Helicobacter pylori Infection Induces Genetic Instability of Nuclear and Mitochondrial DNA in Gastric Cells

Ana Manuel Dantas Machado,1,2 Ceu Figueiredo,2,3 Eliette Touati,4 Valdemar Máximo,2,3 Sonia Sousa,2 Valérie Michel,4 Fátima Carneiro,2,3 Finn Cilius Nielsen,5 Raquel Seruca,2 and Lene Juel Rasmussen1

Abstract Purpose: Helicobacter pylori is a major cause of gastric carcinoma. To investigate a possible link between bacterial infection and genetic instability of the host genome, we examined the effect of H. pylori infection on known cellular repair pathways in vitro and in vivo. Moreover, various types of genetic instabilities in the nuclear and mitochondrial DNA (mtDNA) were examined.

Experimental Design: We observed the effects of H. pylori infection on a gastric cell line (AGS), on C57BL/6 mice, and on individuals with chronic gastritis. In AGS cells, the effect of H. pylori infection on base excision repair and mismatch repair (MMR) was analyzed by reverse transcription-PCR, Western blot, and activity assays. In mice, MMR expression was analyzed by reverse transcription-PCR and the CA repeat instabilities were examined by Mutation Detection Enhancement gel electrophoresis. Mutation spectra in AGS cells and chronic gastritis tissue were determined by PCR, single-stranded conformation polymorphism, and sequencing. H. pylori vacA and cagA genotyping was determined by multiplex PCR and reverse hybridization.

Results: Following H. pylori infection, the activity and expression of base excision repair and MMR are down-regulated both in vitro and in vivo. Moreover, H. pylori induces genomic instability in nuclear CA repeats in mice and in mtDNA of AGS cells and chronic gastritis tissue, and this effect in mtDNA is associated with bacterial virulence.

Conclusions: Our results suggest that H. pylori impairs central DNA repair mechanisms, inducing a transient mutator phenotype, rendering gastric epithelial cells vulnerable to the accumulation of genetic instability and thus contributing to gastric carcinogenesis in infected individuals.

The Gram-negative bacterium Helicobacter pylori colonizes the gastric mucosa of half of the world’s population (1). H. pylori induces chronic gastric inflammation, which can progress through chronic atrophic gastritis, intestinal metaplasia, and dysplasia toward gastric carcinoma. Only a small number of H. pylori-infected individuals develop tumors (1, 2) and complex interactions between bacterial and host factors may contribute to the development of gastric carcinoma (1, 3).

H. pylori virulence factors include the cag pathogenicity island (cag PAI), encoding a type IV secretion system, which translocates CagA into the host epithelial cells following bacterial attachment (1). The bacterial CagA effector protein interferes with host cell functions such as growth, adhesion, motility, and invasion (4–6). VacA cytotoxin is another virulence factor that is inserted in the host cell membrane inducing, among other cellular effects, cytoplasmic vacuolation (1). However, the activity of the cytotoxin differs considerably among strains because of sequence variations in the vacA gene (1). Combination of the signal sequence s1 with midregion m1 is considered the genotype most associated with disease (1). H. pylori strains containing a functional type IV secretion system, CagA-positive and VacA-cytotoxic, are frequently associated with increased risk for gastric carcinoma development, as well as for its precursor lesions (1, 7).

Experimental data suggest that H. pylori infection induces DNA damage and mutation and/or inhibits DNA repair (8–11). H. pylori also induces hypoxia-inducible factor-1α (12) that in turn inhibits mismatch repair (MMR) activity by decreasing both hMSH2 and hMSH6 expression (13).

