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ORIGINAL ARTICLE

CagA EPIYA polymorphisms in Colombian *Helicobacter pylori* strains and their influence on disease-associated cellular responses

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Abstract

AIM: To investigate the influence of the CagA diversity in *Helicobacter pylori* (*H. pylori*) strains from Colombia on the host cell biology.

METHODS: Eighty-four *H. pylori-cagA* positive strains with different Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs patterns, isolated from patients with gastritis (n = 17), atrophic gastritis (n = 17), duodenal ulcer (n = 16), intestinal metaplasia (n = 16) and gastric cancer (n = 18), were included. To determine the integrity of the cag pathogenicity island (*cag*PAI) we evaluated the presence of *cagA*, *cagT*, *cagE*, and *cag10* genes by polymerase chain reaction. AGS gastric epithelial cells were infected with each strain and assayed for translocation and tyrosine phosphorylation of CagA by western blot, secretion of interleukin-8 (IL-8) by enzyme-linked immuno sorbent assay after taking supernatants from cocultures and cell elongation induction. For cell elongation quantification, coculture photographs were taken and the proportion of "hummingbird" cells (> 15 μm) was determined.

RESULTS: Overall 72% (60/84) of the strains were found to harbor a functional *cag*PAI. Levels of phosphorylated CagA were significantly higher for isolates from duodenal ulcer than the ones in strains from gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer (49.1% ± 23.1% *vs* 21.1% ± 19.5%, $P < 0.02$; 49.1% ± 23.1% *vs* 26.2% ± 14.8%, $P < 0.045$; 49.1% ± 23.1% *vs* 21.5% ± 19.5%, $P < 0.043$ and 49.1% ± 23.1% *vs* 29.5% ± 27.1%, $P < 0.047$ respectively). We observed variable IL-8 expression levels ranging from 0 to 810 pg/mL and from 8.8 to 1442 pg/mL at 6 h and 30 h post-infection, respectively. *cag*PAI-defective strains did not induce detectable levels of IL-8 at 6 h post-infection. At 30 h post-infection all strains induced IL-8 expression in AGS cells, although *cag*PAI-defective strains induced significantly lower levels of IL-8 than strains with a functional *cag*PAI (57.1 ± 56.6 pg/mL *vs* 513.6 ± 338.6 pg/mL, $P < 0.0001$). We did not observe differences in the extent of cell elongation induction between strains with a functional or a defective *cag*PAI in 6 h cocultures. At 24 h post infection strains with functional *cag*PAI showed high diversity in the extent of hummingbird phenotype induction ranging from 7% to 34%. *cag*PAI defective strains induced significantly lower levels of elongation than strains with functional *cag*-PAI with one or more than one EPIYA-C motif (15.1% ± 5.2% *vs* 18.9% ± 4.7%, $P < 0.03$; and 15.1% ± 5.2% *vs* 20.0% ± 5.1%, $P < 0.003$ respectively). No differences were observed in cellular elongation induction

or IL-8 expression among *H. pylori* strains bearing one and more than one EPIYA-C motifs, neither at 6 h nor at 24 h of coculture. There were no associations between the levels of induction of cell elongation or IL-8 expression and number of EPIYA motifs or pathology.

CONCLUSION: The present work describes a lack of association between *H. pylori* CagA protein EPIYA motifs variations from Colombian isolates and disease-associated cellular responses.

Key words: *Helicobacter pylori*; *cagA* 3′ region; CagA protein; Interleukin 8; Cell elongation; Glu-Pro-Ile-Tyr-Ala

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infects more than 50% of the world's population[1]. This pathogen has been associated with the development of chronic gastritis, duodenal ulcers and gastric cancer[2], and was classified as a type I carcinogen by the International Agency for Research on Cancer[3].

One of the most important virulence factors of *H. pylori* is the *cag* pathogenicity island (*cag*PAI), which encodes for a type Ⅳ secretion system (T4SS)[4]. Also encoded in the *cag*PAI is the CagA protein, which is translocated into gastric epithelial cells through the T4SS[5], where it undergoes phosphorylation by members of the SRC and Abl families of kinases on tyrosine residues within the C-terminal Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs[6-8]. Phosphorylated CagA interacts with the cellular phosphatase SHP-2[9], which in turn activates several signaling pathways involved, among others, in actin cytoskeletal rearrangements, leading to cell elongation (also known as the "hummingbird phenotype")[10,11]. Translocated CagA can also induce a proinflammatory response, resulting in the expression of interleukin-8 (IL-8) through the activation of nuclear factor κ B (NF-κB)[12-14].

CagA varies in size, and this variation has been shown to be due to EPIYA motifs repeats within the C-terminal region of the protein[15,16]. Four types of EPIYA motifs have been described (A, B, C and D) based on the sequence flanking the motif[17]. Western *H. pylori* isolates have shown to harbor combinations of type A, B and C motifs, while East Asia isolates harbor combinations of type A, B and D motifs[17,18].

