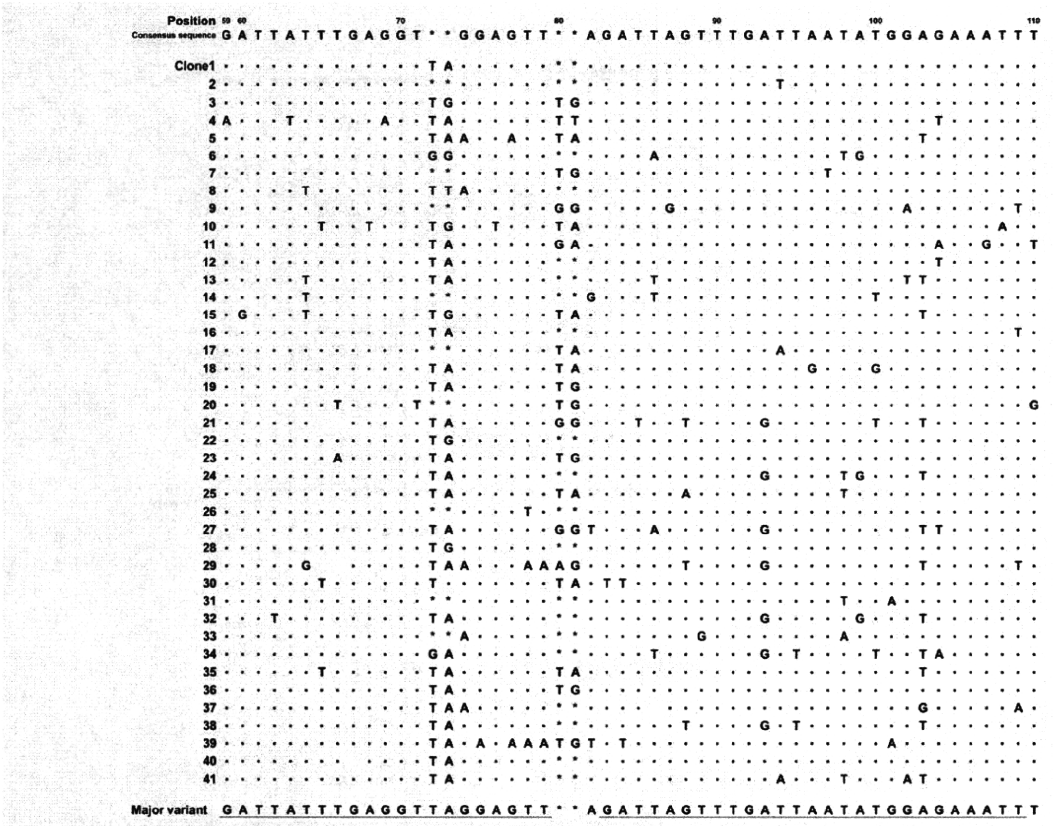

Figure 1. The most frequent Alu sequences after bisulfite conversion and location of CpG sites analyzed by pyrosequencing. The sequence obtained by virtual bisulfite conversion of a consensus sequence in the database is shown at the top. The 41 sequences are obtained by virtual bisulfite treatment of the sequences obtained. A dot shows no variation from the sequence at the top. Two consecutive asterisks show a CpG site. The most frequent sequence (shown at the bottom) was identified as the major variant. The sequences used for bisulfite pyrosequencing are underlined.

Statistical analysis

A difference in mean methylation levels or mean global 5-methylcytosine content was analyzed by the Welch *t* test. Correlation between the global 5-methylcytosine content by LCMS and that by HPLC-UV, correlation of methylation levels among repetitive DNA elements and correlation between age and methylation level were analyzed using Pearson's product-moment correlation coefficient. All the analyses were performed using SPSS (SPSS, Chicago, IL), and the results were considered significant when p values less than 0.05 were obtained by a two-sided test.

Results

Identification of the major variants of individual repetitive DNA elements

A major variant was identified for each of the three repetitive DNA elements to measure methylation levels of as many repeat units as possible. Based on the consensus sequence in the database, each element was amplified by PCR with low stringency, and clones obtained were sequenced (Supporting Information Fig. 1). Since Alu and LINE1 were more variable than Satα, more clones were sequenced for Alu (41 clones) and LINE1 (19 clones) than for Satα (12 clones). After virtual conversion by bisulfite treatment of the sequences obtained, the most frequent sequence was identified as the major variant for each repetitive element (Supporting Information Fig. 2).