Human MMR is initiated via the recognition of a mismatch by one of two protein complexes, hMSH2/hMSH6 or hMSH3/hMSH2.
hMSH2/hMSH3 (14), which subsequently recruit the hMLH1/hPMS2 complex (14). Defects in human DNA MMR result in genetic instability and predisposition to several types of epithelial cancer (15). Microsatellite instability (MSI) is a hallmark of MMR-deficient cells and is frequently encountered in colorectal and gastric adenocarcinomas (14, 16). Besides the role of nuclear DNA mutations, mutations in the mitochondrial DNA (mtDNA) are common in a broad range of cancers, including gastric carcinoma (17, 18). Mutations in the mtDNA may have severe effects on the mitochondrial oxidative phosphorylation pathway (17). Repair of mtDNA has not been fully characterized but is known to involve multiple pathways (19). The base excision repair (BER) is initiated by DNA glycosylases, such as hOGG1, which recognize and remove the damaged base leaving an abasic (AP) site. hAPE1 follows and cleaves the AP site, giving way to the other enzymes that will insert and ligate new nucleotides to the DNA chain (20).

To clarify the relation between H. pylori infection and genomic instability, we analyzed the status of major DNA repair activities in infected human cells and in mice. We show that nuclear MMR activity is down-regulated on infection. In addition, we show that H. pylori infection induces DNA damage and/or inhibits DNA repair. We believe that our article will be of interest to Clinical Cancer Research because it adds novelty to the field of H. pylori pathogenesis by showing that H. pylori infection, both in vivo and in vitro, induces a decrease in repair activity and a transient mutator phenotype, contributing to epithelial gastric genomic instability and to its neoplastic transformation.

Human AGS gastric adenocarcinoma cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, 200 μg/mL streptomycin, and 200 IU/mL penicillin (Invitrogen) at 37°C under 5% CO2 humidified atmosphere. Infections were done with H. pylori strains 26695 (ATCC 700392, cag PAI−, VacA s1/m1), and H. pylori insertion mutants with inactivation of the vacA (60190vacA−), cagE (60190cagE−), or vacA (60190vacA−) genes. The SS1 strain, able to chronically colonize the mouse stomach, was used for in vivo experiments. Bacteria were grown in trypticase soy agar supplemented with 5% sheep blood (BioMérieux) and incubated for 48 h at 37°C under microaerophilic atmosphere.

**Infection of gastric cells**
For the 24 h infection, 80% confluent AGS cells were washed three times in PBS and incubated in antibiotic-free medium. Control groups (48 h) of H. pylori were collected and the bacterial cells were added to the monolayer at a multiplicity of infection (MOI) of 100 bacteria per cell. Cultures were maintained at 37°C under a 5% CO2 humidified atmosphere.

For the 5-day infection, 30% confluent AGS cells were treated similarly as for the 24 h infection. Every 24 h, cells were washed three times with PBS and fresh medium and bacteria were added at a MOI of 10. In parallel experiments, 6 × 10⁶ mol/L H2O2 was added to AGS cells every 24 h. Control cells were processed similarly in the absence of bacteria.

**Infection of mice**
Six-week-old specific pathogen-free C57BL/6 male mice (Charles River Laboratories) were infected orogastrically once with 10⁶ colony-forming units of H. pylori strain SS1 (n = 15). Control groups of uninfected mice (n = 15) were given peptone trypsin broth alone. After 3, 6, and 12 months, 5 uninfected mice and 5 H. pylori SS1-infected mice were sacrificed. The serologic H. pylori status was controlled for each mouse and stomach was isolated as already described (11).

**Patients with chronic gastritis**
In total, 99 H. pylori-infected individuals with chronic gastritis (94 males and 5 females; age, 43.6 ± 6.2 years; range, 30-62 years) were studied. Individuals were recruited among shipyard workers who had undergone standard gastroscopy as part of a screening program for premalignant lesions of the gastric mucosa. Individuals with evidence of past or present peptic ulcer disease were excluded from this study. All procedures were in accordance with the institutional ethical standards. All samples were delinked and unidentified from their donors. All individuals provided written informed consent.

**Histology**
Biopsy specimens from antral and corpus mucosa and surgical specimens were formalin-fixed, embedded in paraffin, and stained with H&E, Alcian blue-periodic acid Schiff, and modified Giemsa. Only cases with adequately sized biopsy specimens of both antral and corpus mucosa were accepted for histologic assessment according to the updated Sydney system (21). Histologic slides were examined by an experienced pathologist (F.C.) blinded for the clinical information of the patients.

