Helicobacter pylori-infection introduces DNA double-strand breaks in host cells.

Running title: H. pylori-induced DNA damage responses.

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Gastric cancer is an inflammation-related malignancies related to long standing acute and chronic inflammation caused by infection with the human bacterial pathogen, *Helicobacter pylori*. Inflammation can result in genomic instability. However, there are considerable data that *H. pylori* itself can also produce genomic instability both directly and through epigenetic pathways. Overall, the mechanisms of *H. pylori*-induced host genomic instabilities remains poorly understood. We used microarray screening of *H. pylori*-infected human gastric biopsies to identify candidate genes involved in *H. pylori*-induced host genomic instabilities. We found upregulation of *ATM* expression in vivo in gastric mucosal cells infected with *H. pylori*. Using gastric cancer cell lines, we confirmed that the *H. pylori*-related ATM activation was due to the accumulation of DNA double-stranded breaks (DSBs). DSBs were observed following infection with both *cag* pathogenicity island (PAI)-positive and -negative strains, but the effect was more robust with *cag* PAI-positive strains. These results are consistent with the fact that both *cag* PAI-positive and -negative infections are associated with gastric carcinogenesis but the risk is higher in individuals infected with *cag* PAI-positive strains.
Introduction

Gastric cancer is categorized as an inflammation-related malignancy. The roles for direct bacterial-host interactions other than through the production of inflammation in the molecular pathogenesis of gastric cancer remains poorly understood. *H. pylori* contains a number of putative virulence factors associated with an increased risk of a clinical outcome such as peptic ulcer or gastric cancer and considerable effort has been expended in study of the effect of these virulence factors on altering intracellular signaling pathways possibly involved in cancer pathogenesis. However, the bulk of the evidence is consistent with the notion that these putative virulence factors enhance the severity of the mucosal inflammatory response which may be largely responsible for the virulence factor-related increased risk of gastric cancer (1-3).

Cancer is often thought of as a genetic disease. Recently Toller et al. using AGS cells, a gastric cancer cell line, described the presence of double-strand DNA breaks (DSBs) following infection with *H. pylori* (8). DSBs are the most severe type of DNA damage and can trigger chromosomal aberrations such as deletions, insertions, and translocations which are a major cause of loss of heterozygocity. Maintenance of genomic integrity is aimed at both preventing and repairing DSBs when they occur. In mammals, the presence of DSBs is recognized by the MRE11–RAD50–NBS1 (MRN) complex (9-11) which captures the DNA ends resulting in activation of ATM (ataxia-telangiectasia mutated) kinase, a member of the phosphatidylinositol 3-kinase-related kinase family (11, 12). The association of ATM with the MRN complex results in activation by phosphorylation of serine residue 1981 resulting in phosphorylation of downstream targets involved in DNA repair and cell cycle checkpoints including CHK2 kinase and p53 (12, 13). Activated CHK2 can inhibit downstream targets resulting in production of the cell cycle checkpoint. ATM pathways also contribute to stabilization of the tumor suppressor protein p53, leading to cell cycle arrest at G1 phase (14) during which DSBs can be repaired by the DSB repair pathways such as by non-homologous end-joining and homologous
recombination (15). Non-homologous end-joining involves the simple ligation between
two DNA ends independent of the chromosome locus; this repair sometimes results in
chromosome aberrations, such as deletions and translocations. As such, non-homologous
end-joining provides a relatively error-prone repair and is used in the V(D)J
recombination and class switch recombination in the immunoglobulin gene locus to
obtain the required diversity of immunoglobulins and class switch from IgM (15).

Homologous recombination occurs between two homologous sequences, in most
cases between two sister chromosomes after DNA replication. The BRCA2-RAD51
complex is the central player for homologous recombination and catalyzes the homology
search and strand exchange reaction (15, 16). RAD54 and RAD54B support the function
of RAD51 (17, 18). Since homologous recombination occurs between two identical
sequences, homologous recombination is relatively error-free. The clinical importance of
dNA damage responses and DSB repair pathways is illustrated by the fact that
individuals with mutated genes such as ATM, MRE11, NBS1, CHK2, BRCA2 and
RAD51 exhibit chromosome instabilities and an increased incidence of a variety of
cancers (12, 14). Thus, DSB repair plays a crucial role in the maintenance of genomic
integrity.