Primers for bisulfite pyrosequencing (Alu in Fig. 1; and Supporting Information Table 2) were designed based on the major variant, covering the most frequent sequences at the CpG site. The Alu methylation level was measured at two CpG sites, ALU1 (position +80 in the consensus sequence) and ALU2 (+197), which represented five and six, respectively, of the 41 sequences. The LINE1 methylation level was measured at three CpG sites, LINE1-1 (+138), LINE1-2 (+206) and LINE1-3 (+270), which represented seven, eight and 11, respectively, of the 19 sequences. The SATα methylation level was measured at one CpG site (+360) that was common to all the 12 sequences.

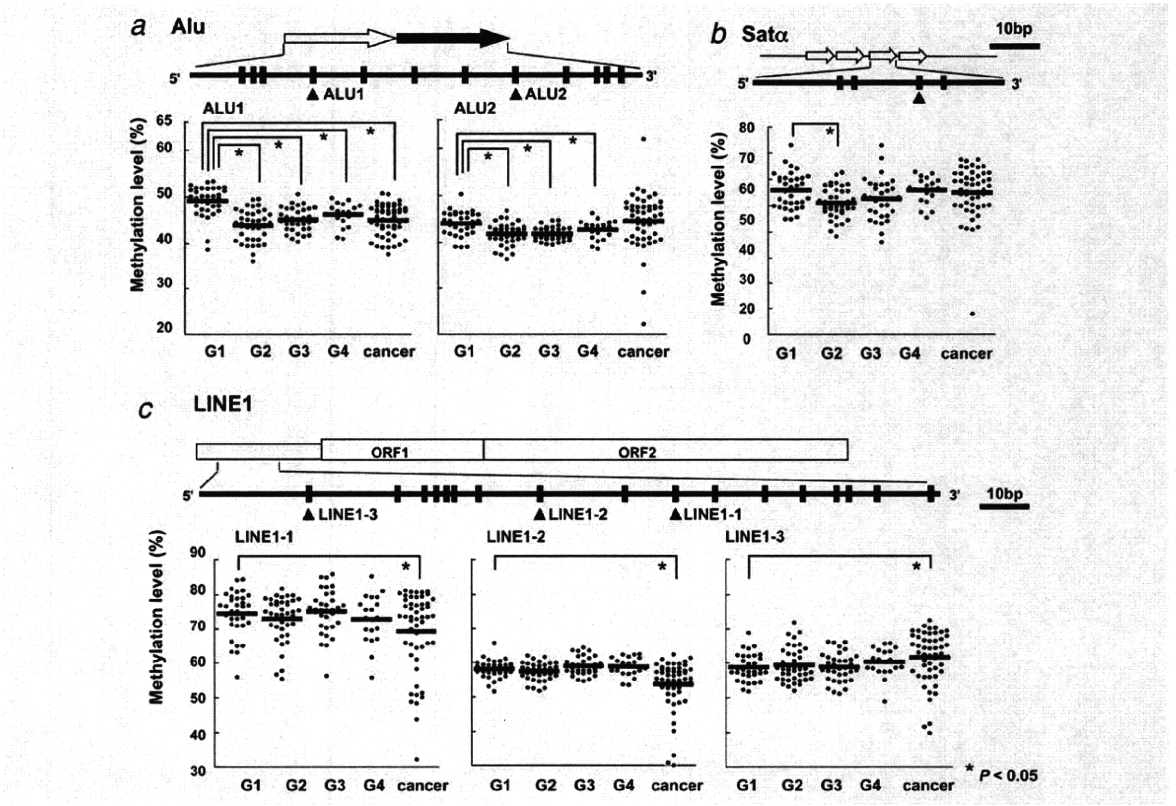

Figure 2. Methylation levels of the three repetitive DNA elements in gastric mucosae of G1-G4 and gastric cancers. Vertical ticks, individual CpG sites; arrowheads, locations of the measured CpG site. A horizontal line in a chart represents a mean methylation level for each group. (*a*) Distribution of methylation levels at two CpG sites of Alu. Compared with G1, the methylation level was decreased in G2, G3 and G4. The methylation level was decreased in cancers at ALU1 but not at ALU2. The top arrows show two duplicated arms of an Alu unit. (*b*) Distribution of methylation levels at one CpG site of Satα. Compared with G1, the methylation level was decreased in G2 and tended to be decreased in G3. The methylation level was not decreased in G4 and cancers. (*c*) Distribution of methylation levels at three CpG sites of LINE1. Compared with G1, the methylation level was not decreased in G2, G3 and G4 at LINE1-1, LINE1-2 and LINE1-3. The methylation level was decreased in cancers at LINE1-1 and LINE1-2 but was increased at LINE1-3. ORF: open reading frame.