**Analysis of gene expression**
RNA was extracted using either the TriPure Isolation Reagent (Roche Diagnostics) or the TRIzol extraction (Invitrogen) and purified on RNeasy mini-columns (Qiagen). cDNA was synthesized using random hexamers and the Advantage RT-for-PCR Kit (Clontech). Gene expression was analyzed by reverse transcription-PCR using primers shown in Supplementary Table S1 as well as described elsewhere (22, 23). Amplified fragments were visualized by electrophoresis, quantified with the Quantity One Software (Bio-Rad), and normalized to GAPDH expression.

**Analysis of protein expression**
Protein levels were analyzed by Western blots. Proteins were detected with polyclonal antibodies against hMLH1 (Calbiochem), hMSH2 (Calbiochem), hMSH6 (Santa Cruz Biotechnology), or actin (Neomarkers). Proteins were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).
Proliferation and apoptosis assay

The proliferative indexes were determined as a function of 5-bromodeoxyuridine incorporation (24). The TUNEL assay (In situ Cell Death Detection Kit; Roche Diagnostics) was used to detect apoptotic cell death. The percentages of 5-bromodeoxyuridine-positive cells and apoptotic cells were determined using a Leica DM IRE2 fluorescence microscope.

Analysis of MMR activity

MMR activity in extracts of AGS cells uninfected or infected with H. pylori for 5 days was assayed using a 2-bp loop heteroduplex substrate as described previously (25). Plaques were scored as white, blue, or mixed (mixed plaques signify no repair). Repair efficiency was determined with the following: repair efficiency (%) = 100 \times [1 - (% mixed plaques in extract-treated sample) / (% mixed plaques in extract-untreated sample)].

Analysis of nuclear MSI in vitro.

After 5 days of infection, DNA was extracted using the SDS/protease K method. Nuclear MSI status was determined using the HNPPCC MSI Test kit (Roche Diagnostics) according to the manufacturer’s instructions. For each sample, 100 ng genomic DNA was used as a template to amplify the microsatellite loci D5S346 (APC), BAT25, BAT26, D17S250 (Mfd15CA), and D2S123 by PCR. The PCR products were analyzed using an ABI 377 sequence analyzer and GeneScan 3.0 (Perkin-Elmer) software.

Analysis of mtDNA instability in vitro.

After 5 days of infection, DNA was extracted using the SDS/protease K method to determine the frequency of mutations in the D-loop region, comprising the D310 [C6] and D514 [CA5] repeats as well as in the complex I ND1 dehydrogenase subunit 1 (ND1), complex IV cytochrome c oxidase subunit I (COI), and complex V ATP synthase subunit 6 (ATPas6) genes. PCR amplifications were done using primers shown in Supplementary Table S1. The amplified fragments were cloned into the pCR2.1 vector (Invitrogen). Approximately 90 white colonies were selected as well as 2 blue colonies as control. Colony PCR amplifications were done using the following primers: forward 5’-CAGGAAACAGCTATGACCAT-3’ and reverse 5’-GTAATACGACTCAC-TATAGGCC-3’. The amplified fragments were purified and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and an ABI Prism 377 DNA Sequencer (Perkin-Elmer). To determine mismatches, the sequences were used as queries against a mitochondrial database (6).

Analysis of nuclear DNA instability in CA repeats loci in vivo.

Genomic DNA was isolated from stomach of mice infected and noninfected and sacrificed after 6 and 12 months as described previously (11). Instabilities at the D6Mit59, D7Mit91, and D19Mit36 loci were evaluated using a PCR-based assay (26) using primer sequences determined from the Mouse Genome Informatics database (7). The PCR products were separated on a Mutation Detection Enhancement gel (BioWhittaker Molecular Applications) gel in the presence of 15% urea and 5% glycerol. The DNA bands were visualized by silver-nitrate staining (Amersham Biosciences). To carry out the Mutation Detection Enhancement gel analysis in CA repeats loci, DNA was isolated from the stomach of 5 mice infected or noninfected and sacrificed at 6 and 12 months as described previously (11). For each analyzed condition, a pool of DNA containing DNA from each of the 5 mice in equal proportion was done. Each lane of the gels reported in the Fig. 2D corresponds to one pool of DNA.

