Research Article

Analysis of \textit{cagA} in \textit{Helicobacter pylori} Strains from Colombian Populations with Contrasting Gastric Cancer Risk Reveals a Biomarker for Disease Severity

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Abstract

\textbf{Background:} \textit{Helicobacter pylori} infection is a risk factor for the development of gastric cancer, and the bacterial oncoprotein CagA contributes to gastric carcinogenesis.

\textbf{Methods:} We analyzed \textit{H. pylori} isolates from persons in Colombia and observed that there was marked variation among strains in levels of CagA expression. To elucidate the basis for this variation, we analyzed sequences upstream from the CagA translational initiation site in each strain.

\textbf{Results:} A DNA motif (AATAAGATA) upstream of the translational initiation site of CagA was associated with high levels of CagA expression. Experimental studies showed that this motif was necessary but not sufficient for high-level CagA expression. \textit{H. pylori} strains from a region of Colombia with high gastric cancer rates expressed higher levels of CagA than did strains from a region with lower gastric cancer rates, and Colombian strains of European phylogeographic origin expressed higher levels of CagA than did strains of African origin. Histopathologic analysis of gastric biopsy specimens revealed that strains expressing high levels of CagA or containing the AATAAGATA motif were associated with more advanced precancerous lesions than those found in persons infected with strains expressing low levels of CagA or lacking the AATAAGATA motif.

\textbf{Conclusions:} CagA expression varies greatly among \textit{H. pylori} strains. The DNA motif identified in this study is associated with high levels of CagA expression, and may be a useful biomarker to predict gastric cancer risk.

\textbf{Impact:} These findings help to explain why some persons infected with \textit{cagA}-positive \textit{H. pylori} develop gastric cancer and others do not. Cancer Epidemiol Biomarkers Prev; 20(10); 2237–49. ©2011 AACR.

Introduction

\textit{Helicobacter pylori} is a gram-negative bacterium that persistently colonizes the human gastric mucosa. Most \textit{H. pylori}-infected persons remain asymptomatic, but the presence of \textit{H. pylori} is associated with an increased risk of gastric cancer. The clinical outcome of \textit{H. pylori} infection is determined by a combination of bacterial, host, and environmental factors (1–6).

An important virulence factor produced by some \textit{H. pylori} strains but not others is the CagA protein (7, 8). Epidemiologic studies have shown that infection with \textit{cagA}-positive strains is associated with an increased risk of gastric cancer, compared with infection with \textit{cagA}-negative strains (9–14). CagA is an antigenic protein that is translocated into host cells by a type IV secretion apparatus (15–19). Upon entry into host cells, CagA is tyrosine phosphorylated by Src kinases at conserved EPIYA motifs within the CagA protein. CagA interacts with multiple host cell components and causes numerous alterations in cell signaling and morphology (17, 20–23).

The incidence of gastric cancer varies considerably throughout the world. Within Colombia, 2 regions located less than 200 km apart in the state of Narino have a markedly different incidence of gastric cancer. In a study conducted in 1976, the incidence of gastric cancer in the Andean mountainous region was estimated to be 150 cases per 100,000 inhabitants, whereas in the coastal region, gastric cancer rates were estimated to be 6 cases per 100,000 inhabitants (24, 25). The \textit{H. pylori} infection rates are very similar in the populations inhabiting these regions (26). The prevalence of \textit{cagA}-positive strains in the low-risk region is slightly (~9%) lower than that found in the high-risk region (27), but this difference is unlikely to account for the 25-fold difference in gastric cancer rates. CagA proteins with a greater number of EPIYA motifs

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undergo more tyrosine phosphorylation and potentially have increased intracellular activity compared with proteins with fewer EPIYA motifs (28–30), but the number of CagA EPIYA motifs is not significantly different in strains from the low-risk and high-risk regions of Colombia (31). Recently, it was noted that cagA-positive strains found in the low- and high-risk regions of Colombia differ in phylogeographic origin (32). Multilocus sequence typing (MLST) analysis indicated that all of the tested H. pylori strains from the high-risk region were of European origin, whereas the majority of strains from the low-risk region were of African origin (32).

In this study, we investigated CagA expression in H. pylori strains isolated from Colombian patients, and observed that there is considerable variation in the levels of CagA expressed by different strains. We identified a motif upstream of the CagA ATG initiation site that is present more frequently in strains expressing high levels of CagA than in strains expressing low levels of CagA, and we show experimentally that this motif is a determinant of CagA expression levels. We report that H. pylori strains of European and African phylogeographic origin differ in the levels of CagA expressed. Finally, we report that, in comparison with H. pylori strains expressing low levels of CagA, strains expressing higher levels of CagA are associated with more advanced precancerous lesions in gastric biopsy samples.

Materials and Methods

**Bacterial strains**

H. pylori strains used in this study were isolated from antral mucosa biopsy samples from subjects in the state of Nariño, Colombia, and H. pylori strain 26695 (33) was used as a reference strain. The strains analyzed in this study were randomly selected from a previously characterized set of Colombian strains (32) using a blinded code. All strains were cagA-positive and contain type s1m1 vacA (32). Several strains were excluded from analysis due to contamination or failure to grow in liquid broth culture. The 36 strains analyzed in this study (17 from subjects in a region of low cancer risk and 19 from subjects in a region of high cancer risk) are shown in Table 1. H. pylori strains were grown in room air supplemented with 5% CO₂ at 37°C. For routine growth, H. pylori strains were maintained on trypticase soy agar plates supplemented with sheep blood. In addition, H. pylori strains were grown in modified Brucella broth containing 5% FBS (BB-FBS) or on BB-FBS agar plates. When necessary, BB-FBS agar plates were supplemented with metronidazole (7.5 μg/mL) or chloramphenicol (5 μg/mL). Escherichia coli strains were grown on Luria Bertani medium. When necessary, the E. coli culture medium was supplemented with ampicillin (50 μg/mL) or chloramphenicol (25 μg/mL).