A positive association between the number of EPIYA motifs repeats and the phosphorylation of CagA protein has been reported[17,19]. Several studies have shown that strains with higher numbers of EPIYA-C motifs are more closely associated with gastric cancer[20-22].

IL-8 expression in gastric tissue has been reported to correlate with the histopathological severity in *H. pylori*-positive patients[23,24]. Furthermore, it has been shown that strains with higher number of EPIYA-C motifs significantly increased IL-8 expression in gastric epithelial cells[25]. As with IL-8 expression, CagA proteins with higher number of EPIYA motifs, and especially EPIYA-C motifs, have shown to potentiate cell elongation in AGS cells[17,19,26,27].

The aim of this study was to evaluate the possible association between CagA EPIYA motifs variations in *H. pylori* isolates from Colombia with the phosphorylation of CagA protein, the expression of IL-8 and cell elongation induction in gastric epithelial cells. Associations between disease severity and *H. pylori*-induced cellular responses *in vitro* were also evaluated.

MATERIALS AND METHODS

H. pylori strains

In total, 84 *cagA*-positive and 6 *cag*PAI-negative strains obtained from the stock collection at the Instituto Nacional de Cancerología, in Bogotá, Colombia were included in the study. *CagA*-positive strains were isolated from patients diagnosed with gastritis (n = 17), atrophic gastritis (n = 17), duodenal ulcer (n = 16), intestinal metaplasia (n = 16) and gastric cancer (n = 18). Isolates' *cagA* genotyping was reported previously[28] and EPIYA motifs combinations used in this study are summarized in Figure 1. *cagA*-positive reference strain NCTC 11 637, with an ABCCC polymorphism, was used as a positive control.

Detection of cagPAI genes

H. pylori genomic DNA was obtained from plate cultures of each isolate using DNAzol (Invitrogen) extraction method according to the manufacturer's instructions. The primers used in this study are listed in Table 1. To determine the integrity of *cag*PAI we evaluated the presence of *cagA, cagT, cagE* and *cag10* genes. All PCR reactions were performed in a volume of 25 μL containing 10 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 25 pmol of the primers, 100 ng of *H. pylori* genomic DNA and 1U Taq polymerase. The polymerase chain reaction (PCR) conditions for each reaction were previously described[29,30]. Positive (strain 11637) and negative controls (strain 3062) for the *cag*PAI were included in each run. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

Culture of H. pylori strains

H. pylori strains were grown on blood agar plates, supplemented with 7% horse serum (Invitrogen), 1% Vitox (Oxoid), and Campylobacter selective supplement (Oxoid), at 37 ℃ in a 10% CO_2-humidified atmosphere for

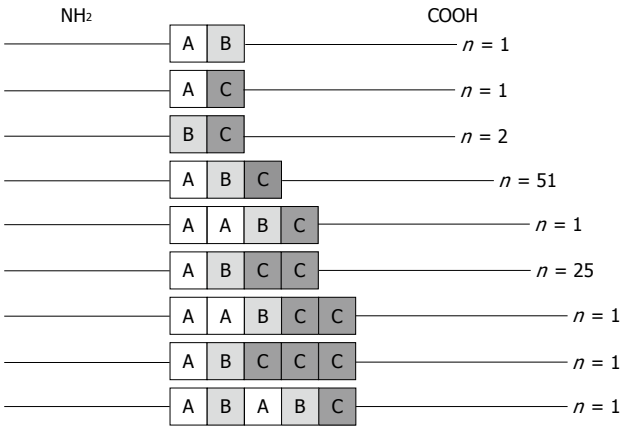

Figure 1 ***Helicobacter pylori*** **CagA Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs variations included in this study.** Eighty-four *cagA*-positive strains isolated from Colombian patients were evaluated. All strains possessed Western EPIYA motifs (A, B and C)[17] ranging from 2 to 5 in number.

3 d. Grown plates were subcultured into brucella broth (DIFCO) containing 10% horse serum (Invitrogen) and Campylobacter selective supplement (Oxoid), and were incubated under microaerophilic and shaking conditions for 24 h. Overnight cultures were set to an optical density of 0.1 at 600 nm (approximately 1.2×10^8 bacteria/mL) by dilution. Brucella broth was discarded after centrifugation of liquid cultures at 7000 rpm for 10 min and bacteria were resuspended in serum- and antibiotic-free RPMI medium (GIBCO) prior to infection.

Co-culture assays

AGS epithelial cells were seeded into 6-well plates (4×10^5 cells/well) or 25 cm^2 flasks (5×10^5 cells) and grown in RPMI 1640 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen) and 2.5 μg/mL amphotericin (GIBCO) at 37 ℃ in a 5% CO_2 atmosphere for 24 h. Eighty percent confluent cell cultures were then washed with phosphate buffered saline (PBS), and serum- and antibiotic-free RPMI was added to the wells. Sixteen hours serum-starved cell cultures were infected with *H. pylori* suspensions at a multiplicity of infection (MOI) of 100. Cocultures were incubated at 37 ℃ in a 5% CO_2-humidified atmosphere.

