SHORT COMMUNICATION

Epigenetic Mechanisms of ATM Activation after Helicobacter pylori Infection

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Gastric cancer (GC) is the second leading cause of cancer-related mortality worldwide. The disease develops from the accumulation of several genetic and epigenetic changes. Among other risk factors, Helicobacter pylori infection is considered the main driving factor of GC development. H. pylori infection increases DNA damage levels and leads to epigenetic dysregulation, which may favor gastric carcinogenesis. An early step in double-strand break repair is the recruitment of ataxia-telangiectasia mutated serine/threonine kinase (ATM) to the damaged site, where it plays a key role in advancing the DNA damage checkpoint process. H. pylori infection has been associated with the introduction of double-strand breaks in epithelial cells, triggering damage signaling and repair response involving ATM. Thus, the current study analyzed the effect of H. pylori infection on the DNA damage response sensor, ATM, in gastric epithelial cells and in biopsy specimens from patients with GC. In this study, we identified that H. pylori infection stimulated DNA damage, and therefore induced ATM in a virulence factor—dependent manner. In addition, we found that H. pylori might activate ATM through histone H3 and H4 hyperacetylation and DNA promoter hypomethylation. Our findings show a mechanism associating ATM signaling induction with H. pylori infection. (Am J Pathol 2018, 188: 329–335; https://doi.org/10.1016/j.ajpath.2017.10.005)

Since the discovery of Helicobacter pylori by Warren and Marshall1 in 1982, an increasing number of studies have shown a strong association between bacterial infection and gastric disease, including gastric cancer (GC).2 This has led the International Agency for Research on Cancer to classify H. pylori as a type I carcinogen.3 The ability of H. pylori to colonize the human stomach and to promote chronic infection can be attributed to the production of specific bacterial products that collectively are referred to as virulence factors. These factors modulate the pathogen—host interaction, and determine bacterial pathogenicity. In addition, the presence of these virulence factors allows the bacteria to remain in the host’s gastric mucosa, causing an inflammatory response, which may favor GC development.4 The type IV secretion system is encoded by a pathogenicity island called cag Pathogenicity Island (CagPAI) that is responsible for injection of CagA into the host cell. The presence of CagPAI enhances a strong inflammatory response in gastric epithelial cells, and drastically increases the risk of atrophic gastritis and GC.5,6 The production of vacuolating cytotoxin (VacA), encoded by the vacA gene, is another important virulence factor used by H. pylori to induce cytoplasmic vacuolization in gastric cells.7,8 Once internalized, VacA induces cytochrome C release from mitochondria, promoting apoptosis. Moreover, VacA binds to the cellular epithelium by interacting with tyrosine phosphatase proteins and enhancing pore formation in cell membranes.9 This disrupts cell-to-cell communication and inhibits T-cell activity at the site of infection, thus allowing bacterial persistence. The modulation of T cells causes a proinflammatory response mediated by NF-kB, leading to IL-8 induction.10–12 The

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interaction between a host and bacteria harboring \(cagA^+\) and \(vacA\) s1m1 genotype increases the risk of GC up to 87-fold.\(^{13}\)

Besides chronic inflammation, \(H.\) pylori infection increases reactive oxygen species and DNA damage levels and also leads to epigenetic dysregulation, which may favor gastric carcinogenesis.\(^{14}\) The initial step in double-strand break (DSB) repair is detection of the DNA damage through recruitment of the MRE11-RAD50-NBS1 complex, which in turn, recruits and activates ataxia–telangiectasia mutated serine/threonine kinase (ATM) to the damaged site.\(^{15}\) ATM phosphorylates and activates other DSB repair machinery proteins, including H2AX, which becomes phosphorylated (\(\gamma\)H2AX) near breaks in DNA.\(^{16}\) Some studies have suggested that ATM can regulate the cellular response induced by oxidative stress, acting both as a sensor and as a mediator.\(^{17}\) \(H.\) pylori infection has been associated with the introduction of DSBs in epithelial and mesenchymal cells, triggering a damage-signaling and repair response involving ATM, ATR, and CHK2 repair factors with subsequent accumulation of \(\gamma\)H2AX.\(^{18-20}\) Both in vitro and in vivo studies have suggested that the induction of DSB by \(H.\) pylori may contribute to genetic instability and chromosomal aberrations, which are hallmarks of GC.

The analysis of epigenetic code (ie, DNA methylation or specific histone modifications), might be a powerful way to examine if changes in the transcriptional pattern of the host cell are associated with bacterial infection. Indeed, it already has been shown that pathogens can induce histone modifications such as acetylation and phosphorylation of the N-terminal tails of histones H3 and H4.\(^{21}\) There is complementary evidence showing that DSB processing occurs in the context of chromatin, through histone modifications, to promote accessibility to the repair factors on DNA damage sites in chromatin.\(^{22}\) Because only a few publications have reported that \(H.\) pylori was able to affect DNA repair through modulation of host cell chromatin, we were interested in determining the impact of \(H.\) pylori infection on ATM regulation through epigenetic modification.