**Human subjects**

The H. pylori strains used in this study were obtained from 36 male subjects between the ages of 39 and 60 years with dyspeptic symptoms who underwent gastrointestinal tract endoscopy in 2 public hospitals in the State of Nariño, Colombia (32). The hospitals are located in 2 cities with contrasting gastric cancer risks: Túquerres in the Andes Mountains where gastric cancer incidence is high, and Tumaco on the coast where gastric cancer incidence is low. The 36 individuals included in this study were part of a larger series of subjects that has been described previously (32).

Gastric mucosa biopsy samples from the 36 subjects were obtained from the antrum, incisura angularis, and corpus, and embedded in paraffin for histology. One additional antral biopsy from each subject was immediately frozen in glycerol and thiglycolate for H. pylori culture, and kept at ~80°C. The samples were shipped on dry ice to Vanderbilt University (Nashville, Tennessee) for analysis. All participants provided informed consent and all samples were sent to Vanderbilt University without identifiers. The protocol was approved by the Committees on Ethics of Universidad del Valle and Hospital Departamental in Nariño, Colombia, and by the Institutional Review Board at Vanderbilt University.

**Mutagenesis of the cagA promoter region in H. pylori 26695**

To facilitate introduction of unmarked mutations into H. pylori, a cat-rdxA cassette was synthesized and cloned into pUC57 vector (Genscript). This cassette confers resistance to chloramphenicol mediated by the chloramphenicol acetyl-transferase (cat) gene from Campylobacter coli, and susceptibility to metronidazole is mediated by an intact rdxA gene (HP0954) from H. pylori 26695 (34). To generate unmarked mutant strains, we used a method that is a variant of counterselection methods used previously in H. pylori (35, 36). As a first step, the rdxA gene, which confers resistance to metronidazole, and approximately 0.5 kb of flanking DNA on each side, was PCR-amplified from H. pylori 26695 and cloned into pGEMT-Easy to yield pMM670. By using pMM670 as the template for inverse PCR and then ligating the PCR product, we generated a modified plasmid (pMM672) in which the coding region of rdxA was deleted. H. pylori 26695 transformed with pMM672 (which is unable to replicate in H. pylori) and metronidazole-resistant colonies were selected (34). PCR analysis confirmed that the rdxA locus was deleted from a mutant strain designated H. pylori 26695ΔrdxA.

We next modified H. pylori 26695 ΔrdxA to generate a strain (26695 cagA::cat-rdx-9) containing a cat-rdxA cassette immediately downstream of the ATG initiation site of the cagA gene. To generate this mutant, primers 5’-GTCGTTTGTAGAATTGTTAGATCTTTAGG-3’ and 5’-TATCCCAACCATCCCCACCCAG-3’ were utilized to PCR amplify a DNA fragment from H. pylori 26695 comprising approximately 650 bp upstream and 650 bp downstream of the cagA translational start site. This PCR product was cloned into pGEMT-Easy (Promega), and the resultant plasmid was used as a template for inverse PCR with primers that introduced a BamHI site immediately before the ATG start site.
following the \textit{cagA} translation start site. The resultant plasmid was digested with \textit{Bam}HI and a cassette encoding the \textit{cat-\textit{rdxA}} cassette (described above) was ligated into the restriction site. The \textit{cagA::cat-\textit{rdxA}} plasmid, which is unable to replicate in \textit{H. pylori}, was transformed into \textit{H. pylori} 26695 \textit{D\textsubscript{rdxA}}, and single colonies resistant to chloramphenicol (5 \textmu g/mL) but sensitive to metronidazole (7.5 \textmu g/mL) were selected. Transformant 26695 \textit{cagA::cat\textsubscript{rdxA}}-9 was selected for further studies. Immuno- blot analysis revealed the loss of CagA expression in this strain, and the insertion of the \textit{cat-\textit{rdxA}} cassette into the \textit{cagA} gene was confirmed by PCR amplification and DNA