CagA phosphorylation assays

After 6 h of coculture the medium was removed and cells were washed with PBS containing 1.0 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$ and scraped from the flasks into 3 mL PBS containing 1 mmol/L sodium vanadate, harvested by centrifugation at 1000 *g* by 10 min, resuspended in 100 μL of PBS-sodium vanadate and lysed with 4 × Laemli sample buffer [0.2 TRIS-HCl pH 6.8, 0.4 mmol/L ditiothreitol, 8% sodium dodecyl sulfate (SDS), 40% glycerol, 0.4% bromophenol blue]. Cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 6% resolving gel and a 4% stacking gel. Proteins were then transferred onto nitrocellulose membranes by semidry transfer. For protein detection, membranes were probed with a 1:1000 dilution of anti-phospho-tyrosine monoclonal antibody (Santa Cruz Biotechnologies) followed by a 1:4000 dilution of HRP-conjugated goat anti-mouse (Zymax, Invitrogen). Blots were developed using the Amersham ECL detection reagents (GE Healthcare). Membranes were subsequently stripped (using a 62.5 mmol/L TRIS-HCL pH 6.8, 100 μmol/L ß-2-Mercaptoethanol solution, 2% SDS at 50 ℃ for 30 min) and reprobed with 1:1000 polyclonal anti-CagA antibody (Santa Cruz Biotechnologies) followed by 1:60 000 HRP-conjugated goat anti-rabbit secondary antibody (Zymax, Invitrogen) and developed as described above. Densitometry was performed using a Gel Doc GS-670 (Biorad) and results were expressed as the ratio of phosphorylated CagA to total CagA multiplied by 100.

IL-8 assay

Medium samples from 6 h and 30 h co-cultures were collected, centrifuged at 7000 rpm for 10 min to discard unattached bacteria or cells, and supernatants were stored at -80 ℃ until further use. IL-8 concentration was measured using an IL-8 Human ELISA kit (Invitrogen) according to the manufacturer's instructions. Uninfected AGS cells were used as a negative control.

Cellular elongation assay

Six hours and twenty-four h cocultures were examined by differential interference contrast microscopy with a Leica DM IL phase contrast inverted microscope (Leica). For this, 3 randomly chosen 20 × fields were photographed with a MD800-CK camera for microscope (Amscope). Hummingbird cells were measured and counted with the software ImageJ v1.44c (developed by Wayne Rasband at the National Institutes of Health, Bethesda, MD, United States and available at http://rsb.info.nih.gov/ij/). Hummingbird cells are characterized by the formation of needle-like projections[11]. We defined hummingbird phenotype as cells with needle-like projections > 15 μm. Uninfected AGS cells were used as a negative control.

Statistical analysis

Mann Whitney *U* test was used for statistical analysis. A *P* value < 0.05 was considered statistically significant. All data were analyzed with the software Graphpad Prism 5 (Graphpad Software, Inc.). All experiments were run in duplicates.

RESULTS

cagPAI status, CagA expression and tyrosine phosphorylation

From the 84 *cagA*-positive strains, 74 (88.1%) tested positive for *cagE,* 72 (85.7%) for *cagT* and 68 (81%) for *cag10*. Overall, 67 (79.8%) tested positive for all four *cag*PAI genes by PCR, and were therefore predicted, on the basis of this limited testing, to have an intact *cag*PAI. The remaining 17 strains, which tested negative for one or more genes, were collectively predicted to have a partial (*i.e.*, in-

Table 1 Primers used for the detection of *Cag*PAI genes

Gene	Primer	Sequence 5′-3′	Product size (bp)	Reference
cagE	101	TTGAAAACTTCAAGGATAGGATAGAGC	510	[16]
	102	GCCTAGCGTAATATCACCATTACCC		
cagT	cagTF	ATGAAAGTGAGAGCAAGTGT	823	[30]
	cagTR	TCACTTACCACTGAGCAAAC		
cag10	cag10F	ATGGAAGACTTTTTGTATAA	2208	[30]
	cag10R	TCACAGTTCGCTTGAACCCA		

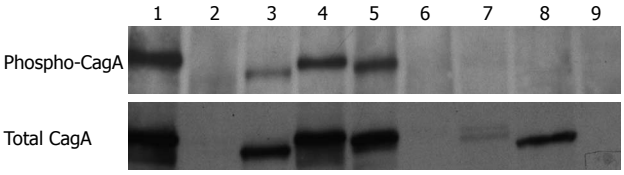

Figure 2 Phosphorylation of CagA protein in AGS cells after coculture with *Helicobacter pylori CagA*-positive strains. Cell lysates were evaluated by western blot using anti-phosphotyrosine or anti-CagA antibodies. A representative assay is shown. Lane 1: 11 637 control strain with a functional *cag*PAI and 5 Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs (ABCCC); Lanes 2 and 6: Isolates with a defective *cag*PAI lacking expression of CagA; Lanes 7 and 8: Isolates with a defective *cag*PAI expressing CagA, with absence of CagA phosphorylation; Lanes 3-5: Isolates with a functional *Cag*PAI with three (ABC; lane 3) or four (ABCC; lanes 4 and 5) EPIYA motifs; Lane 9: Uninfected AGS cells.

complete) *cag*PAI. Bacterial lysates of the 84 strains were assessed by Western blot with anti-CagA antibodies, from which 75 (89.3%) expressed the CagA protein. The nine strains lacking CagA expression harbored a partial *cag*PAI.