Here, we examined human H. pylori-infected human gastric mucosa to identify
novel tumor suppressors affected by H. pylori-infection with an emphasis on those
resulting in suppression of genome instability. Specifically, we used microarray screening
of human H. pylori-infected gastric biopsies to identify genes involved in the DNA
damage response to DSBs. We then used in vitro studies to confirm the presence of DSBs
and examine the effect of cag pathogenicity island (cag PAI) on H. pylori-induced
chromosome instabilities.

Materials and Methods

Gastric mucosae, bacterial strains, human cell lines and medium.
Gastric mucosal biopsies were obtained from volunteers in Bhutan and in Oita, Japan as described previously (19). *H. pylori* stains were cultivated in Brucella broth with 10% fetal bovine serum (FBS) in an anaerobic jar with an anaeropack (Mitsubishi Gas Chemical, Japan). Strain TN2GF4, an East-Asian type *H. pylori*, was used as the wild type strain. *cag* PAI−, *vacA*, and *cag* PAI− *vacA*− double mutants were derived from TN2GF4 (20). AGS cells were cultured in RPMI1640 with 10% FBS. In addition, human primary fibroblasts, BR1 (control), AT2KY (*ATM*-deficient), and AT3OS (*ATM*-deficient), were cultured in DMEM with 15% FBS and also used in this study.

**Total RNA isolation.**

The total RNA was isolated from the gastric mucosa of each individual kept in RNAlater (Invitrogen, USA) and purified using SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. RNA samples were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality was confirmed with an Experion System (Bio-Rad Laboratories, Hercules, CA).

**Gene expression microarrays.**

cRNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligomicroarray according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner and relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1). The microarray data was registered in gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/info/linking.html); the accession number is GSE47797.

**Histological analysis.**
Biopsy specimens were fixed in 10% (v/v) neutralized buffered formalin, embedded in paraffin, and then stained with hematoxylin–eosin (HE) and Giemsa. Specimens were examined without knowledge of the experimental results. The histological features of chronic gastritis, including inflammation (lymphocyte infiltration), activity of gastritis (neutrophil infiltration), mucosal atrophy, and intestinal metaplasia, were determined based on the updated Sydney System.

**Immunohistochemistry.**

Immunohistochemistry was performed as described previously (21). Briefly, after antigen retrieval and inactivation of endogenous peroxidase activity, tissue sections were incubated either with rabbit monoclonal phospho-S1981 ATM (1/200; ab81292, Abcam) or rabbit polyclonal anti-*H. pylori* (1/200; B0471, DAKO) with diluting solution (DAKO, Denmark) overnight at 4°C. After washing, the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (Nichirei, Japan), followed by incubation with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, USA). Peroxidase activity was detected using H$_2$O$_2$/diaminobenzidine substrate solution.

**In vitro infection and Western blot analysis.**

AGS cells were seeded at approximately 25% confluence in T-25 flasks and incubated overnight. Afterward, indicated *H. pylori* strains were added at an MOI of approximately 100. After 24 hours, cells were harvested with trypsin/EDTA. Western blot was performed with anti-γ-H2AX (1/2,000; Millipore), anti-Tubulin (1/5,000; Sigma), anti-ATM (1/2,000; Abcam), and rabbit monoclonal anti-phospho-S1981 ATM (1/2,000; Abcam), anti-CHK2 (1/2,000; Abcam), and anti-phospho-T68 CHK2 (1/1,000; Cellsignaling). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies
(1/5,000; Jackson) were used as secondary antibodies, and bands were visualized with NBT/BCIP solution (Roche).

**Immunofluorescent staining.**

Immunofluorescent staining was performed as described previously. Briefly, AGS cells were seeded at approximately 50% confluence on glass coverslips. Then indicated *H. pylori* strains were added. After the indicated time of incubation, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The method of immunofluorescent staining was as published previously(22). Briefly, cells were permeabilized with 0.25% Triton X-100 in PBS, and γ-H2AX was detected with mouse monoclonal anti-γ-H2AX (1/200; Millipore). *H. pylori* were detected with rabbit polyclonal anti-*Helicobacter pylori* (1/200; DAKO). Alexa 488-conjugated goat anti-mouse antibody and Alexa 594-conjugated goat anti-rabbit antibody (1/400; Molecular Probes) were used as secondary antibodies in PBS with 0.15% glycine and 0.5% bovine serum albumin. Nuclear staining was done with DAPI and fluorescent signals were visualized with a Keyence BZ-9000 fluorescent microscope.

