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 (cagPAI) 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 cagPAI. 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. cagPAI-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 cagPAI-defective strains induced significantly lower levels of IL-8 than strains with a functional cagPAI (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 cagPAI in 6 h cocultures. At 24 h post-infection strains with functional cagPAI showed high diversity in the extent of hummingbird phenotype induction ranging from 7% to 34%. cagPAI defective strains induced significantly lower levels of IL-8 than strains with a functional cagPAI (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 cagPAI in 6 h cocultures. At 24 h post-infection strains with functional cagPAI showed high diversity in the extent of hummingbird phenotype induction ranging from 7% to 34%. cagPAI defective strains induced significantly lower levels of elongation than strains with functional cagPAI 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.

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**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 1 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-kB (NF-kB)[^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^-^19^]. 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 *cag*4-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’ *cag*4 genotyping was reported previously[^28^] and EPIYA motifs combinations used in this study are summarized in Figure 1. *cag*4-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 cagPAI we evaluated the presence of *cagA*, *cagT*, *cagF* 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 MgCl2, 200 μmol/L dNTPs, 25 pmol of the primers, 100 ng of *H. pylori* genomic DNA and 1 U Taq polymerase. The polymerase chain reaction (PCR) conditions for each reaction were previously described[^29^]. Positive (strain 11637) and negative controls (strain 3062) for the *cagPAI* 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 °C in a 10% CO2-humidified atmosphere for
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 x 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 x 10^5 cells/well) or 25 cm^2 flasks (5 x 10^4 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°C in a 5% CO2 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°C in a 5% CO2-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 CaCl2 and 0.5 mmol/L MgCl2, 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 x Laemmli sample buffer [0.2 TRIS-HCL pH 6.8, 0.4 mmol/L dithiothreitol, 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 semidy transfer. For protein detection, membranes were probed with a 1:1000 dilution of antiphospho-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 β-2-Mercaptoethanol solution, 2% SDS at 50°C 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°C 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 II phase contrast inverted microscope (Leica). For this, 3 randomly chosen 20 x 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 [10]. 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 cagPAI genes by PCR, and were therefore predicted, on the basis of this limited testing, to have an intact cagPAI. 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 CagPAI genes

| Gene | Primer | Sequence 5'→3' | Product size (bp) | Reference |
|------|--------|----------------|------------------|-----------|
| cagE | 101    | TTGAAAACTTCAAGGATAGGATAGGC | 510              | [16]      |
|      | 102    | GCCTAGCGTAATATCACCATTACCC  |                  |           |
| cagT | cagTF  | ATGAAAGTGAGAGCAGTGT       | 823              | [30]      |
|      | cagTR  | TCCTTACCACTGAGCAGAAC      |                  |           |
| cag10| cag10F | ATGGGAAGACTTTTTGTATAA     |                  |           |
|      | cag10R | TCACAGTTCGGTTACACCACA     | 2208             | [30]      |

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-phosphoryrosine or anti-CagA antibodies. A representative assay is shown. Lane 1: 11 637 control strain with a functional cagPAI and 5 Glu-Pro-5 Tyr-Ala (EPIYA) motifs (ABCC); Lanes 2 and 6: Isolates with a defective cagPAI lacking expression of CagA; Lanes 7 and 8: Isolates with a defective cagPAI expressing CagA, with absence of CagA phosphorylation; Lanes 3-5: Isolates with a functional CagPAI with three (ABC; lane 3) or four (ABCC; lanes 4 and 5) EPIYA motifs; Lane 9: Uninfected AGS cells.

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

The 84 cagA-positive and 6 cagPAI-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 cagA 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 cagPAI components not detected by the PCR of the selected cagPAI genes. This showed, as previously observed by Argent et al., that PCR prediction of cagA 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 cagPAI and classified them, along with the partial-cagPAI strains, as cagPAI-defective isolates, as described above.

CagPAI-negative and cagPAI-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 cagPAI-negative and cagPAI-defective strains induced significantly lower levels of IL-8 than strains with a functional cagPAI 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 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).
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 cagPAI in 6 h cocultures (Figure 4C). At 24 h post infection strains with functional cagPAI showed high diversity in the extent of hummingbird phenotype induction ranging from 7% to 34%. CagPAI-negative and cagPAI-defective strains induced significantly lower levels of elongation than strains with functional cagPAI 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 cagPAI-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 cagPAI 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 cagPAI, nor with the expression and delivery of CagA into epithelial AGS cells. About 28% of the strains were found to have a defective cagPAI, and 10%, in addition of being cagPAI-defective,
Table 2  Induction of interleukin-8 expression and cell elongation by cagPAI-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 |
| 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.

