Molecular and Cellular Pathobiology

Inflammatory Processes Triggered by Helicobacter pylori Infection Cause Aberrant DNA Methylation in Gastric Epithelial Cells

Tohru Niwa1, Tetsuya Tsukamoto2, Takeshi Toyoda2, Akiko Mori1, Harunari Tanaka2, Takao Maekita3, Masao Ichinose3, Masae Tatematsu2, and Toshikazu Ushijima1

Abstract

Altered patterns of DNA methylation associated with Helicobacter pylori (HP) infection of gastric epithelial cells are thought to contribute to gastric cancer risk. However, it is unclear whether this increased risk reflects an infection-associated inflammatory response or the infection itself. In this study, we sought to clarify mechanisms in a gerbil model of gastric cancer where we showed that HP infection is causally involved in induction of aberrant DNA methylation. By genome-wide screening, CpG islands that were aberrantly methylated in gerbil gastric cancer cell lines were isolated, and 10 islands were shown to be specifically methylated only in gastric mucosae infected with HP. By temporal analysis, methylation levels in gastric epithelial cells started to increase at 5 to 10 weeks after infection and reached high levels by 50 weeks. When HP was eradicated, methylation levels markedly decreased 10 and 20 weeks later, but they remained higher than those in gerbils that were not infected by HP. Expression levels of several inflammation-related genes (CXCL2, IL-1β, NOS2, and TNF-α) paralleled the temporal changes of methylation levels. Significantly suppressing inflammation with the immunosuppressive drug cyclosporin A did not affect colonization by HP but blocked the induction of altered DNA methylation. Our findings argue that DNA methylation alterations that occur in gastric mucosae after HP infection are composed of transient components and permanent components, and that it is the infection-associated inflammatory response, rather than HP itself, which is responsible for inducing the altered DNA methylation. Cancer Res; 70(4); 1430–40. ©2010 AACR.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGI) is one of the major inactivating mechanisms of tumor-suppressor genes and is deeply involved in human carcinogenesis (1). Nevertheless, there is only limited information on its inducers and induction mechanisms. Chronic inflammation, known to promote certain types of cancers (2), is one of the possible inducers of aberrant methylation. The presence of aberrant methylation is frequently observed in healthy individuals (5, 10). In addition, eradication of HP infection induces aberrant methylation and what mechanisms are involved.

In human gastric mucosa, the presence of Helicobacter pylori (HP) infection, a well-known inducer of chronic inflammation and gastric cancers (8, 9), is associated with high methylation levels or high incidences of methylation (5, 10–12). In addition, among individuals without HP infection, noncancerous gastric mucosae of gastric cancer patients have higher methylation levels than gastric mucosae of healthy individuals (5, 10). In addition, eradication of HP leads to a decreased incidence of CDH1 (E-cadherin) promoter methylation (11, 13, 14). These findings suggest that HP infection induces aberrant methylation in gastric mucosae and indicate that levels of accumulated methylation are associated with gastric cancer risk. However, because infection experiments are impossible in humans, it needs to be clarified in animal models whether or not HP infection induces methylation and what mechanisms are involved.

HP infection in humans is best modeled in Mongolian gerbils (Meriones unguiculatus). As in man, HP infection induces severe inflammation in gerbil gastric mucosae and promotes gastric carcinogenesis induced by administration of N-methyl-N-nitrosourea (MNU) or N-methyl-N′-nitrosoguanidine (15). The incidence of gastric cancers in gerbils depends on the duration of HP infection, and eradication of HP significantly reduces the incidence (16), as in man (17, 18). Thus, we can expect that the gerbil model is also useful in analyzing whether HP infection induces aberrant methylation and what mechanisms are involved in vivo. However, unfortunately,
little information is available for the gerbil genome, and the genetic and molecular analysis of this model has been
hampered.

