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

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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 noncancerous tissues of patients with inflammation-associated cancers, such as liver cancers, ulcerative colitis–associated colon cancers, and gastric cancers (3–7). However, a causal role of chronic inflammation in methylation induction remains to be established.

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In human gastric mucosae, 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 representational difference analysis (MS-RDA). Using the CGIs isolated, 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 maintained in RPMI 1640 supplemented with 10% fetal bovine serum 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 previously (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 and stomachs were resected. Because it was difficult to identify cancers macroscopically in gastric mucosae with severe hyperplasia, we dissected an area of gastric cancer tissue by an apparatus for laser microdissection (ASLMD, Leica Microsystems) after histologic confirmation. For temporal analysis of methylation levels, male gerbils were inoculated with *HP* (ATCC 43504) at 5 wk of age. Eradication therapy was done at 55 wk of age by administering amoxicillin, clarithromycin, and lansoprazole by gavage (20). Gerbils that had *HP* after the eradication therapy were excluded from analysis. As a vehicle control, 0.5% of carboxymethyl cellulose was given by gavage. To suppress gastritis, gerbils were administered with 250 μg/mL cyclosporin A (CsA; Neoral, Novartis Pharma) in drinking water for 20 wk. The stomach was resected and cut along the greater curvature. From the posterior wall of the pyloric region (pyloric antrum), which contains the pyloric glands, gastric epithelial cells (GEC) were isolated by the gland isolation technique (21). The anterior wall of the pyloric region was further cut into two pieces: one for RNA and DNA extraction from a sample with mucus and mucosal and submucosal layers and the other for histologic analysis. Whole blood was obtained from the inferior vena cava. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Human clinical samples. Human gastric mucosae were obtained by endoscopic biopsy from 10 *HP*-negative (five men and five women; average age 42.4 y, ranging from 29 to 56 y) and 10 *HP*-positive (four men and six women; average age 42.4 y, ranging from 23 to 53 y) healthy volunteers, whose *HP* status had been judged by a serum anti-*HP* antibody test (SBS). Gastric cancer samples were obtained from surgical specimens from 14 patients who underwent gastrectomy due to early gastric cancers (seven men and seven women; average age 65.9 y, ranging from 47 to 79 y). Sampling 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 proteinase 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-d $(T)_{12-18}$ 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 *jhpr3* gene of *HP* was carried out and normalized to the gerbil *Il4* gene (Supplementary Table S1).

Methylation-sensitive representational difference analysis. MS-RDA is a subtraction method that can identify differentially methylated loci between two genomes independent of genomic information (23) and was done using *Hpa*II or *Sac*II 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 DNA with *Sss*I methylase (New England Biolabs) and amplifying genomic DNA with ϕ29 DNA polymerase (GenomiPhi DNA Amplification Kit, GE Healthcare), respectively (25). One microgram of DNA digested with *Bam*HI 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 *Bam*HI 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 described previously (22). Quantitative MSP (qMSP) was done

Table 1. CGIs methylated in gerbil gastric cancer cell lines and *HP*-infected GECs

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

*Conserved regions identified in the human database.

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 S2 and Supplementary Fig. S1) and the *Alu* repeat was used for human DNA (27).

Statistical analysis. Statistical analyses were conducted with SPSS 13.0J (SPSS Japan, Inc.). To evaluate significant difference 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 *Hpa*II and *Sac*II 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 from *HP*-infected gerbils (Supplementary Fig. S2). 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

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.

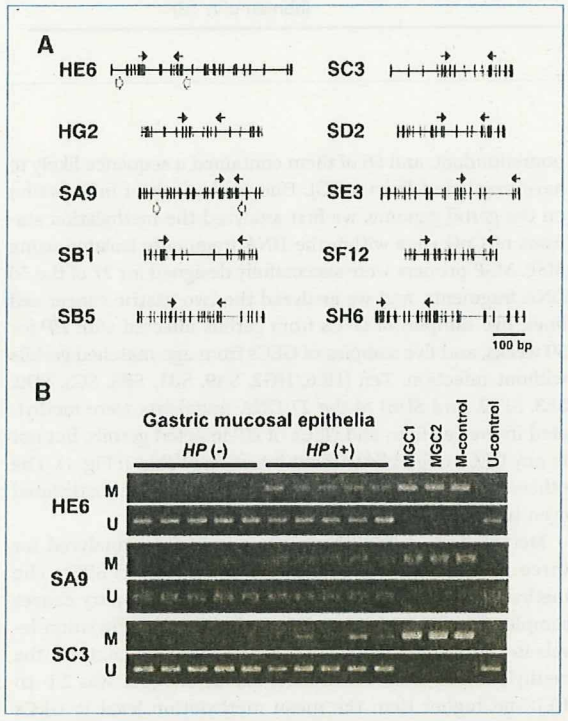

Figure 1. Isolation of CGIs that were aberrantly methylated in gerbil gastric cancers and GECs. A, a CpG map of the fragment isolated by MS-RDA. Vertical lines, individual CpG sites; arrows, positions of MSP primers; open arrows (HE6 and SA9), positions of bisulfite sequencing primers. B, representative results of MSP analyses in GECs from gerbils with and without *HP* infection for 50 wk and gastric cancer cell lines. M, MSP using a primer pair specific to methylated DNA; U, MSP using a primer pair specific to unmethylated DNA; M-control, genomic DNA treated with *SssI* methylase; U-control, DNA amplified with GenomiPhi.

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,*

Il6, Il7, 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, Il1b, Nos2*, and *Tnf*, paralleling inflammatory cell markers (Fig. 4B). The *Cox2, Ifng, Il2, Il4*, and *Il6* expression did not parallel the methylation levels after *HP* eradication, and the *Il7* 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 SB1 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