Figure 4  Influence of cagPAI status and CagA Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs variations on interleukin-8 expression and cell elongation. CagPAI-negative strains (n = 6), cagPAI-defective strains (n = 24) and functional-cagPAI 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.
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 cagPAI\[^{1,2,36}\].

Strains bearing an intact cagPAI 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\[^{28}\], 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\[^{36}\], 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\[^{70}\]. 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)\[^{12,28}\]. 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\[^{32}\]. In contrast, two studies, one involving isogenic cagA mutants and the second involving independent strains, found that IL-8 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/cagPAI-defective and cagPAI-functional strains. Strains bearing a functional cagPAI induced variable levels of IL-8 expression at 6 h of coculture, whereas cagA-negative and cagPAI-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 cagPAI-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\[^{19,40}\]. 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\[^{45}\] 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)\[^{28,43,49}\]. Moreover, Sgouras et al\[^{46}\] 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\[^{71}\]. 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\[^{51}\]. 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\[^{15,47}\]. Moreover, Schneider et al\[^{48}\] 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\[^{6,7}\]. Strains carrying biologically more active CagA have been associated with an increased risk of developing gastric carcinoma\[^{17,18,22}\]. H. pylori-mediated cell elongation is potentiated by CagA proteins with higher number of EPIYA motifs\[^{9,27}\]. Furthermore, proteins harboring higher number of EPIYA-C repeats increase hummingbird cells in AGS cells\[^{8,26}\]. 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\(^ {22,27,41,48}\).

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 II-8, II-1β and tumor necrosis factor-α, affect cytokine production upon *H. pylori* infection, increasing the risk of developing gastric diseases\(^{38}\). In addition to host genetic factors, environmental factors (e.g., dietary and smoking habits) may also play an important role in *H. pylori* pathogenesis\(^{40,50}\). 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.

**Peer review**

The authors investigate 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.

**REFERENCES**

1. **Kusters JG**, van Vliet AH, Kuipers EJ. Pathogenesis of Helicobacter pylori infection. _Clin Microbiol Rev_ 2006; 19: 449-490 [PMID: 16847081 DOI: 10.1128/CMR.00054-05].

2. **Atheyon JC**. The pathogenesis of Helicobacter pylori-induced gastrointestinal-duodenal diseases. _Ann Rev Pathol_ 2006; 1: 63-96 [PMID: 18039108 DOI: 10.1146/annurev.pathol.1.110304.10125].

3. **Schistosomes, liver flukes and Helicobacter pylori**. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. _IARC Monogr Eval Carcinog Risks Hum_ 1994; 61: 1-241 [PMID: 7715068].

4. **Wen S, Moss SF**. Helicobacter pylori virulence factors in gastric carcinogenesis. _Cancer Lett_ 2009; 282: 1-8 [PMID: 19111390 DOI: 10.1016/j.canlet.2008.11.016].

5. **Backert S, Ziska E, Brinkmann V, Zamir-Sh‐Antd U, Faunon‐nier A, Jungblut PR, Naumann M, Meyer TF. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. _Cell Microbiol_ 2000; 2: 155-164 [PMID: 11207572 DOI: 10.1046/j.1462-5822.2000.00043.x].

6. **Poppe M, Feller SM, Römer G, Wessler S. Phosphorylation of Helicobacter pylori CagA by c-Abl leads to cell motility. Oncogene_ 2007; 26: 3462-3472 [PMID: 17160020 DOI: 10.1038/sj.onc.1210139].

7. **Selbach M, Seoane S, Hauck CR, Meyer TF, Backert S. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. _J Biol Chem_ 2002; 277: 6775-6778 [PMID: 11788577 DOI: 10.1074/jbc.C100754200].

8. **Stein M, Bagnoli F, Halenbeck R, Rappuoli R, Fanti WJ, Covacci A. c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. Mol Microbiol_ 2002; 43: 971-980 [PMID: 11929545 DOI: 10.1046/j.1365-2958.2002.02781.x].

