute to the genesis of *H. pylori*-associated diseases, they do not explain the entire process and do not distinguish between the two key outcomes. Thus, studies focusing on new putative bacterial virulence determinants are still desirable. Furthermore, identification of bacterial factors implicated in the evolution of *H. pylori* infection is one of the steps in understanding the interaction between the bacterium and the host cell.

Like the *cag* PAI, the plasticity region of *H. pylori* displays some characteristics of a PAI, i.e., large size and a lower percentage of G+C DNA than in the rest of the bacterial genome. This region also contains 46 and 48% of the genes that are unique to *H. pylori* strains 26695 and J99, respectively. These strain-specific genes may encode factors contributing to

different disease outcomes, thus becoming virulence factor candidates.

In fact, in a previous study, Occhialini et al. (23) found that the *JHP947* gene was present more frequently in strains isolated from patients with gastric adenocarcinoma (64.7%; 11 of 17) than in those isolated from patients with gastritis alone (34.6%; 9 of 26). In this study, in which a large number of patients was evaluated, we confirmed the association between the presence of *JHP947*-positive *H. pylori* strains and gastric carcinoma. However, in contrast to the study of Occhialini et al., the present methodology did not permit definitive conclusions to be reached regarding the association between the *JHP940* gene and gastric carcinoma or between the *HP986* gene and gastritis. These discordant results may be explained by differences between the plasticity regions of *H. pylori* strains isolated in distinct geographic areas, and they show the need to study different populations. However, there is also considerable geographic diversity among *H. pylori* gene sequences, and therefore failure to obtain a PCR product with a single pair of PCR primers does not necessarily mean that a gene is absent. It seems possible that one or both PCR primers might not anneal optimally with the corresponding sequences in these Brazilian isolates. Further experimental work needs to be done in order to investigate whether the *JHP940* and *HP986* genes are really absent from the Brazilian isolates. We tried, unsuccessfully, to amplify these genes with another set of PCR primers. In the future, Southern hybridizations could be used to confirm the results for these two genes.

In this study we demonstrated for the first time an association between the presence of the *JHP947* gene and duodenal ulcer. Therefore, the *JHP947* gene, as well as the *cagA* gene, which is a marker of the *cag* PAI, may be implicated in the development of both duodenal ulcer and gastric carcinoma.

In a case control study carried out in Brazil, Queiroz et al. (29) observed a significant association between *cagA*-positive status and distal gastric carcinoma. In addition, an association between infection with a *cagA*-positive *H. pylori* strain and duodenal ulcer was shown in another case control study with Brazilian children (27). Several other studies have demonstrated such associations (7, 9, 10, 18, 25, 26, 34). Infection with *cagA*-positive *H. pylori* strains that contain the *cag* PAI elicits a marked inflammatory response with a higher density of polymorphonuclear cells on the gastric mucosa, which has been considered a risk factor for the development of these severe diseases.

Our results showed that the presence of the *JHP947* gene was associated with the *cagA*-positive genotype. However, the high degree of gastritis that was associated with the presence of both genes in the univariate analysis did not remain associated with the *JHP947* gene in the full model of logistic regression, showing that the severity of the microscopic alterations of the gastric mucosae was associated only with the presence of the *cagA* gene. Salama et al. (30) analyzed 15 strains by whole-genome microarray and found genes (strain-specific and also candidate virulence genes) that were coinherited with the *cag* PAI, like the *JHP947* gene, but they did not identify genes associated with specific diseases; this may be due to the small sample size and the lack of clinical information for many of the strains evaluated. Thus, although the *JHP947* gene is a true gene which was previously found to be expressed by reverse transcription-PCR (23), its putative function does not seem to be correlated with the severity of the gastric inflammation caused by *H. pylori* infection. The findings that we observed, however, provide a framework for future studies that would more mechanistically delineate the role of this locus in the diseases associated with *H. pylori*. It also has to be emphasized that although the plasticity region of *H. pylori* is an unstable locus, Israel et al. demonstrated in a recent study (14) that the *JHP947* gene, which was present in the original J99 *H. pylori* strain, remained present 6 years later.

In conclusion, this study confirmed the association of a new ORF with gastric carcinoma in a large number of strains and demonstrated that this gene was also associated with duodenal peptic ulcer. In addition to the *cag* PAI and VacA cytotoxin, which are recognized virulence markers, the *JHP947* gene is a novel virulence marker candidate of *H. pylori*. Functional analysis of the gene potentially identified as a novel pathogenicity marker will provide insight into its role in the virulence of *H. pylori* and the evolution of the infection.

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