**Material and Methods**

**Cultured Cell Lines**

Human gastric adenocarcinomas AGS and HGC27 were purchased from the Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil). Cell lines were cultured as adherent monolayers. The cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 200 \(\mu\)g/mL streptomycin, and 200 IU/mL penicillin (Invitrogen, Carlsbad, CA) at 37°C under a 5% \(CO_2\) humidified atmosphere.

**Patients**

Tumor samples and adjacent healthy gastric tissue were obtained from 76 GC patients from the Southeast and North of Brazil. The study was approved by the Ethics Committee of Sao Francisco University. Written informed consent was obtained from all patients before specimen collection. The rapid urease test and PCR were used to test for the presence of \(H.\) pylori. Total RNA was isolated using the RNeasy tissue kit (Qiagen, Valencia, CA). Reverse transcription was performed using random priming and Superscript Reverse Transcriptase (Life Technologies) according to the manufacturer’s guidelines. Quantitative real-time PCR was performed using Power SYBR Green Master Mix (Thermo Scientific, Waltham, MA), 10 mmol/L of each primer, and 20 ng of cDNA in an ABI PRISM 7500 thermocycler (Applied Biosystems, Foster City, CA). Primer sequences are complementary to internal gene sequence reading in the 5’ to 3’ direction. Variations in RNA input were corrected by analyzing the expression of the 18S rRNA housekeeping gene. The \(\Delta\Delta CT\) method was used for relative quantification.

**Bacterial Infections**

\(H.\) pylori wild-type strain P12\(^{23}\) (\(cagPAI^+\), \(vacA\) s1m1) and \(H.\) pylori mutants with inactivation of the \(cagPAI\) (\(\Delta cagPAI\)) and \(vacA\) (\(\Delta vacA\)) were gifts from Dr. Rainer Haas (Ludwig-Maximilians-Universität München, Munich, Germany). Two independent \(H.\) pylori strains derived from Donostia Hospital (San Sebastian, Spain) patients (\(Tox^+\) : \(cagA^+\) /\(vacA\) s2m2; \(Tox^+\) : \(cagA^+\) /\(vacA\) s1m1) were grown for 48 hours in selective medium (pylori-Gelose; BioMérieux, Marcy-l’Étoile, France) at 37°C under microaerophilic conditions. The infections were performed at 80% cellular confluence. The bacteria were added to the cellular monolayer at a multiplicity of infection of 100 bacteria per cell, and co-cultured for 4 hours.

**Immunofluorescence Analysis**

Infected and uninfected AGS cells were grown on coverslips, fixed with 4% paraformaldehyde at 18°C to 21°C (room temperature) for 15 minutes, and then permeabilized with phosphate-buffered saline containing 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 10 minutes. The cells were blocked with 1% bovine serum albumin for 20 minutes before incubation with primary antibodies (anti-\(\gamma\)H2AX and anti–phospho-S1981 ATM; Cell Signaling Technology, Beverly, MA) at room temperature for 1 hour. After washing with phosphate-buffered saline, cells were incubated with the secondary antibody Alexa Fluor–conjugated goat anti-rabbit IgG at room temperature for 1 hour. After a final wash with phosphate-buffered saline, coverslips were mounted with antifading mounting medium containing DAPI.

**Automated \(\gamma\)H2AX and pATM Foci Counting**

Eight-bit tiff files were merged to multichannel images using ImageJ software version 1.51 (NIH, Bethesda, MD; [http://imagej.nih.gov/ij]). Foci counting and intensity measurements
were performed with Focinator software version 2-10 (created by S.O., Essen, Germany; γH2AX channel with noise, 0; cut-off, 11; phospho-ATM (pATM)-channel with noise, 0; cut-off, 16; and for both regions of interest threshold method, Li) as previously described.24

**ATM mRNA Expression Analysis**

Total RNA was extracted with TRIzol (Life Technologies). Reverse transcription was performed using random priming and Superscript Reverse Transcriptase (Life Technologies) according to the manufacturer’s guidelines. Quantitative real-time PCR was performed using Power SYBR Green Master Mix (Thermo Scientific), 10 μmol/L of each primer, and 20 ng of cDNA in an ABI PRISM 7500 thermocycler (Applied Biosystems). Variations in RNA input were corrected by 20 ng of cDNA in an ABI PRISM 7500 thermocycler (Applied Biosystems). Variations in RNA input were corrected by

**Western Blot Analysis**

Western blots were performed following standard procedures. Total protein was extracted with lysis buffer, and 20 μg of the protein was separated on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase was detected with specific antibodies (Abcam, Cambridge, MA). Horseradish peroxidase–linked anti-rabbit or horseradish peroxidase–linked anti-mouse secondary antibodies (DAKO Corporation, Hamburg, Germany) were used at a 1:2000 dilution. Signal was detected by chemiluminescence using enhanced chemiluminescence substrate (Amersham Bioscience, Little Chalfont, England).