**Pulsed-field gel electrophoresis.**

AGS cells were infected with either wild type or *cag* PAI-mutated *H. pylori* strains. After 24 hours of incubation, cells were harvested by trypsinization, and plugs, 0.5% (w/v) agarose containing $2.5 \times 10^5$ cells in PBS, were prepared with a CHEF disposable plug mold (BioRad). The condition of pulsed-field gel electrophoresis was as previously published (23). Briefly, plugs were incubated in lysis buffer [100 mM EDTA, 1% (w/v) sodium lauryl sarcosyn, 0.2% (w/v) sodium deoxycholate, 1 mg/mL proteinase K] at 37°C for 24 hours. Pulsed-field gel electrophoresis was performed at 13°C for 23 hours in 0.9% (w/v) agarose containing 0.25×TBE buffer using a Biometra Rotaphor (Biometra).
The parameters were: voltage 180–120 V log; angle from 120° to 110° linear; interval 30 s to 5 s log. Gels were stained with ethidium bromide and analyzed using a Typhoon FLA7000 scanner (GE healthcare). Band intensities were quantified using ImageQuant (GE Healthcare). The amount of broken DNA was calculated as the intensity of DNA in the migrated fraction over the intensity of DNA in the well and the migrated fraction. Means and standard errors were determined from the results of three independent experiments.

FACS analysis.
Cells were harvested by trypsinization and fixed overnight with 70% ethanol at 4°C. After the ethanol was washed away, the cells were washed with PBS containing 0.1% Tween-20, 0.5 μg/mL propidium iodide and 0.4 mg/mL RNase for overnight. The cells were analyzed on a FACS caliper (Becton Dickinson). As a control, cells were treated with 1 μg/mL mitomycin C for 24 hours.

Results
ATM was activated by infection with *H. pylori* in human gastric epithelial cells in vivo and in cultured cells.
Tumor suppressors affected by *H. pylori*-infection were identified using microarray screening to compare the gene expression profiles of gastric mucosa obtained from individuals with *H. pylori* gastritis and from uninfected controls. The emphasis was on identifying genes related to DNA damage responses against DSBs. The gene expression patterns of the genes related to DNA damage response against DSBs are summarized in Table S1. Gene expression of *ATM* and *MDC1* was increased 180% in biopsies of those with *H. pylori* gastritis compared to uninfected controls (P<0.05). In contrast, *MRE11* expression was similar in infected and normal mucosa but was reduced in biopsies with
intestinal metaplasia (a mucosa to which *H. pylori* rarely attaches) compared to uninfected controls. *ATM* was also higher in *H. pylori* gastritis than in biopsies from uninfected individuals (*P* = 0.053) or with intestinal metaplasia. Among the genes involved in the DNA damage response to DSBs, significant alterations were seen in *ATM*, *MDC1*, and *MRE11* (Table S1). Moreover, none of the genes involved in other DNA repair pathways, such as mismatch repair, nucleotide excision repair, and base excision repair, showed altered gene expression in biopsies obtained from *H. pylori*-infected individuals (either gastritis or intestinal metaplasia) compared to uninfected controls (Table S1). Otherwise, further data was registered in gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/info/linking.html. The accession number is GSE47797). These results are consistent with the notion that the DNA damage response against DSBs was activated when *H. pylori* attached to host mucosal cells (eg, *H. pylori* gastritis). It was decreased following the development of intestinal metaplasia, a mucosa to which *H. pylori* rarely attaches.

