

Regulation of Gastric Carcinogenesis by *Helicobacter pylori* Virulence Factors

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Abstract

Helicobacter pylori is the strongest known risk factor for gastric adenocarcinoma, and strains that possess the *cag* secretion system, which translocates the bacterial effector CagA into host cells, augment cancer risk. *H. pylori* strains that express the vacuolating cytotoxin or the outer membrane protein OipA are similarly associated with severe pathologic outcomes. We previously reported that an *in vivo* adapted *H. pylori* strain, 7.13, induces gastric adenocarcinoma in rodent models of gastritis. In the current study, we used carcinogenic strain 7.13 as a prototype to define the role of virulence constituents in *H. pylori*-mediated carcinogenesis. Mongolian gerbils were infected with wild-type strain 7.13 or *cagA*⁻, *vacA*⁻, or *oipA*⁻ mutants for 12 to 52 weeks. All infected gerbils developed gastritis; however, inflammation was significantly attenuated in animals infected with the *cagA*⁻ but not the *vacA*⁻ or *oipA*⁻ strains. Gastric dysplasia and cancer developed in >50% of gerbils infected with either the wild-type or *vacA*⁻ strain but in none of the animals infected with the *cagA*⁻ strain. Inactivation of *oipA* decreased β -catenin nuclear localization *in vitro* and reduced the incidence of cancer in gerbils. OipA expression was detected significantly more frequently among *H. pylori* strains isolated from human subjects with gastric cancer precursor lesions versus persons with gastritis alone. These results indicate that loss of CagA prevents the development of cancer in this model. Inactivation of *oipA* attenuates β -catenin nuclear translocation and also decreases the incidence of carcinoma. In addition to defining factors that mediate *H. pylori*-induced cancer, these results provide insight into mechanisms that may regulate the development of other malignancies arising within the context of inflammatory states. [Cancer Res 2008;68(2):379–87]

Introduction

Gastric cancer is the second leading cause of cancer-related death worldwide (1). The strongest identifiable risk factor for the development of this malignancy is infection with the pathogen *Helicobacter pylori*, which led the IARC and the WHO to classify this bacterial species as a type I carcinogen (1). *H. pylori* colonizes

the stomach of approximately half of the world's population, yet only a fraction of infected individuals ever develop gastric cancer (1).

One strain-specific *H. pylori* constituent that augments cancer risk is the cytotoxin-associated gene (*cag*) pathogenicity island (1), a genetic locus that encodes a type IV bacterial secretion system. Upon delivery into host cells by the *cag* secretion system, the product of the terminal gene in the island, CagA, undergoes Src-dependent tyrosine phosphorylation and activates a eukaryotic phosphatase (SHP-2), leading to dephosphorylation of host cell proteins and cellular morphologic changes (2, 3). CagA has also been shown to dysregulate β -catenin signaling (4, 5) and apical-junctional complexes (6), events that have been linked to increased cell motility and oncogenic transformation in a variety of models (7, 8).

Another *H. pylori* locus linked to an enhanced risk for gastric cancer is *vacA*, which encodes a secreted toxin (VacA; ref. 9). *vacA* is present in virtually all *H. pylori* strains (9); however, cytotoxin activity is dictated by variations in *vacA* gene structure (9). *In vitro*, VacA induces the formation of intracellular vacuoles (9) and can induce apoptosis (10). Vacuolating activity is significantly associated with the presence of the *cag* island (1); however, not all persons infected with *cag*⁺ toxigenic strains develop gastric cancer, suggesting that additional *H. pylori* constituents are important in pathogenesis.

In vivo, ~15% to 20% of *H. pylori* bind to gastric epithelial cells (11). Sequence analysis has revealed that an unusually high percentage of the *H. pylori* genome (4%) is predicted to encode outer membrane proteins (OMP; ref. 12), which may function as adhesins and contribute to pathogenesis. One such OMP that is associated with disease outcome is OipA (13–15). Expression of OipA is regulated by slipped strand mispairing within a CT rich dinucleotide repeat region located in the 5' region of the gene (13, 16). Recent reports have shown that OipA coregulates proinflammatory cytokine expression and mediates adherence of *H. pylori* to gastric epithelial cells (13–21).

We recently reported that an *in vivo* adapted *H. pylori* strain (7.13) reproducibly induces gastric cancer in Mongolian gerbils, compared with its progenitor strain B128, which fails to induce cancer in this model (4). Because strain 7.13 is easily transformable, we used this isolate as a prototype to define the role of virulence constituents in *H. pylori*-induced gastric inflammation and cancer. We also extended these results into the natural niche of this pathogen by examining expression of OipA among *H. pylori* strains harvested from infected human subjects that either did or did not possess gastric cancer precursor lesions. The results show that CagA and OipA mediate the development of gastric carcinogenesis, providing a framework for understanding how malignancies arise within the context of *H. pylori*-induced inflammation.

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Materials and Methods

Animals and *H. pylori* challenge. All procedures were approved by the Institutional Animal Care Committee of Vanderbilt University. Multiple cohorts of male Mongolian gerbils, ages 4 to 8 weeks, were orogastrically challenged with sterile *Brucella* broth, *H. pylori* wild-type strain 7.13, or isogenic mutant strains 7.13 *cagA*⁻, 7.13 *vacA*⁻, or 7.13 *oipA*⁻ strains and were sacrificed between 12 and 52 weeks postinoculation (4). Immediately after sacrifice, blood was obtained via cardiac puncture, and serum was stored at -20°C until used for detection of anti-*H. pylori* antibodies via ELISA, as described previously (4). One half of the glandular stomach was fixed for histologic examination, and the other half was homogenized in sterile PBS, plated on selective Trypticase soy agar plates, and incubated for 3 to 5 days for *H. pylori* culture (4).

Linear strips extending from the squamocolumnar junction through proximal duodenum were fixed in 10% neutral-buffered formalin, paraffin-embedded, stained with H&E, and indices of inflammation and injury were scored by a single pathologist (E. Johnston) blinded to treatment groups (4). The following variables were graded on a 0 to 3 scale in the gastric antrum and body: acute inflammation, chronic inflammation (mononuclear cell infiltration independent of lymphoid follicles), cryptitis, and colonization density of *H. pylori* in the gastric glands and foveolae or adjacent to the epithelium (4, 22). Lymphoid follicle formation was graded on a 0 to 2 scale as previously described (4, 22). Dysplastic mucosa was graded as 0 (absent), 1 (focal), or 2 (extensive) and consisted of irregular, angulated, and occasionally cystically dilated glands with enlarged overlapping hyperchromatic nuclei. Carcinoma was defined as irregular, angulated, cystically dilated glands with occasional cribriform architecture in the submucosa and muscularis propria, spreading laterally to the surface mucosal component (4, 22). Paraffin-embedded sections were also stained with an anti-*H. pylori* antibody (DakoCytomation) as previously described (23).