Analysis of mtDNA instability in vivo.

DNA was extracted from gastric antral biopsy specimens using the method described by Boom et al. (27). Briefly, biopsy specimens were homogenized in guanidinium isothiocyanate with a sterile micropestle. DNA was captured onto silica particles, washed, and then eluted in 100 μL of 10 mmol/L Tris-HCl (pH 8.3). DNA from blood samples was isolated using a standard proteinase K digestion and phenol/chloroform extraction procedure.

---

6 http://www.mitomap.org
7 http://www.informatics.jax.org
Six mitochondrial genes encoding ND1, ND3, ND4, ND5, COI, and ATPase6, as well as two regions of the D-loop, D310 [C6] and D514 [CA5], were analyzed by the PCR single-stranded conformation polymorphism method as described previously (18). All cases showing band shifts were submitted to a second analysis (new PCR amplifications and single-stranded conformation polymorphism analysis using the initial DNA samples), and only reproducible bands were considered. Only cases with mtDNA alterations in the biopsy specimen and not in blood DNA were considered to harbor somatic mutations.

Abnormal bands, as well as the corresponding normal bands, detected by single-stranded conformation polymorphism were recovered from Mutation Detection Enhancement gels and submitted to PCR reamplification with the original set of primers. Reamplification products were purified and sequenced as described previously. Sequencing was done in both strands using the original primers.

H. pylori vacA and cagA genotyping

H. pylori vacA and cagA genotyping was done by multiplex PCR and reverse hybridization on a line probe assay as described earlier (28). Strains were grouped as the highest (vacA s1m1 cagA+) or lowest (vacA s2m2 cagA-) virulence-associated genotype. Cases infected with multiple H. pylori strains (n = 20) or infected with genotypes that could not be assigned to the highest or low virulence group (n = 6) were not included in the analysis.

Statistical analysis

Associations between H. pylori infection and the presence and type of mtDNA mutations as well as the frequencies of mtDNA mutations were assessed by the x^2 and Fisher’s exact test. MMR, hypoxia-inducible factor-1α, and BER levels, MMR activity, and proliferation and apoptosis assay results were expressed as mean ± SD of at least two independent experiments and analyzed by Student’s t test. The differences between data sets were considered significant at P values < 0.05.

Results

Analysis of MMR gene expression in vitro.

Previous reports using cells infected at high MOI have suggested that H. pylori affect MMR gene expression and cause increased cell death as well as growth inhibition (8, 10). Expression of MMR proteins are affected by cell growth (29) and bacterial-induced growth inhibition could lead to decreased MMR expression. To minimize the effect of H. pylori-induced cell death, we infected human gastric carcinoma AGS cells with the virulent H. pylori strains 60190 and 26695 for 24 h at a MOI of 100 or for 5 days at a MOI of 10. After 24 h of infection, there was a small, if any, decrease in MMR mRNA levels compared with control cells, particularly for hMSH2 and hMSH6 genes (Fig. 1A). In cells infected for 5 days with strain 60190, the decrease in MMR levels was more evident (Fig. 1B); hPMS1, hPMS2, hMSH2, hMSH3, and hMSH6 mRNA levels were reduced 1.5- to 3-fold. Western blot analysis confirmed that hMSH2 and hMSH6 levels were affected by H. pylori infection (Fig. 1C). To investigate the role of H. pylori virulence factors in MMR expression, cells were infected with H. pylori strains lacking a functional type IV secretion system (cagE mutant), cagA, or vacA genes. No significant differences were observed between the parental strain and the mutants, with the exception of hMSH6 (Fig. 1D).

In cells treated with H2O2, we observed a similar decrease in
MMR levels (Fig. 1B), suggesting that *H. pylori* infection affects expression of MMR genes comparable with an oxidative stress agent. We examined if the decrease in MMR expression in infected cells was caused by *H. pylori*-induced regulation of hypoxia-inducible factor-1α transcription factor. Only minor changes in hypoxia-inducible factor-1α levels were observed (Supplementary Fig. S1) and there were no major effects of the type IV secretion system, cagA, and vacA virulence factors, indicating that hypoxia-inducible factor-1α expression is not significantly affected by *H. pylori* infection.