However, it is known that following DSB induction, the DNA damage response against DSBs is not strongly regulated at the transcripational level (24). Thus, the overrepresentation of *ATM* transcripts in the gastritis samples might simply represent tissue infiltration with T- and B-lymphocytes both of which have high physiological levels of DSB repair proteins. To confirm whether *ATM* was activated in gastric mucosal cells, we performed immunohistochemistry of the gastric mucosa from individuals with gastritis and with intestinal metaplasia. First, the presence of *H. pylori*-infection was confirmed with anti-*H. pylori* staining (Fig. S1A). Then, we examined *H. pylori* positive tissues for phospho-S1981 *ATM*, the activated form of *ATM*. Phosphorylated *ATM* was observed in the epithelial cells of gastric biopsies with *H. pylori* gastritis (Fig. 1A,B Fig. S1B). In contrast, only a few epithelial cells in biopsies with intestinal metaplasia showed phosphorylated *ATM* (Black arrows in Fig. 1C).
Next, we used AGS cells infected with *H. pylori* to examine whether *H. pylori* infection induced ATM-dependent DNA damage responses in vitro. Activated ATM proteins were identified using anti-Phospho-S1981 ATM antibody. Because ATM phosphorylates serine residue 139 of histone H2AX, this phosphorylation is often called γ-H2AX and thus the appearance of γ-H2AX was also analyzed. After infection, ATM-activation was observed and correlated with the appearance of γ-H2AX (Fig. 1D). We also quantified the correlation between ATM activation and the appearance of γ-H2AX and confirmed this correlation (Fig. 1E). To confirm that γ-H2AX-formation was ATM-dependent, ATM-deficient primary fibroblasts isolated from patients with Ataxia telangiectasia (JCRB Cell Bank, Osaka, Japan) were infected with *H. pylori* and the appearance of γ-H2AX was analyzed. As expected, γ-H2AX foci were not observed in ATM-deficient fibroblasts after *H. pylori*-infection (Fig. 1F). In contrast γ-H2AX foci were evident in ATM-proficient fibroblasts (Fig. 1G). These results support the notion that *H. pylori*-infection activates the ATM-dependent DNA damage response. In a time course experiment, AGS cells were infected with *H. pylori* and the appearance of γ-H2AX was analyzed over 48 hours (Fig. S2). γ-H2AX foci first appeared at 3 hours after infection and continued to be expressed over the entire 48 hour experiment. Overall, we conclude *H. pylori*-infection causes DSBs in infected host cells and these DSBs trigger the activation of ATM.

**Role of *H. pylori* cagA and cag PAI in the ATM-dependent DNA damage response.**

To explore whether *H. pylori* virulence factors were involved in DSB formation in host cells, various *H. pylori*-mutated strains were infected with AGS cells and the accumulation of DSBs was analyzed. Using the targeting knockout technique, *cagA*, *cag* PAI single, *vacA* single, and *vacA* cag PAI double mutants generated from the strain TN2GF4 and the mutants were used to infect AGS cells at an MOI of 100. γ-H2AX foci formation was analyzed following infection with wild type and mutant *H. pylori* after 24
hours. γ-H2AX foci were strongly induced by wild type *H. pylori* but not following infection with *cagA*-, *cag PAI* -or *vacA* -cag PAI- double mutants (Fig. 2A-C) suggesting that the *cag PAI* may be involved in the formation of γ-H2AX. Since the *cag PAI* acts upstream of CagA, we used *cag PAI* strains as the *cag*-negative strain. We quantified the appearance of γ-H2AX-positive cells following infection with wild type and with *cag PAI* - single mutants. Accumulation of phosphorylated ATM was not observed after infection with *cag*-negative strains (Fig 2c) which supports the notion that the appearance of γ-H2AX is largely correlated with ATM kinase activity. With wild type *H. pylori*, the appearance of γ-H2AX-positive cells was significantly increased (p <0.01) but was not observed following infection with the *cag PAI* -mutants (Fig. 2B).

To confirm that γ-H2AX represented the presence of DSBs in host cells, we used pulsed-field gel electrophoresis to examine for the presence of broken DNA fragments. In this experiment, AGS cells were infected with wild type or *cag PAI* -mutants at MOI = 6.3-400 and broken DNAs were fractionated by pulsed-field gel electrophoresis based on molecular size. After infection with wild type *H. pylori*, an accumulation of DSBs was observed at the lower MOI (Fig. 2D). Accumulation of DSBs after the infection with *cag PAI* -mutants was present but reduced compared to wild type infection (Fig. 2D). Next we quantified the accumulation of DSBs after infection and also analyzed the effect of *cagA* -strain as well as wild type, and *cag PAI* strain. At an MOI of 200 and 400, the accumulation of DSBs in wild type was significantly greater than with *cagA* - and *cag PAI* -mutants (P<0.05) (Fig. 2E). A similar tendency for DSB formation was confirmed when the appearance of γ-H2AX was assessed using western blot (Fig. 2F).