\begin{table}[h]
\centering
\begin{tabular}{lllll}
\hline
Strain & \text{CagA expression}\textsuperscript{a} & \text{Number of AATAAGATA motifs}\textsuperscript{b} & \text{Region}\textsuperscript{c} & \text{MLST type}\textsuperscript{d} & \text{Histopathology score}\textsuperscript{e} \\
\hline
5001 & 1.0 & 0 & Low & hpAfrica1 & 2.3 \\
5004 & 1.4 \pm 0.2 & 0 & Low & hpAfrica1 & 2.3 \\
5005 & 0.4 \pm 0.2 & 0 & Low & hpAfrica1 & 1.7 \\
5007 & 1.4 \pm 0.3 & 0 & Low & hpAfrica1 & 2.0 \\
5009 & 1.4 \pm 0.2 & 1 & Low & hpEurope & 1.7 \\
5010 & 0.5 \pm 0.3 & 0 & Low & hpAfrica1 & 2.0 \\
5016 & 1.8 \pm 0.2 & 0 & Low & hpAfrica1 & 3.5 \\
5017 & 0.9 \pm 0.3 & 0 & Low & hpAfrica1 & 2.3 \\
5018 & 1.6 \pm 0.5 & 1 & Low & hpAfrica1 & 3.5 \\
5022 & 0.9 \pm 0.3 & 0 & Low & hpEurope & 4.5 \\
5024 & 1.9 \pm 0.1 & 0 & Low & hpAfrica1 & 2.0 \\
5028 & 1.1 \pm 0.4 & 1 & Low & hpEurope & 3.8 \\
5032 & 2.3 \pm 0.2 & 1 & Low & hpEurope & 3.5 \\
5038 & 3.2 \pm 0.5 & 1 & Low & hpEurope & 3.5 \\
5043 & 3.7 \pm 0.7 & 1 & Low & hpEurope & 5.3 \\
5051 & 2.4 \pm 0.2 & 1 & Low & hpAfrica1 & 2.0 \\
5054 & 1.6 \pm 0.6 & 0 & Low & hpAfrica1 & 2.3 \\
5056 & 4.0 \pm 0.8 & 1 & High & hpEurope & 4.5 \\
5057 & 2.4 \pm 0.4 & 1 & High & hpEurope & 3.5 \\
5068 & 2.4 \pm 0.4 & 1 & High & hpEurope & 4.3 \\
5069 & 3.5 \pm 0.6 & 1 & High & hpEurope & 4.3 \\
5072 & 0.7 \pm 0.2 & 1 & High & hpEurope & 3.5 \\
5074 & 5.1 \pm 1.1 & 1 & High & hpEurope & 4.7 \\
5080 & 0.5 \pm 0.3 & 1 & High & hpEurope & 3.5 \\
5082 & 4.4 \pm 1.3 & 1 & High & hpEurope & 4.8 \\
5086 & 4.8 \pm 1.1 & 1 & High & hpEurope & 4.6 \\
5090 & 1.0 \pm 0.2 & 0 & High & hpEurope & 2.0 \\
5091 & 1.9 \pm 0.2 & 1 & High & hpEurope & 4.5 \\
5093 & 3.5 \pm 0.3 & 1 & High & hpEurope & 4.5 \\
5095 & 2.0 \pm 0.2 & 1 & High & hpEurope & 2.0 \\
5096 & 1.5 \pm 0.1 & 0 & High & hpEurope & 4.3 \\
5097 & 1.0 \pm 0.3 & 1 & High & hpEurope & 4.4 \\
5100 & 2.2 \pm 0.1 & 1 & High & hpEurope & 2.3 \\
5104 & 4.7 \pm 0.6 & 1 & High & hpEurope & 3.5 \\
5112 & 2.2 \pm 0.1 & 1 & High & hpEurope & 4.3 \\
5114 & 0.9 \pm 0.3 & 0 & High & hpEurope & 4.4 \\
\hline
\end{tabular}
\caption{Expression of CagA in Colombian \textit{H. pylori} strains}
\end{table}

\textsuperscript{a}CagA expression was analyzed by immunoblotting, and relative levels of expression were quantified by comparing the CagA signal intensity of each strain with that of a standard (CagA from \textit{H. pylori} strain 5001). For each strain, the CagA signal was calculated for 3 independent biological samples, and the average of these values is presented. Values represent mean \pm SEM.

\textsuperscript{b}The number of AATAAGATA motifs upstream of the ATG translation initiation site is shown.

\textsuperscript{c}\textit{H. pylori} strains were isolated from regions of Colombia with either high risk or low risk for the development of gastric cancer.

\textsuperscript{d}MLST analysis (32) was previously used to characterize the ancestral origin of the \textit{H. pylori} strains. \textit{hpAfrica1} denotes isolates classified as either West African (\textit{hpWAfrica}) or South African (\textit{hpSAfrica}).

\textsuperscript{e}Histopathology scores describing the severity of lesions observed in gastric tissue from which the \textit{H. pylori} strains were isolated.
sequencing. To introduce alterations into the cagA promoter region, 26695 cagA::catrdx-9 was transformed with various plasmids described below, and transformants resistant to metronidazole were selected.

**cagA promoter swapping experiments**

DNA fragments encompassing approximately 0.5 kb upstream and 0.7 kb downstream from the cagA transcription start site (0.6 kb upstream and 0.6 kb downstream of the ATG initiation site) were PCR amplified from Colombian strains using primers 5'-GCAAAAAA- CAAAACCCAGCTGA-3' and 5'-ACCACTAGGGCCC- TCCATTtTTTTC-3', and the amplified products were cloned into the pGEMT-Easy (Promega) vector. The resultant plasmids, which are unable to replicate in *H. pylori*, were then used to transform *H. pylori* strain 26695 cagA::catrdx-9 (described above). Transformations were plated onto metronidazole-containing BB-FBS agar plates, and metronidazole-resistant clones were isolated. These metronidazole-resistant colonies represent recombination events resulting in the removal of the cat-rdxA cassette from the cagA ORF of *H. pylori* strain 26695 cagA::catrdx-9. As the removal of the cat-rdxA insertion should result in the restoration of an intact cagA gene, all *H. pylori* transformants were subsequently screened for CagA expression. To ensure that the 26695 cagA promoter region had been replaced with the corresponding wild-type Colombian sequences, the cagA promoter regions of transformants expressing CagA were sequenced.

**Targeted mutagenesis of cagA promoter region**

To generate mutations in the promoter region of cagA, a 1.2 kb fragment encompassing 0.5 kb of DNA upstream and 0.7 kb downstream of the cagA transcriptional start site was PCR amplified from *H. pylori* strain 5056 with primers 5'-GCAAAAAAACAACCCAAGCTGA-3' and 5'-ACCACTAGGGCCC-TCCATTtTTTTC-3' and cloned into the plasmid vector pGEMT-Easy. Targeted mutagenesis was carried out using the Quick-Change mutagenesis kit (Agilent Technologies). The introduction of mutations into plasmids was confirmed by DNA sequencing. These plasmids were then used to transform *H. pylori* strain 26695 cagA::catrdx-9 and transformants were selected as described above.