To functionally characterize the effect of *H. pylori* infection on MMR, we measured MMR activity (25). Whereas 46.6% of actively proliferating cells after infection by *H. pylori* there is a decrease (5.4% on the fifth day of infection) in infection in our experimental system. The results show that proliferation and induces mitochondrial-mediated apoptosis in infection not only impairs MMR gene and protein expression but also down-regulates the activity of the MMR pathway.

Because it is known that *H. pylori* infection affects cell proliferation and induces mitochondrial-mediated apoptosis (1), we determined how cultured cells responded to *H. pylori* infection in our experimental system. The results show that there is a decrease (5.4% on the fifth day of infection) in actively proliferating cells after infection by *H. pylori* compared with the uninfected control culture as well as (3.5% on the fifth day) increase in apoptotic cells (Supplementary Fig. S2). *H. pylori* infection induced minor changes in cellular proliferation and death, probably not relevant to influence our study of repair mechanisms.

**Analysis of MMR gene expression in vivo.** To validate our *in vitro* data in an *in vivo* model, we studied the MMR gene expression in C57BL/6 mice (Fig. 2B). At 3 months post-infection, we observed a decrease in MMR gene expression compared with the uninfected control animals, which is similar to that observed in *in vitro* after 5 days of infection (Fig. 1). As suggested from *in vitro* results, the *H. pylori*-induced MMR down-regulation is independent of the functionality of the type IV secretion system, because *H. pylori* SS1 strain has a nonfunctional cag PAI (30). In mice infected for 12 months, there was no evident decrease in MMR gene expression, suggesting a recovery of gene expression after extended exposure of mice to *H. pylori*.

**Nuclear MSI and mtDNA instability.** It has been reported that human cells infected with *H. pylori* at high MOI (>100) show moderate MSI in one (CA)13 repeats (10). We infected AGS cells with *H. pylori* at MOI of 10 for 5 days and measured MSI using the five microsatellite markers recommended by the National Cancer Institute for diagnosis of hereditary nonpolyposis colorectal cancer. We did not detect MSI in *H. pylori*-infected AGS cells compared with noninfected control cells (Fig. 2C). Our results suggest that at least for 5-day infection with a low MOI, nuclear MMR activity, although diminished, does not lead to comparable levels of MSI in nDNA as those observed in hereditary nonpolyposis colorectal cancer patients carrying germ-line defects of hMSH2 and hMLH1.

Previously, we have shown that induction of a gastric mutagenic effect in C57BL/6 mice, infected with *H. pylori* strain SS1 for 6 months, was mainly attributable to oxidative DNA damage related to the observed chronic gastritis. This mutagenic activity was not observed if the infected mice were examined after 12 months (11). We analyzed if genetic instabilities might occur in three loci carrying (CA)n repeats. After 6 months post-infection, variation was missing (loci D6Mit59 and D19Mit36) and additional bands (locus D7Mit91) were observed in the Mutation Detection Enhancement gel migration patterns when comparing DNA isolated from infected and noninfected mice (Fig. 2D, left). This results indicate the induction of instabilities in CA repeats at 6 months post-infection, which were not observed after 12 months post-infection (Fig. 2D, right). These data correlate with the induction of point mutations (11) and with the decreased level of MMR components during *H. pylori* infection for 3 months (Fig. 2B). In contrast, after a longer period of infection, there was no significant alteration in MMR levels (Fig. 2B) and mutagenicity (11), consistent with the absence of genetic instabilities in loci carrying (CA)n repeats (Fig. 2D, right).