Overall, our results suggest that although both *cag*-positive and -negative *H. pylori* are capable of inducing DSBs in host cells, however, *cag*-positive strains show greater activity.

**Infection with *H. pylori* activates the DSB-induced cell cycle checkpoint.**
Since ATM is involved in the DSB-induced cell cycle checkpoint, such induction of DSB-induced cell cycle checkpoint might be expected following *H. pylori*-induced DNA damage. To address this, AGS cells were infected with *H. pylori* and the cell cycle status was investigated. First, cell cycle status was analyzed by FACS. Cells were infected at an MOI of 100 either with wild type *H. pylori*, *cag* PAI- or *vacA* mutants, and after 24 hours cells were stained with popidium iodide (PI). As a positive control of cell cycle arrest, cells treated with mitomycin C were used. Cells infected with wild type and *vacA* mutants showed reduced G1 and increased S phase, suggesting cell cycle arrest might have occurred (Fig. 3A). However, cells infected with a *cag* PAI- strain showed less cell cycle arrest compared to wild type or *vacA* strains (Fig. 3A). These results suggest that cells infected with *H. pylori* can activate cell cycle checkpoint caused by the presence of DSBs.

To confirm whether the DSB-induced cell cycle checkpoint is activated, the status of ATM and CHK2 were analyzed. Both phosphorylated ATM and CHK2 were observed after *H. pylori*-infection (Fig. 3B) suggesting that it is likely that the progression of cell cycle is arrested by the ATM-CHK2-dependent pathway following *H. pylori* infection. In summary, we showed that infection with *cag*-positive *H. pylori* causes a greater accumulation of DSBs than with *cag*-negative infections and that the presence of DSB formation results in activation of an ATM-dependent cell cycle checkpoint.

**Downregulation of RAD51 after infection with *cag*-positive *H. pylori*.**

To examine the cellular responses against *H. pylori*-induced DNA damage, microarray screening was done using AGS cells infected with wild type *H. pylori* and mutants to compare the gene expression profiles of infected cells and uninfected controls. Interestingly, gene expression of *RAD51*, *RAD54*, and *RAD54B*, key factors in DSB repair via homologous recombination, were reduced compared to uninfected controls and in cells infected with *cag*-negative strains (Fig. 3C). Reduction of the RAD51 protein level was confirmed by western blot (Fig. 3D). We tested several anti-RAD51 and
anti-RAD54B antibodies to detect these proteins but failed to detect them by western blot.

Since homologous recombination is only activated in S phase (25), one possibility is that
the reduction of RAD51-expression was due to cell cycle arrest at G1 phase. However,
cells infected with cag-positive strains showed reduced G1 and increased S phase (Fig
3A). These results showed that cells infected with H. pylori could enter S phase followed
by cell cycle arrest. In summary, we showed that infection with cag-positive H. pylori
causes a greater accumulation of DSBs and reduction of RAD51 than did infection with
cag-negative H. pylori.

Discussion
H. pylori was officially recognized as a Class I human carcinogen in relation to gastric
cancer by The World Health Organization (WHO) in 1994. Gastric carcinogenesis is
tightly related to H. pylori-induced chronic inflammation associated progressive mucosal
damage. H. pylori-infection is considered necessary but insufficient cause for gastric
carcinogenesis as the presence of the infection does not automatically result in a high
incidence of gastric cancer. Gastric carcinogenesis requires the activation of oncogenes
and the inactivation of various tumor suppressors resulting in genomic instability. Since
genomic instabilities occur randomly, the effect is probabilistic. Indeed, there are data
that H. pylori per se also have the ability to cause genomic instabilities both directly and
through epigenetic pathways in the gastric mucosa of infected hosts (7, 8, 26). Moreover,
epidemiological studies have shown that both cag PAI positive and negative infections
are associated with development of gastric cancer with presence of the cag PAI
approximately doubling the risk of gastric cancer (27, 28). Although gastric
carcinogenesis requires genomic instabilities, molecular mechanism regarding how H.
pylori induces genomic instabilities remain poorly understood, particularly in relation to
the functions of the cag PAI. In this study, we addressed whether H. pylori induces
genomic instabilities and found that the infection both cag-positive and negative strains
induced accumulation of DSBs the effect was greater with cag-positive strains compared to cag-negative strains.