***H. pylori* strains and cell culture.** AGS and MKN28 human gastric epithelial cells were grown in RPMI 1640 (Life Technologies Bethesda Research Laboratories) with 10% fetal bovine serum (FBS; Sigma) and 20 µg/mL gentamicin in 5% CO₂ at 37°C. The *H. pylori* rodent-adapted wild-type strain 7.13, or 7.13 *cagA*⁻, 7.13 *vacA*⁻, or 7.13 *oipA*⁻ mutants were grown in *Brucella* broth with 10% FBS for 18 h, harvested by centrifugation, resuspended in cell culture medium without antibiotics, and added to gastric cells at a bacteria:cell concentration of 100:1. Isogenic *cagA* (HP0547) and *vacA* (HP0887) null mutants were constructed within strain 7.13 by insertional mutagenesis, using *aphA* (4), and recombinants were selected on *Brucella* agar with kanamycin (25 µg/mL). The isogenic *oipA* (HP0638) null mutant was constructed within strain 7.13 by insertional mutagenesis as previously described (13), using a chloramphenicol resistance gene cassette (*cat*), and recombinants were selected on *Brucella* agar with chloramphenicol (10 µg/mL).

To control for potential clonal variation of the mutants, Western blots against CagA, VacA, and OipA were performed on multiple *H. pylori* strain 7.13 *cagA*⁻, *vacA*⁻, and *oipA*⁻ clones (*n* = 3 for each) as previously described (4, 9, 13), and immunoblotting confirmed that each mutant clone lacked expression of the targeted protein. These *cagA*⁻, *vacA*⁻, and *oipA*⁻ clones were tested using *in vitro* assays that detect specific phenotypes induced by these particular genes: assessment of cell elongation (hummingbird phenotype) for *cagA*, cell vacuolation for *vacA*, and β-catenin nuclear translocation for *oipA* as previously described (4, 9). The phenotype of each clone was identical to each of the sister clones tested in all regards other than the expected loss of the corresponding phenotype (e.g., loss of cell elongation for all *cagA*⁻ mutants, loss of vacuolation for all *vacA*⁻ mutants, and loss of β-catenin nuclear translocation for all *oipA*⁻ mutants; data not shown). Multiple single colony *cagA*⁻, *vacA*⁻, and *oipA*⁻ mutant isolates recovered after infection into gerbils were also confirmed by PCR (13, 24) to retain the appropriate mutation and by Western blot to lack expression of the corresponding protein.

DNA sequencing. *H. pylori* genomic DNA was extracted as described previously (4). *oipA* was amplified from genomic DNA using primer pair

5'-CAAGCGCTTAACAGATAGGC-3' and 5'-AAGCGTTTTCTGCTGAAGC-3' and primer pair 5'-TTCTTCTATAGGCACATTGC-3' and 5'-TACAAAACAATCAAAGAGCG-3'. A single 1.2 Kb and 450 bp product were detected, respectively, and purified (MinElute PCR purification kit; Qiagen) and sequence determination was performed by the Vanderbilt University DNA sequencing facility using BigDye Terminator chemistry and analyzed on ABI's 3730×1 DNA Analyzer.

Western analysis. Gastric cell lysates were harvested and protein concentrations were quantified by the Bradford assay (Pierce; ref. 4). Proteins (20 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Pall Corporation). Levels of phosphorylated CagA were determined using an anti-pY99 antibody (1:300; Santa Cruz), and actin levels were determined using an antiactin (C-11) antibody (1:500; Santa Cruz). Primary antibodies were detected using goat anti-rabbit (1:20,000; Sigma) or donkey anti-goat (1:5,000; Sigma) horseradish peroxidase-conjugated secondary antibodies, visualized by the enhanced chemiluminescence detection system (Cell Signaling), and quantified using the ChemiGenius system (Syngene). Densitometric analysis of multiple Western blots was then performed as previously described (4).

Bacterial adherence assay. AGS cells were plated in 10-cm polypropylene tissue culture plates (Nunc) at 3 × 10⁶ cells per plate and allowed to grow for 24 h to subconfluency. *H. pylori* were added to cells at a bacteria:cell ratio of 100:1. *H. pylori*:gastric cell cocultures were washed after 4 h with PBS (pH 7.6) thrice to remove nonadherent bacteria, and total cell lysates were harvested. Serial 10-fold dilutions of cell extracts were cultured on Trypticase Soy Agar plates with 5% sheep blood and were incubated for 3 to 5 days at 37°C under microaerobic conditions.

Immunofluorescence. Gastric epithelial cells were cultured on glass cover slides, and cells treated with or without *H. pylori* were washed twice with PBS, permeabilized, and fixed with ice-cold methanol at -20°C as described (4). Slides were incubated in 3% bovine serum albumin (Sigma) for 10 min and incubated with rabbit anti-β-catenin antibody (1:100; Sigma) overnight at 4°C. Washed slides were then incubated with goat anti-rabbit IgG-Cy2 (1:100; Molecular Probes) at room temperature for 30 min (4). For each sample, at least 100 cells were evaluated by an independent observer unaware of experimental conditions.

***H. pylori* clinical strains.** The 22 *H. pylori* clinical strains used in this study were randomly selected from a larger population of isolates harvested from male patients between ages 40 and 59 years enrolled in an ongoing prospective study in Colombia designed to study mechanisms of *H. pylori* carcinogenesis *in vivo* (25). Exclusion criteria included previous gastrectomy or concomitant systemic illness (25). The protocol was approved by the Universidad del Valle Ethics Committee. Three gastric biopsy specimens were obtained from the antrum, incisura angularis, and corpus during endoscopy and were used for culture and histology as previously described (4). For culture, gastric antral biopsy specimens were placed immediately in liquid nitrogen and stored at -70°C. To isolate *H. pylori*, specimens were thawed, placed in 250 µL of normal saline, and homogenized using a tissue grinder (Micro Kontes) as described (4). Fifty microliters were plated onto Skirrow's plates with 5% sheep blood and incubated for 96 h under microaerobic conditions as described (4). OipA expression was determined by Western blot on bacterial lysates as previously described (18).

For histology, gastric tissues were fixed in buffered formalin and embedded in paraffin. Four-micrometer-thick sections were cut and mounted on ProbeOn-Plus slides (Fisher Scientific) for H&E staining. For all analyses, pathologists were unaware of *H. pylori* OipA expression results. Histopathologic diagnoses were assessed independently by two pathologists (P. Correa and M.B. Piazuelo) following published guidelines (26, 27), and subjects were stratified into those with nonatrophic gastritis versus those with gastric atrophy or intestinal metaplasia.

Statistical analysis. Gerbils were considered to be successfully infected if results from histologic examination of tissue, culture, and serology for *H. pylori* whole cell antigens were concordantly positive. Mann-Whitney U and Student's *t* tests were used for statistical analyses of intergroup comparisons. A log-rank test was used to determine differences between disease outcome between groups. Significance was defined as a *P* value of <0.05.