**Chromatin Immunoprecipitation Coupled with Quantitative PCR**

Chromatin was immunoprecipitated from cell culture and *H. pylori* co-culture by using an EpiTect Chromatin Immunoprecipitation One-Day Kit (Qiagen) following the manufacturer’s instructions. Briefly, the samples were cross-linked with 1% paraformaldehyde at 37°C and harvested. The cells were then lysed, and the DNA was sonicated and incubated with 2 μg of each antibody (recognizing H3ac, H4ac, and IgG; Qiagen) at 4°C overnight. The chromatin/DNA complex was recovered with protein A beads and the DNA was extracted and purified. The DNA from immunoprecipitation or control (no chromatin input) was used for quantitative PCR analysis with primers specific for the ATM promoter region (GPH1002696; Qiagen).

**Methylation Pattern**

DNA samples from culture and *H. pylori* co-culture were extracted using a Qiagen Blood and Tissue Kit (Qiagen) following the manufacturer’s instructions. The DNA was treated with restriction enzymes included in the DNA Restriction EpiTect Methyl II Kit (Qiagen) to generate methylated and unmethylated fractions. After digestion, the DNA was analyzed by quantitative PCR using specific primers recognizing the ATM promoter region (Qiagen).

**Data Evaluation**

Data are presented as means ± SEM. Statistical significance (*P* value) was calculated using the *t*-test. The *γ*H2AX and pATM foci were counted using Focinator software version 2-10, and statistical significance was calculated using one-way analysis of variance. *P* < 0.05 was considered significant. Chromatin immunoprecipitation—quantitative PCR was analyzed according to the following formula: ΔΔ* Ct* (normalized IP) = [Δ* Ct* (IP) − (Δ* Ct* [input] − log2 [1%])].

**Results**

*H. pylori* Infection Increases the Level of DNA DSBs in Gastric Cells

The impact of *H. pylori* on DNA damage was investigated in gastric epithelial cell lines. *γ*H2AX, a typical marker to examine DNA damage after genotoxic stress, was visualized by an immunofluorescence-based foci assay 4 hours after *H.
pylori infection (Figure 1A). There was an increase in γH2AX foci number in cells infected with the H. pylori Tox− strain (Figure 1B). The uninfected cells (control) had an average number of 5.08 foci per nucleus. The infection with the less-virulent strain, Tox−/C0, showed a significantly increased amount of γH2AX foci (11.98 per nucleus). Interestingly, the cells infected by the Tox+ strain showed an even higher number of foci (13.94 per nucleus).

ATM Is Up-Regulated by H. pylori Infection

To investigate whether H. pylori–induced DNA damage is associated with the activation of ATM, the level of mRNA was measured by quantitative PCR and total and phosphorylated forms of ATM were measured by Western blot and immunofluorescence, respectively. The ATM mRNA level increased in AGS and HGC27 cells infected by the more virulent strain (Tox+) of H. pylori (Figure 2, A and B). The expression levels of ATM also were investigated in gastric cells infected by either wild-type or mutant H. pylori strains lacking cagPAI or vacA to evaluate whether ATM induction occurs in a virulence factor–dependent manner. Data show that the deletion of cagPAI and vacA, independently, strongly reduced ATM expression (Figure 2C), indicating that H. pylori induction of ATM requires a direct interaction between the bacterial type IV secretion system and vacA cytotoxic effects and their host cells. In a human context, higher levels of ATM expression were observed in H. pylori–positive tumor biopsy specimens when compared with healthy tissues (Figure 2D).

The total and phosphorylated forms of ATM also were observed in gastric cells infected by H. pylori (Figure 2, E and F), indicating the activation of ATM. Figure 2G represents the pATM mean foci count per single nucleus. The uninfected cells (control) had an average number of 0.91 foci per nucleus. The infection with the less-virulent strain, Tox−, showed a significantly increased amount of pATM foci (5.40 per nucleus), whereas the cells infected by the Tox+ strain showed an even higher number of foci (6.01 per nucleus). Together, these results suggest that H. pylori triggers DSBs, and subsequently activates ATM as a response to DNA damage.