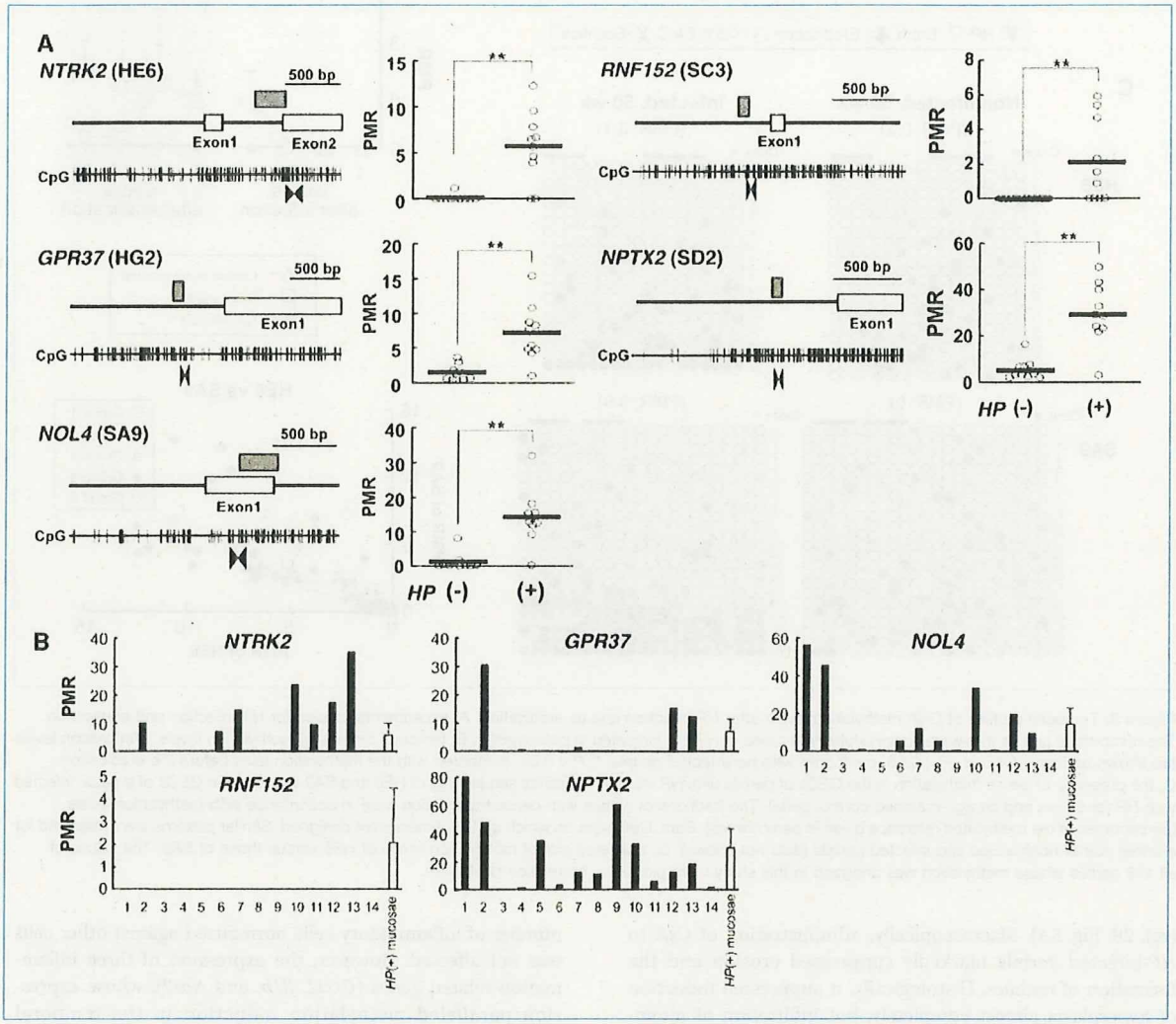

Figure 2. Methylation of homologous regions in human gastric mucosae. A, methylation levels in human gastric mucosal biopsies. Left, genomic structures and the regions analyzed by qMSP. Vertical lines, individual CpG sites; gray box, regions with homology between gerbil and man; open boxes, exons; faced 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.

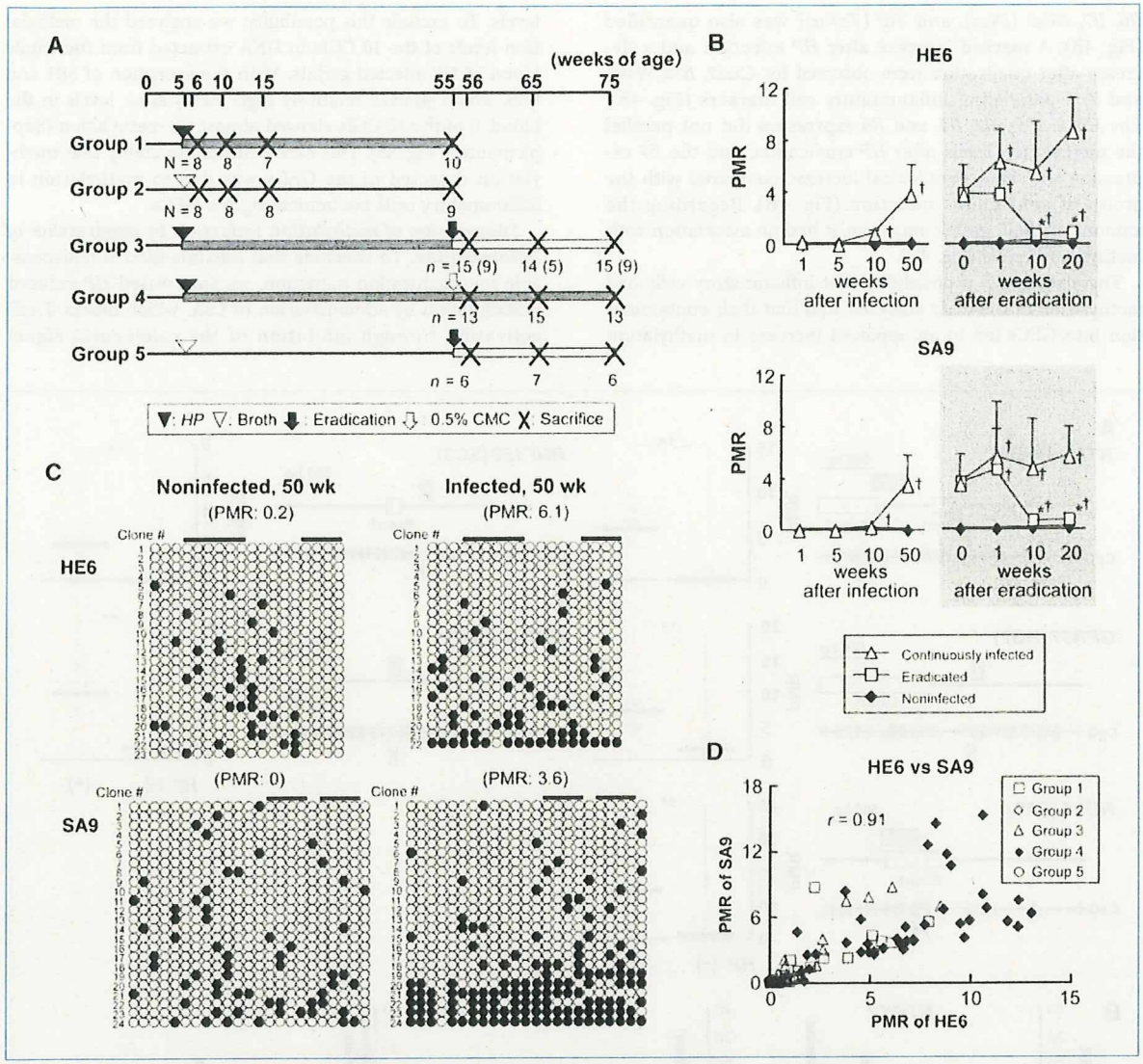

Figure 3. Temporal profiles of DNA methylation levels after *HP* infection and its eradication. A, experimental design for *HP* infection and eradication. The numbers of gerbils that were successfully eradicated of *HP* are indicated in parentheses. B, temporal profiles of methylation levels. Methylation levels are shown as mean + SD. †, $P < 0.05$, compared with noninfected gerbils; *, $P < 0.05$, compared with the methylation level before the eradication. C, the presence of dense methylation in the GECs of gerbils with *HP* infection. Bisulfite sequencing of HE6 and SA9 was done in GECs of a gerbil infected with *HP* for 50 wk and an age-matched control gerbil. The fractions of clones with dense methylation were in accordance with methylation levels (percentages of the methylated reference given in parentheses). Bars, CpG sites on which qMSP primers were designed. Similar patterns were observed for another pair of noninfected and infected gerbils (data not shown). D, scattered plot of methylation levels of HE6 versus those of SA9. The values of all 149 gerbils whose methylation was analyzed in this study were plotted. *r*, correlation coefficient.