9. **Higashi H, Tsutsui M, Muto S, Sugiyama T, Asama T, Hatakeyama M, Hatakeyama M, S. HP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. Science_ 2002; 295: 683-686 [PMID: 11743164 DOI: 10.1126/science.1067147].

10. **Backert S, Selbach M. Role of type IV secretion in Helicobacter pylori pathogenesis. _Cell Microbiol_ 2008; 10: 1573-1581 [PMID: 18410539 DOI: 10.1111/j.1462-5822.2008.01156.x].

11. **Segal ED**, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. _Proc Natl Acad Sci USA_ 1999; 96: 14559-14564 [PMID: 10588574 DOI: 10.1073/pnas.96.25.14559].

12. **Brandt S, Kwok T, Hartig R, König W, Backert S. NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. _Proc Natl Acad Sci USA_ 2005; 102: 9300-9305 [PMID: 15972330 DOI: 10.1073/pnas.0409735102].

13. **Lamb A, Yang XD, Tsang YH, Li JD, Higashi H, Hatakeyama M, Peak RM, Blanke SR, Chen LF. Helicobacter pylori CagA activates NF-kappaB by targeting TAK1 for TRAF6-mediated Lys 63 ubiquitination. _EMBO Rep_ 2009; 10: 1242-1249 [PMID: 19820695 DOI: 10.1038/embob.2009.210].

14. **Sharma SA**, Tummuru MK, Blaser MJ, Kerr LD. Activation of IL-8 gene expression by Helicobacter pylori is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. _J Immunol_ 1998; 160: 2401-2407 [PMID: 9498753].

15. **Covacci A, Censi S, Bugnoli M, Petracca R, Burroni D,
Macchia G, Massone A, Papini E, Xiang Z, Figura N. Molecular characterization of the 128-kDa immunodominant antigen of Helicobacter pylori associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci USA 1993; 90: 5791-5795 [PMID: 8358809 DOI: 10.1073/pnas.90.12.5791]

Tummuru MK, Cover TL, Blaser MJ. Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytokinin production. Infect Immun 1993; 61: 1799-1809 [PMID: 8478069]

Higashi H, Tsutsunami R, Fujita A, Yamazaki S, Asaka M, Azuma T, Hatakeyama M. Biological activity of the Helicobacter pylori virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc Natl Acad Sci USA 2002; 99: 14428-14433 [PMID: 12391297 DOI: 10.1073/pnas.222375399]

Backert S, Tegtmeyer N, Selbach M. The versatility of Helicobacter pylori CagA effector protein functions: The master key hypothesis. Helicobacter 2010; 15: 163-176 [PMID: 20553737 DOI: 10.1111/j.1537-2965.2010.00759.x]

Argent RH, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of Helicobacter pylori. Gastroenterology 2004; 127: 514-523 [PMID: 15300584 DOI: 10.1053/j.gastro.2004.06.006]

Azuma T, Yamakawa A, Yamazaki S, Fukuta K, Ohtani M, Ito Y, Dojo M, Yamazaki Y, Kuriyama M. Correlation between variation of the 3′ region of the cagA gene in Helicobacter pylori and pylori disease and outcome in Japan. J Infect Dis 2002; 186: 1621-1630 [PMID: 12447379 DOI: 10.1086/345374]

Quiroga AJ, Huertas A, Combita AL, Bravo MM. Variation in the number of EPIYA-C repeats in CagA protein from Colombian Helicobacter pylori strains and its ability middle to induce hummingbird phenotype in gastric epithelial cells. Biomedica 2010; 30: 251-258 [PMID: 20890572 DOI: 10.1035/j.gastro.2008.03.041]

Quiroga AJ, Huertas A, Combita AL, Bravo MM. Variation in the number of EPIYA-C repeats in CagA protein from Colombian Helicobacter pylori strains and its ability middle to induce hummingbird phenotype in gastric epithelial cells. Biomedica 2010; 30: 251-258 [PMID: 20890572]

Ando T, Kusugami K, Ohsuga M, Shinoda M, Sakakibara Naito M, Saito H, Fukatsu A, Ichiyama S, Ohta M. Interleukin-8 and cytokine expression in vivo to cagA+ Helicobacter pylori. Am J Gastroenterol 2006; 101: 1621-1630 [PMID: 16905431 DOI: 10.1111/j.1572-0241.2006.00633.x]