In this study, we aimed to show that HP infection is causally involved in induction of aberrant DNA methylation and
to clarify a critical factor involved. For this, we first isolated CGIs that could be methylated in gerbil gastric cancers by a
genome-wide screening method, methylation-sensitive representation-difference analysis (MS-RDA). Using the CGIs iso-
lated, we then showed that methylation was induced specifically in gerbils with HP infection and that inflammation
induced by HP infection, not HP itself, was critically involved in methylation induction.

Materials and Methods

Cell lines. Two gerbil gastric cancer cell lines, MGC1 and
MGC2, were established from a single gastric cancer induced in a
gerbil by MNU and HP infection (19). They were main-
tained in RPMI 1640 supplemented with 10% fetal bovine se-
rum on a type I collagen-coated dish (Asahi Techno Glass).
Although we did not check the cross-contamination of cell
lines biochemically or genetically just before use, they had
the same morphology and growth rates as described previ-
ously (19).

Animal experiments and sample preparation. Male
Mongolian gerbils (MGS/Sea) were purchased from Kyudo.
To induce gastric cancers, male gerbils were administrated
with 30 ppm of MNU (Sigma-Aldrich) in drinking water for
a week at 7, 9, 11, 13, and 15 wk of age, and then inoculated
with HP (ATCC 43504, American Type Culture Collection) by
gavage at 17 wk of age (20). At 57 wk, gerbils were sacrificed
without removal of the stomach due to early gastric cancers (seven men and seven
women; average age 65.9 y, ranging from 47 to 79 y). Sam-
pling was conducted under the approval of Institutional
Review Boards.

Nucleic acid extraction. From tissue sections, DNA was
extracted by heating the dissected sections at 100°C for 20
min at pH 12, followed by phenol/chloroform extraction
(22). From isolated glands, DNA was extracted by protease
K digestion and the phenol/chloroform method. From the
whole blood, DNA was extracted with a QuickGene DNA
whole blood kit (Fujifilm). RNA was isolated with Isogen
(Wako).

Quantitative PCR for gene expression analyses and
HP detection. To analyze gene expression levels, cDNA was
synthesized from 2 μg of DNase-treated RNA with an oligo-
(dT)12–18 primer. Real-time PCR using gene-specific primers
(Supplementary Table S1) and SYBR Green Real-time PCR
Master Mix (TOYOBO) was done, and the amplification curve
of a sample was compared with curves of standard DNA
samples with known copy numbers. Standard DNA samples
were prepared by serial dilution of a PCR product or a plasmid
containing a cloned PCR fragment after its quantification.
Gene expression levels were normalized to that of
Gapdh. To measure the amount of HP, real-time PCR using
specific primers for the jhpjpr3 gene of HP was carried out and
normalized to the gerbil Ih4 gene (Supplementary Table S1).

Methylation-sensitive representational difference analysis.
MS-RDA is a subtraction method that can identify differ-
entially methylated loci between two genomes independent
of genomic information (23) and was done using HpaII or
SacII methylation-sensitive restriction enzyme as described
previously (24). The final PCR product was cloned into pGEM
T-Easy (Promega) and sequenced. If a DNA fragment had a
CpG score ≥0.65 and G + C content ≥55%, the fragment was
considered to be derived from a CGI. To identify homologous
regions in mice and men, database searches were carried out at
a GenBank web site.