Table 1. Colonization efficiency of wild-type and isogenic mutant *Helicobacter pylori* strains in Mongolian gerbils

Duration of infection (wk)	12 to 16	24 to 30	40 to 52
Infected/challenged	n/n (%)	n/n (%)	n/n (%)
<i>Brucella</i> broth control	0/19 (0%)	0/5 (0%)	0/13 (0%)
7.13	18/24 (75%)	19/20 (95%)	9/13 (70%)
7.13 <i>cagA</i> ⁻	18/30 (60%)	20/25 (80%)	17/20 (85%)
7.13 <i>vacA</i> ⁻	13/17 (76%)	9/10 (90%)	17/17 (100%)

Results

Colonization efficiency and density of wild-type *H. pylori*, *cagA*⁻, or *vacA*⁻ mutants. The presence of the *cag* island and production of the vacuolating cytotoxin augment the risk of *H. pylori*-induced cancer in humans. Therefore, we sought to directly determine if loss of these virulence factors altered pathologic responses in animals infected with *H. pylori*. Success of *H. pylori* challenge was determined by quantitative culture, *H. pylori* immunostaining, and serum ELISA to detect anti-*H. pylori* antibodies. No animals challenged with *Brucella* broth alone had detectable evidence of *H. pylori*. There were no differences in colonization efficiency between any of the *H. pylori* challenged groups (Table 1). Colonization density was evaluated by histologic examination of *H. pylori* present in H&E-stained sections as well as by *H. pylori* immunostaining (Fig. 1). For all time points, colonization density was significantly higher in gerbils infected with the 7.13 *cagA*⁻ mutant strain compared with wild-type 7.13, and these results were confirmed by quantitative culture (data not shown). Colonization was higher in the gastric antrum compared with the corpus with all strains (data not shown).

Inactivation of *CagA* attenuates the severity of inflammation induced by *H. pylori*. We next sought to define the kinetics of inflammation and carcinogenesis and the role of bacterial virulence factors in these processes by infecting gerbils with *H. pylori* strain 7.13, 7.13 *cagA*⁻, or 7.13 *vacA*⁻ mutant strains for 12 to 52 weeks. There was no evidence of gastric inflammation or injury in uninfected control animals ($n = 37$) up to 52 weeks postchallenge (Fig. 2). All gerbils infected with *H. pylori* developed significantly higher inflammatory scores compared with uninfected controls ($P < 0.05$). This response, however, was significantly attenuated ($P < 0.05$) in gerbils infected with the *cagA*⁻ mutant strain compared with those infected with wild-type 7.13 or the 7.13 *vacA*⁻ mutant strain. Severe chronic active gastritis developed exclusively in gerbils infected with wild-type *H. pylori* or the *vacA*⁻ mutant and was characterized by ulceration and transmural acute inflammation with peritoneal abscess formation. This was accompanied by an abundant lymphoplasmacytic infiltrate involving antral mucosa and submucosa with extension into subserosal tissue and the adjacent pancreas, and lymphoid follicles.

In contrast, gerbils infected with the *cagA*⁻ mutant developed only mild-to-moderate chronic antral gastritis, which was characterized by lymphoid aggregates, occasional lymphoid follicle formation, and rare mucosal active inflammation. The inflammatory response was confined to the mucosa and occasionally involved the muscularis mucosae, in contrast to the transmural inflammatory infiltrate observed in animals infected with wild-type *H. pylori* or the *vacA*⁻ isogenic mutant strain (Figs. 2 and 3).

Development of gastric dysplasia and cancer is dependent on *CagA*. Based on the known association between the *cag* island, toxigenicity, and gastric cancer, we next investigated the effects of *CagA* and *VacA* on carcinogenesis *in vivo* by infecting gerbils with wild-type or *cagA*⁻ or *vacA*⁻ 7.13 mutant strains. Gastric dysplasia and adenocarcinoma developed in wild-type and *vacA*⁻ mutant *H. pylori*-infected gerbils by 12 to 16 weeks postinoculation (Fig. 4).

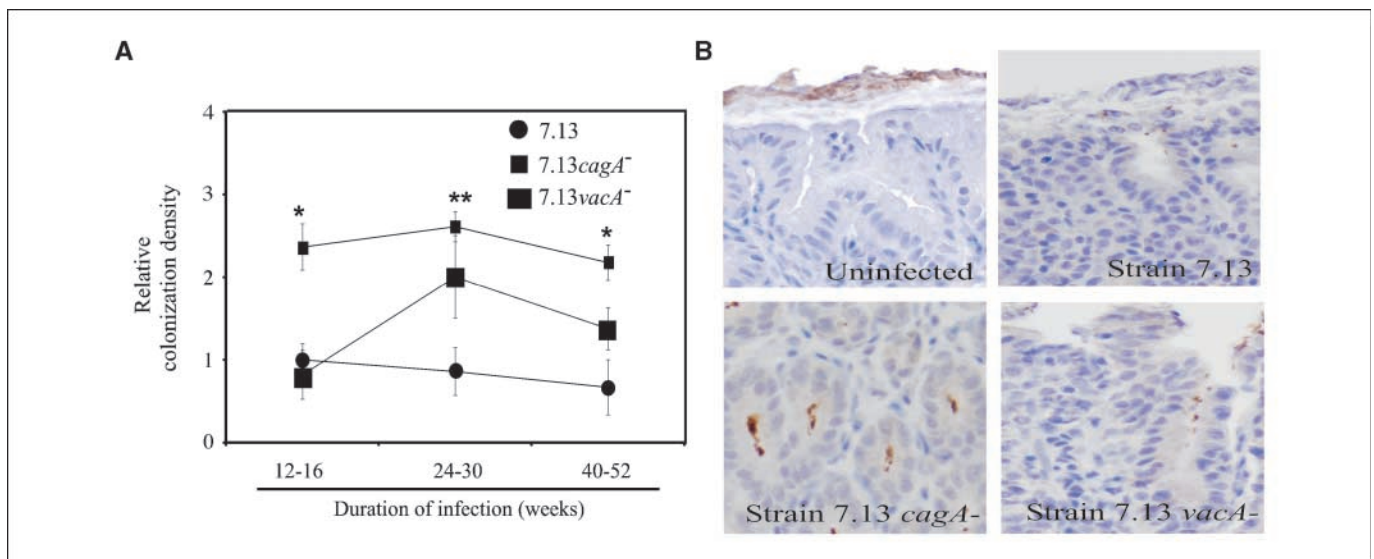


Figure 1. Colonization density within antral mucosa of Mongolian gerbils challenged with sterile *Brucella* broth or *H. pylori* strains 7.13, 7.13 *cagA*⁻, or 7.13 *vacA*⁻ for 12 to 52 wk. **A**, colonization density was measured by histologic examination as described in the text. No *H. pylori* were identified in any broth-challenged controls. Points, mean; bars, SE. *, $P \leq 0.05$ compared with *H. pylori* strain 7.13 and 7.13 *vacA*⁻. **, $P \leq 0.05$ compared with *H. pylori* strain 7.13. **B**, representative *H. pylori* immunohistochemical stains are shown for each treatment group at 12 wk postchallenge; magnification, $\times 20$.