Once CagA is expressed, it is delivered into host cells *via* the T4SS and becomes phosphorylated by host cell kinases[11]. Seventy-three out of the seventy-five CagA-expressing strains were evaluated for CagA phosphorylation in coculture with AGS cells (Figure 2). From these, in 58 strains (79.4%) CagA was phosphorylated during infection. In 15 strains CagA was not phosphorylated, including eight CagA-expressing strains bearing a partial *cag*PAI. The remaining seven strains lacking CagA phosphorylation were predicted to have an "intact" *cag*PAI according to *cagT, cagE,* and *cag10* PCR results. This last result indicates that PCR detection of selected *cag*PAI genes is not sufficient to predict the functionality of the *cag*PAI. In addition, and as described below, these seven strains failed to induce IL-8 secretion in AGS cells, which supports this conclusion. Based on this *in vitro* characterization of the strains, we grouped isolates bearing a partial *cag*PAI (*i.e.*, strains which tested negative for one or more *cag*PAI genes) and strains with a non-functional *cag*PAI (*i.e.*, strains showing no CagA phosphorylation nor induction of IL-8 secretion) as strains with a defective *cag*PAI. In summary, 24 out of the 84 strains (28.5%) were found to harbor a defective *cag*PAI: 17 strains with a partial *cag*PAI and 7 strains with a non-functional *cag*PAI.

We further evaluated the association degree between the levels of CagA phosphorylation and the histopathological diagnoses for strains with functional *cag*PAI. Interestingly, the mean of CagA phosphorylation in strains from duodenal ulcer was shown to be significantly higher than the ones in strains from gastritis, atrophic gastritis, intestinal metaplasia and gastric cancer ($P < 0.02$, 0.045, 0.043 and 0.047 respectively; Figure 3A).

We also investigated the relationship between the number of EPIYA-C motifs and the levels of CagA phosphorylation. Isolates from this study ranged from one to three EPIYA-C motifs (Figure 1). CagA-expressing *H. pylori* strains bearing one and more than one EPIYA-C motifs were grouped together. Although higher levels of CagA phosphorylation were observed in strains with more than one EPIYA-C motif in comparison with strains with one EPIYA-C motif, this difference was not significant (Figure 3B).

Influence of cagPAI status and cagA polymorphisms on IL-8 expression in AGS cells

The 84 *cagA*-positive and 6 *cag*PAI-negative strains isolated from Colombian patients were tested for IL-8 induction in AGS cells. *CagA*-positive strains induced variable expression levels of IL-8 ranging from 0 to 810 pg/mL and from 8.8 to 1442 pg/mL at 6 h and 30 h post-infection, respectively. Ten out of the 67 strains classified by genotyping to bear an intact *cag*PAI did not induce IL-8 expression after 6 h of coculture with AGS cells. Three of these strains showed CagA translocation and phosphorylation in contrast to the remaining seven strains, in which no phosphorylation was observed. These last seven strains were likely to bear defects in other *cag*PAI components not detected by the PCR of the selected *cag*PAI genes. This showed, as previously observed by Argent *et al*[19], that PCR prediction of *cag*PAI intactness is a poor test for the presence of a T4SS capable of inducing IL-8 expression in AGS. We therefore considered these strains to have a non-functional *cag*PAI and classified them, along with the partial-*cag*PAI strains, as *cag*PAI-defective isolates, as described above.

*Cag*PAI-negative and *cag*PAI-defective strains did not induce detectable levels of IL-8 at 6 h post-infection (Figure 4A). At 30 h post-infection all strains induced IL-8 expression in AGS cells, although *cag*PAI-negative and *cag*PAI-defective strains induced significantly lower levels of IL-8 than strains with a functional *cag*PAI with one or more than one EPIYA-C motif ($P < 0.001$; Figure 4B).