The presence of Alu and Satα hypomethylation in *H. pylori*-infected gastric mucosae

The Alu methylation level was measured by bisulfite pyrosequencing in gastric mucosae (G1, G2, G3 and G4) and cancers (Fig. 2*a*; Supporting Information Table 3). In the normal control group (G1), the methylation level was 49.2 ± 3.2% (mean ± SD) at ALU1 and 44.1 ± 2.5% at ALU2. The mean methylation level at ALU1 was decreased in G2 (decreased to 89.2% of that in G1, $p < 0.05$), G3 (decreased to 91.9%, $p < 0.05$) and G4 (decreased to 94.1%, $p < 0.05$), and remained low in cancers (90.9% of that in G1, $p < 0.05$). Similarly, the mean methylation level at ALU2 was decreased in G2, G3 and G4 (decreased to 94.8, 95.0 and 97.1% of that in G1; $p < 0.05$, < 0.05 and < 0.05, respectively). However, a decrease was not observed in cancers (100.9% of that in G1, $p = 0.61$).

The methylation level of SATα was 55.9 ± 6.8% in G1 (Fig. 2*b*; Supporting Information Table 3). The mean methylation level was decreased in G2 (decreased to 91.6% of that in G1; $p < 0.05$) and had a tendency to be decreased in G3 (decreased to 94.3%, $p = 0.08$). However, in contrast with Alu, it was not decreased in G4 (100.0% of that in G1, $p = 0.98$) or cancers (98.2% of that in G1, $p = 0.63$).

The absence of LINE1 hypomethylation in *H. pylori*-infected gastric mucosae

The methylation levels at LINE1-1, LINE1-2 and LINE1-3 were 74.5 ± 6.6%, 58.0 ± 2.6% and 58.7 ± 3.9%, respectively, in G1 (Fig. 2*c*; Supporting Information Table 3). The mean methylation levels at LINE1-1, LINE1-2 and LINE1-3 were not decreased in G2, G3 or G4, respectively. The methylation levels at these CpG sites were highly variable in cancers, but the mean methylation levels at LINE1-1 and LINE1-2 were significantly decreased and that at LINE1-3 was significantly increased (LINE1-1, 93.2% of that in G1, $p < 0.05$; LINE1-2, 92.4% of that in G1, $p < 0.05$; LINE1-3, 105.1% of that in G1, $p < 0.05$).

No decrease of global 5-methylcytosine content in *H. pylori*-infected gastric mucosae and its high variability in gastric cancers

The global 5-methylcytosine content was measured by LCMS for five samples of G1, 17 of G2, 18 of G3, six of G4 and 27 cancer samples, because of the large amount of DNA necessary for the analysis. The global 5-methylcytosine content in G1 was 4.6 ± 0.8% and was not decreased in G2, G3 and G4 (100.3%, 92.5% and 100.7% of that in G1; $p = 0.96$, 0.31 and 0.94, respectively). In cancers, the global 5-methylcytosine content was highly variable (4.3 ± 1.4%), some showing no decrease and the others showing marked decrease (Fig. 3).

To confirm that the high variability observed was due to that of the 5-methylcytosine content in cancer cells and not due to high variability of the population of cancer cells in cancer tissues, the global 5-methylcytosine content was analyzed in gastric cancer cell lines and was again shown to have high variability (2.8–7.5%). The global 5-methylcytosine content obtained by the current LCMS method was in good accordance with that obtained by the conventional HPLC-UV method (n = 8, correlation coefficient = 0.95, $p < 0.05$).

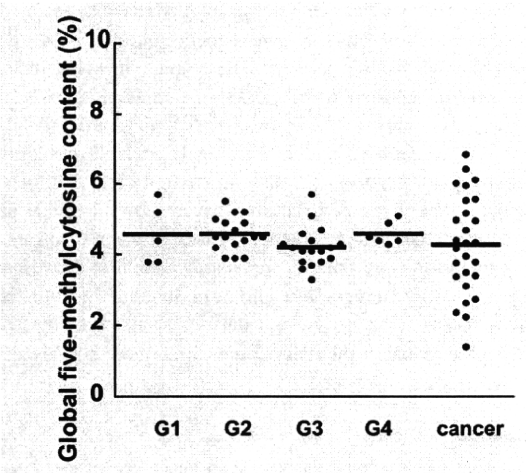

Figure 3. Distribution of global 5-methylcytosine content in G1-G4 and cancers. A horizontal line represents the mean global 5-methylcytosine content for each group. The global 5-methylcytosine content was at a similar level among G1, G2, G3 and G4. In cancers, the global 5-methylcytosine content was highly variable, some showing no decrease and others showing marked decease.