(ref. 28; Fig. 5A). 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

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

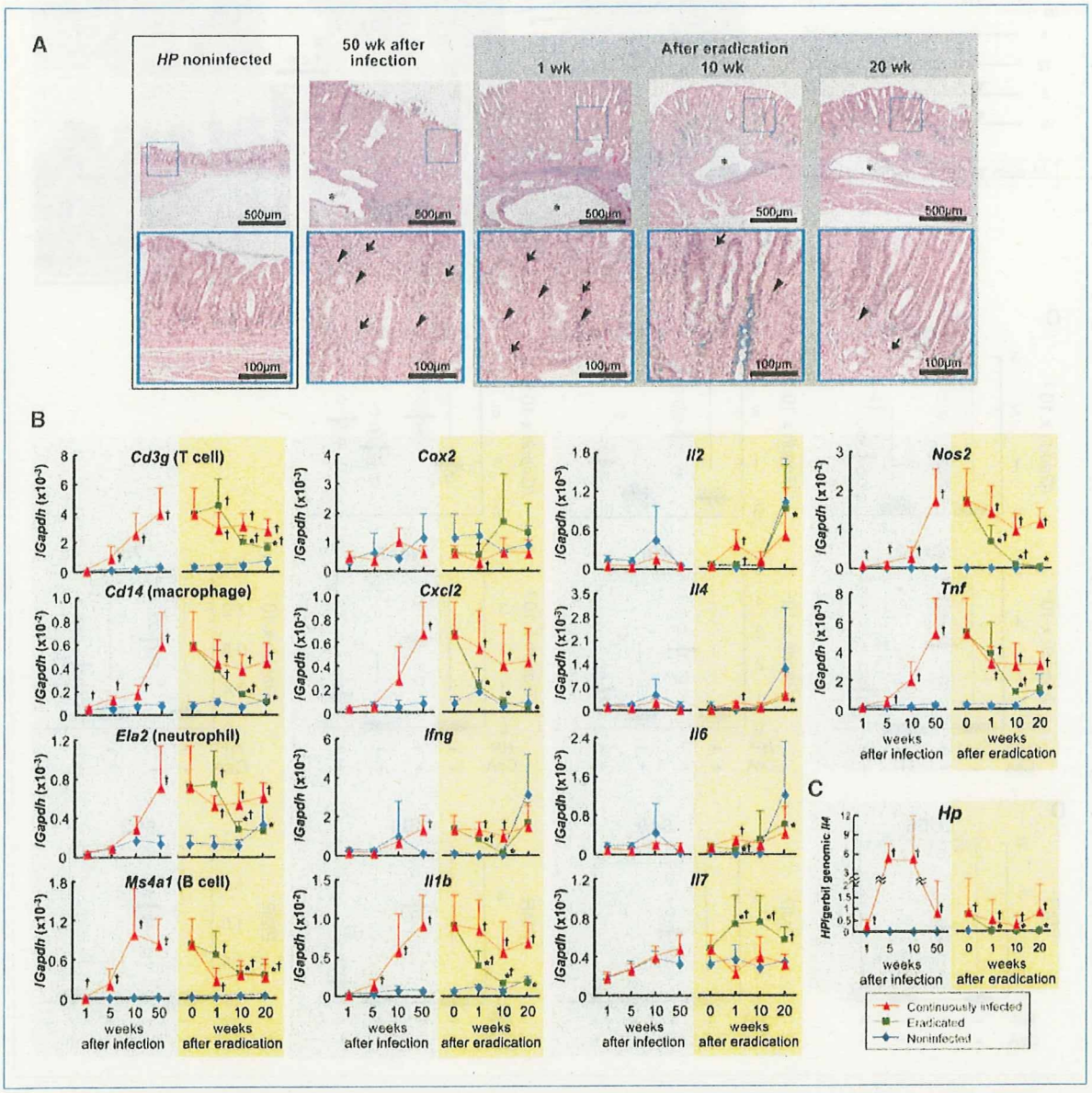

Figure 4. Changes in inflammation after *HP* infection and its eradication. A, histologic changes in gastric mucosa before and after *HP* eradication. Sections were stained with hematoxylin, eosin, and Alcian blue. Infiltration of numerous mononuclear cells (arrowheads) and polymorphonuclear cells (arrows) 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.