We found that ATM was activated in vivo in infected human gastric mucosa and that the effect was specifically related to cells infected with *H. pylori* (Fig. 1A,B). We also showed that activated ATMs and γ-H2AX are both present in *H. pylori*-infected human gastric epithelium. These results support the notion that *H. pylori* can introduce DNA breakages of the host chromosome in the human stomach. γ-H2AX is present in DSBs and also at single-stranded breaks caused by DNA replication damage. In in vitro experiments we confirmed that γ-H2AX formation was the result of DSB formation caused by *H. pylori*-infection. The prior study suggested that γ-H2AX formation from single-stranded breaks is mostly ATR kinase-dependent, while γ-H2AX from DSBs is mostly ATM kinase-dependent (13). The fact that γ-H2AX was not observed in *H. pylori* infected ATM-deficient cell lines is consistent with *H. pylori* infection related γ-H2AX formation being ATM-dependent (Fig. 1D-G). From these results, we conclude that activated ATM signals present in *H. pylori*-infected gastric epithelial cells in vivo (Fig. 1B) occur in response to the presence of *H. pylori*-induced DSBs. Since ATM and other factors involved in ATM-dependent DNA damage responses are known to be strong tumor-suppressors, it is likely that the ATM-dependent response occurs in response to *H. pylori*-induced DSBs in order to prevent or reduce chromosome aberrations.

Previously, Toller et al. showed DSBs induction by cag-positive and cag PAI-negative strains in vitro were comparable. They also showed that DSB production was dependent on *H. pylori*-host cell contact and on BabA adhesion. We found that generally accumulations of DSBs after infection with cag-positive *H. pylori* were significantly greater than among those infected with cag-negative strains, such as cagA and cag PAI-negative strains, particularly after infection with MOI's between 200 to 400 (Fig. 2D,E). This result differs from the previous studies (8). One possibility is a difference in the genetic background of *H. pylori* strains used in those studies. To address
this, further investigation will be required. At least, from our results, gene products from 
_\text{cag} \text{PAI}_ appear to play an important role in the accumulation of DSBs in infected host 
cells. We also found that the expression of \text{RAD51} was reduced after the infection with 
_\text{cag}-positive strains_ and this was not simply due to the cell cycle arrest at G1 phase (Fig. 
3A,C,D). These findings suggest a new function of CagA resulting in the inactivation of 
\text{RAD51}. The higher accumulation of DSBs after infection with _\text{cag}-positive strains_ may 
in part be related to the reduced activity of DSB repair via homologous recombination. 
Future experiments are needed to address the molecular mechanism how CagA is 
involved in inactivation of \text{RAD51}. However, since we observed that the DSB 
accumulation also occurred even in the absence of _\text{cag} \text{PAI}_ function, we agree with 
Toller’s and co-worker’s suggestion that DSB formation can occur in a _\text{cag} \text{PAI}_-independent manner. Our results are also consistent with the previous 
epidemiological studies showing that both _\text{cag}-positive and negative infections_ are 
associated with development of gastric cancer and that the presence of the _\text{cag} \text{PAI}_ 
approximately doubles the risk of gastric carcinogenesis compared to infection with a 
_\text{cag}-negative strain_ (27-29).

One strength of this study is that the in vivo data was similar in samples from two 
widely different areas (Japan and Bhutan), however, it must be noted that _\text{cag}-negative 
infection_ are rare in both areas and the in vitro data with _\text{cag}-negative strains_ could not be 
confirmed in vivo. That confirmation must await future experiments using tissues with 
contain samples from _\text{cag}-negative infections_.