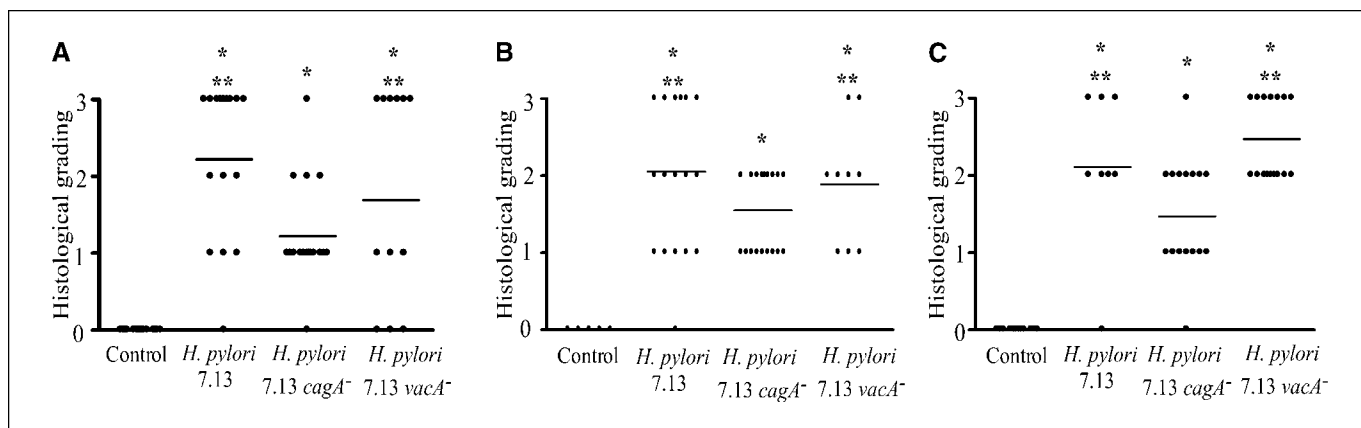


Figure 2. Severity of inflammation and injury within the gastric antra of Mongolian gerbils inoculated with *H. pylori* is dependent on CagA. Comparison of gastric antral inflammation in Mongolian gerbils infected with broth alone or with *H. pylori* strains 7.13, 7.13 *cagA*⁻, or 7.13 *vacA*⁻ for 12 to 16 wk (A), 24 to 30 wk (B), and 40 to 52 wk (C). Inflammation was significantly attenuated in gerbils infected with the *H. pylori* 7.13 *cagA*⁻ mutant compared with wild-type 7.13 for all time points. Inflammation was determined by histologic examination, as described in the text, and data are presented as scatter plots with mean values. *, $P \leq 0.05$ for infected gerbils compared with broth control. **, $P \leq 0.05$ for strain 7.13 and 7.13 *vacA*⁻ compared with the *H. pylori* 7.13 *cagA*⁻ mutant strain.

There were no statistically significant differences in the development of dysplasia or cancer between gerbils infected with wild-type or *vacA*⁻ mutant *H. pylori* at any time point. Gastric lesions in gerbils infected with wild-type 7.13 or the *vacA*⁻ mutant were histologically indistinguishable. Tumors were characterized by irregular, angulated, dilated glands invading into the submucosa and muscularis propria and were accompanied by surface antral dysplasia, marked chronic active gastritis, and occasional abscess formation (Fig. 4). In contrast, dysplasia or adenocarcinoma was not observed in any of the gerbils infected with the *cagA*⁻ *H. pylori* mutant at any time point (Fig. 4). These results suggest that CagA, along with severe inflammation, is essential for the development of gastric dysplasia and adenocarcinoma in this model of *H. pylori*-induced gastric cancer.

Effect of OipA on *H. pylori*-induced injury in vitro and in Mongolian gerbils. Adherence is critical for *H. pylori*-induced

injury, and we have previously shown that strain 7.13 adheres more avidly to gastric epithelial cells *in vitro* than its progenitor noncarcinogenic strain B128 (4). Because OipA is an *H. pylori* OMP that mediates adherence (13–15), we next focused on the role of this virulence-associated *H. pylori* OMP in gastric carcinogenesis.

We first sought to determine if OipA was expressed by carcinogenic strain 7.13 or its noncarcinogenic parental strain B128. Western blot analysis showed that OipA was selectively expressed by the carcinogenic strain 7.13 (Fig. 5A). Because OipA expression is regulated by the number of tandem CT dinucleotide repeats located in the 5' region of the gene (13, 16), we performed sequence analysis of *oipA* in strains 7.13 and B128. These results revealed that *oipA* was in frame and "on" in strain 7.13 but was out of frame in strain B128 due to a two bp insertion in the 5' CT-rich repeat region, resulting in translation of a premature

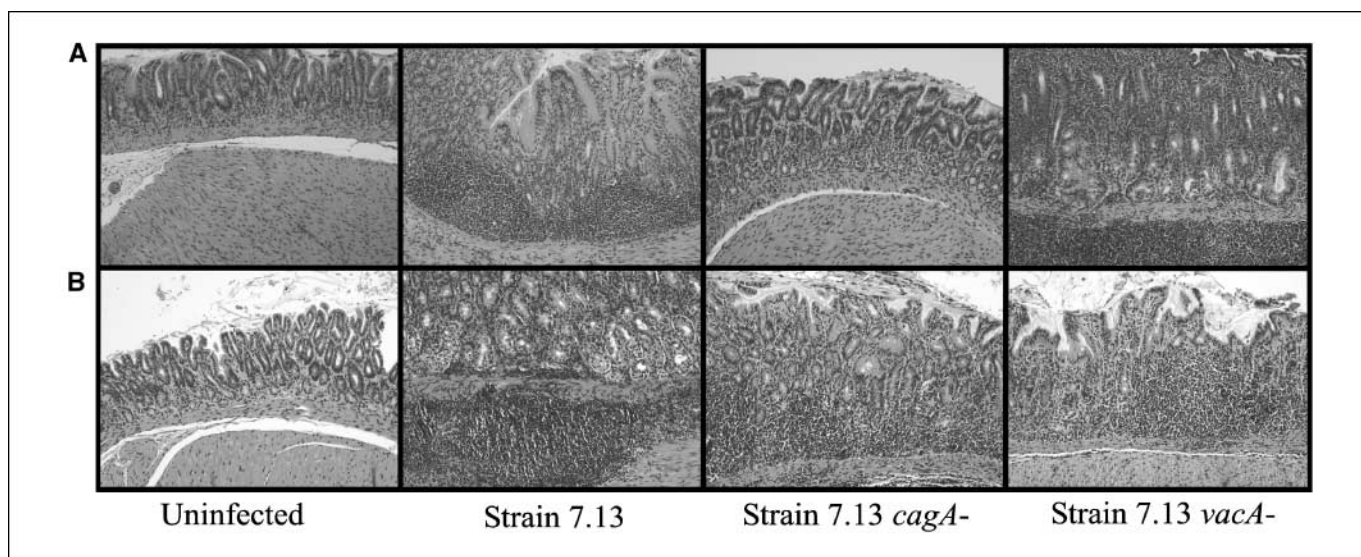


Figure 3. Gastric inflammation and injury in gerbils infected with or without *H. pylori* for 12 to 52 wk. Representative H&E stains are shown for (A) 16 wk and (B) 52 wk postinfection. Magnification, $\times 10$.

