Expression and Interleukin 8 Secretion at the polymorphic carboxyl-terminus of the protein [2]. CagAylated by host kinases on tyrosine residues within EPIYA motifs terleukin 8 (IL-8) production by epithelial cells [3, 4]. Addition- can stimulate proinflammatory cytokine secretion, including in- the host cells, which are able to activate Nod1, resulting in IL-8 induction [5].

Patients infected with *H. pylori* strains that express CagA are at increased risk of gastric premalignant lesions and gastric carcinoma [6, 7]. Furthermore, the magnitude of the risk for premalignant and malignant gastric lesions increases as the number of EPIYA-C motifs of the infecting strain increases [8].

Previously, Loh et al showed that *H. pylori* strains originating from a region in Colombia characterized by a high risk for gastric carcinoma expressed higher levels of CagA than did *H. pylori* strains isolated from a region with low risk for gastric carcinoma [9, 10]. This variability was attributed to DNA motifs that exhibited sequence heterogeneity in the promoter region of the cagA gene [9, 10]. In particular, the presence of the DNA motif AATAAGATA upstream of the cagA ATG initiation codon and located 59 bp downstream of the transcription start site (according to strain 26695) has been identified in *H. pylori* strains expressing high levels of CagA [9, 10]. Additionally, the presence of this AATAAGATA motif and strains that express high CagA levels were associated with more-advanced precancerous lesions in the population at high risk for gastric carcinoma [9].

As there are currently no data on sequence variation in the cagA promoter of *H. pylori* strains from other regions of the world, we characterized the cagA promoter and addressed the relationships between strain-specific promoter sequences and CagA protein expression in *H. pylori* strains from northern Portugal. This is an area where the risk for gastric carcinoma is high, the prevalence of *H. pylori* is almost 85% in adults, and the proportion of strains positive for CagA is 62% [11]. Furthermore, we evaluated the influence of promoter sequence variation and CagA expression on both gastric histopathology and IL-8 secretion induced by *H. pylori* in gastric epithelial cells.

**MATERIALS AND METHODS**

**Patient Materials**

A total of 46 *H. pylori* cagA–positive isolates were obtained from individuals who were part of a case-control study involving first-degree relatives of 27 case patients with early onset gastric carcinoma and 19 controls [12]. The individuals were between 19 and 68 years old (mean age, 48 years), and the female to male ratio was 2.07:1 (Supplementary Table 1). All individuals underwent high-definition upper gastrointestinal endoscopy at Centro Hospitalar do Porto (CHP; Porto, Portugal). Biopsy specimens were collected from the antrum, incisura, and corpus for histopathological evaluation, and an additional antrum biopsy specimen was used for *H. pylori* culture. The study was approved by the ethical committee of CHP, and written informed consent was received from all participants.
Characterization of the cagA Promoter Region

The cagA promoter was characterized by polymerase chain reaction analysis and sequencing of a region that covers 524 bp upstream and 162 bp downstream of the cagA transcription start site. Nucleotide sequences were submitted to GenBank under accession numbers KF441746–57, KT716332, and KT716333.

Evaluation of CagA Expression

CagA expression was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of equal amounts of H. pylori protein, followed by Western blot using the sc-28368 antibody (Santa Cruz Biotechnology, California). CagA expression was quantified for each isolate and normalized for CagA expression of H. pylori 60 190. This strain was chosen since it is negative for the +59 AATAAGATA motif, which, when present, is associated with enhanced CagA expression [9].

Quantification of IL-8 Secretion

AGS human gastric epithelial cells (ATCC CRL-1739) were infected with H. pylori at a multiplicity of infection of 100. After 24 hours, supernatants were collected and used to evaluate IL-8 secretion, using an enzyme-linked immunosorbent assay (BioLegend, California), following the manufacturer’s instructions.

Statistical Analyses

Comparisons between the cagA variants and CagA protein expression or IL-8 secretion were performed with the Student t test and 1-way analysis of variance. The relationship between the −10 promoter sequences and the +59 motif was addressed with the Fischer exact test. The relationship between CagA expression and IL-8 secretion was evaluated by the Pearson correlation analysis. Differences were considered statistically significant at P values of <.05.