To determine whether *H. pylori* induces mtDNA instability in *in vitro*, we measured mutations in the D-loop region containing the mononucleotide D310 [C6] and dinucleotide D514 [C5A] repeats. The overall frequency of mutational events was higher in AGS cells infected with *H. pylori* strain 60190 (27.4%) than in noninfected control cells (11.2%; P = 0.020; Fig. 3A). Furthermore, we observed differences regarding the spectrum of mtDNA mutations (Supplementary Table S2). Cells infected with *H. pylori* 60190 showed a significantly higher number of transitions (17.8%) than uninfected control cells (5.6%; P = 0.036). The two transition events GC->AT and AT->GC were also observed in H2O2-treated AGS cells. Interestingly, insertions were only detected in *H. pylori* 60190-infected cells (4.1% Supplementary Table S2).

To further clarify the role of *H. pylori* virulence factors in the induction of mtDNA mutations, we infected AGS cells with *H. pylori* strains lacking cagE, cagA, or vacA and found that these bacterial mutants induced significantly lower overall mutation frequencies (10.7%, 11.2%, and 12.0%, respectively) than cells infected with the parental strain (27.4%; P = 0.012, 0.014, and
Patients with chronic gastritis harboring mtDNA mutations (Table 1) were more frequently infected with strains of the highest virulence-associated vacA s1m1 and cagA* genotypes (80.0%) than patients without mtDNA mutations (50.0%; P = 0.045; Table 1). These results are in agreement with the in vitro data, suggesting that H. pylori infection induces mtDNA mutations, particularly transitions. These mutations affect both D-loop and coding genes, showing a preference for complex I genes. The load of mtDNA mutations varies according to H. pylori genotypes, suggesting that virulent strains are more prone to induce mtDNA mutations.

Table 1. Relationship between H. pylori genotypes and mtDNA mutations among individuals with chronic gastritis (P < 0.05, Fisher’s exact test)

<table>
<thead>
<tr>
<th>H. pylori genotype*</th>
<th>Mutations in mtDNA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>vacA s1m1 cagA+</td>
<td>29 (50.0)</td>
<td>12 (80.0)</td>
</tr>
<tr>
<td>vacA s2m2 cagA-</td>
<td>29 (50.0)</td>
<td>3 (20.0)</td>
</tr>
</tbody>
</table>

*Of the 99 cases with chronic gastritis, 20 were infected with multiple strains, four were infected with vacA s1m2 genotype, and two were infected with vacA s1m1 cagA* genotypes, and those were excluded from the analysis.

0.024, respectively; Fig. 3A). Interestingly, we observed a decrease in transition mutations in AGS cells infected with the cagE mutant (6.7%) compared with those infected with the parental H. pylori strain 60190 (17.8%; P = 0.046; Supplementary Table S2). No significant differences in transition mutations were found between cells infected with H. pylori 60190 and cells infected with the cagA and vacA mutants (P = 0.106 and 0.052, respectively; Supplementary Table S2).

To verify if H. pylori was able to induce mutations in coding regions of the mtDNA as well as in the D-loop region, we measured mutations in the ND1, COI, and ATPase6 genes. In ND1 and COI genes, the frequency of mutational events was higher in AGS cells infected with H. pylori (30.4% and 25.7%, respectively) than in noninfected control cells (10.9%, P < 0.0001 and 15.2%, P = 0.0038, respectively; Fig. 3B). As to the ATPase6 gene mutational frequency, no significant differences were observed between infected and noninfected cells (P = 0.78; Fig. 3B). Interestingly, we observed increased transition mutations in both ND1 and COI (26.6% and 24.5%, respectively) genes in cells infected with H. pylori compared with uninfected cells (10.3%, P = 0.0002 and 14.0%, P = 0.004, respectively; Supplementary Table S2).

To validate our in vitro data in an in vivo model, we took advantage of a well-characterized series of gastric biopsies from 99 H. pylori-infected patients with chronic gastritis. In these samples, we determined the frequency of mutations in mtDNA in both two D-loop regions (D310 [C6] and D514 [C5]) and coding regions (ND1, ND3, ND4, ND5, COI, and ATPase6 genes). Twenty (20.2%) of the 99 gastric biopsy specimens harbored mtDNA mutations. Two cases had mutations in both D-loop and coding regions. In the D-loop, mutations were found in 17 cases and those occurred only at the D310 [C6] repeat. In the coding regions studied, mutations were observed in 5 cases and affected only complex I gene ND1. This is in accordance with the results obtained in vitro where we show that, after 5 days of infection with H. pylori, the highest increase in mutation frequency was observed in the ND1 gene (Fig. 3B). We also show that the majority of mutations detected in patients were transition mutations (Supplementary Table S3), supporting our in vitro analysis of mtDNA mutations (Fig. 3; Supplementary Table S2).