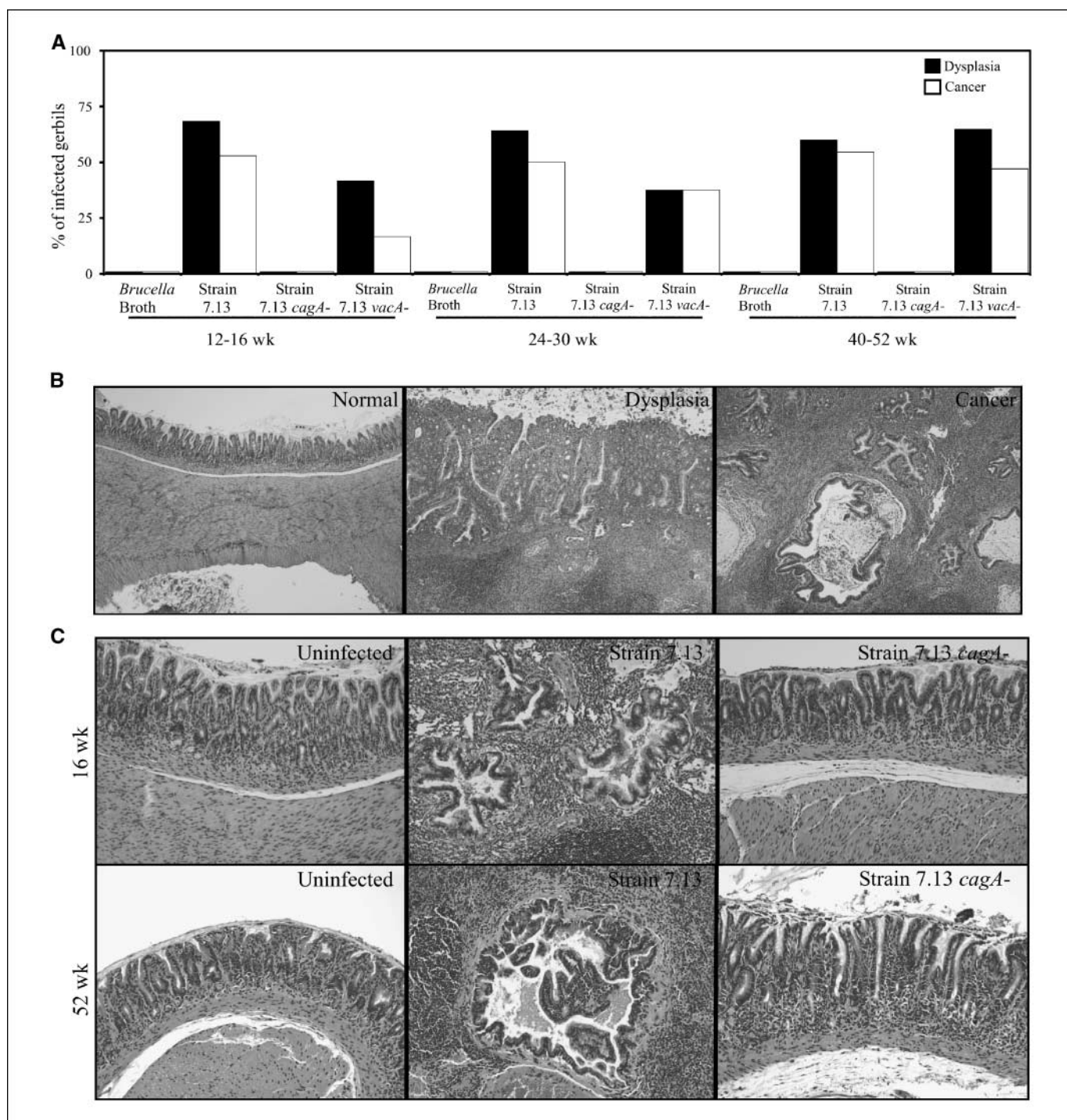


Figure 4. The development of gastric dysplasia and adenocarcinoma is dependent on the bacterial virulence factor CagA. **A**, chronology of gastric dysplasia (black columns) and adenocarcinoma (white columns) in Mongolian gerbils challenged with broth alone or with *H. pylori* strains 7.13, 7.13 *cagA*⁻, or 7.13 *vacA*⁻. Results are expressed as a percentage of infected gerbils with dysplasia or adenocarcinoma at 12 to 16 wk, 24 to 30 wk, and 40 to 52 wk postinoculation. **B**, representative H&E stains are shown for the progression of gastric injury from normal mucosa to dysplasia to adenocarcinoma with invasion through the submucosa; magnification, $\times 4$. **C**, invasive carcinoma developed in gerbils infected with *H. pylori* strain 7.13 as early as 16 wk postchallenge and persisted through 52 wk. No dysplasia or adenocarcinoma developed in gerbils infected with strain 7.13 *cagA*⁻ at any time point. No gastric injury was seen in uninfected control gerbils. Magnification, $\times 10$.

stop codon. The remaining *oipA* sequence between the two strains was identical.

oipA expression is significantly associated with the presence of the *cag* pathogenicity island (13–15, 28). Because OipA has recently

been shown to also mediate adherence of *H. pylori* to gastric epithelial cells (14, 15), we next compared binding avidity and CagA translocation efficiency between wild-type strain 7.13 and a 7.13 *oipA*⁻ isogenic mutant strain. We first examined CagA expression