A previous report has suggested a positive association between the number of EPIYA-C motifs and IL-8 expression[25]. We therefore evaluated *H. pylori*-IL-8 induction according to the number of EPIYA-C motifs in each strain. There were no differences in IL-8 expression

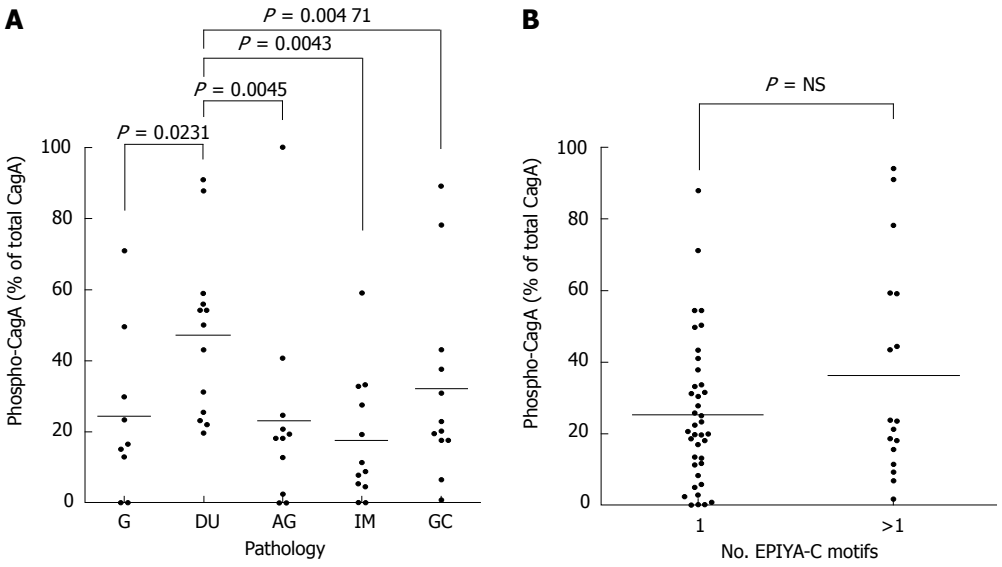

Figure 3 CagA-protein phosphorylation and its relationship to Glu-Pro-Ile-Tyr-Ala-C (EPIYA-C) motifs and disease severity. Sixty functional-*cag*PAI strains were cocultured with AGS cells for 6 h. Coculture lysates were assessed by Western blot and levels of CagA phosphorylation were determined by densitometry. A: Evaluation of CagA phosphorylation levels according to the pathology from which strains were isolated; B: Relationship between the number of EPIYA motifs and the levels of CagA phosphorylation. G: Gastritis; DU: Duodenal ulcer; AG: Atrophic gastritis; IM: Intestinal metaplasia; GC: Gastric cancer. NS: Not significant

among *H. pylori* strains bearing one and more than one EPIYA-C motifs, neither at 6 h nor at 30 h of coculture, suggesting a lack of association between CagA EPIYA-C motifs variations in *H. pylori* isolates from Colombia and IL-8 induction (Figure 4A and B).

Influence of cagPAI status and cagA polymorphisms on hummingbird phenotype induction

AGS cells were cocultured with the same 84 strains tested for IL-8 expression and evaluated for hummingbird phenotype formation. We did not observe differences in the extent of cell elongation induction between strains with a functional or a defective *cag*PAI in 6 h cocultures (Figure 4C). At 24 h post infection strains with functional *cag*PAI showed high diversity in the extent of hummingbird phenotype induction ranging from 7% to 34%. *Cag*PAI-negative and *cag*PAI-defective strains induced significantly lower levels of elongation than strains with functional *cag*PAI with one or more than one EPIYA-C motif (P = 0.032 and 0.003 respectively; Figure 4D).

Similarly to IL-8 expression, no differences were observed in cellular elongation induction among *H. pylori* strains bearing one and more than one EPIYA-C motifs, neither at 6 h nor at 24 h of coculture (Figure 4C and D). Unexpectedly, three *cag*PAI-defective strains induced elongation in more than 20% of the cells.

H. pylori-induced cellular responses and their association to disease severity

To assess the degree of association between the disease severity and IL-8 or cell elongation induction, strains with a functional *cag*PAI were grouped by the pathology from which they were isolated.

No differences were found in IL-8 expression among pathology groups, although small variations among IL-8 mean values were observed (Table 2). A slight increase in IL-8 mean values after 30 h of coculture in the direction Atrophic Gastritis, Intestinal Metaplasia, Gastric Cancer was observed. However, differences among groups were not significant. Interestingly, the two strains that showed the highest induction were isolated from patients diagnosed with gastric cancer.

Hummingbird phenotype induction had no significant association to disease severity either (Table 2). As in IL-8 induction, the two strains showing the highest cell elongation induction belonged to the gastric cancer group. However, these two strains were different from those inducing the highest IL-8 levels.

DISCUSSION

In Colombia, a country with high incidence of gastric cancer, 72% to 90% of *H. pylori* isolates harbor the *cagA* gene[31,32], a virulence factor associated with more severe disease[33]. It has been shown that the number of EPIYA-C motifs on CagA is associated with the levels of CagA tyrosine phosphorylation, SHP-2 binding activity and cytoskeletal alterations[17,26]. In this study we have evaluated the biological activities of *cagA*-positive Colombian strains on gastric epithelial cells according to the CagA polymorphisms, and their potential association with the severity of gastroduodenal diseases.