We next analyzed if there was a relationship between mtDNA mutations and the virulence of the infecting H. pylori strain. Patients with chronic gastritis harboring mtDNA mutations were more frequently infected with strains of the highest virulence-associated vacA s1m1 and cagA* genotypes (80.0%) than patients without mtDNA mutations (50.0%; P = 0.045; Table 1). These results are in agreement with the in vitro data, suggesting that H. pylori infection induces mtDNA mutations, particularly transitions. These mutations affect both D-loop and coding genes, showing a preference for complex I genes. The load of mtDNA mutations varies according to H. pylori genotypes, suggesting that virulent strains are more prone to induce mtDNA mutations.

Analysis of BER gene expression. Because H. pylori infection induces oxidative stress in human cells (1) and oxidative DNA damage is repaired by the BER pathway (20), we examined BER gene expression in infected AGS cells. We measured the expression of two key players of the mitochondrial BER pathway, hOGG1 and hAPE1, to explore a possible association between BER activity and mutations in mtDNA on infection. As previously mentioned, DNA glycosylases as hOGG1 begin the BER process by removing a damaged base, leaving an AP site, which is cleaved by hAPE1 (20). Our results show that H. pylori infection caused a significant decrease in hAPE1 expression (Fig. 4A) but not in hOGG1 expression (Fig. 4B).

**Discussion**

Although a causal relationship exists between H. pylori infection and the development of gastric carcinoma (31), the molecular mechanisms underlying this observation have remained elusive. Because impairment of genomic integrity often leads to carcinogenesis, we focused on the response of the host DNA repair pathways to H. pylori infection.

H. pylori infection leads to down-regulation of MMR gene expression in AGS cells in a manner similar to that occurring in cells treated with H2O2. In mice chronically infected with H. pylori infection induces oxidativestressinhumancells(1)andoxidativeDNA damage is repaired by the BER pathway (20), we examined BER gene expression in infected AGS cells. We measured the expression of two key players of the mitochondrial BER pathway, hOGG1 and hAPE1, to explore a possible association between BER activity and mutations in mtDNA on infection. As previously mentioned, DNA glycosylases as hOGG1 begin the BER process by removing a damaged base, leaving an AP site, which is cleaved by hAPE1 (20). Our results show that H. pylori infection caused a significant decrease in hAPE1 expression (Fig. 4A) but not in hOGG1 expression (Fig. 4B).
H. pylori, we observed a similar decrease in MMR expression. After 12 months, however, the expression of MMR genes were comparable with uninfected cells, indicating that MMR mRNA levels recover after long-term exposure of mice to H. pylori. This could be explained by elimination of cells with high level of DNA lesions due to catastrophic cell death. In mice, infection causes active gastritis with metaplastic changes and infiltration of inflammatory cells in the gastric mucosa (11) that could promote oxidative DNA damage. It is known that H. pylori infection leads to DNA damage of the epithelial cells due to production of ROS (1). DNA damage caused by H. pylori may likely result from both a direct DNA-damaging effect by oxygen radicals and an ineffective repair. Accordingly, in the absence of an efficient MMR system, DNA lesions can accumulate, acting as early events culminating in malignancy.