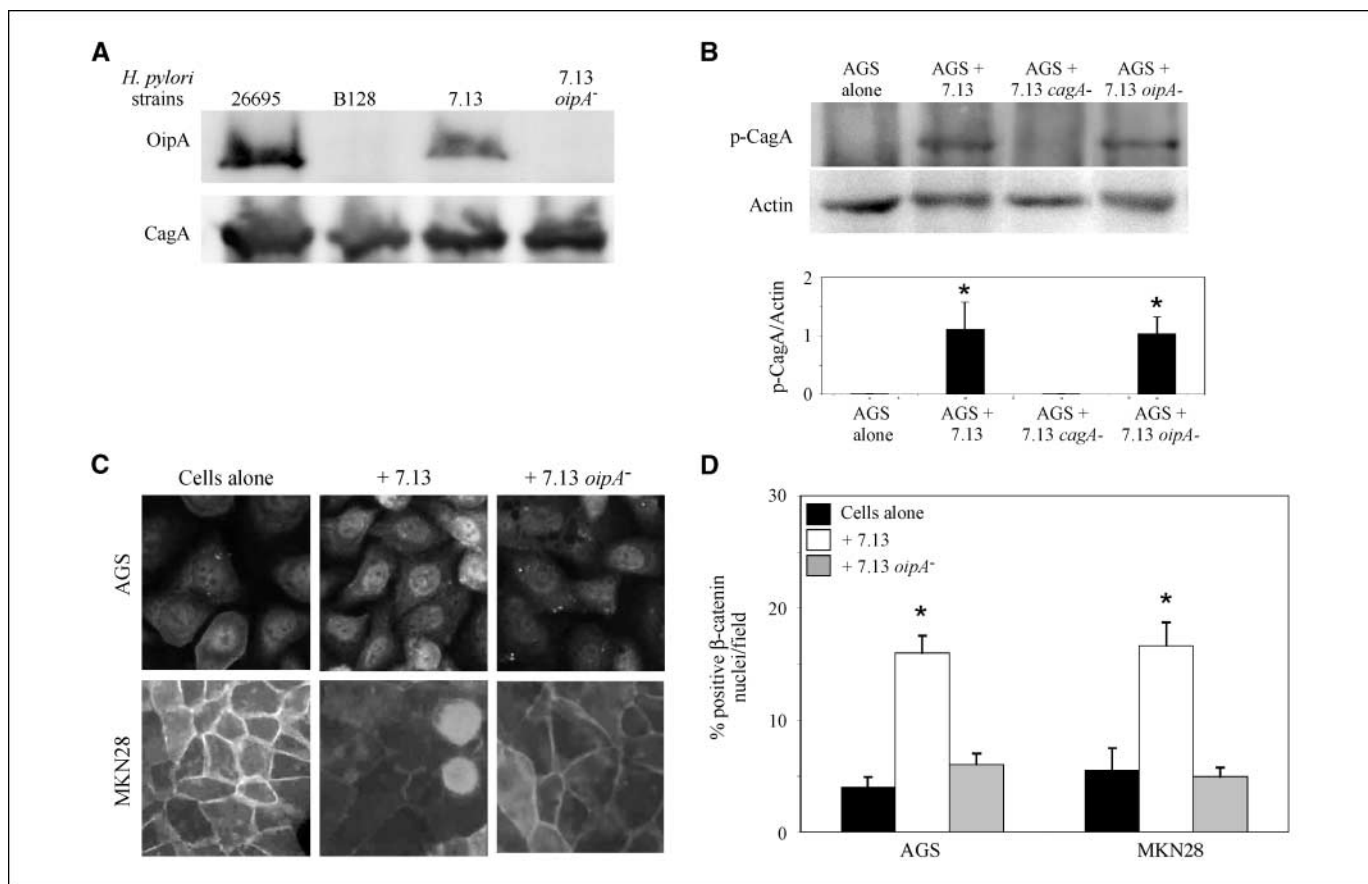


Figure 5. OipA is expressed in carcinogenic *H. pylori* strain 7.13 and is linked to β -catenin nuclear localization. **A**, lysates from *H. pylori* strains 26695 (positive control), B128, 7.13, and the 7.13 *oipA*⁻ mutant grown overnight in broth cultures were used for Western blot analysis using an anti-OipA or an anti-CagA antibody. **B**, lysates from AGS cells cocultured with broth alone, *H. pylori* strain 7.13, or the 7.13 *cagA*⁻ or *oipA*⁻ mutant strains were used for Western blot analysis using antiphospho-tyrosine or antiactin antibodies. A representative blot is shown. Densitometric analysis of multiple Western blot repetitions performed on at least three occasions is shown below representative blot. Columns, mean; bars, SD. *, $P < 0.05$ versus AGS cells alone. **C**, AGS or MKN28 human gastric epithelial cells incubated with medium alone, strain 7.13, or the 7.13 *oipA*⁻ mutant for 6 h (AGS cells) or 24 h (MKN28 cells) were immunostained with an anti- β -catenin antibody and visualized by fluorescence microscopy. Representative images are shown. **D**, AGS or MKN28 cells were cultured in the absence or presence of *H. pylori* strain 7.13 or its isogenic *oipA* mutant derivative for 6 h (AGS cells) or 24 h (MKN28 cells). The percentage of cells with nuclear β -catenin is shown for each sample per 100 cells evaluated. Columns, mean; bars, SD. *, $P < 0.05$ versus uninfected gastric cells alone.

in the wild-type strain 7.13 and *oipA*⁻ mutant by Western blot analysis (Fig. 5A). There were no differences in levels of CagA expression between the two strains, indicating that any alterations in phenotype induced by inactivation of *oipA* were not due to a decrease in CagA expression. We found that, similar to previous reports (14, 15), wild-type strain 7.13 binds significantly more avidly than its *oipA*⁻ mutant to AGS gastric epithelial cells (data not shown). Of interest, however, inactivation of *oipA* did not alter CagA phosphorylation (Fig. 5B), findings that are consistent with a recent report investigating the effect of *oipA* disruption on CagA phosphorylation (14).

Previous studies have shown that β -catenin nuclear translocation is dependent on the presence of CagA within host cells (4, 5, 7). Because *oipA* expression is associated with the presence of the *cag* island, we next determined if loss of *oipA* alters β -catenin nuclear localization induced by wild-type strain 7.13. AGS or MKN28 human gastric epithelial cells were infected with *H. pylori* strain 7.13, the 7.13 *oipA*⁻ mutant, or medium alone. β -catenin cytosolic and nuclear staining increased in each cell line after infection with strain 7.13 (Fig. 5C). In contrast, compared with parental wild-type *H. pylori* strain 7.13, the 7.13 *oipA*⁻ mutant had a significantly

($P = 0.013$) reduced capacity to induce cytosolic or nuclear translocation of β -catenin (Fig. 5C and D), and β -catenin localization was similar in uninfected and *H. pylori oipA*⁻ mutant-infected cells (Fig. 5C). Because our data in Fig. 5A and B show that inactivation of *oipA* does not alter CagA expression or phosphorylation, these findings indicate that *H. pylori*-induced β -catenin activation requires multiple *H. pylori* disease-associated virulence constituents.