Although we included 84 *cagA*-positive strains in this study, the presence of this gene did not show strict concordance with the integrity of *cag*PAI, nor with the expression and delivery of CagA into epithelial AGS cells. About 28% of the strains were found to have a defective *cag*PAI, and 10%, in addition of being *cag*PAI-defective,

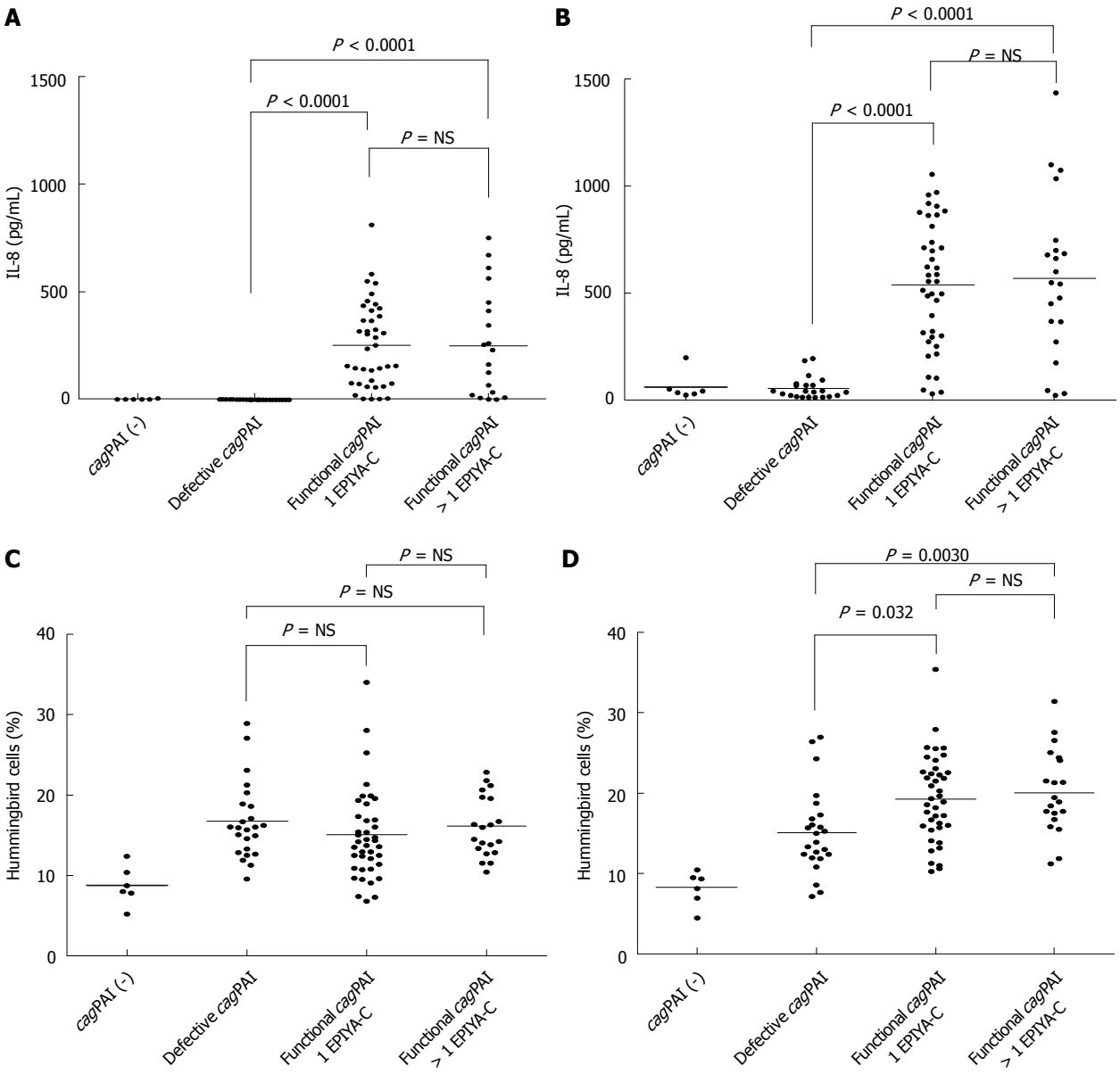

Figure 4 Influence of *cag*PAI status and CagA Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs variations on interleukin-8 expression and cell elongation. *Cag*PAI-negative strains (n = 6), *cag*PAI-defective strains (n = 24) and functional-*cag*PAI strains with either one (n = 40) or more than one (n = 20) EPIYA-C motifs were cocultured with AGS cells. (A) 6 h and (B) 30 h coculture supernatants were collected and assessed for interleukin-8 concentration by ELISA. Mean values are represented by horizontal lines within the scatterplots. Experiments for each strain were run in duplicates; (C) 6 h and (D) 24 h coculture photographs were taken and the percentage of hummingbird cells was determined. Mean values are represented by horizontal lines within the scatterplots. Experiments for each strain were run in duplicates. NS: Not significant.