Methylation analysis. Fully methylated and fully
unmethylated controls were prepared by methylating genomic
daDNA with Sso1 methylase (New England Biolabs) and ampli-
ifying genomic DNA with d29 DNA polymerase (GenomiPhi
DNA Amplification Kit, GE Healthcare), respectively (25).
One microgram of DNA digested with BamHI was treated
with sodium bisulfite and suspended in 80 μL of Tris-EDTA
(TE) buffer as described previously (22). In the case of
paraffin-embedded samples, DNA was treated with sodium
bisulfite without BamHI digestion and suspended in 20 μL
of TE buffer. One microliter of aliquot was used as a template
for methylation-specific PCR (MSP) and bisulfite sequencing.
Conventional MSP and bisulfite sequencing were done
with specific primer sets (Supplementary Table S2) as de-
scribed previously (22). Quantitative MSP (qMSP) was done
by real-time PCR using primers specific to DNA molecules methylated at a locus and to a repeat sequence. Methylation levels were expressed as a percentage of the methylated reference, which was obtained as [(number of methylated fragments of a target CGI in sample) / (number of repeat sequences in sample)] / [(number of methylated fragments of a target CGI in SssI-treated DNA) / (number of repeat sequences in SssI-treated DNA)] × 100. As a repeat sequence, the B2 repeat was used for gerbil DNA (ref. 26; Supplementary Table S1) and the B2 repeat was used for human DNA (27).

Statistical analysis. Statistical analyses were conducted with SPSS 13.0J (SPSS Japan, Inc.). To evaluate significant differences between two independent groups of sample data, the Mann-Whitney U test was used. Spearman’s rank correlation coefficient (r) was used to measure correlation.

Results

Identification of CGIs specifically methylated by HP infection in GECs of Mongolian gerbils. To identify CGIs methylated in GECs of gerbils with HP infection, we adopted the strategy of a genome-wide screening in cancers and high-sensitivity analysis in GECs. The genome-wide screening was done by MS-RDA using a pool of two gerbil gastric cancer cell lines (MGC1 and MGC2) as the driver and GECs of noninfected gerbils as the tester. The final products of two series of MS-RDA using HpaII and SacI were cloned and 180 DNA fragments were sequenced. One hundred three of them were nonredundant, and 56 of them contained a sequence likely to have originated from a CGI. Due to the lack of information on the gerbil genome, we first analyzed the methylation statuses of CpG sites within the DNA fragments isolated using MSP, MSP primers were successfully designed for 27 of the 56 DNA fragments, and we analyzed the two gastric cancer cell lines, five samples of GECs from gerbils infected with HP for 50 weeks, and five samples of GECs from age-matched gerbils without infection. Ten (HE6, HG2, SA9, SB1, SB5, SC3, SD2, SE3, SF12, and SH6) of the 27 DNA fragments were methylated in the cell lines and GECs of HP-infected gerbils, but not in any GECs of gerbils without infection (Table 1; Fig. 1). The others were methylated only in the cell lines or methylated even in GECs of gerbils without infection.

Methylation in primary gastric cancers was analyzed for three randomly selected CGIs (HE6, SA9, and SB5). The methylation levels of HE6 and SB5 in eight primary cancer samples were similar to or below the mean methylation levels in GECs with HP infection for 50 weeks. In contrast, the methylation level of SA9 in most cancer samples was 2.1- to 19.1-fold higher than the mean methylation level in GECs of HP-infected gerbils. These results suggested that HP infection induced aberrant methylation of multiple but specific CGIs in gerbil GECs, and that methylation of some of these CGIs was associated with growth advantage of the cells.

Methylation of the corresponding CGIs in human samples. To examine whether or not these CGIs are also methylated in humans by HP infection, conserved regions of the