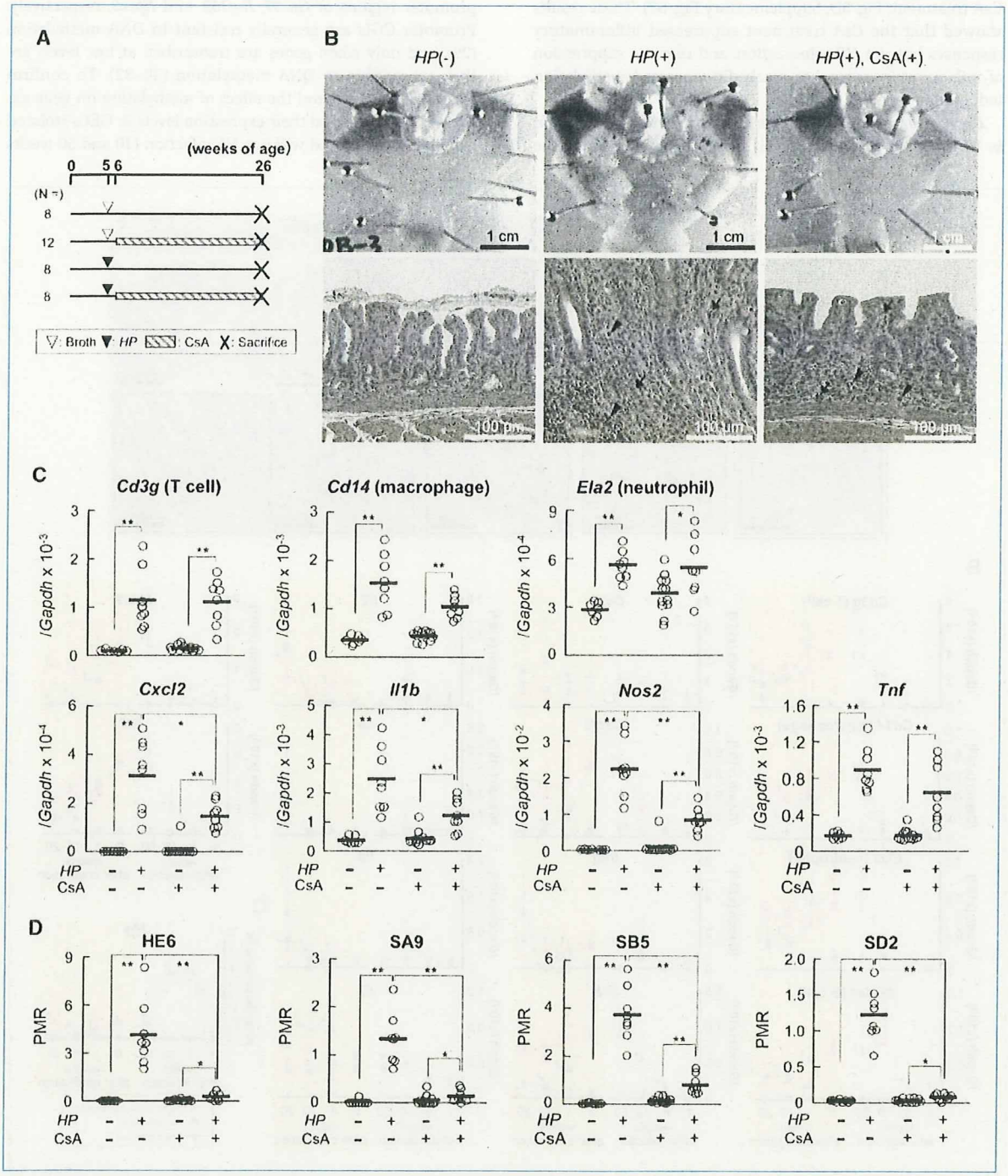

Figure 5. Suppression of inflammation and methylation induction by CsA treatment. A, experimental design for CsA treatment and *HP* infection. B, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in *HP*-infected gerbils without 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 noninfected and infected gerbils (Supplementary Fig. S8). *Rnf152* 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 genes 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 *Dnmt1, Dnmt3a*, and *Dnmt3b* 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*. 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 mucosae 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 mucosae without *HP* infection correlate with gastric cancer risk (5, 10), and this fact is also in line with the hypothesis that permanent methylation in gastric mucosae 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^{-4} to 10^{-3}/*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 mucosae. As a response to *HP* infection, *Rnf152* was downregulated whereas *Gpr37* and *Nptx2* were not.

Promoter CGIs of *GPR37* and *NPTX2* were highly methylated in human gastric mucosae 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 mucosae (RefExA database[4]), 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 mucosae with *HP* infection (5, 45). Therefore, although most methylation identified here was

[4] 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-RDA, 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 method, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Methylation destiny

Moira takes account of histones and RNA polymerase II

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Aberrant DNA methylation is deeply involved in various human disorders. Contrary to our initial expectation, aberrant methylation is now known to possess several unique characteristics different from mutations, including target gene specificity. Specific cancers have methylation of specific genes and specific inducers of methylation, such as *Helicobacter pylori* infection, induce methylation of specific genes. Mechanistically, it has been known that low levels of transcription of a gene promote its methylation. Multiple studies have shown that high levels of trimethylation of histone H3 lysine27 in normal cells are associated with a risk of becoming methylated during carcinogenesis. We recently demonstrated that genes with high levels of binding of RNA polymerase II, regardless of transcription levels, are resistant to induction of aberrant methylation. Now, epigenetic destiny can be predicted by these factors and interference with these factors might be able to change the destiny.

Key words: epigenetics, aberrant DNA methylation, *H. pylori*, tobacco smoking, histone modification, RNA polymerase II

Abbreviations: CGI, CpG island; *H. pylori*, *Helicobacter pylori*; MeDIP, methylated DNA immunoprecipitation; H3K27me3, trimethylation of histone H3 lysine27; PRC, polycomb repressive complex; DNMT, DNA methyltransferase; H3K9me3, trimethylation of histone H3 lysine9; H3Ac, acetylation of histone H3; H3K4me3, trimethylation of histone H3 lysine4; TSS, transcription start site; NFR, nucleosome free region; Pol II, RNA polymerase II

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Introduction

DNA methylation of a promoter CpG island (CGI) causes silencing of its downstream gene by multiple mechanisms.[1] When aberrant methylation occurs in promoter CGIs of genes involved in human disorders, such as tumor-suppressor genes, it inactivates these genes and is causally involved in human disorders.[2-4] To inactivate tumor-suppressor genes, aberrant methylation is an alternative mechanism to point mutations and chromosomal losses.[5,6] Historically, inactivation of tumor-suppressor genes by mutations was discovered more than a decade earlier than inactivation by aberrant methylation, and characteristics of aberrant methylation were assumed to be similar to those of mutations.

However, recent studies have revealed that aberrant DNA methylation possesses unique characteristics different from mutations,[7] such as deep involvement of chronic inflammation in its induction,[8] target gene specificity in its induction,[9,10] the presence at high levels in non-cancerous tissues[11-13] and a large number of affected genes in a single cancer cell.[14-16] In a generally accepted multistep carcinogenesis model, a mutation is induced in random genes, with some preference of expressed genes, in a population of cells, and a cell that accidentally harbored mutation of a specific gene, such as tumor-suppressor gene, is selected.[17] In contrast, methylation is now recognized to be induced in specific genes in specific types of cancers[14,18,19] and by specific inducers, such as *Helicobacter pylori* (*H. pylori*) infection[11] and tobacco smoking.[10] Here, we will focus on the presence of target gene specificity in methylation induction and the mechanisms involved in it.

DNA Methylation of Specific Genes in Cancers

The presence of target gene specificity in DNA methylation induction was initially indicated by the presence of methylation of specific genes in cancer cells.[14,18,19] A pioneering study of 1,184 non-biased CGIs using restriction landmark genomic scanning revealed that some specific CGIs

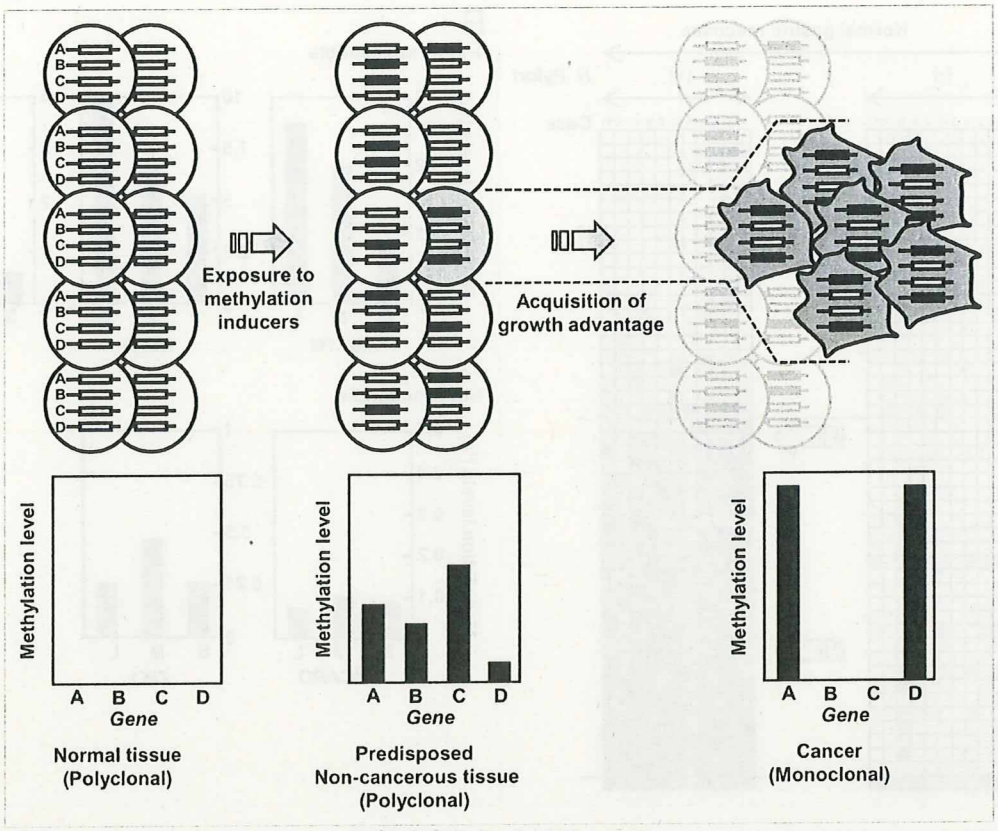

Figure 1. Different meaning of aberrant DNA methylation in non-cancerous and cancer tissues. Cells in an entirely normal tissue contain no aberrant methylation, and, by exposure to methylation inducers, cells come to harbor aberrant methylation of specific genes. A cancer, consisting of many cancer cells, develops from a single precursor cell that contains aberrant methylation of a tumor-suppressor gene (gene D). Since aberrant methylation of a tumor-suppressor gene confers growth advantage, all the cancer cells have its methylation even if it is not a specific target for methylation induction and is rarely induced in non-cancerous tissues. In contrast, methylation of genes whose inactivation does not confer growth advantage (genes A, B and C) is stochastically carried over into a cancer tissue. Therefore, a methylation pattern in a cancer tissue reflects events that incidentally happened in its single precursor cell, and target gene specificity can be assessed only by analyzing a large number of cancers. In contrast, a methylation pattern in a non-cancerous tissue reflects events that happened in any of the many cells in the tissue, and target genes have high levels of methylation.