**Western blot analysis**

To compare the levels of CagA expression in *H. pylori* Colombian strains, the bacterial strains were inoculated into BB-FBS broth and grown overnight. Cultures were then inoculated into fresh BB-FBS to an initial OD600 of 0.1. Broth cultures were grown for 15 hours and harvested; the OD600 of the *H. pylori* cultures at the time of harvest was approximately 0.5 to 0.6, corresponding to cultures in logarithmic phase. *H. pylori* cells were lysed using NP-40 lysis buffer (37, 38), and each sample (5 μg of protein) was subjected to SDS-PAGE and Western blot analysis. CagA expression was analyzed using a 1:10,000 dilution of a CagA-specific polyclonal antibody generated against amino acids 1-300 (Santa Cruz Biotechnology), or a 1:30,000 dilution of an independent polyclonal anti-CagA antiserum generated against a larger segment of CagA (amino acids 1–880). Goat anti-rabbit horseradish peroxidase (1:6,000) was used as a secondary antibody and immunoreactive bands were visualized using a chemiluminescence kit (GE healthcare). Following CagA analysis, blots were treated with Restore Western Blot Stripping Buffer (Thermo), and reprobed with antiserum generated against soluble proteins of *H. pylori* (anti-HP, 1:10,000; ref. 39). To quantify levels of CagA expression, we compared the CagA signal in each sample with the CagA signal of a reference strain (strain 5001). Densitometry was done using ImageJ software (NIH), and background signal levels were subtracted from measurements of CagA-specific signal intensities. For each strain, 3 independent samples were analyzed, and the average of these values was calculated.

**Real-time PCR**

Total RNA was isolated from *H. pylori* using Trizol Reagent (Gibco) according to manufacturer’s instructions. The RNA was digested with RQ1 RNase-free DNase (Promega), and the RNA samples were then subjected to a clean-up step using RNAeasy columns (Qiagen). cDNA synthesis was done on 100 ng of purified RNA using the first-strand cDNA synthesis kit (Biorad). As controls, first-strand cDNA reactions were carried out in parallel without reverse transcriptase. The cDNA and control reactions were diluted 1:20 and used in real-time PCR reactions. Real-time PCR was done using an ABI Real-Time PCR machine, with SYBR green as the fluorochrome. Abundance of transcript was calculated using the ΔΔCt method, with each transcript signal normalized to the abundance of the 16S rRNA internal control. The normalized transcript signal for each sample was then compared with similarly normalized values obtained with the 26695 wild-type strain. The primers used for real-time analysis are as follows:

16S rRNA: 5'-GGAGTACCGTCGCAAGATTAAA-3' and 5'-CTACGCGATTCTTCAATGTCAA-3'; cagA: 5'-GAGTCATAATGGCATAAGACCTGA-3' and 5'-TTG-TGCAAGAAATTTCAATGTCAA-3'; HP1588 (encoding a hypothetical protein): 5'-GCTGTCATTGTTGCGAA-TGC-3' and 5'-TGCAATATCAATCGCTGTCCATA-3'; ureA (encoding urease subunit A): 5'-GAAAGACAT-CACTATCAACGAAGC-3' and 5'-GTACCGGCC-AATGCAATCAA-3'.

**Analysis of histopathology**

For each of the 36 subjects from whom *H. pylori* strains were isolated, a global gastric histologic diagnosis was determined independently by 2 pathologists (P.C. and M. B.P.). This diagnosis was based on analysis of all gastric biopsies from antrum, incisura angularis, and corpus, according to the updated Sydney system for the classification of gastritis (40) and to the Padova International
Classification for dysplasia (41). Both pathologists were blinded to the geographic origins of the subjects and data pertaining to *H. pylori* isolates. Global diagnoses were assigned on an ordinal scale from (1) to (6), as follows: (1) mild to moderate nonatrophic gastritis; (2) severe nonatrophic gastritis; (3) multifocal atrophic gastritis without intestinal metaplasia; (4) intestinal metaplasia (IM); (5) dysplasia; and (6) adenocarcinoma. A previously validated histopathology scoring system taking into account the extent and degree of atrophic, metaplastic, and dysplastic changes was also used to quantify differences in morphologic variables within each global diagnosis category. The assignment of these values was accomplished as described previously (32).

**Results**

**Expression of CagA in Colombian *H. pylori* strains**

This study began with an analysis of CagA expression in *H. pylori* strains isolated from subjects in Colombia. We detected considerable variation in the levels of CagA expressed by these strains. For example, immunoblotting studies revealed that CagA expression in strains 5001, 5004, 5005, and 5007 was lower than CagA expression in strains 5043, 5056, 5074, and 5093 (Fig. 1A), and this difference was recapitulated when the samples were immunoblotted with a different CagA antiserum (data not shown). The relative level of CagA expression was quantified by comparing the CagA signal of each *H. pylori* strain to the CagA signal of a strain selected as a reference (strain 5001). As shown in Table 1, the relative expression levels of CagA expression in the panel of Colombian strains ranged from a minimum of 0.4 to a maximum of 5.1.

**Strain-specific sequences upstream from cagA influence levels of CagA expression**

We next sought to determine whether the observed heterogeneity in levels of CagA expression among Colombian strains was attributable to variations in sequences upstream of the cagA ATG translation initiation site. As a first approach, we amplified 1.2 kb sequences (0.5 kb upstream of the cagA transcriptional start site) from the 8 Colombian strains described above, and introduced these sequences into *H. pylori* strain 26695 cagA::catrdx-9. These experiments resulted in replacement of the 26695 sequence with a different CagA antiserum (data not shown). The relative level of CagA expression was quantified by comparing the CagA signal of each *H. pylori* strain to the CagA signal of a strain selected as a reference (strain 5001). As shown in Table 1, the relative expression levels of CagA expression in the panel of Colombian strains ranged from a minimum of 0.4 to a maximum of 5.1.

![Figure 1. Heterogeneity among *H. pylori* strains in expression of CagA.](image-url)

**A.** *H. pylori* strains were cultured in broth and lysed as described in the Methods. Cell extracts were standardized by protein concentration and immunoblotted to detect CagA. Blots were stripped and reprobed with an anti-*H. pylori* antiserum. **B.** *H. pylori* 26695 cagA::catrdx-9 was transformed with 1.2 kb DNA fragments derived from the indicated Colombian strains (500 bp upstream and 700 bp downstream of the cagA transcriptional start site). The resulting transformants were cultured as described in the Methods, and were analyzed for CagA expression as described above. C. Analysis of transformants depicted in (B). Nucleotide numbers are relative to the cagA transcriptional start site, and designate the chromosomal region in *H. pylori* 26695 that was replaced with sequences derived from Colombian strains. Vertical lines indicate the cagA transcriptional start site and the ATG translation initiation site. D, this schematic illustrates the predicted cagA transcriptional start site (TS), an AT-rich inverse repeat identified in the cagA promoter region of *H. pylori* 26695, and the ATG translation initiation site.