For further details see the Supplementary Materials.

RESULTS AND DISCUSSION

Sequence Heterogeneity in the cagA Promoter Region

In Portuguese strains, the region upstream from the cagA start codon presented high heterogeneity. The differences included variation in copy number and/or sequence of the motifs located −344, −53, −10, and +59 bp from the transcription start site, defined according to the nucleotide position in H. pylori 26 695 (GenBank accession number KT716333; Figure 1). Differences were also observed in the number of TGN repeats immediately upstream of the −10 motif and that corresponds to an extended −10 promoter sequence. Detailed information on the frequency and type of variation of the different motifs is provided in Figure 1A. Our data are in agreement with observations reported for Colombian strains, which display variability mostly in these motifs [9]. In contrast, all strains in this study contained the −53 motif, and 1 strain had 2 copies of the +59 motif. Additionally, in 3 strains (6.1%), we detected a T → C mutation in the third nucleotide of the +59 motif.

We also identified 4 novel motifs (Figure 1B) at positions −321 bp (AATA), which was present in 34 (73.9%) of the strains; −280 bp (CAn repeat), which was present as 2 (in 1 strain [2.2%]), 3 (in 17 [36.9%]), or 4 (in 28 [60.9%]) copies; −217 bp (AACCA), which was absent (in 1 strain [2.2%]) or present as 1 (in 12 [26.1%]) or 2 (in 33 [71.7%]) copies; and at position +10 bp (TGCA), which was present in 21 strains (45.6%). An insertion of 18 nucleotides in H. pylori strain 26 695 (293–311 bp upstream from the transcription start site) was also detected (Figure 1B).

Relationship Between cagA Promoter Sequence Variation and CagA Expression

To investigate whether sequence variation in the cagA promoter had an influence in the levels of CagA expression, we performed Western blot analyses for each H. pylori isolate. The relative CagA protein expression in the Portuguese strains ranged between 0.02 and 6.92. H. pylori strains containing the +59 motif expressed higher levels of CagA than strains that did not contain the motif (P = .003; Figure 2B). This finding concurs with what was observed in Colombian strains [9]. Additionally, we observed that strains containing the −10 TATAATGA sequence expressed higher levels of CagA than did strains containing other −10 nucleotide sequences (P = .012; Figure 2A). One possible explanation for this finding is that the majority of the strains having the −10 TATAATGA sequence (36 of 38 [94.7%]) also contained the +59 motif, while only about half of the strains with other sequences in the −10 promoter region (13 of 25 [52.0%]) contained the +59 motif (P < .001). No additional relationships between particular −10 sequences and CagA expression were observed (Supplementary Figure 1F).

When considering strains that express CagA at levels of at least 2 times that of H. pylori 60 190 as high CagA-expressing strains, the presence of the +59 motif was found in all of the high CagA-expressing strains (20 of 20 [100%]), while all 11 strains lacking this motif expressed relatively low CagA levels (P = .001). No relationships were observed between variation in other promoter motifs and CagA protein expression (Supplementary Figure 1).

Relationship Between cagA Promoter Variation, CagA Expression, and Histopathological Findings

No significant differences were observed between the levels of chronic inflammation, polymorphonuclear activity, or atrophy and the +59 motif (Supplementary Table 2). Interestingly, individuals with intestinal metaplasia were all infected with H. pylori strains containing the +59 motif (P = .009; Figure 2C). However, no significant differences were detected in CagA expression between strains infecting individuals with and those infecting individuals without intestinal metaplasia (Supplementary Figure 2). Similarly, CagA expression was not related to the histopathological parameters. Our findings contrast with those
for the Colombian population, where strains that contained the +59 motif and expressed high levels of CagA were associated with more-advanced precancerous lesions than the ones detected in patients infected with strains without the +59 motif and expressing low CagA levels [9]. One of the reasons that may underlie the differences between studies could be the fact that ours included predominantly females (31 subjects [67%]), while the study that was conducted with Colombian patients included only males [9], and it is known that gastric carcinoma rates in men are double those of women [13]. Furthermore, it was shown that the phylogeographic origin of the strains is also an important contributor for the expression of CagA [9]. Yet another possibility is that there are differences between the 2 populations with regard to other gastric carcinoma risk factors, such as salt consumption. Interestingly, it was demonstrated that high salt concentration can upregulate CagA expression [10].