were methylated at high incidences in specific tumor types among seven tumor types.[14] Analysis of promoter CGIs of mostly tumor-suppressor genes also showed that some CGIs were methylated at high incidences in specific tumor types.[18] A comprehensive analysis of colon cancers using the modern technology of methylated DNA immunoprecipitation (MeDIP)-microarray analysis revealed that most methylated genes were located within defined genomic clusters, were associated with common sequence motifs, belonged to specific functional categories, and had low transcription levels already in normal cells.[19]

However, analysis of cancer cells always raises a question about the role of a gene inactivated in a cancer. "Did the inactivation confer a growth advantage to a cell with it, and thus was the cell selected?" Since inactivation of different sets of genes is expected to confer growth advantage to cells of different tissues, DNA methylation of different sets of genes can be simply explained by functional selection, rather than by methylation induction of specific genes in a specific tissue context (**Fig. 1**). To avoid this limitation, analysis of non-cancerous tissues where functional selection has not taken place yet is a good solution. Also, it enables us to analyze numerous independent events in different cells while analysis of a cancer, a monoclonal lesion, provides information on the events that took place in a single precursor cell of the cancer.

The Presence of Aberrant DNA Methylation in Non-Cancerous Tissues

Again, going back to the era of mutations, it was a challenging idea to analyze mutations in non-cancerous tissues. Since mutations are present only in a very minor fraction of cells in non-cancerous tissues (1 of 10^3 to 10^5 cells), they cannot be detected by ordinary sequencing techniques and their frequency can be measured only by special methods that introduce positive selection of mutants.[20] Instead, if a sequence polymorphism is detected in a cancer tissue, it can be established as a mutation by confirming its absence in the surrounding non-cancerous tissue of the same individual.[21]

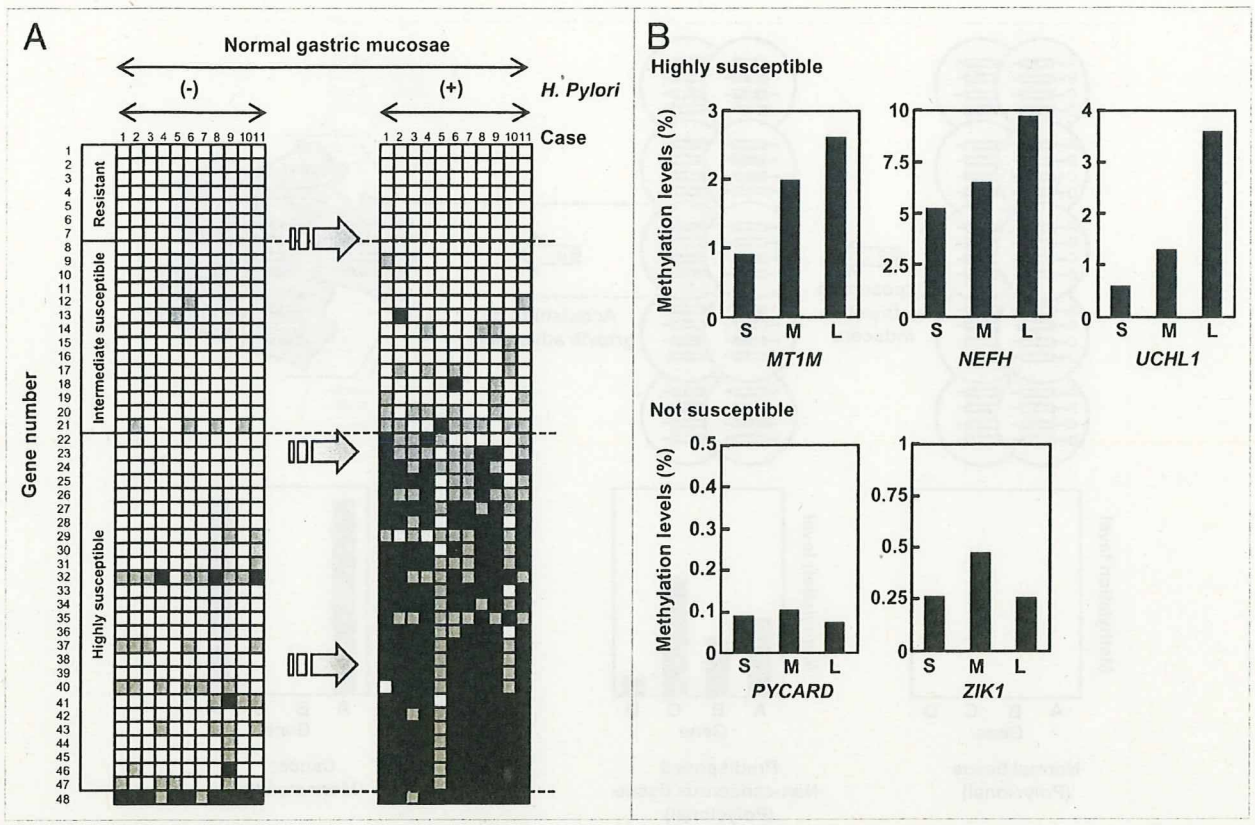

Figure 2. Target gene specificity in DNA methylation induction in non-cancerous tissues. (A) Methylation profile of the 48 genes in normal gastric mucosae with and without *H. pylori* infection (modified from Nakajima et al.[9]). The methylation status of each gene is represented as unmethylated (white), weakly methylated (gray), and highly methylated (black). Seven genes (gene 1 to 7) were completely resistant to aberrant methylation induction. Fourteen genes (gene 8 to 21) were slightly susceptible to methylation induction. Twenty-six genes (gene 22 to 47) were highly susceptible to methylation induction. (B) The correlation between smoking history and aberrant methylation in specific genes (modified from Oka et al.[10]). Average methylation levels in non-cancerous esophageal mucosae of individuals with short (S, no or smoking duration <21 years), middle (M, smoking duration; <40 years but more than 21 years), and long (L, smoking duration is more than 40 years) smoking history. *MT1M*, *NEFH* and *UCHL1* were considered to be susceptible to methylation induction by smoking.