Consistent with the observation of Toller et al., DSBs were only produced in cells to 
which \text{H. pylori} attached such as gastric epithelial cells or primary fibroblasts suggesting 
a requirement for a direct bacterial-host interaction rather than release of DNA damaging 
components. Together with these results suggest that DSB formation is a direct response 
to the host-bacterial interaction and provides a potential mechanism for why eradication 
of the infection is associated with a marked reduction in the risk of metachronous cancers
in the most high risk group of patients who have already had one early gastric cancer removed endoscopically (30).
Conflict of interests

KH, TU, YT, MW, NY, KY, SS, MM, and YY do not have conflict of interests. DYG is an unpaid consultant for Novartis in relation to vaccine development for treatment or prevention of *H. pylori* infection. DYG is also a paid consultant for RedHill Biopharma regarding novel *H. pylori* therapies and for Otsuka Pharmaceuticals regarding diagnostic breath testing. DYG has received royalties from Baylor College of Medicine patents covering materials related to $^{13}$C-urea breath test.

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Author Contributions

KH designed project and have done most of experiments. TU and MW performed some pathological experiments. YT, NY, and KY performed some of the celliological experiments. SS, MM, DYG & YY supervised this project. KH, DYG & YY wrote this manuscript.
References


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Figure legends.

FIG 1. Analysis of ATM activation caused by the infection with *Helicobacter pylori*. (A-C) Immunohistochemistry (IHC) of gastric mucosa. The tissue section was stained with anti-phospho-S1981 ATM antibody. (A) The pyloric region of stomach from the uninfected individual. (B) The pyloric region isolated from the individual with gastritis. (C) Pyloric region isolated from the individual with intestinal metaplasia. (D) Immunofluorescent staining of activated ATM in vitro. AGS cells were infected with *H. pylori*. After 24 hours of incubation, cells were fixed and phospho-S1981 ATM (Red) and γ-H2AX, phospho-Ser139 histone H2AX (Green) were stained. Nuclear was stained with DAPI (Blue). (E) The quantification of ATM-activation and the appearance of γ-H2AX after the infection with wild type *H. pylori* after 24 hours. * represents the statistically significant by the student t-test (P<0.05). (F) ATM-deficient fibroblasts were infected with *H. pylori*, and γ-H2AX (Green) and *H. pylori* (red) were stained. (G) ATM-proficient fibroblasts were infected with *H. pylori*, and γ-H2AX (Green) and *H. pylori* (red) were stained.

FIG 2. Analysis of γ-H2AX induced by the infection with *H. pylori*. (A) Immunofluorescent staining of γ-H2AX in vitro. AGS cells were infected with indicated genotype of *H. pylori* strains, and γ-H2AX (Green) and *H. pylori* (Red) were stained. (B) Percentage of γ-H2AX-positive cells after the infection with *H. pylori*. Percentages of γ-H2AX-positive cells were determined by counting more than 200 cells. Mean and SE were determined from four independent experiments. (C) Western blot analysis of phospho-S1981 ATM and γ-H2AX. AGS cells were infected with the indicated genotype of *H. pylori* strains, and phospho-S1981 ATM and γ-H2AX were stained. ATM and tubulin were loading control. * was the non-specific. (D,E) Analysis of DSBs by pulsed-field gel electrophoresis (PFGE). (D) DSBs after the infection with wild type and
cag PAI-mutated stains. Intact DNA was stacked in the well, while broken DNA was migrated into the gel. Various sizes of DNAs (from 500 kb to at least 5.7 Mb) were compacted in one band, indicated as “Broken DNA”. (E) Quantification of DSBs from the results of PFGE. (F) Western blot analysis of γ-H2AX. AGS cells were infected with wild type and cag PAI-mutated stains, and the appearance of γ-H2AX was analyzed.

FIG 3. Analysis of DNA damage responses after the infection with H. pylori. (A) Cell cycle analysis after infection with H. pylori. Indicated genotype of H. pylori strains were infected and cell cycle state were analyzed with propidium iodide (PI)-staining in a flowcytometer. Mitomycin C (MMC) is a control. (B) Analysis of ATM-CHK2-dependent cell cycle checkpoint. Cells are either treated with MMC or infected with H. pylori and Phosphorylation state of ATM and CHK2 were analyzed with antibodies that can recognize phosphorylated-specific antigens. (C) Microarray analysis of H. pylori-infected AGS cells. Cells were infected with indicated genotypes of H. pylori strains, and gene expression profiles related to DNA repair, DNA damage responses, and cell cycle control were compared. (D) Western blot analysis of RAD51. Protein accumulations of RAD51 were analyzed at 24 hours after infection of wild type and indicated mutants of H. pylori strains. Actin is loading control.
FIG 1.