Peek RM, Miller GG, Tham KT, Perez-Perez GI, Zhao X, Fajardo CA. Lack of association with pathogenesis. Helicobacter 2010; 16: 3936-3943 [PMID: 20712055 DOI: 10.3748/wjg.v16.i31.3936]

Tummuru MK, Sharma SA, Blaser MJ. Helicobacter pylori pibB, a homologue of the Bordetella pertussis toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. Mol Microbiol 1995; 18: 867-876 [PMID: 8852091 DOI: 10.1111/j.1365-2958.1995.8050867.x]

Maeda S, Yoshida H, Ikemoue T, Oghura K, Kanai F, Kato N, Shiratori Y, Omata M. Structure of cag pathogenicity island in Japanese Helicobacter pylori isolates. Gut 1999; 44: 336-341 [PMID: 10026317 DOI: 10.1136/gut.44.4.336]

Bravo LE, van Doorn LJ, Realpe JL, Correa P. Virulence-associated genotypes of Helicobacter pylori: do they explain the African enigma? An J Gastroenterol 2002; 97: 2893-2842 [PMID: 12425557 DOI: 10.1111/j.1572-0241.2002.00731.x]

Quiroga AJ, Cittelly DM, Bravo MM. [BabA2, oipA and cagE Helicobacter pylori genotypes in Colombian patients with gastroduodenal diseases]. Biomedica 2005; 25: 325-334 [PMID: 16276680]

Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peak RM, Choy PH, Stemmermann GN, Nomura A. Infection with Helicobacter pylori strain CagA-5 is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res 1995; 55: 2111-2115 [PMID: 7745310]

Kaiser F, Khan AA, Hussain MA, Carroll IM, Ahmed N, Tiwari S, Shouye D, Das B, Alam M, Ali SM, Habibullah CM, Sierra R, Megraud F, Sechi LA, Ahmed N. The cag pathogenicity island of Helicobacter pylori is disrupted in the majority of patient isolates from different human populations. J Clin Microbiol 2004; 42: 5302-5308 [DOI: 10.1128/JCM.42.11.5302-5308.2004]

Jones KR, Joo YM, Jang S, Yoo YJ, Lee HS, Chung IS, Olsen CH, Whitmire JM, Merrell DS, Cha JH. Polymorphism in the CagA EPIYA motif impacts development of gastric cancer. J Clin Microbiol 2009; 47: 959-968 [PMID: 19152528 DOI: 10.1128/JCM.02330-08]

Nguyen LT, Uchida T, Tsukamoto Y, Trinh TD, Ta L, Mai HB, Le HS, Ho DQ, Hoang HH, Matsuishi T, Okimoto T, Kodama M, Murakami K, Fujikoa T, Yamaoka Y, Moriayama M. Clinical relevance of cagPAI intactness in Helicobacter pylori isolates from Vietnam. Eur J Clin Microbiol Infect Dis 2010; 29: 651-660 [PMID: 20372956 DOI: 10.1007/s10096-010-0909-z]

Backert S, Schwarz T, Miehlke S, Kirsch C, Sommer C, Kwok T, Gerhard M, Goebel UB, Lelh N, Koenig W, Meyer TF. Functional analysis of the cag pathogenicity island in Helicobacter pylori isolates from patients with gastritis, peptic ulcer, and gastric cancer. Infect Immun 2004; 72: 1043-1056 [PMID: 14742552 DOI: 10.1128/IAI.72.2.1043-1056.2004]

Argent RH, Thomas RJ, Letley DP, Rittig MG, Hardie KR, Atherton JC. Functional association between the Helicobacter pylori virulence factors VacA and CagA. J Mol Microbiol 2008; 57: 145-150 [PMID: 18201978 DOI: 10.1099/ jmm.0.047465-0]

Viala J, Chapat C, Boneca IG, Cardona SE, Morran AP, Athman R, Mémet S, Huerre MR, Coyle AJ, DiStefano PS, Sansonetti PJ, Labigne A, Bertin J, Philippot DJ, Ferro RL. NodI responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immune 2004; 5: 1166-1174 [PMID: 15489856 DOI: 10.1038/nri1311]

Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkinson HC, Le Bourhis L, Karrar A, Vialar J, Mak J, Hutton ML, Davies J, Crick PJ, Hertzog PJ, Philippot DJ, Girardin SE, Whitchurch CB, Ferrero RL. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cell Microbiol 2010; 12: 372-385 [PMID: 19888899 DOI: 10.1111/j.1462-5822.2009.01404.x]