In contrast, researchers in cancer epigenetics field became aware that aberrant DNA methylation could be detected in a minor fraction of cells, even in non-cancerous tissues.[22-26] Different from mutations, methylation is physiologically present in various regions of the genome and, to demonstrate that methylation of a genomic region is *aberrant*, its absence in the corresponding normal tissue needs to be established. Even adopting this stringent criterion, aberrant methylation was detected in histologically normal non-cancerous liver tissues of patients with a liver cancer[22] and in non-cancerous gastric epithelia of patients with a gastric cancer.[23] Possible aberrant methylation was detected in Barrett's esophagus,[24] colonic mucosae of patients with ulcerative colitis[25] and gastric tissue of gastric cancer patients.[26]

To connect the presence of aberrant DNA methylation in non-cancerous tissue to cancer risk, we systematically collected samples from gastric tissues of entirely healthy individuals and non-cancerous gastric tissues of gastric cancer patients, and quantified methylation levels in individual samples.[11,13] Methylation levels were about 5- to 300-fold higher in the latter samples than in the former samples, among individuals without *H. pylori* infection. At the same time, *H. pylori* infection, a potent risk factor for gastric cancers,[27] was associated with temporarily high levels of methylation.[11,28] Other studies also showed that aberrant methylation is already accumulated in non-cancerous tissues, and that the accumulation is associated with cancer risk in multiple types of cancers,[12] such as esophageal,[29] breast[30] and renal cancers.[31]

Target Gene Specificity of Methylation Induction in Non-Cancerous Tissues

It is now clear that aberrant DNA methylation is present in non-cancerous tissues. And, we can analyze methylation induction in a large number of cells, although methylation levels are expected to be low, compared with those in cancers (Fig. 1). However, only limited numbers of specific inducers of aberrant methylation have been established so far,[8] including *H. pylori* infection,[9,11] hepatitis virus[32]

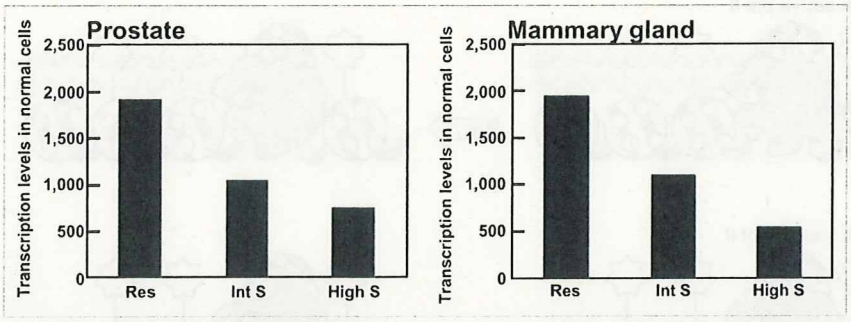

Figure 3. Low transcription levels of genes susceptible to DNA methylation induction (modified from Takeshima et al.[39]). Genes were classified into those resistant (Res), intermediately susceptible (Int S), and highly susceptible (High S) to methylation induction during carcinogenesis. Their transcription levels in normal prostatic epithelial cells (left) and normal mammary epithelial cells (right) were measured by expression microarray. A gradual decrease of transcription levels in genes with higher susceptibility was observed.

and tobacco smoking.[10] In the case of *H. pylori* infection, we recently demonstrated that inflammation induced by it is critical for methylation induction.[33] To reveal the target gene specificity in aberrant DNA methylation induction by *H. pylori*, we sensitively analyzed methylation of 48 genes, which can be methylated at least in gastric cancer cell lines,[34] in human gastric mucosae with and without *H. pylori* infection (**Fig. 2A**).[9] It was clearly shown that some genes were susceptible to methylation induction by *H. pylori* infection while others were resistant. The susceptible genes had lower transcription levels in normal gastric mucosae than the resistant genes. Target gene specificity by tobacco smoking was also present in esophageal mucosae. When we quantified methylation levels of 13 genes, which can be methylated in esophageal cancers, methylation levels of only five genes had significant correlations with duration of tobacco smoking (**Fig. 2B**).[10]

Role of Low Transcription in Target Gene Specificity

Regarding the mechanisms underlying the target gene specificity, low transcription in normal cells was proposed in the early 2000s.[35-38] As mechanistic analyses in vitro, Song et al. demonstrated that disruption of promoter activity (thus low transcription levels) of a transfected gene leads to aberrant DNA methylation of promoter CGIs in a cancer cell line.[35] Using an endogenous gene demethylated by a DNA demethylating agent, 5-aza-2'-deoxycitidine, de Smet et al. demonstrated that the demethylated gene becomes remethylated when it is not transcribed.[36] As for findings in vivo, we showed that most genes methylated in pancreatic cancers and malignant melanomas had no or low transcription levels in their normal counterpart cells.[37,38] Genome-wide studies using microarrays in colorectal, prostate, and breast cancers also showed that genes with low transcription in normal cells tend to be methylated in cancers (**Fig. 3**).[19,39] Even using genes methylated in non-cancerous tissues, genes susceptible to aberrant methylation had lower transcription levels than resistant genes.[9]

Role of Histone Modifications in Target Gene Specificity

As another mechanism for the target gene specificity, histone modifications have drawn a lot of attention over the last couple of years. Using selected genes, three groups demonstrated that genes methylated in cancers are pre-marked by trimethylation of histone H3 lysine 27 (H3K27me3) in embryonic stem cells[40-42] and normal corresponding tissue.[42] Premark by H3K27me3 of genes that will become methylated in cancers was further confirmed using genes identified by DNA methylation microarray analysis.[39,43,44] H3K27me3 is known to be recognized by a polycomb repressive complex (PRC).[45-47] A component of PRC2, EZH2, and, that of PRC1, CBX7, are known to interact with DNA methyltransferases (DNMTs),[48,49] and there is a possibility that H3K27me3 functions as a recruiting signal for DNMTs. Another representative repressive histone modification, trimethylation of histone H3 lysine 9 (H3K9me3), in normal cells was not associated with genes that become methylated during carcinogenesis.[39]

Regarding histone modifications of active chromatin, we observed that genes resistant to aberrant DNA methylation tend to have acetylation of histone H3 (H3Ac) and trimethylation of histone H3 lysine4 (H3K4me3) in normal cells.[39] Active histone modifications are known to be recognized by proteins involved in transcriptional activation, such as ATP-dependent chromatin remodeling complex, SWI/SNF[50] and the basal transcription factor, TFIID,[51] and are associated with high levels of transcription. The resistance of genes with active histone modifications to methylation induction may be dependent upon high levels of gene transcription.

Role of RNA Polymerase II Binding, Active or Stalled, in Target Gene Specificity

Although genes with low transcription levels are susceptible to DNA methylation induction, many such genes are still resistant. Even if limited to genes that have low transcription and H3K27me3 in normal cells, 16% of them are still resistant to methylation induction during carcinogenesis.[39] This indicates that there are additional factors that confer resistance to methylation induction. At individual gene