We next sought to define the role of OipA in our rodent model of carcinogenesis by infecting Mongolian gerbils with wild-type strain 7.13 or its *oipA*⁻ derivative for 12, 16, and 24 weeks. Neither colonization efficiency nor density was statistically different between the two treatment groups (data not shown). Further, there were no differences in severity of inflammation between the two groups at these time points (Fig. 6A). All gerbils developed moderate-to-marked chronic active gastritis with frequent lymphoid follicle formation and occasional ulceration (Fig. 6A). However, there was a significant attenuation ($P = 0.0026$) in the development of dysplasia and carcinoma in gerbils infected with the *oipA*⁻ mutant compared with the wild-type strain (Fig. 6B). At 16 weeks postchallenge, 56% of gerbils infected with wild-type

strain 7.13 developed dysplasia and/or adenocarcinoma versus 9% of gerbils infected with the *oipA*⁻ mutant, which only developed dysplasia. Cancer did not develop in gerbils infected with the 7.13 *oipA*⁻ mutant until 24 weeks postinfection, at which time, 27% had adenocarcinoma, compared with 44% of gerbils infected with the wild-type strain 7.13. All cancers that developed in gerbils infected with the 7.13 *oipA*⁻ mutant strain were focal and involved only the superficial submucosa, compared with more widely extensive lesions that developed in gerbils infected with the wild-type 7.13 strain. These results indicate that loss of OipA significantly attenuated the development of adenocarcinoma and lessened the severity of lesions that were observed.

***H. pylori* OipA expression is associated with gastric carcinogenesis in humans.** To more completely understand the role of OipA in gastric carcinogenesis, we focused on the natural niche of *H. pylori* and examined OipA expression and risk of gastric cancer precursor lesions among a population of *H. pylori* isolates harvested from persons residing in Colombia, South America. *H. pylori*-infected subjects were stratified into those with gastritis only ($n = 9$) or those with gastric atrophy or intestinal metaplasia ($n = 13$), lesions known to confer an increased risk for gastric cancer. Of the 9 strains isolated from persons with gastritis only, 2 (22%) expressed OipA versus 8 (62%) of 13 strains harvested from patients with gastric atrophy or intestinal metaplasia ($P = 0.037$; Fig. 6C). The concordance of these findings with our animal results suggests that OipA plays a role in *H. pylori*-induced gastric carcinogenesis.

Discussion

A biological consequence of long-term *H. pylori* colonization is an increased risk of gastric adenocarcinoma. Our current results have identified a potential mechanism that may help explain the increased cancer risk conferred by *H. pylori* by capitalizing on a rodent model that recapitulates human disease, using an *H. pylori* strain that rapidly induces gastric cancer, defining the bacterial constituents required for these effects, and extending these results into the natural niche of this pathogen (human gastric mucosa).

H. pylori strains that possess a functional *cag* island clearly increase disease risk, and our current results show that the effector molecule encoded by this locus (CagA) is required for induction of dysplasia and adenocarcinoma in gerbil gastric mucosa. These findings extend the results of Shibata et al. and Rieder et al. (29, 30) who showed that CagA was related to the severity of gastritis in infected gerbils. Our current findings have not only confirmed that CagA is required for the development of severe inflammation but also for dysplasia and cancer. Further, our results now implicate OipA as a bacterial factor that may also play a role in cancer development.

Previous studies have reported a strong association between OipA expression and presence of the *cag* island (13–15, 28), and in *cag*⁺ strains, *oipA* is typically “on”, eventuating in expression of the full-length OipA protein. Our current data, as well as those from other investigators (14), show that OipA does not facilitate phosphorylation of intracellular CagA. Our results do,

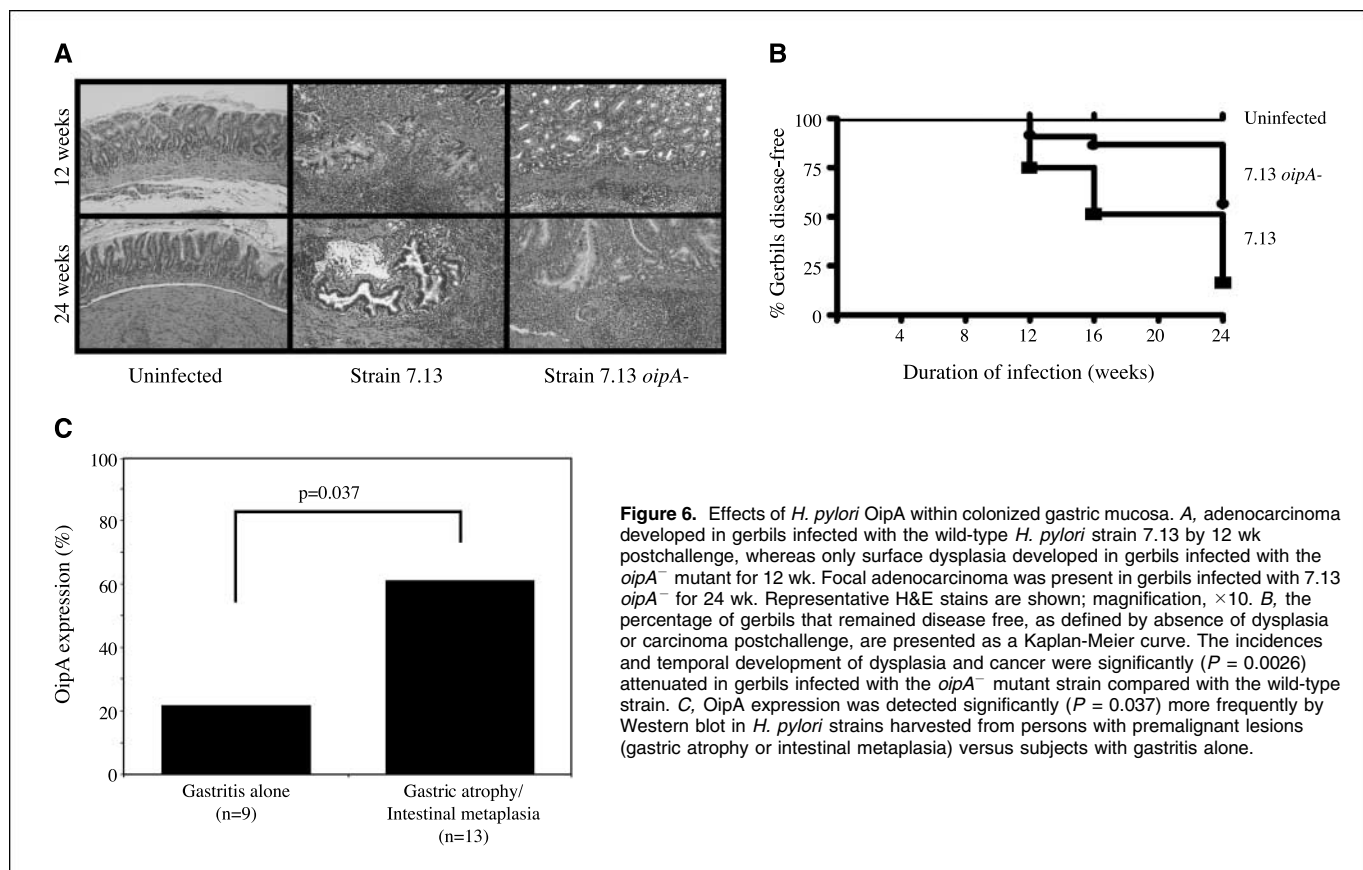


Figure 6. Effects of *H. pylori* OipA within colonized gastric mucosa. **A**, adenocarcinoma developed in gerbils infected with the wild-type *H. pylori* strain 7.13 by 12 wk postchallenge, whereas only surface dysplasia developed in gerbils infected with the *oipA*⁻ mutant for 12 wk. Focal adenocarcinoma was present in gerbils infected with 7.13 *oipA*⁻ for 24 wk. Representative H&E stains are shown; magnification, $\times 10$. **B**, the percentage of gerbils that remained disease free, as defined by absence of dysplasia or carcinoma postchallenge, are presented as a Kaplan-Meier curve. The incidences and temporal development of dysplasia and cancer were significantly ($P = 0.0026$) attenuated in gerbils infected with the *oipA*⁻ mutant strain compared with the wild-type strain. **C**, OipA expression was detected significantly ($P = 0.037$) more frequently by Western blot in *H. pylori* strains harvested from persons with premalignant lesions (gastric atrophy or intestinal metaplasia) versus subjects with gastritis alone.