Crabtree JE, Farmery SM, Lindley IJ, Figura N, Peichl P, Tompkins DS. CagA/cytotoxic strains of Helicobacter pylori and interleukin-8 in gastric epithelial cell lines. J Clin Pathol 2008; 61: 78-85 [PMID: 17293193 DOI: 10.1136/jcp.2007.054835]
42 Owen RJ, Sharp S, Lawson AJ, Durrani Z, Rijpkema S, Kidd M. Investigation of the biological relevance of Helicobacter pylori cagE locus diversity, presence of CagA tyrosine phosphorylation motifs and vacuolating cytotoxin genotype on IL-8 induction in gastric epithelial cells. *FEMS Immunol Med Microbiol* 2003; 36: 135-140 [PMID: 12738382 DOI: 10.1016/S0928-8244(03)00022-1]  
43 Reyes-Leon A, Atherton JC, Argent RH, Puente JL, Torres J. Heterogeneity in the activity of Mexican Helicobacter pylori strains in gastric epithelial cells and its association with diversity in the cagA gene. *Infect Immun* 2007; 75: 3445-3454 [PMID: 17439024 DOI: 10.1128/IAI.01951-06]  
44 Sgouras DN, Panayotopoulou EG, Papadakos KS, Martinez-Gonzalez B, Roumbani A, Panayiotou J, vanVliet-Constatinidou C, Mentis AF, Roma-Giannikou E. CagA and VacA polymorphisms do not correlate with severity of histopathological lesions in Helicobacter pylori-infected Greek children. *J Clin Microbiol* 2009; 47: 2426-2434 [PMID: 19535517 DOI: 10.1128/JCM.00159-09]  
45 Steinschi LA, Correa P, Peek RM, Camargo MC, Piazuelo MB, Romero-Gallo J, Hobbs SS, Krishna U, Delgado A, Mera R, Bravo LE, Schneider BG. CagA C-terminal variations in Helicobacter pylori strains from Colombian patients with gastric precancerous lesions. *Clin Microbiol Infect* 2010; 16: 369-378 [PMID: 19456839 DOI: 10.1111/j.1469-0691.2009.02811.x]  
46 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; 12: 354-362 [PMID: 16489633]  
47 Strömborg E, Ededo A, Lundin BS, Bergin P, Brisslert M, Svennerholm AM, Lindholm C. Down-regulation of epithelial IL-8 responses in Helicobacter pylori-infected duodenal ulcer patients depends on host factors, rather than bacterial factors. *Clin Exp Immunol* 2005; 140: 117-125 [PMID: 15762883 DOI: 10.1111/j.1365-2249.2005.02736.x]  
48 Panayotopoulou EG, Sgouras DN, Papadakos KS, Petraki K, Breurec S, Michopoulos S, Mantzaris G, Papatheodoridis G, Mentis A, Archimandritis A. CagA and VacA polymorphisms are associated with distinct pathological features in Helicobacter pylori-infected adults with peptic ulcer and non-peptic ulcer disease. *J Clin Microbiol* 2010; 48: 2237-2239 [PMID: 20392907 DOI: 10.1128/JCM.00162-10]  
49 Sgouras DN, Panayotopoulou EG, Papadakos KS, Petraki K, Michopoulos S, Mantzaris G, Papatheodoridis G, Mentis A, Archimandritis A. CagA and VacA polymorphisms do not correlate with severity of histopathological lesions in Helicobacter pylori-infected Greek children. *J Clin Microbiol* 2009; 47: 2426-2434 [PMID: 19535517 DOI: 10.1128/JCM.00159-09]  
50 Lu HS, Umeda M, Murata-Kamiya N, Zhang HM, Higashi H, Hatakeyama M. Structural and functional consequence of CagA multimerization in cells. *J Biol Chem* 2006; 281: 3204-3212 [PMID: 16554210 DOI: 10.1074/jbc.M606172200]  
51 Torres J, Correa P, Ferreccio C, Hernandez-Suarez G, Herrero R, Cavazza-Porro M, Dominguez R, Morgan D. Gastric cancer incidence and mortality is associated with altitude in the mountainous regions of Pacific Latin America. *Cancer Causes Control* 2013; 24: 249-256 [PMID: 23224721 DOI: 10.1007/s10552-012-114-8]