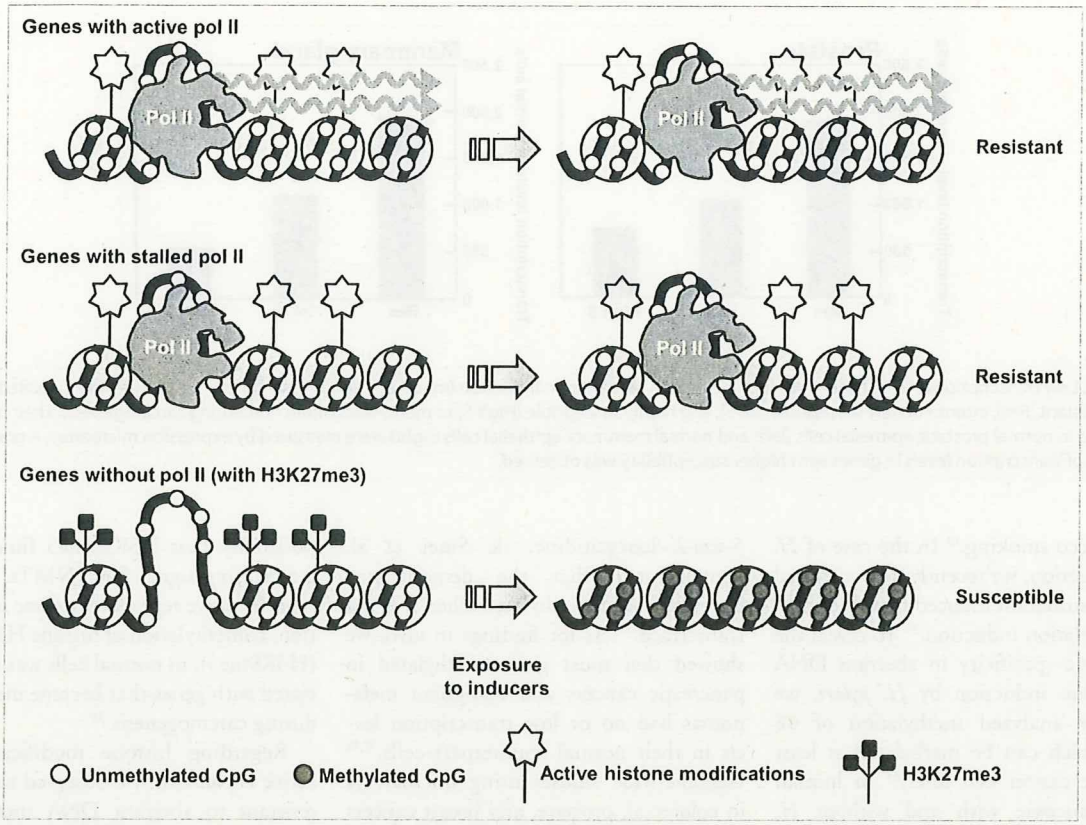

Figure 4. A scheme of the instructive mechanism of aberrant DNA methylation induction. Both genes with active Pol II and genes with stalled Pol II are resistant to aberrant methylation induction. In contrast, genes without Pol II but with H3K27me3 are highly susceptible to aberrant methylation induction.

levels, SP1/3 and MLL have been reported to be involved in resistance of the *APRT* and *Hoxa9* genes, respectively, to methylation induction.[52-54]

A region just upstream of a transcription start site (TSS), designated as a nucleosome-free region (NFR),[55] is most resistant to DNA methylation induction,[56] indicating that something there is associated with resistance to methylation induction. Recent studies showed that RNA polymerase II (Pol II) is stalled at NFRs for some genes with low transcription levels,[57,58] and we decided to focus on Pol II as a factor that confers resistance to methylation induction. Genome-wide analysis of histone modifications and Pol II binding in normal cells revealed that, even among genes with low transcription, high levels of Pol II binding and active histone modifications were associated with resistance to methylation induction during carcinogenesis.[39] By multivariate analysis, Pol II binding had stronger influence on the resistance than active histone modifications. These results showed that the presence of Pol II, active (high transcription levels) or stalled (low transcription levels), is associated with resistance to methylation induction during carcinogenesis (Fig. 4).

Pol II forms a large complex with several general transcription factors,[59] and such a large complex around NFRs might inhibit the recruitment of DNMTs. Further analysis is needed to establish cause-consequence relationship between the presence of Pol II and resistance to DNA methylation induction, and to clarify molecular mechanisms of why genes with high Pol II binding are resistant to methylation induction.

Concluding Remarks

The presence of target gene specificity in DNA methylation induction indicates that a methylation profile specific to a carcinogenic factor can be used as a methylation fingerprint that tells past exposure to the factor. Since target genes are premarked by the presence of H3K27me3 and the absence of Pol II binding, methylation fingerprints are likely to be present for various inducers of aberrant methylation. Methylation fingerprints in individual tissues are likely to become available in the coming years and, if such fingerprints are also present in peripheral leukocytes, a new field of epigenetic epidemiology will be opened up.

The fact that genes with active transcription are resistant to DNA methylation induction can be rephrased as "iron (a gene) rusts (is methylated) from disuse (without Pol II binding)," or "use it, or lose it." An important implication is that we might be able to protect a gene from becoming methylated by bringing Pol II to it. Although the distribution of H3K27me3 is likely to be predetermined

by cell types, the distribution of Pol II is considered to be modifiable, for example by inducing gene transcription. If we can develop a method, including use of chemicals, which can change the thread by Goddess Moira, it is likely to be a novel method for disease prevention by keeping our epigenome fresh.

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Review Article

Aberrant DNA methylation in contrast with mutations

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Aberrant DNA methylation is known as an important cause of human cancers, along with mutations. Although aberrant methylation was initially speculated to be similar to mutations, it is now recognized that methylation is quite unlike mutations. Whereas the number of mutations in individual cancer cells is estimated to be ~80, that of aberrant methylation of promoter CpG islands reaches several hundred to 1000. Although mutations of a specific gene are very few in non-cancerous (thus polyclonal) tissues (usually at 1×10^{-5}/cell), aberrant methylation of a specific gene can be present up to several 10% of cells. Mutagenic chemicals and radiation are well-known inducers of mutations, whereas chronic inflammation is deeply involved in methylation induction. Although mutations are induced in mostly random genes, methylation is induced in specific genes depending on tissues and inducers. Methylation is potentially reversible, unlike mutations. These characteristics of methylation are opening up new fields of application and research. (*Cancer Sci* 2010; 101: 300–305)

Aberrant DNA methylation is deeply involved in human carcinogenesis,[1–3] and is often described as ''genome-overall hypomethylation and regional hypermethylation''. Genome-overall hypomethylation was discovered in the early 1980s[4,5] and has been shown to induce genomic instability and promote carcinogenesis.[6–8] Regional hypermethylation denotes methylation of normally unmethylated CpG islands (CGI) and, in particular, methylation of a promoter CGI is known to silence its downstream gene by multiple mechanisms, including aberrant nucleosome formation.[9,10] Inactivation of a tumor-suppressor gene was first discovered for *RB* in 1993,[5,11] and now a wide variety of tumor-suppressor genes, including *CDKN2A* (p16), *MLH1*, and *CDH1* (E-cadherin), are known to be inactivated by aberrant methylation.[2] In many types of cancers, aberrant promoter methylation is frequently observed and in some types of cancers, such as gastric cancers, aberrant methylation is more frequent than mutations in inactivating mechanisms of specific tumor-suppressor genes.[12]

In the 1990s, investigators found that tumor-suppressor genes can be inactivated by aberrant methylation of promoter CGI, and that most CGI analyzed by conventional methods were kept unmethylated, even in cancers. This made them think that genes with aberrant methylation of promoter CGI were tumor-suppressor genes. Some investigators were inspired that they could identify tumor-suppressor genes if they could identify aberrant methylation by genome-wide screening methods.[13–16] Actually, these methods contributed to the identification of important CGI in diagnostic purposes and isolation of tumor-suppressor genes.[3] In addition, the fact that aberrant methylation of promoter CGI is an alternative to a mutation for inactivation of tumor-suppressor genes made many investigators think that epigenetic alterations would share similar features with mutations in other aspects, such as their frequencies in cancer and non-cancerous tissues, inducers, and target genes.