<table>
<thead>
<tr>
<th>Clone name</th>
<th>GenBank accession no.</th>
<th>Genomic location deduced from analyses using human or mouse genome database</th>
<th>Nucleotide position in human or mouse sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE6</td>
<td>AB429514</td>
<td>Exon 2 of Ntrk2 gene*</td>
<td>16,449,514–16,449,840 bp in NT_023935.17 (human chr. 9)</td>
</tr>
<tr>
<td>HG2</td>
<td>AB429515</td>
<td>Exon 1 of Gpr37 gene*</td>
<td>49,589,571–49,589,704 bp in NT_007933.14 (human chr. 7)</td>
</tr>
<tr>
<td>SA9</td>
<td>AB429516</td>
<td>Exon 1 of Nol4 gene*</td>
<td>13,292,105–13,292,430 bp in NT_010966.13 (human chr. 18)</td>
</tr>
<tr>
<td>SB1</td>
<td>AB429517</td>
<td>Intergenic region between Sp4 and Sp8 genes*</td>
<td>20,698,454–20,698,697 bp in NT_007819.16 (human chr. 7)</td>
</tr>
<tr>
<td>SB5</td>
<td>AB429513</td>
<td>Not identified</td>
<td>Not identified</td>
</tr>
<tr>
<td>SC3</td>
<td>AB429518</td>
<td>Promoter region of Rnf152 gene*</td>
<td>7,352,575–7,352,875 bp in NT_025028.13 (human chr. 18)</td>
</tr>
<tr>
<td>SD2</td>
<td>AB429519</td>
<td>Promoter region of Nptx2 gene*</td>
<td>23,480,374–23,480,422 bp in NT_007933.14 (human chr. 7)</td>
</tr>
<tr>
<td>SE3</td>
<td>AB429520</td>
<td>Intron 1 of Slc35f1 gene*</td>
<td>39,311,942–39,312,270 bp in NT_001838990.2 (human chr. 7)</td>
</tr>
<tr>
<td>SF12</td>
<td>AB429521</td>
<td>Intergenic region between Cntn1 and Pdzm4 genes*</td>
<td>53,513,634–53,513,936 bp in NT_039621.7 (mouse chr. 15)</td>
</tr>
<tr>
<td>SH6</td>
<td>AB429522</td>
<td>Intergenic region between Sox1 and Loc729095 gene*</td>
<td>213,253–213,298 bp in NT_027140.6 (human chr. 13)</td>
</tr>
</tbody>
</table>

*Conserved regions identified in the human database.
10 gerbil CGIs in humans were searched for. Eight of the 10 CGIs were found to be conserved between gerbils and humans (marked in Table 1), and five were located in the vicinities of genes (Fig. 2A, left). When the methylation levels of these five CGIs were quantified in human gastric mucosal biopsies, all of them had 5- to 48-fold higher methylation levels in individuals with HP infection ($n = 10$) than in those without ($n = 10$; right). Their methylation levels had close correlation with each other (correlation coefficient = 0.70–0.88; Supplementary Table S3).

The methylation levels of the five CGIs were then analyzed in primary human gastric cancers. NTRK2, GPR37, NOL4, and NPTX2 had methylation in seven, three, four, and five, respectively, of 14 cancers analyzed, using the average methylation level of mucosal biopsies of HP-infected healthy volunteers as a threshold. There was no case with methylation of RNF152 (Fig. 2B). These results showed that some of these CGIs were also methylated in human gastric cancers.

**Induction of DNA methylation by chronic HP infection.** Using the 10 CGIs isolated by MS-RDA, the effect of HP infection on methylation induction was analyzed at 1, 5, 10, and 50 weeks after HP infection (Fig. 3A). The methylation levels of HG2, SB5, and SD2 started to increase at 5 weeks after infection. At 10 weeks, CGIs other than SE3 and SH6 showed significantly higher methylation levels than those of the noninfected gerbils (3.2- to 85.0-fold). At 50 weeks, all the CGIs showed significantly higher methylation levels (14.3- to 215-fold; Fig. 3B; Supplementary Fig. S3). These results suggested that chronic HP infection, not acute HP infection, was responsible for methylation induction.

The presence of dense methylation (methylation of a majority of CpG sites on a single DNA molecule) was confirmed by bisulfite sequencing of HE6 and SA9 in GECs of two gerbils with HP infection and two without. Densely methylated DNA molecules were detected only in HP-infected gerbils (Fig. 3C). The vast majority of DNA molecules were either largely unmethylated or largely methylated, and the fraction of methylated DNA molecules was in accordance with methylation levels measured by qMSP. The methylation levels of the 10 CGIs closely correlated with each other (average correlation coefficient = 0.87; range 0.70–0.95; Fig. 3D; Supplementary Table S4).