Defects in MMR not only generate point mutations but also lead to generalized genomic instability (32). In fact, in stomach, MSI is observed in ~20% of sporadic gastric carcinoma cases. We show the occurrence of MSI in DNA isolated from mouse stomach after 6 months of infection but not after 12 months. This is similar to that observed previously for point mutations (11) and in accordance with the decrease of MMR protein levels seen after shorter periods and not after prolonged infection. These findings may be explained by compensatory mechanisms possibly due to an increase in gastric epithelial cell proliferation or a change in the balance between apoptosis and cell proliferation after infection (11). Accordingly, induction of epithelial cell proliferation and apoptosis were observed by proliferating cell nuclear antigen and activated caspase-3 immunohistochemistry experiments on the gastric mucosa on H. pylori-infected mice for 12 months.8 Taken together, our data suggest that H. pylori generates a transient mutator state, which might be critical for cellular transformation (Fig. 5), for the survival of just one mutated cell may lead the path to malignancy.

Mutations in the mitochondrial genome have been detected in nearly every type of cancer investigated to date (17), including gastric preneoplastic lesions (33). Previous studies have found that transition mutations are the major mutational events in mtDNA of gastric carcinomas (18). In agreement with these results, we observed an increase in mutation frequencies in both the mitochondrial D-loop region and in several genes encoding subunits of the electron transport chain when we infected human cells with H. pylori 60190. These mutations were mostly transition mutations and preferentially affected complex I ND1 and complex IV COI genes. This is in agreement with the data obtained from chronic gastritis-infected patients. It was also evident in this population that the H. pylori genotype influences the number of mtDNA mutations, with the more virulent strains inducing a greater load of mutations.

Our data suggest that, by increasing the mutation frequency of specific coding genes in the mitochondrial genome, H. pylori may interfere with the activity of the electron transport chain and therefore impair oxidative phosphorylation. Oxidative phosphorylation impairment may increase ROS generation and lead to even greater mtDNA damage.

Transition mutations that are known to be caused by oxidative damage, dominated in H. pylori-infected cells. The BER system is known to repair oxidative DNA damage in mtDNA (19). We did not detect any significant change in hOGG1 expression, whereas hAPE1 was down-regulated in infected gastric cells. During BER, AP sites are generated by hOGG1 and then repaired by hAPE1. An imbalance between generation and repair of AP site has been shown to be mutagenic by generating excess AP sites that can be converted into mutation by DNA polymerases or other repair enzymes (34). Other studies have reported an increase in APE1 levels during H. pylori infection (35, 36) in experiments using high MOI and shorter infection time (35) or using H. pylori protein extracts instead of live bacteria (36). Supporting our data, Meira et al. (37) showed that H. pylori infection in mice deficient in BER alkyadenine DNA glycosylase (Aag) activity may lead to development of precancerous stages. These authors suggested a model where increased levels of cellular damage and death due to ROS led to increased inflammation that consequently led to production of more ROS and tumor-promoting cytokines (37).

The multistep model of gastric carcinoma development postulates that carcinogenesis is initiated by inflammation caused by H. pylori infection as well as by dietary exposure to salt and nitrate, which cause DNA damage (38). To investigate this further, we have identified three mechanisms by which H. pylori may provoke a loss of genomic integrity and promote transformation. These molecular mechanisms include (a) increase in ROS and decrease repair activity, (b) mutations in mtDNA, and (c) induction of a transient mutator phenotype, which may generate mutations in the nuclear genome on short-term infections with H. pylori. We suggest that H. pylori-induced carcinogenesis is the combinatorial result of at least these three linked mutagenic phenomena (Fig. 5).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Rachel Thomas, Richard Argent, and John Atherton (University of Nottingham) for the mutants H. pylori strains used in this study and Keshav K. Singh, Lennart Friis-Hansen, Maria José Oliveira, and Jorge Lima for critical reading of the article and helpful suggestions.

---

8 Unpublished data.
References

19. Larsen NB, Rasmussen M, Rasmussen LJ. Nuclear and mitochondrial DNA repair: similar pathways? Mitochon-
30. Crabtree JE, Ferrero RL, Kusters JG. The mouse coloni-
Helicobacter pylori Infection Induces Genetic Instability of Nuclear and Mitochondrial DNA in Gastric Cells


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/9/2995

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2009/05/20/1078-0432.CCR-08-2686.DC1

Cited articles
This article cites 37 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/9/2995.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/15/9/2995.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.