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Comparative analysis of sequences upstream from cagA

Next, we analyzed the nucleotide sequences upstream from cagA in strains expressing high levels of CagA and strains expressing low levels of CagA, in an effort to identify sequence differences that might account for the differences in CagA expression. Specifically, we sequenced and analyzed an approximately 1.2 kb region (0.5 kb upstream and 0.7 kb downstream of the transcriptional start site) from the 8 strains described above (4 strains expressing relatively high levels of CagA and 4 strains expressing relatively low levels of CagA). Several differences among strains were identified upstream of the cagA transcriptional start site (Fig. 2A). These differences include variations in the number of copies of a TGGATC motif (1 or 2 copies) located 344 bp upstream of the transcriptional start site, and variation in the number of copies of a TAATGA motif (0, 1, 2 copies) located within an AT-rich region of the cagA promoter that contains an inverted repeat (Fig. 2A). The predicted −10 promoter sequences also differed among the strains; the observed −10 sequences included TATAATGA, TATATGCA, TATTATGA, and TATTATAA (Fig. 2B). In addition, there was variation among strains in the presence of a TGN motif.
AATAAGATA motif (Fig. 3B). Consistent with previous results (Fig. 1), the introduction of wild-type 5056 DNA into *H. pylori* strain 26695 *cagA*::catrdx-9 resulted in a marked increase in CagA expression compared with what was observed in wild-type *H. pylori* 26695 (Fig. 3C and D). In contrast, transformants harboring 5056 DNA containing mutations Mut1 and Mut2 expressed significantly lower levels of CagA (Fig. 3C). This provided evidence that the AATAAGATA motif influences levels of CagA expression.

To verify that alterations in the AATAAGATA motif resulted in alterations of *cagA* transcription, real-time PCR was done to measure *cagA* transcript levels in the aforementioned *H. pylori* 26695 transformants. Consistent with results obtained at the protein level, *H. pylori* strains transformed with DNA from wild-type strain 5056 showed a significant increase in *cagA* mRNA levels compared with that of wild-type strain 26695 (Fig. 3E). In contrast, little or no increase in *cagA* transcript levels was observed in transformants harboring 5056 sequences that contain mutations to either the TAA or GATA portions of the AATAAGATA motif. This provided further evidence that the AATAAGATA motif influences levels of CagA transcription.

**Targeted mutagenesis of additional nucleotides surrounding the AATAAGATA motif**

*H. pylori* strain 26695 consistently expresses relatively low levels of CagA (Fig. 1B), but an analysis of nucleotide sequences upstream of the 26695 *cagA* ATG initiation site revealed the presence of the AATAAGATA motif that is associated with high CagA expression (Fig. 3A). Similarly, we found that some Colombian strains failed to produce high levels of CagA, despite harboring the AATAAGATA motif (Table 1). We sought to elucidate the reason for this apparent discrepancy by further studying strain 26695. Careful inspection of the 26695 sequence revealed that even though it contained the AATAAGATA motif, it differed from strains expressing high levels of CagA in nucleotides just downstream of the AATAAGATA motif. For example, a nucleotide sequence CCGAATAGGTAT immediately follows the AATAAGATA motif in strain 26695. This provided further evidence that the AATAAGATA motif influences levels of CagA transcription.

**Targeted mutagenesis of additional nucleotides surrounding the AATAAGATA motif**

Next, we sought to experimentally test whether the AATAAGATA motif influenced the level of CagA expression. To do this, we started with a cloned 1.2 kb DNA segment (0.5 kb upstream and 0.7 kb downstream of the *cagA* transcriptional start site) derived from strain 5056, which, when introduced into strain 26695 *cagA*::catrdx-9, resulted in higher levels of CagA expression than what was observed in wild-type *H. pylori* 26695 (Fig. 1B). Two separate site-specific mutations were introduced into the AATAAGATA motif of the cloned 5056 DNA. These nucleotide changes involved changing the AATAAGATA motif to either AATAATAG (designated Mut1) or AATAATAG (designated Mut2; Fig. 3A). The 5056 sequences (wild-type and mutant derivatives) were introduced into *H. pylori* strain 26695 *cagA*::catrdx-9 by natural transformation. A minimum of 355 bp upstream of the transcriptional start site (459 bp upstream of the ATG initiation site) was introduced into *H. pylori* 26695 *cagA*::catrdx-9, resulting in the introduction of the 5056 *cagA* promoter plus wild-type or mutant variants of the AATAAGATA motif (Fig. 3B). Consistent with previous results (Fig. 1), the introduction of wild-type 5056 DNA into *H. pylori* strain 26695 *cagA*::catrdx-9 resulted in a marked increase in CagA expression compared with what was observed in wild-type *H. pylori* 26695 (Fig. 3C and D). In contrast, transformants harboring 5056 DNA containing mutations Mut1 and Mut2 expressed significantly lower levels of CagA (Fig. 3C). This provided evidence that the AATAAGATA motif influences levels of CagA expression.

To verify that alterations in the AATAAGATA motif resulted in alterations of *cagA* transcription, real-time PCR was done to measure *cagA* transcript levels in the aforementioned *H. pylori* 26695 transformants. Consistent with results obtained at the protein level, *H. pylori* strains transformed with DNA from wild-type strain 5056 showed a significant increase in *cagA* mRNA levels compared with that of wild-type strain 26695 (Fig. 3E). In contrast, little or no increase in *cagA* transcript levels was observed in transformants harboring 5056 sequences that contain mutations to either the TAA or GATA portions of the AATAAGATA motif. This provided further evidence that the AATAAGATA motif influences levels of CagA transcription.