Table 2 Induction of interleukin-8 expression and cell elongation by *cag*PAI-functional *Helicobacter pylori* strains according to the histopathological diagnosis

Pathology	IL-8 (pg/mL)				Elongation			
	6 h		30 h		6 h		24 h	
	mean	95%CI	mean	95%CI	mean	95%CI	mean	95%CI
Gastritis	256.1	(145.1-367.1)	594.5	(419.5-769.6)	15.3%	(10.7-19.9)	18.98%	(15.7-22.3)
Atrophic gastritis	224.7	(58.7-390.6)	463.7	(244.9-682.5)	14.9%	(12.2-17.6)	19.84%	(16.9-22.7)
Intestinal metaplasia	192.7	(89.0-296.4)	532.1	(398.2-666.0)	15.32%	(12.2-18.4)	17.7%	(18.5-20.6)
Gastric cancer	265.5	(107.5-423.5)	589.5	(355.4-823.5)	16.3%	(12.8-19.9)	19.55%	(15.9-23.1)
Duodenal ulcer	278.9	(154.8-402.9)	572.2	(343.6-800.8)	14.72%	(13.6-15.8)	19.8%	(17.2-22.4)

IL-8: Interleukin-8.

did not express the CagA protein. It is likely, that the promoter region of the *cagA* gene was disrupted in these strains, as previously reported for isolates from different human populations[34]. These results reinforce previous reports indicating that the presence of the *cagA* gene alone is not an accurate marker for an intact *cag*PAI[19,34-36].

Strains bearing an intact *cag*PAI showed high variability in CagA phosphorylation levels. *In vitro* experiments have shown that the number of EPIYA-C motifs is associated with the degree of CagA phosphorylation[26], and some studies with clinical isolates have also reported this association[19,27]. In our study we observed higher levels of CagA phosphorylation in strains with more than one EPIYA-C motifs than in strains with one EPIYA-C motif, although the differences were not significant. Considering that it has been proposed that CagA EPIYA motifs polymorphisms influence the degree of virulence as well as the oncogenic potential of individual *cagA*-positive strains[26], we evaluated the association between phosphorylation levels and histopathological diagnosis. We observed significant higher levels of CagA phosphorylation in strains from duodenal ulcer patients when compared to strains from the other pathologies, which is in agreement with a previous study reporting a similar behavior in strains isolated from duodenal ulcer patients[37]. It has been shown that *H. pylori*-induced inflammatory response is triggered upon CagA translocation into the host cell, where it activates NF-κB leading to IL-8 expression[12,13]. Furthermore, CagA-mediated IL-8 induction has been shown to be time- and strain-dependent. There is evidence demonstrating the importance of CagA for IL-8 expression in long incubation periods (24-48 h)[12,25]. However, the role of CagA in short incubation periods has been controversial. One study has found that isogenic *cagA*-mutant strains induce lower levels of IL-8 expression than their parental strains after 6 h and 9 h of co-culture[12]. In contrast, two studies, one involving isogenic *cagA* mutants and the second involving independent strains, found that IL-8 expression was not affected after 6 h of incubation[19,38]. Given these contradictory results, we tested IL-8 induction after 6 h and 30 h post-infection. We observed a clear CagA-dependent IL-8 expression pattern, as evidenced by the differences in IL-8 induction between *cagA*-negative/*cag*PAI-defective and *cag*PAI-functional strains. Strains bearing a functional *cag*PAI induced variable levels of IL-8 expression at 6 h of coculture, whereas *cagA*-negative and *cag*PAI-defective strains failed to induce IL-8 secretion. Furthermore, we confirmed the importance of CagA on IL-8 induction after long incubation periods, although we also detected low levels of IL-8 expression for *cagA*-negative and *cag*PAI-defective strains. These low levels of IL-8 induction at 30 h are probably the effect of other delayed responses like the CagA-independent IL-8 expression mechanism, in which *H. pylori* peptidoglycan translocated through bacterial membrane vesicles into epithelial cells activates, *via* Nod1, NF-κB resulting in IL-8 expression[39,40]. Furthermore, Crabtree *et al*[41] also reported low levels of secretion of IL-8 by *cagA*-negative strains after 24 h of infection. Taken together, our results support the concept of *H. pylori* time-dependent IL-8 induction, highlighting the importance of CagA for both, short and long, incubation periods.