**Decrease in methylation levels after HP eradication.** HP was eradicated at 50 weeks after infection, and the methylation levels of the 10 CGIs were measured in GECs of the gerbils before and 1, 10, and 20 weeks after the eradication (Fig. 3A). Complete absence of HP was confirmed by PCR of HP genomic DNA (Fig. 4C). At 1 week after eradication, no decrease in methylation was observed (Fig. 3B; Supplementary Fig. S3). At 10 weeks after eradication, in contrast, the methylation levels of the 10 CGIs decreased to 9% to 32% of those before the eradication (significant for 9 of the 10 CGIs, except for SH6). An additional 10 weeks (20 weeks after eradication) did not lead to a further decrease in methylation levels. Importantly, the methylation levels after the decrease due to eradication were still significantly ($P < 0.01$ for two CGIs, and $P < 0.05$ for seven CGIs) higher than those in gerbils without any HP infection in their life.

**Close association between methylation induction and inflammation, and not HP itself.** HP infection is known to induce severe inflammation in gastric mucosae in gerbils, as in humans. Histologic analysis revealed that infiltration of polymorphonuclear cells and mononuclear cells started at 5 to 10 weeks after HP infection, and it became severe at 50 weeks (Fig. 4A; Supplementary Fig. S4). After eradication, a decrease in infiltration was not clear at 1 week, but was marked by 10 and 20 weeks (Fig. 4A). These histologic findings were paralleled by expression of inflammatory cell markers [Cd3g, Cd14, Ela2, and Ms4a1 (Cd20) for T cell, macrophage, neutrophil, and B cell, respectively] in gastric tissues containing both mucosal and submucosal layers (Fig. 4B). Although Ms4a1 expression decreased after eradication, gerbils without eradication (continuous infection) also showed a similar decrease, indicating that the decrease in Ms4a1 expression (B-cell infiltration) was independent of HP eradication.

To explore the components of inflammation associated with methylation induction, the expression of inflammation-related genes [Cox2, Cxcl2 (MIP-2), Ifng, Il1b, Il2, Il4,
II6, II7, Nos2 (iNos), and Tnf (Tnf-α) was also quantified (Fig. 4B). A marked increase after HP infection and a decrease after eradication were observed for Cxcl2, II1b, Nos2, and Tnf, paralleling inflammatory cell markers (Fig. 4B). The Cx2, Ifng, II2, II4, and II6 expression did not parallel the methylation levels after HP eradication, and the II7 expression showed a paradoxical increase compared with the group of continuous infection (Fig. 4B). Regarding the amount of HP in gastric mucosae, it had no association with methylation levels (Fig. 4C).

There remained a possibility that inflammatory cells had methylation of the CGIs analyzed, and that their contamination into GECs led to an apparent increase in methylation levels. To exclude this possibility, we analyzed the methylation levels of the 10 CGIs in DNA extracted from the whole blood of HP-infected gerbils. With the exception of SBI and SB5, which showed relatively high methylation levels in the blood, 8 of the 10 CGIs showed almost no methylation (Supplementary Fig. S5). This excluded the possibility that methylation detected in the GECs was due to methylation in inflammatory cells contaminating the GECs.

**Suppression of methylation induction by suppression of inflammation.** To conclude that inflammation is indispensable for methylation induction, we suppressed HP-induced inflammation by administration of CsA, which blocks T-cell activation through inhibition of the calcineurin signal transduction pathway. The methylation levels of the CGIs in the CsA-treated group were significantly lower than those in the control group, except for SBI and SB5 (Fig. 4C).