H. pylori–Induced ATM Activation through H3 and H4 Hyperacetylation

To assess a possible effect of specific changes in histone modifications occurring at the ATM promoter during H. pylori
infection, chromatin immunoprecipitation—quantitative PCR experiments were performed. It was investigated whether ATM up-regulation corresponded to high levels of histone H3 and H4 acetylation at the ATM promoter region. The results showed that the acetylation states of H3 and H4 were modulated upon infection. Histones H3 and H4 were highly acetylated after 4 hours of infection with Tox + and P12 strains (Figure 3A), in agreement with the ATM expression pattern, suggesting that H3 and H4 acetylation levels are predictive of H. pylori-mediated ATM gene induction.

**H. pylori Regulates ATM Expression through Promoter Region Hypomethylation**

Changes in DNA methylation patterns are associated with the development of various diseases, including cancer. Environmental factors such as bacterial infections can alter these patterns. Because ATM was up-regulated by H. pylori infection, AGS cells infected with a Tox + H. pylori strain were used to address this idea and determine the methylation level at the ATM promoter region.

It has been shown that H. pylori infection can change the DNA methylation pattern of oncogenes, tumor suppressor genes, and genes involved in cell growth and DNA repair, which contributes to an increased risk for developing GC.25–28 In this work, we were able to determine quantitatively the extent of ATM promoter methylation (Figure 3B). Together with histone acetylation findings, we suggest that ATM up-regulation may be caused by a strong epigenetic modulation during H. pylori infection. The lack of in vivo validation is a limitation of the present study, although the in vitro data indicate the effects of bacterial infection on ATM hypermethylation.

**Discussion**

Gastric carcinogenesis is a multistep process triggered by H. pylori and characterized by activation of oncogenes and the inactivation of tumor suppressors, resulting in genomic instability. It has been shown that H. pylori infection is associated with introduction of DSBs, which activate ATM and γH2AX formation.18–20 Considering that GC is primarily an epigenetic disease,29 we addressed whether H. pylori infection activates ATM through epigenetic mechanisms.

Initially, we found that H. pylori strains harboring cagA+/vacA s1m1 (Tox +) increased γH2AX foci, which may alter genome integrity through DNA DSBs, leading to genomic instability and eventually cancer. DSBs are the most harmful type of DNA damage and have been shown to be induced by H. pylori infection in gastric epithelial cells.18 Inappropriate repair of DSBs is a prominent factor for malignant tumors owing to genomic instability.30 In agreement with the current work, higher γH2AX levels have been shown to correlate with H. pylori infection in AGS co-culture.18 In addition, in a study based on human samples, Xie et al31 also found that high expression of γH2AX appeared to correlate with H. pylori infection, which implied that cells infected by the bacterium may accumulate oncogenic changes and continue proliferating with high levels of DNA damage, which may favor carcinogenesis.

Consistent with previous data, these results indicated that ATM is activated by H. pylori infection in vitro18,20 and in vivo,20 supporting that γH2AX DSBs are mostly ATM-dependent. There is some discrepancy in the literature regarding the association of DSB induction and virulence factors cagPAI and vacA. Although Toller et al18 showed that H. pylori virulence factors cagPAI and vacA are dispensable for DSB induction; Hanada et al20 reported an accumulation of DSBs after infection with cagA + strains. Similarly, our data indicated that ATM expression is dependent on cagPAI and vacA. This discrepancy may be owing to the differences in the genetic background of the H. pylori strains.

Histone acetylation is an important mechanism of gene regulation used by many bacteria during infection. For instance, Listeria monocytogenes deacetylates H4 and alters the gene expression pattern of the host at the beginning of infection.32 Similarly, it has been suggested that H. pylori...
infection is associated with histone modifications with consequent changes in the gastric epithelium that affect the pathogenicity of the bacterium. These findings suggest a common strategy among bacteria to maintain their survival. Very few reports are available on host cell histone acetylation induced by *H. pylori*, and none of them shows histone acetylation in the ATM promoter region. In the current study, we found epigenetic regulation of ATM gene transcription by *H. pylori* through histone acetylation in AGS cells.

The delicate host–pathogen interplay can have different outcomes. The identification of the mechanisms by which *H. pylori* affects host cell response will allow a better understanding of GC carcinogenesis and the development of more efficient therapeutic strategies for the patient. In this work, we identified that *H. pylori* infection stimulates DNA damage with consequent induction of an ATM response in a virulence factor–dependent manner, *in vitro* and *in vivo*. In addition, we showed that ATM regulation is mediated by epigenetic alterations, such as hyperacetylation of histones H3 and H4 and hypomethylation of its promoter region. Our findings show a mechanism associating ATM signaling induction with *H. pylori* infection.

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**References**