However, recent findings by high-resolution genome-wide analysis of DNA methylation and by many other approaches have shown that aberrant DNA methylation has many unique features different from mutations (here, point mutations and small base deletions) (Table 1). In this review, we will summarize the contrasts between these two kinds of alterations: aberrant DNA methylation and mutations.

Number of alterations in a cancer cell

Recent use of high-throughput sequencing and high-resolution microarray technologies has illuminated detailed genetic and epigenetic alterations in cancer cells.

Assessment of the role of genetic alterations in carcino genesis. The assessment of whether a specific sequence alteration is a mutation and what the role of a mutation is in carcinogenesis is relatively straightforward. If a possible sequence change is specifically present in cancer tissues but not in non-cancerous tissues, it is a somatic mutation. If the mutation alters the amino acid sequence of an encoded protein, it is a candidate for a driver mutation.[17,18] Comparison between the incidence of mutations with amino acid alteration and that of silent mutations can provide information on whether there is a selection bias for cells with a mutation of the gene in carcinogenesis. Mutations that drive the initiation, progression, or maintenance of a cancer are classified as driver mutations, and mutations that simply accompany carcinogenesis or are produced as a result of transformation are classified as passenger mutations.

Number of driver and passenger mutations in cancers. As high-throughput sequencing becomes more powerful, a wider selection of genes has been analyzed for broader ranges of cancers. By sequencing more than 20 000 transcripts in breast and colon cancers, it was estimated that approximately 80 non-silent mutations are present in a typical cancer, and that <15 genes are likely to be driver mutations.[18] By sequencing of a wide variety of cancers for selected genes (518 protein kinases), it was shown that lung cancers harbor more mutations than colon and gastric cancers, and that one-third of cancers did not have any somatic mutations in these kinases.[17] The presence of a limited number of driver mutations and a large number of passenger mutations was confirmed in these studies.

Assessment of the role of "aberrant" methylation in carcino genesis. In contrast to mutations, assessment of the biological significance of ''aberrant'' DNA methylation is very difficult. At least, the effect of methylation on gene silencing and the role

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Table 1. Comparison between mutations and DNA methylation

	Mutation	DNA methylation	References
Number of alterations per cancer cell	~80	Several hundred to 1000	(18,23,27–30)
Frequency of alterations of a specific gene in non-cancerous tissues	10^{-5}/cell, up to 10^{-3}/cell	0.1 to several % up to several 10% of cells	(44,46)
Inducers	Mutagenic chemicals, radiation, oxygen radical	Chronic inflammation, aging	(45,56)
Target gene	Random	Specific	(18,27,37,61)
Reversibility	Irreversible	Reversible	(18,61,70–73)

Detailed explanations are in individual sections.

of the silencing in carcinogenesis need to be assessed separately and precisely.

To assess the effect on gene silencing, the location of a methylated region and the CpG density of the region are critically important.[19,20] The methylation status of promoters with high CpG density, namely promoter CGI, has a clear association with decreased transcription whereas that of promoters with low CpG density are unclear. Depending on the relative position against a transcription start site (TSS), the degree of association between DNA methylation and decreased gene expression is different. Methylation of a 200–300-bp upstream region of a TSS has been known to be consistently associated with repressed transcription.[1–3,21] The region is now known as a "nucleosome-free region" (NFR), which lacks a nucleosome[9] and whose DNA methylation leads to formation of nucleosome(s) and represses transcription.[10] Recent genome-wide studies also support the idea that methylation of NFR is consistently associated with low gene transcription.[19,20,22,23] At the same time, methylation of a far upstream region and exon 1 can also be associated with decreased transcription via methylation of the NFR. On the other hand, methylation of a gene body is occasionally associated with increased gene expression.[22–25] It is noteworthy that, even within a CGI, the methylation status of different regions is occasionally heterogeneous and investigators should analyze an appropriate region.[3]

Even if limited to DNA methylation that causes gene silencing, the role of the DNA methylation in carcinogenesis needs to be carefully assessed. As described below, there are hundreds to 1000 genes with methylation of their NFR in cancer cells, and it is likely that most of them are passengers. Also as described below, genes without expression in normal cells tend to become methylated in cancers, and such genes without expression are unlikely to be tumor-suppressor genes. To establish a gene with methylation of its NFR in cancers as a tumor-suppressor gene, we need mutation analysis of the gene in cancers and functional analysis of the gene after its transduction into cancer cells and expression at a physiological level and after its knock down in normal cells. Most tumor-suppressor genes are known to be inactivated by homozygous mutation, by combination of methylation and mutation, or by methylation of all copies, and methylation is more frequent than mutations.[26]

Number of methylation of CGI in NFR in cancers. Detailed pictures of CGI aberrantly methylated in cancers are becoming clear by microarray analysis combined with methylated DNA immunoprecipitation or methylated-CpG island recovery assay using methylated-DNA binding domain proteins.[23,27–30] As normalization of signals obtained by microarray is still under development[23,31–35] and CGI in various positions against TSS and various regions within CGI have been analyzed so far, it is difficult to compare different reports at this time.

According to our previous studies focusing on methylation of NFR in promoter CGI,[23,34] large fractions of them were methylated in gastric cancer cell lines (Table 2). Although there is controversy about how methylation in cell lines reflects that in primary cancers,[35,36] it seems safe to estimate that one-third to one-half of CGI methylated in cell lines are also methylated in primary cancers. We currently estimate that several hundred to 1000 NFR in promoter CGI are methylated in a primary cancer cell. If not limited to NFR, 216–848 of 27 800 CGI are reported to be methylated in primary lung squamous cell cancers.[30] If limited to methylation of NFR that can be detected by re-expression after treatment with a demethylating agent, the number decreases markedly, such as to less than 1/100.[23] These show that a large number of NFR and other CGI are methylated in cancers, which is in line with pioneering studies.[37,38] The large number is in sharp contrast to the number of mutations in a cancer.

Methylation of a specific gene in a large fraction of cells in non-cancerous tissues

DNA methylation shows a sharp contrast to mutations also in the fraction of cells with an alteration of a specific gene in non-cancerous tissues. Moreover, the degree of accumulation of aberrant DNA methylation can be associated with cancer risk.

Meaning of the fraction of cells with an alteration in cancer and non-cancerous tissues. The fraction of cells with an alteration (mutation or methylation) of a specific gene is often compared between cancer and non-cancerous tissues. However, the meaning of the fraction is entirely different in the two kinds of tissues.

Not to mention, a cancer develops after multiple processes of clonal selection (Fig. 1). In non-cancerous tissues, no selection for a cell with an alteration has been imposed yet, and thus the fraction of cells with the alteration is mainly determined by the frequency with which the alteration is induced. The frequency

Table 2. Estimated number of methylated CpG islands (CGI)

Cell lines	Nucleosome-free region	CGI (not restricted to promoters)
Stomach cancer	641–1205 of 9624 (6.6–12.5%)	3768–7310 of 30 533 (12.3–23.9%)
Prostate cancer	501–800 of 8930 (5.6–8.6%)	5593–7638 of 34 405 (16.3–22.2%)
Breast cancer	480–673 of 8866 (5.4–7.6%)	4118–4755 of 34 424 (12.0–13.8%)

The number of nucleosome-free regions and CGI analyzed are different in individual experiments because the number of probes assessed as functional was different in each experiment.