Correlation of hypomethylation among individual repetitive elements and the lack of correlation between hypomethylation and age

Correlations of the methylation levels among individual repetitive DNA elements were significant but weak (correlation coefficients = 0.15 - 0.55, $p < 0.05$; Supporting Information Table 4). Correlation between the global 5-methylcytosine content and the methylation level of individual repetitive elements was not significant, except for ALU2 (Supporting Information Table 5).

Hypermethylation of some CpG islands is known to be observed in an age-dependent manner.[37] Therefore, we analyzed association between hypomethylation of the three repetitive DNA elements and age within G1, whose members had no influence of *H. pylori* infection (Table 1). However, we observed no association. We also analyzed association between hypomethylation and gender, but again there was no association. Hypomethylation of LINE1 (LINE1-2 and LINE1-3) was marginally associated with intestinal-type histology, but that of Alu and Satα was not (Supporting Information Table 6).

Discussion

Our study showed that Alu and Satα hypomethylation was already present in *H. pylori*-infected gastric mucosae and that Alu, but not Satα, hypomethylation persisted after *H. pylori* infection discontinued and was also present in cancers. In contrast, LINE1 hypomethylation was present only in cancers. It was strongly indicated that hypomethylation is induced in gastric mucosae by *H. pylori* infection at Alu and Satα repetitive elements as an early event during gastric carcinogenesis whereas LINE1 hypomethylation is induced as a result of cellular transformation. To our knowledge, the presence of Alu and Satα hypomethylation in *H. pylori*-infected gastric mucosae during gastric carcinogenesis is shown here for the first time. Regarding hypomethylation of a specific repetitive element in noncancerous tissues that in liver, tissues exposed to hepatitis B virus[38] has been reported. Because hypomethylation is

Table 1. Lack of association between hypomethylation of the three repetitive DNA elements and age (or gender)

	ALU1	ALU2	LINE1–1	LINE1–2	LINE1–3	SATα
Age						
r	0.14	−0.30	−0.24	0.13	0.02	0.07
p	0.45	0.08	0.17	0.48	0.93	0.71
Gender						
Male (n = 16)	48.2 ± 4.1	44.0 ± 2.2	74.2 ± 7.5	58.5 ± 1.4	59.3 ± 4.1	58.4 ± 7.8
Female (n = 18)	50.1 ± 2.0	44.3 ± 2.8	74.8 ± 5.8	57.5 ± 3.3	58.3 ± 3.8	53.7 ± 5.0
p	0.12	0.74	0.79	0.25	0.47	0.05

r, correlation coefficient. To avoid confounding effects of *H. pylori* infection, the analyses were conducted in *H. pylori*-negative healthy volunteers (G1).

known to lead to genomic instability,[5] precise understanding of the timing of occurrence of hypomethylation is important as a fundamental basis to understand gastric carcinogenesis.

Alu and Satα hypomethylation showed different profiles in G1–G4, which are considered to represent the time course of gastric carcinogenesis.[19–22] Satα methylation levels were significantly decreased in G2 and tended to be decreased in G3, but not after disappearance of *H. pylori* infection (G4), whereas Alu hypomethylation persisted. The dynamics of Satα were reminiscent of hypermethylation of many protein-coding genes, which is potently induced by *H. pylori* infection and decreases after eradication of *H. pylori*.[13,39] As a mechanism for the different profiles of Alu and Satα, we can hypothesize that their hypomethylations are induced in different cell types. If methylation is induced in stem cells, it is expected to persist even after *H. pylori* infection discontinues whereas methylation induced in progenitor cells can disappear.[14] There is a possibility that Alu hypomethylation is relatively more easily induced in gastric stem cells than Satα hypomethylation. As a mechanism of how *H. pylori* infection induces hypomethylation of Alu and Satα, insufficiency of maintenance DNA methylation can be considered. It is known that expression levels of DNA methyltransferases are lower in gastric epithelial cells with *H. pylori* infection than those without in humans and gerbils.[40,41]

The finding here is important as a fundamental basis of gastric carcinogenesis associated with *H. pylori* infection. Alu is distributed throughout the genome,[40] and its hypomethylation could possibly lead to chromosomal instability as an early event during gastric carcinogenesis, as is known in mice.[4–6] From a clinical viewpoint, we initially expected that hypomethylation could be used as a cancer risk marker such as hypermethylation of CpG islands.[13,14] However, Alu hypomethylation had only low sensitivity and specificity in distinguishing healthy volunteers and gastric cancer patients among *H. pylori*-negative individuals (Fig. 2*a*), and use of hypomethylation as a risk marker was considered not to be realistic. Nevertheless, the early occurrence of Alu hypomethylation and its possible involvement in chromosomal instability suggested a possibility that suppression of hypomethylation induction can be used as a novel target of cancer prevention.