**Targeted mutagenesis of additional nucleotides surrounding the AATAAGATA motif**

*H. pylori* strain 26695 consistently expresses relatively low levels of CagA (Fig. 1B), but an analysis of nucleotide sequences upstream of the 26695 *cagA* ATG initiation site revealed the presence of the AATAAGATA motif that is associated with high CagA expression (Fig. 3A). Similarly, we found that some Colombian strains failed to produce high levels of CagA, despite harboring the AATAAGATA motif (Table 1). We sought to elucidate the reason for this apparent discrepancy by further studying strain 26695. Careful inspection of the 26695 sequence revealed that even though it contained the AATAAGATA motif, it differed from strains expressing high levels of CagA in nucleotides just downstream of the AATAAGATA motif. For example, a nucleotide sequence CCGAATAGGTAT immediately follows the AATAAGATA motif in strain 26695. This provided further evidence that the AATAAGATA motif influences levels of CagA transcription.

**Targeted mutagenesis of additional nucleotides surrounding the AATAAGATA motif**

Next, we sought to experimentally test whether the AATAAGATA motif influenced the level of CagA expression. To do this, we started with a cloned 1.2 kb DNA segment (0.5 kb upstream and 0.7 kb downstream of the *cagA* transcriptional start site) derived from strain 5056, which, when introduced into strain 26695 *cagA*::catrdx-9, resulted in higher levels of CagA expression than what was observed in wild-type *H. pylori* 26695 (Fig. 1B). Two separate site-specific mutations were introduced into the AATAAGATA motif of the cloned 5056 DNA. These nucleotide changes involved changing the AATAAGATA motif to either AATAATAG (designated Mut1) or AATAATAG (designated Mut2; Fig. 3A). The 5056 sequences (wild-type and mutant derivatives) were introduced into *H. pylori* strain 26695 *cagA*::catrdx-9 by natural transformation. A minimum of 355 bp upstream of the transcriptional start site (459 bp upstream of the ATG initiation site) was introduced into *H. pylori* 26695 *cagA*::catrdx-9, resulting in the introduction of the 5056 *cagA* promoter plus wild-type or mutant variants of the AATAAGATA motif (Fig. 3B). Consistent with previous results (Fig. 1), the introduction of wild-type 5056 DNA into *H. pylori* strain 26695 *cagA*::catrdx-9 resulted in a marked increase in CagA expression compared with what was observed in wild-type *H. pylori* 26695 (Fig. 3C and D). In contrast, transformants harboring 5056 DNA containing mutations Mut1 and Mut2 expressed significantly lower levels of CagA (Fig. 3C). This provided evidence that the AATAAGATA motif influences levels of CagA expression.

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of not only the AATAAGATA motif, but also additional nucleotides located downstream of this motif in determining the levels of CagA expressed.

**Analysis of CagA expression and the AATAAGATA motif in strains from 2 regions of Colombia**

The *H. pylori* strains used in these studies originated from 2 regions in Colombia (Túquerres and Tumaco), characterized by a high risk or low risk for the development of gastric cancer, respectively. Therefore, we sought to determine whether there were any detectable differences in levels of CagA expression in strains from the 2 regions. As shown in Figure 4A, strains isolated from patients in the high-risk region expressed higher levels of CagA than did strains isolated from the low-risk region (*P* = 0.028). We also detected differences in the presence of the AATAAGATA motif in strains from the 2 regions (Fig. 4B). The AATAAGATA motif was detected more commonly in *H. pylori* strains from the high-risk region (where strains typically express high levels of CagA), than in strains from the low-risk region. Among 19 strains from the high-risk region, 16 possessed this motif. In contrast, fewer than half of the strains from the low-risk region (7 of 17) possessed the AATAAGATA motif (*P* = 0.014; Table 1, Fig. 4B).

**Analysis of CagA expression and the AATAAGATA motif in strains of European or African phylogeographic origins**

MLST analysis was previously used to determine the phylogeographic origin of Colombian *H. pylori* strains, and all of the strains analyzed were determined to be
either European or African in origin (32). As shown in Figure 4C, strains expressing the highest levels of CagA were European in origin, and the strains of African origin expressed relatively low levels of CagA \((P = 0.0187)\). The AATAAGATA motif was present more commonly in European strains (21 of 25 strains) than in African strains (2 of 11 strains) \((P = 0.0003; \text{Fig. } 4D)\). When comparing African strains (all from the low risk region) with European strains from the same low-risk region, the AATAAGATA motif was again found to be present more commonly in European strains (5 of 6 strains), compared with African strains (2 of 11 strains; \(P = 0.03)\). Thus, in comparison with strains of African origin, strains of European origin are more likely to contain the AATAAGATA cagA motif and express high levels of CagA.

**Correlation of CagA expression and the AATAAGATA motifs with histopathology scores**

All of the Colombian \(H.\) pylori strains analyzed in the current study were isolated from gastric biopsies, and the severity of gastric pathology (focusing on premalignant lesions) was scored as described in the Methods. To determine whether levels of CagA expression are associated with severity of gastric disease, we compared the histopathology score of each patient with the levels of CagA expressed by the corresponding \(H.\) pylori strain. As shown in Figure 5A, there was a significant correlation between these 2 parameters \((r_s = 0.453, P = 0.0057)\). \(H.\) pylori strains expressing high levels of CagA were consistently associated with high histology scores, and the lowest histology scores were associated with strains expressing low levels of CagA (Fig. 5A). Figure 5B analyzes levels of CagA expression in strains from patients with gastric histologic diagnoses that are considered either precancerous (histology score \(\geq 3\), indicative of atrophic gastritis with and without intestinal metaplasia or dysplasia) or nonprecancerous (histology score <3, indicative of nonatrophic gastritis). The level of CagA expression in \(H.\) pylori strains from patients with precancerous lesions was significantly higher than the level of CagA expression in \(H.\) pylori strains from patients with nonprecancerous lesions \((P = 0.02, \text{Fig. } 5B)\). Representative images illustrating gastric lesions of varying severity are shown in Figure 5C.