**Figure 2.** Methylation of homologous regions in human gastric mucosae. A, methylation levels in human gastric mucosal biopsies. Left, genomic structures and the regions analyzed by qMSP. Vertical lines, individual CpG sites; gray box, regions with homology between gerbil and man; open boxes, exons; faced arrowheads, positions of primers for qMSP. Right, result of qMSP analyses. Methylation levels were quantified in 10 healthy volunteers without HP infection and 10 with HP infection. Bold horizontal bars, average. **, *P < 0.01. B, methylation levels in primary gastric cancers. Fourteen primary gastric cancer samples and a pool of 10 mucosal biopsies of HP-infected healthy volunteers were analyzed. For the gastric mucosae, their mean methylation level and SD are shown. PMR, percentage of the methylated reference.
Macroscopically, administration of CsA to HP-infected gerbils markedly suppressed erosion and the formation of nodules. Histologically, it suppressed induction of hyperplasia almost completely, but infiltration of mononuclear and polymorphonuclear cells remained (Fig. 5B). Importantly, the number of HP colonized in the stomach was not affected by the CsA treatment (Supplementary Fig. S6). The expression levels of inflammatory cell markers (Cd3g, Cd14, and Ela2) were not reduced, indicating that the number of inflammatory cells normalized against other cells was not affected. However, the expression of three inflammation-related genes (Cxcl2, Il1b, and Nos2), whose expression paralleled methylation induction in the temporal analysis, was significantly reduced by the CsA treatment (Fig. 5C).

The DNA methylation levels of the 10 CGIs were markedly reduced in GECs of CsA-treated gerbils (0% to 28% of methylation levels of GECs from HP-infected gerbil without the treatment).
CsA treatment; Fig. 5D; Supplementary Fig. S7). These results showed that the CsA treatment suppressed inflammatory responses but not HP colonization, and that the suppression of inflammatory responses markedly repressed methylation induction.

Expression analysis of genes with promoter methylation in HP-infected GECs. HG2, SC3, and SD2 were located in the promoter regions of Gpr37, Rnf152, and Nptx2, respectively. Promoter CGIs are generally resistant to DNA methylation (29), and only when genes are transcribed at low levels are they susceptible to DNA methylation (30–32). To confirm the low expression and the effect of methylation on gene expression, we analyzed their expression levels in GECs isolated from gerbils with and without HP infection (10 and 50 weeks).

Figure 4. Changes in inflammation after HP infection and its eradication. A, histologic changes in gastric mucosa before and after HP eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) did not change at 1 wk after eradication but markedly decreased at 10 and 20 wk. However, the presence of fibrosis and heterotopic proliferative glands (*) did not differ. B, temporal profiles of expression of inflammatory cell markers and inflammation-related genes. Red, green, and blue lines, gerbils with continued infection, gerbils with eradication, and those without any HP infection, respectively. C, numbers of HP in the gerbil stomach. Real-time PCR of HP-specific DNA using DNA extracted from gastric tissues containing mucus was done. Values are shown as mean ± SD. †, P < 0.05, compared with noninfected gerbils; *, P < 0.05, compared with the expression level before eradication.
Figure 5. Suppression of inflammation and methylation induction by CsA treatment. A, experimental design for CsA treatment and HP infection. B, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in HP-infected gerbils without the CsA treatment and were markedly suppressed by the CsA treatment. Infiltration of mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) was also severe in HP-infected gerbils without the CsA treatment and was repressed in CsA-treated animals. Gastric mucosae of HP-negative gerbils with CsA treatment showed no abnormal changes (data not shown). C, expression of inflammatory cell markers and inflammation-related genes. The expression of inflammatory cell markers normalized to Gapdh expression was not reduced. However, the expression of three inflammation-related genes (Cxcl2, Il1b, and Nos2) was significantly reduced by the CsA treatment. D, methylation levels in GECs. The CsA treatment markedly suppressed methylation induction by HP infection. Bold horizontal bar, average. *, P < 0.05; **, P < 0.01.
after infection) and in gastric cancer cell lines. All the three genes showed low expression levels in the GECs of non-infected and infected gerbils (Supplementary Fig. S8). Braf expression was significantly decreased in HP-infected gerbils compared with noninfected gerbils (44% and 25% at 10 and 50 weeks, respectively, after infection; P < 0.001). None of the three methylation were expressed in cancer cell lines with complete methylation of these CGIs (Fig. 1B; Supplementary Fig. S8, top).