We observed a lack of association between the number of EPIYA-C motifs and the level of IL-8 induction after coculture with AGS cells, even in prolonged incubation times. Neither an increasing number of EPIYA motifs nor an increasing number of EPIYA-C motifs had a boost effect on IL-8 expression. There are reports in the literature supporting our findings[42-44]. Reyes-Leon *et al*[43] reported no differences in IL-8 induction between Mexican *H. pylori* strains bearing one EPIYA-C motif and those with two or more C motifs. Interestingly, Mexican and Colombian *H. pylori* populations share common predominant polymorphisms (ABC and ABCC)[28,43,45]. Moreover, Sgouras *et al*[44] observed no differences in the levels of secreted IL-8 induced by individual isogenic subclones expressing CagA protein with different number of EPIYA-C motifs isolated from the same patient. It is worth noting the contrast of our results with those found by Argent *et al*[25], in which they observed a direct association between the number of EPIYA-C motifs and IL-8 expression in microevolved *H. pylori* strains from England. Discrepancies between studies may be explained by contrasting the geographical origin of the strains in each study. Our strains were isolated in Colombia and Argent strains were isolated in England, which are regions with high and mild gastric cancer risks, respectively[46]. It has recently been shown that *H. pylori* strains from different geographical and gastric cancer risk regions have distinct IL-8 induction behaviors in AGS cells[27]. Cellular inflammatory response in AGS cells was shown to be independent of the pathology from which strains were isolated, although the two strains showing the highest IL-8 expression levels were isolated from patients diagnosed with gastric cancer. These results are in agreement with previous studies showing that *H. pylori* strains isolated from different gastric pathologies varied in their ability to induce IL-8 expression in AGS cells, but did not associate to disease severity[19,47]. Moreover, Schneider *et al*[27] reported in a recent study involving *cagA*-positive *H. pylori* strains isolated from Colombian patients, that IL-8 expression induced by isolates from precancerous lesions did not differ from that induced by isolates from nonatrophic gastritis.

The induction of hummingbird phenotype upon infection with *cagA*-positive *H. pylori* strains has long been proposed as one of the mechanisms contributing to CagA oncogenic transformation[8,17]. Strains carrying biologically more active CagA have been associated with an increased risk of developing gastric carcinoma[17,19,22]. *H. pylori*-mediated cell elongation is potentiated by CagA proteins with higher number of EPIYA motifs[19,27]. Furthermore, proteins harboring higher number of EPIYA-C repeats increase hummingbird cells in AGS cells[17,26]. Our results disagree with these statements, as neither of both CagA molecular variations groups affected hummingbird

phenotype formation, but are in agreement with previous studies suggesting a lack of association between the number and type of EPIYA motifs and cellular elongation in *H. pylori* clinical isolates[22,37,43,48].

We found no differences in cell elongation induction when evaluating strains according to the pathology from which they were isolated. These results are in agreement with the results reported by Backert *et al*[37], in which strains isolated from German patients with different gastric pathologies showed no differences in cell elongation in AGS cells.

It is also important not to take *H. pylori* infection as the ultimate factor involved in gastric carcinogenesis, as there are many environmental and host factors associated with the disease. Polymorphisms in cytokine genes, such as *IL-8*, *IL-1β* and tumor necrosis factor-α, affect cytokine production upon *H. pylori* infection, increasing the risk of developing gastric diseases[2]. In addition to host genetic factors, environmental factors (*e.g.*, dietary and smoking habits) may also play an important role in *H. pylori* pathogenesis[49,50]. More interestingly, a recent study based on epidemiological and geographical data has proposed altitude as a surrogate for host, bacterial and environmental factors associated with gastric cancer risk[51].

In conclusion, we have reported a lack of association between *H. pylori* CagA protein EPIYA motifs variations from Colombian isolates and disease-associated cellular effects. Taken together, these results suggest that other factors (*e.g.*, host or environmental) may play a more important role than *H. pylori* CagA protein EPIYA variations in gastric cancer development in Colombia.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infect more than 50% of the world's population. Around 10%-15% of the infected individuals develop gastroduodenal diseases such as chronic gastritis, duodenal ulcers and gastric cancer. Currently, the determinants of the variable clinical outcomes have not been fully elucidated. One of the most important virulence factors of *H. pylori* is the *cag* pathogenicity island (*cag*PAI), which encodes several proteins, including CagA.

Research frontiers

CagA varies in size, and this variation has been shown to be due to Glu-Pro-Ile-Tyr-Ala (EPIYA) repeats within the C-terminal region of the protein. In Western *H. pylori* strains three types of EPIYA motifs have been described (A, B and C) based on the sequence flanking the motif. Strains with higher numbers of EPIYA-C motifs are more closely associated with gastric cancer and with an increased CagA *in vitro* activity, although this is controversial.

Innovations and breakthroughs

In contrast with studies in other populations, this study reports a lack of association between CagA EPIYA motifs variations from *H. pylori* colombian isolates and disease-associated cellular responses or gastroduodenal disease severity.

Applications

These results suggest that other factors (*e.g.*, host or environmental) may play a more important role than *H. pylori* CagA protein EPIYA variations in gastric cancer development in Colombia, a country with a high incidence of gastric cancer.

Terminology

H. pylori CagA protein is a virulence factor encoded in the *cag*PAI of the bacterium which is translocated into gastric epithelial cells through a type IV secretion system. Once in the cell, CagA becomes phosphorylated on tyrosine residues within the EPIYA motifs, mediating in turn the activation of several signaling pathways involved in the expression of pro-inflammatory cytokines such as interleukin-8 or in cell elongation, among others.

Peer review

The authors investigates the role of *cagA H. pylori* gene polymorphisms in the various bacterial-related chronic conditions. The paper is well designed and the results represent novel aspects of the infection consequences.

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