We next analyzed whether the presence of the AATAAGATA motif was associated with histopathology scores (Fig. 6). Strains containing the \(cagA\) AATAAGATA motif were associated with higher histopathology scores, compared with strains in which the motif is absent \((P = 0.025, \text{Fig. } 6A)\). We also analyzed whether there was
any correlation between histology scores and other sites of heterogeneity upstream from \textit{cagA}, including predicted -10 sequences and the previously identified TGGATC and TAATGA motifs (Fig. 2A). As shown in Figure 6, there was no significant correlation between histopathology scores and the number of TGGATC motifs (Fig. 6B), number of TAATGA motifs (Fig. 6C), or type of -10 sequence (Fig. 6D). Therefore, an increased severity of preneoplastic lesions was correlated with both high levels of CagA expression (Fig. 5) and the presence of the AATAAGATA motif (Fig. 6A).

**Discussion**

Gastric cancer develops in a small subset of \textit{H. pylori}-infected persons, and the remainder remains asymptomatic. The risk of gastric cancer is determined by bacterial factors, host factors, and environmental factors (1–6). Numerous studies have shown that \textit{cagA}-positive \textit{H. pylori} strains are associated with an increased risk of gastric cancer compared with strains that lack cagA (9–14). The risk of gastric cancer is also determined by several related bacterial features, including sequence variation within CagA (such as the number and type of EPIYA motifs; refs. 28–30), and the presence or absence of a functional \textit{cag} type IV secretion system (which translocates CagA into host cells; refs. 15–19). In this study, we show that there is considerable variation among \textit{H. pylori} strains in expression of CagA, and we provide evidence that the level of CagA expression may be an additional factor that influences gastric cancer risk.

By comparing DNA sequences upstream of the cagA ATG initiation site, we identified an AATAAGATA DNA motif that was present significantly more frequently in
strains expressing high levels of CagA than in strains expressing low levels of CagA. All of the strains expressing high levels of CagA contained this motif, whereas only 35% of strains expressing low levels of CagA contained this motif. Experimental studies revealed that the AATAAGATA motif is required for high levels of CagA expression, and sequences immediately downstream from this motif are also required for high-level CagA expression. Collectively, these results indicate that the AATAAGATA motif is necessary but not sufficient for high levels of CagA expression. In future studies, it will be important to precisely map the boundaries of the region that is required for high-level CagA expression. Nucleotide sequences located near promoters are often sites for binding of regulatory proteins, which can positively or negatively regulate gene transcription. In this case, since this AATAAGATA motif is located downstream of the cagA transcriptional start site within the 5′ untranslated region (5′-UTR; ref. 42), at a site somewhat distant from the promoter, it seems relatively unlikely that it serves as a binding site for a regulatory protein. Instead, we speculate that presence of the AATAAGATA motif may confer increased transcript stability.

One possible mechanism by which RNA stability is regulated is through base pairing with small regulatory RNA. The existence of numerous H. pylori sRNAs potentially involved in gene regulation has recently been described (45). In most cases, binding of sRNA to the target mRNA, typically in the 5′-UTR, results in reduced stability of the target mRNA through occlusion of the ribosome binding site and degradation of the mRNA transcript (46). However, at least one example of increased transcript stability mediated by sRNAs has been described (47). In this case, it is thought that in the absence of the sRNAs, a hairpin structure in the 5′ UTR forms, and that this hairpin structure forms a recognition site for RNA degradation. Base pairing of the sRNAs to binding sites in the 5′ UTR results prevents the stem loop structure, leading to increased stability of the mRNA transcript. Given the location of the AATAAGATA motif on the 5′ UTR of CagA, we speculate that a similar phenomenon might be operative with cagA.

The H. pylori strains used in this study were isolated from patients in 2 regions of Colombia characterized by either a high risk or low risk for the development of gastric cancer. It was recently shown by MLST analysis that cagA-positive H. pylori strains from the high-risk region are predominantly European in origin, and strains from the low-risk region are either African or European in origin (32). In this study, we observe that H. pylori isolates from the region with a high risk of gastric cancer express higher levels of CagA than do H. pylori isolates from the region with a low cancer risk, and we find that H. pylori isolates of European origin express CagA at higher levels than do H. pylori strains of African origin. Consistent with these observations, the AATAAGATA motif was found predominantly in H. pylori strains from the high-risk region and in strains of European origin. Thus far, there

Figure 6. Relationship between histopathology scores and cagA upstream sequences. DNA sequencing revealed 4 regions upstream of the cagA ATG initiation site that varied among the different Colombian strains (see Figure 2A). The strains vary in the number of copies of the indicated DNA sequences (A–C) or vary in ~10 sequences (D). The histology scores are compared with the number of AATAAGATA motifs (A), TGGATC motifs (B), TAATGA motifs (C), and type of ~10 sequence (D). P values were calculated using t test (A–C) or 1-way ANOVA (D).
are relatively few robust genetic markers that allow differentiation of African and European strains (48). It will be important in future studies to test additional *H. pylori* isolates, including *H. pylori* strains derived from native European or African subjects, and evaluate whether presence of the AATAAGATA consistently correlates with geographic origin of strains and whether this motif provides a robust marker for CagA expression levels. In a previous study, it was noted that Colombian *H. pylori* strains of European origin were associated with increased severity of preneoplastic lesions and increased DNA damage in gastric biopsy samples, compared with Colombian strains of African origin (32). In the current study, we observed that *H. pylori* strains expressing the highest levels of CagA were consistently associated with precancerous lesions; in contrast, only about half of the strains expressing lower levels of CagA were associated with precancerous lesions, and the remainder had nonatrophic gastritis. We propose that the differences in gastric cancer rates between the low- and high-risk regions may be attributed, at least in part, to differences in levels of CagA produced by the corresponding *H. pylori* strains. The differences in the gastric cancer rates in these 2 regions of Colombian are also influenced by many other microbial, host, and environmental factors (49–51). For example, persons in the low-risk region typically consume higher amounts of fruit, fresh vegetables, and seafood compared with persons in the high-risk region. Vitamin C, found in fruits and vegetables, and selenium, found in fish, may provide protection against the development of gastric cancer (52–54). As another example, intestinal parasites are more common in the low-risk region than in the high-risk region, and may also influence gastric cancer rates by diminishing the inflammatory response to *H. pylori* (51, 55).