The absence of DNA methyltransferase upregulation. DNA methyltransferases (Dnmt) are final effectors of maintenance and induction of DNA methylation, and their overexpression is frequently observed in various types of human cancers (33). To analyze possible upregulation of Dnmts by HP infection, expression levels of Dnm1, Dnm3a, and Dnm3b mRNAs were quantified in GECs of gerbils with and without HP infection. Contrary to our initial expectation, the expression levels of the three Dnmts were significantly lower in GECs with HP infection (1/2 to 1/3) than those without (Supplementary Fig. S8, bottom).

Discussion

Our study using a gerbil model showed that HP infection is causally involved in induction of aberrant DNA methylation in GECs. Thus far, a strong association has been shown between the presence of HP infection and high methylation levels or high incidence of methylation in human gastric mucosae (5, 10–12). Taking advantage of an animal model, we were able to conduct an experiment by infecting gerbils with HP and showed that HP infection was the cause of methylation induction.

The critical role of inflammation in methylation induction was shown. Temporal analysis showed that methylation levels were closely associated with infiltration of inflammatory cells, and suppression of inflammation by CsA markedly repressed methylation induction even in the presence of HP infection. These results indicated that HP itself was not necessary for methylation induction once inflammation was induced by it. This finding is important because a direct role of HP is suggested by the facts that the SHP2 oncoprotein is deregulated by injection of virulent factors such as CagA into GECs (34) and HP possesses multiple DNA (cytosine-5) methyltransferases (35).

Among the inflammation-related genes analyzed, the expression levels of Cxcl2, Il1b, Nos2, and Tnf were upregulated in the stomach with HP infection and decreased after eradication, almost paralleling those of methylation levels. In the CsA treatment, in which methylation induction was markedly suppressed, upregulation of Cxcl2, Il1b, and Nos2 by HP infection was significantly suppressed and that of Tnf also had a tendency to be suppressed. These results suggest that some specific inflammation-related genes are cooperatively involved in methylation induction by HP infection. In human ulcerative colitis and hepatitis (cirrhosis), where aberrant methylation is believed to be induced, increased expression of Il8 (human functional homolog of Cxcl2), Il1B, Nos2, and Tnf was also observed (36–39), suggesting that upregulation of these genes is a common feature of methylation-associated inflammation. Especially for human Il1B, its allele with a specific single nucleotide polymorphism is known to be associated with increased gastric cancer risk and increased incidence of CDH1 promoter methylation in gastric cancers (40, 41). Also, increased production of nitric oxide, due to upregulation of a nitric oxide synthase (NOS2) by IL1B or administration of nitric oxide donors, induced methylation of FMR1 and HPRT genes in vitro (42).

This study also clearly shows that methylation in gastric mucosa with HP infection consists of temporary and permanent components, which has been suggested by studies in humans (5, 10). Methylation that disappeared after eradication corresponds to the temporary component, and methylation that did not disappear corresponds to the permanent component. A pyloric gland (mucosal epithelia) is known to be composed of one or a few stem cells, multiple progenitor cells, and a large number of differentiated cells, and it is renewed within 3 to 14 days (43, 44). Temporary methylation is likely to have been induced in progenitor or differentiated cells, which will finally drop off from the gastric epithelium. Permanent methylation is likely to be induced in stem cells, which will remain for life. In humans, methylation levels in gastric mucosa without HP infection reflects methylation in stem cells.