cagA Promoter Region Variation, CagA Expression, and In Vitro IL-8 Secretion

Since we confirmed that strain-specific sequences in the cagA promoter region led to enhanced CagA expression, and because although gastric epithelial IL-8 secretion is a complex process, CagA may be involved [3, 4], we hypothesized that cagA promoter sequences associated with high CagA expression may be associated with enhanced IL-8 secretion. After infecting the gastric AGS cell line with H. pylori isolates (n = 44) and quantifying IL-8 secretion in the culture supernatants, a significant positive correlation between H. pylori CagA expression and...
IL-8 secretion was observed (Pearson correlation, $r_p = .391$; $P = .009$; Figure 2D). Furthermore, infection with strains containing the +59 motif was associated with enhanced IL-8 secretion ($P = .013$; Figure 2E). No significant relationships were observed between any of the other variable motifs in the $cagA$ promoter and IL-8 (Supplementary Figure 3). These results reflect the importance of the +59 motif, not only in enhanced CagA expression but also in the induction of IL-8 secretion from epithelial gastric cells.

A previous study reported increased IL-8 expression when cells were infected with $H. pylori$ strains containing higher number of CagA EPIYA-C motifs, although this association was not found by others [14, 15]. To exclude the possibility that differences in CagA expression and IL-8 secretion were due to strain variability in the number of CagA EPIYA-C phosphorylation motifs, we characterized the 3' EPIYA-encoding region of $cagA$ of the strains. No relationships were found between the number of CagA EPIYA-C motifs and CagA expression ($P = .237$) or IL-8 secretion ($P = .403$; Supplementary Figure 4). Our results are in agreement with those of Papadakos et al, who used isogenic mutant strains with varying number of EPIYA-C motifs and showed that secreted levels of IL-8 were independent of the number of EPIYA C motifs [15].

In conclusion, the $cagA$ promoter region of Portuguese $H. pylori$ strains is genetically heterogeneous, and this heterogeneity influences CagA expression. The AATAAAGATA +59

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Figure 2. Relationship between CagA protein expression, $cagA$ promoter motifs, and interleukin 8 (IL-8) secretion by AGS cells. A and B, Analysis of CagA protein expression in respect to $-10$ sequences and +59 motifs. Statistical significance was determined by the $t$ test. Other sequences stand for the presence of the following: TATAATGA, TATACTGA, TATAGTAA, TATAGTGA, and CATAATAA. C, Relationship between the presence of intestinal metaplasia (IM) and the +59 motif. Statistical significance was evaluated by the Fisher exact test. D, Findings of Pearson correlation analysis, indicating a positive relationship between IL-8 secretion and CagA expression ($r_p = .391$ and $P = .009$). E, Relationship between IL-8 secretion and the +59 $cagA$ promoter motif. Statistical significance was determined by the $t$ test.
motif is associated with high levels of CagA expression and influences in vitro IL-8 secretion by gastric cells. All cases with intestinal metaplasia were infected with *H. pylori* strains containing the +59 motif at the cagA promoter region. Further investigation of populations from other geographic regions at high risk of gastric carcinoma will shed light on the usefulness of cagA promoter variation and CagA expression as markers to predict disease risk.

**Supplementary Data**

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

**Notes**

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16. Gonzalez CA, Figueiredo C, Lowe CB, et al. *Helicobacter pylori* cagA promoter elements associated with high levels of CagA expression and influence in vitro IL-8 secretion by gastric cells. All cases with intestinal metaplasia were infected with *H. pylori* strains containing the +59 motif at the cagA promoter region. Further investigation of populations from other geographic regions at high risk of gastric carcinoma will shed light on the usefulness of cagA promoter variation and CagA expression as markers to predict disease risk.