In summary, many previous studies have shown the importance of CagA in the development of gastric diseases, and specific polymorphisms in CagA (such as the number and type of EPIYA motifs) have been associated with the severity of disease. In this study, we provide evidence that variation among strains in levels of CagA expression is also a factor that correlates with the severity of disease.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

23. Muyskens JB, Guillen LM, Helicobacter pylori CagA disrupts epithelial 
   Gastric cancer in Colombia. III. Natural history of precursor lesions. J 
   Gastric cancer in Colombia. I. Cancer risk and suspect environmental 
   et al. Age at acquisition of Helicobacter pylori infection: comparison of 
   two areas with contrasting risk of gastric cancer. Helicobacter 2004; 
27. Bravo LE, van Doom LJ, Realpe JL, Correa P. Virulence-associated 
   phenotypes of Helicobacter pylori: do they explain the African enigma? 
   Am J Gastroenterol 2002;97:2839–42.
   Influence of EPIYA-repeat polymorphism on the phosphorylation-
   dependent biological activity of Helicobacter pylori CagA. Gastroentero-
   logy 2006;130:1181–90.
   motif is a membrane-targeting signal of Helicobacter pylori virulence 
30. Basso D, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, 
   et al. Clinical relevance of Helicobacter pylori cagA and vacA gene 
31. Sicinski LA, Correa P, Peek RM, Camargo MC, Piazuelo MB, Romero-
   Gallo J, et al. CagA C-terminal variations in Helicobacter pylori strains 
   from Colombian patients with gastric precancerous lesions. Clin 
32. de Sablet T, Piazuelo MB, Shaffer CL, Schneider BG, Asim M, 
   Chaturvedi R, et al. Phylogenetic origin of Helicobacter pylori is a 
   gut.2010.234468.
33. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann 
   RD, et al. The complete genome sequence of the gastric pathogen 
   Helicobacter pylori exploits a unique repertoire of type IV secretion 
   system components for pilus assembly at the bacteria-host cell 
   in the Helicobacter pylori vacA gene with a sucrose sensitivity marker. 
36. Dalidene D, Dalile D, Kerslute B, Burga DE. Contraselectable streptomycin 
   susceptibility determinant for genetic manipulation and analysis of 
   Helicobacter pylori. Appl Environ Microbiol 2006;72: 
   5908–14.
37. Busler VJ, Torres VJ, McClain MS, Tirado O, Friedman DB, Cover TL. 
   Protein–protein interactions among Helicobacter pylori cagA proteins. J 
   Bacteriol 2006;188:4787–800.
38. Loh JT, Torres VJ, Cover TL. Regulation of Helicobacter pylori cagA 
39. Cao P, McClain MS, Forsyth MH, Cover TL. Extracellular release of 
   allergenic proteins by Helicobacter pylori. Infect Immun 1998;66: 
   2984–86.
40. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading 
   of gastritis. The updated Sydney System. International workshop on 
   Gastric dysplasia: the Padova international classification. Am J Surg 
   Pathol 2000;24:167–76.
42. Sphohn G, Beier D, Rappuoli R, Scarlato V. Transcriptional analysis of 
   the divergent cagAB genes encoded by the pathogenicity island of 
43. Sabelnikov AG, Greenberg B, Lacks SA. An extended -10 promoter 
   alone directs transcription of the DprII operon of Streptococcus 
44. Forsyth MH, Cover TL. Mutational analysis of the vacA promoter 
   provides insight into gene transcription in Helicobacter pylori. J Bac-
45. Sharma CM, Hoffmann S, Darfeulle F, Reignier J, Findeiss S, Sittka A, 
   et al. The primary transcriptome of the major human pathogen Heli-
   615–28.
47. McCullen CA, Benhammou JN, Majdaliani N, Gottesman S. Mecha-
   nism of positive regulation by DsrA and RprA small noncoding RNAs: 
   pairing increases translation and protects pool mRNA from degrada-
   180– and 480-base-pair insertions in African and African-American 
49. Camargo MC, Burk RF, Bravo LE, Piazuelo MB, Hill KE, Fontham ET, 
   et al. Plasma selenium measurements in subjects from areas with 
   443–51.
50. Piazuelo MB, Camargo MC, Mora RM, Delgado AG, Peak RM Jr, 
   Correa H, et al. Eosinophils and mast cells in chronic gastritis: possible 
   Intestinal helminthiasis in Colombian children promotes a Th2 
   response to Helicobacter pylori: possible implications for gastric 
   intervention trials in Linxian, China: supplementation with specific 
   vitamin/mineral combinations, cancer incidence, and disease-specific 
   mortality in the general population. J Natl Cancer Inst 1993;85: 
   1483–92.
   vitamin/mineral combinations, cancer incidence, and disease-specific 
   mortality in the general population. J Natl Cancer Inst 1993;85: 
   1483–92.
Analysis of cagA in Helicobacter pylori Strains from Colombian Populations with Contrasting Gastric Cancer Risk Reveals a Biomarker for Disease Severity

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