HG2, SC3, and SD2 were methylated in GECs, although they were located in promoter CGIs, which are generally resistant to DNA methylation (29). Among promoter CGIs, those of genes with low transcription are known to be susceptible to methylation (30, 31, 45), and as expected, all the three genes had low transcription levels in GECs. Transcription levels at 10<sup>−6</sup> to 10<sup>−10</sup>/Gapdh (GAPDH) correspond to 1 to 10 copies of mRNA per cell and are less than 35% of the average expression level of all the genes analyzed by expression microarray (46). Because their methylation levels in GECs of gerbils infected with HP for 10 and 50 weeks were less than a few percent, their methylation was unlikely to have affected the overall expression levels in gastric mucosa. As a response to HP infection, Rnf132 was downregulated whereas Gpr37 and Nptx2 were not.

Promoter CGIs of GPR37 and NPTX2 were highly methylated in human gastric mucosa with HP infection and were frequently methylated in human gastric cancers. Because their tumor-suppressive functions have not been reported and they are not expressed in normal gastric mucosa (RefExA database<sup>4</sup>), their silencing is unlikely to be causally involved in gastric carcinogenesis, and they are considered to be passengers. Likewise, methylated CGIs that were not associated with genes were likely to be passengers. However, it is now known that a lot of passengers and limited number of drivers are methylated to high and small degrees, respectively, in human gastric mucosa with HP infection (5, 45). Therefore, although most methylation identified here was

<sup>4</sup> http://157.82.78.238/refexa/main_search.jsp
considered to be passenger, it is likely that tumor-suppressor genes are also methylated in association with their methylation. Gastric mucosa with accumulation of silencing of various genes, including both drivers and passengers, is considered to form a field where cancers will develop (epigenetic field for cancerization; refs. 7, 10, 47).

As a final effector of methylation induction, we examined overexpression of Dnmts, which are implicated in methylation induction in various human cancers (33). Unexpectedly, all the three Dnmts were downregulated by HP infection. Our recent data in humans also showed that mRNA levels of Dnmts had decreasing tendencies in HP-infected gastric mucosae (45). These results indicate that overexpression of Dnmts is not involved in HP-induced methylation induction, and suggest that local distribution of Dnmts and/or protective factors, such as the presence of RNA polymerase II (48), might be disturbed by inflammation.

Genome-wide screening to isolate DNA fragments methylated by HP infection was done by MS-RAA, which is applicable to any species without genome information. We used cell lines as the driver so that we could avoid heterogeneity of primary samples and aberrant methylation will be present in all the DNA molecules in the driver. This was considered to be essential for a genome-wide screening because most methods cannot detect small differences. Although cell lines might have artificial methylation, we confirmed the presence of specific methylation in GECs, and a high-sensitivity methylation, qMSP, was used for this. As expected, methylation levels of CGIs identified here were small (i.e., a few percent) in GECs with HP infection, showing that the strategy was correct.

In summary, HP infection was causally involved in induction of aberrant DNA methylation, and a critical role of inflammation in the induction was indicated. This model is expected to be useful in analyzing detailed molecular mechanisms for induction of aberrant DNA methylation.

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Dr. Takashi Sugimura for his critical discussion and sustained encouragement.

Grant Support


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 7/24/09; revised 11/10/09; accepted 11/27/09; published Online First 2/2/10.

References


Inflammatory Processes Triggered by *Helicobacter pylori* Infection Cause Aberrant DNA Methylation in Gastric Epithelial Cells

Tohru Niwa, Tetsuya Tsukamoto, Takeshi Toyoda, et al.

*Cancer Res* 2010;70:1430-1440. Published OnlineFirst February 2, 2010.

Updated version: Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-2755

Supplementary Material: Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/02/01/0008-5472.CAN-09-2755.DC1

Cited articles: This article cites 48 articles, 19 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/4/1430.full.html#ref-list-1

Citing articles: This article has been cited by 35 HighWire-hosted articles. Access the articles at: /content/70/4/1430.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.