however, indicate that OipA is required for β -catenin nuclear localization, suggesting that different *H. pylori* constituents can induce nuclear translocation of β -catenin. The specific mechanism(s) through which OipA alters β -catenin signaling are currently under investigation in our laboratory and may involve signaling pathways previously reported to be used by other bacteria. For example, coculture of intestinal epithelial cells with nonpathogenic *Salmonella* leads to activation of β -catenin signaling via AvrA-mediated blockade of β -catenin ubiquitination (31, 32). *Bacteroides fragilis* toxin induces nuclear accumulation of β -catenin in human intestinal cells (33), and bacterial lipopolysaccharide can stimulate nuclear localization of β -catenin in myeloid cells via toll-like receptor 4 (TLR-4)-dependent activation of phosphatidylinositol-3-OH kinase, which subsequently inhibits glycogen synthase kinase 3 β , thereby increasing steady-state levels of free β -catenin (34). Previous data have also shown that treatment of cells with growth factors, such as epidermal growth factor (EGF), resulting in transactivation of the EGF receptor, culminates in increased activity of β -catenin (35), and *H. pylori* *cag*⁺ strains have been shown to selectively transactivate the EGF receptor in a CagA-independent manner (36). Collectively, these data have provided a framework for future studies to define the specific mechanisms through which OipA induces nuclear localization of β -catenin using our *in vitro* and *in vivo* model systems in an effort to provide more detailed insights into *H. pylori*-induced gastric carcinogenesis.

Our human data also support the relationship between OipA and the development of gastric cancer, as OipA expression was significantly related to the presence of gastric cancer precursor lesions among *H. pylori*-infected individuals. Previous studies have suggested that in certain strains of *H. pylori*, OipA may function as a colonization factor. For example, Akanuma et al. (37) reported that an *H. pylori* *oipA*⁻ mutant was unable to colonize gerbils at a 3-week time point. However, this likely reflects differences in *H. pylori* strains used for inoculation as our results in gerbils in conjunction with our results in humans, demonstrating that clinical isolates can clearly lack OipA expression, suggest that OipA is likely not required for colonization or persistence in the gastric mucosa.

The vast majority of *H. pylori* in colonized hosts are free living; however, ~15% to 20% bind specifically to gastric epithelial cells, and adherence is required for prolonged persistence in the stomach and for induction of injury and disease (38, 39). Upon initial inspection, it seems that our *in vitro* and *in vivo* results related to OipA and adherence are not concordant, as inactivation of *oipA* led to an attenuation of *H. pylori* binding to gastric cells *in vitro* but no difference in colonization density within gerbil mucosa. However, the effects of *H. pylori* *in vitro* and *in vivo* can differ considerably. In contrast to isolated cell culture systems that contain only *H. pylori* and gastric epithelial cells, additional factors within the stomach including gastric peristalsis, gastric mucus, and mucosal inflammation may influence the effects of *H. pylori* virulence constituents on adherence. Further, within the stomach, only a fraction of the total *H. pylori* population directly bind to epithelial cells, and our *in vivo* studies that quantified colonization density (histologic examination and quantitative culture) are not optimal for discriminating bacteria bound to gastric epithelial cells versus bacteria free living in the mucus gel layer. Such discrimination would require more sensitive imaging techniques (e.g., electron microscopy). Previous studies have

shown that OipA-mediated binding can be affected by the ratio of bacteria to epithelial cells, which may also alter the level of *H. pylori* binding in the stomach (15). Thus, our results cannot exclude the possibility that inactivation of *oipA* decreases the number of *H. pylori* that adhere to gastric epithelial cells in the stomach.

Although human epidemiologic studies have clearly linked VacA to gastric cancer, our results in gerbils are consistent with a previous study demonstrating that inactivation of *vacA* does not alter *H. pylori*-induced gastric carcinoma in this particular rodent model of disease (40). *In vitro*, VacA exerts multiple effects on epithelial cells, which may potentially influence transformation. VacA can bind to a receptor-type protein tyrosine phosphatase, RPTP β , which regulates cellular proliferation, differentiation, and adhesion to the extracellular matrix (41), events that may lower the threshold for carcinogenesis. VacA has also been shown to induce apoptosis in cell culture systems (10), which could potentially accelerate the progression to gastric atrophy. However, as previously mentioned, the effects of *H. pylori* on gastric epithelial responses *in vitro* and *in vivo* can differ considerably. For example, *H. pylori* decreases epithelial cell proliferation *in vitro* (42) in contrast to increased rates of cell growth found within *H. pylori*-infected gastric mucosa (43).

In addition to inherent differences in model systems, other factors likely underpin our inability to detect an effect of VacA on cancer development. Several *H. pylori* genetic loci, including the *cag* pathogenicity island, *babA*, and *vacA* have been identified for which persons harboring particular alleles have differing risks of gastric cancer. However, not all persons infected with *cag*⁺ *babA*⁺ toxigenic strains develop gastric cancer, suggesting that *H. pylori* constituents are not absolutes but instead reflect degrees of risk. This has highlighted the importance of host factors that influence disease, and in support of this, several studies have shown that polymorphisms within host immune response genes such as interleukin (IL)-1 β , tumor necrosis factor- α , IL-10, IL-8, and TLR-4 also heighten the risk for gastric cancer precursor lesions or adenocarcinoma (44–50). These data suggest that cancer risk is the summation of multiple factors including the polymorphic nature of the bacterial population in the host, the host genotype, and environmental exposures, each affecting overall risk for cancer development, which may help to explain the inability of VacA *per se* to influence gastric carcinogenesis in this model.

In summary, these findings show that chronic *H. pylori*-induced gastric inflammation alone is not sufficient for transformation to occur in Mongolian gerbils, and that *H. pylori* virulence factors play a critical role in carcinogenesis in this model. This study also illustrates the complex interplay of microbial factors that contribute to neoplasia in the context of inflammatory states and provides a model for understanding malignancies that arise within foci of chronic inflammation.

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