In cancers, LINE1 methylation level, which is often used as a surrogate for global hypomethylation,[42,43] was highly variable. The high variability of the LINE1 methylation level in gastric cancers was in good accordance with that reported in bladder and colon cancers.[44,45] It was considered that, because a cancer tissue is monoclonal, its methylation level reflects that of its single precursor cell and, thus, stochastically shows a low or high level. When methylation levels of the three CpG sites analyzed for LINE1 were compared in gastric cancers, methylation levels were decreased at two CpG sites while increased at another site. This suggested that there is a difference in susceptibility to hypomethylation among CpG sites. The difference of susceptibility could be related on the location of a CpG site within LINE1 because the CpG site whose methylation level was increased was located at an edge of LINE1.

The global 5-methylcytosine content in gastric cancers was also highly variable. In contrast, global hypomethylation is generally considered as one of the hallmarks of cancer cells.[1,3] In most studies, global hypomethylation is assessed by hypomethylation of repetitive sequences and not by the global 5-methylcytosine content. The 5-methylcytosine content is already reported to be variable in some cancers.[46] In gastric cancers, only our previous study[39] measured the 5-methylcytosine content, and it was highly variable not only in primary gastric cancers but also in gastric cancer cell lines. Therefore, global hypomethylation measured by the global 5-methylcytosine content was highly variable in gastric cancers.

In conclusion, our data strongly indicated that *H. pylori* infection potently induces Alu and Satα hypomethylation in gastric mucosae as an early event during gastric carcinogenesis and that global 5-methylcytosine content is not always decreased in gastric cancers.

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Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation

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Chronic inflammation is deeply involved in induction of aberrant DNA methylation, but it is unclear whether any type of persistent inflammation can induce methylation and how induction of cell proliferation is involved. In this study, Mongolian gerbils were treated with five kinds of inflammation inducers [*Helicobacter pylori* with cytotoxin-associated gene A (CagA), *H.pylori* without CagA, *Helicobacter felis*, 50% ethanol (EtOH) and saturated sodium chloride (NaCl) solution]. Two control groups were treated with a mutagenic carcinogen that induces little inflammation (20 p.p.m. of *N*-methyl-*N*-nitrosourea) and without any treatment. After 20 weeks, chronic inflammation with lymphocyte and macrophage infiltration was prominent in the three *Helicobacter* groups, whereas neutrophil infiltration was mainly observed in the EtOH and NaCl groups. Methylation levels of eight CpG islands significantly increased only in the three *Helicobacter* groups. By Ki-67 staining, cell proliferation was most strongly induced in the NaCl group, demonstrating that induction of cell proliferation is not sufficient for methylation induction. Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* showed increased expression specifically in the three *Helicobacter* groups. In human gastric mucosae infected by *H.pylori*, *NOS2* and *TNF* were also increased. These data showed that inflammation due to infection of the three *Helicobacter* strains has a strong potential to induce methylation, regardless of their CagA statuses, and increased cell proliferation was not sufficient for methylation induction. It was suggested that specific types of inflammation characterized by expression of specific inflammation-related genes, along with increased cell proliferation, are necessary for methylation induction.

Introduction

Aberrant DNA methylation of promoter CpG islands (CGIs) is deeply involved in human carcinogenesis (1,2). As inducers of aberrant DNA methylation, aging and chronic inflammation have been suggested because methylation was present in colonic tissues of the aged (3) and patients with long-standing ulcerative colitis (4–6), in the liver with chronic hepatitis (7) and in gastric tissues with *Helicobacter pylori* (*H.pylori*)-induced gastritis (8,9). Especially in the stomach, accumulation levels of aberrant methylation correlate with risk of gastric cancers (8,10–12). Chronic inflammation is characterized by transition of inflammatory cell types from polymorphonuclear cells (mainly neutrophils) to mononuclear cells (lymphocytes and macrophages) and persistent cell proliferation (13). However, it is still unclear whether chronic inflammation with infiltration of mononuclear cells and expression of specific genes or simply persistent inflammation is important for methylation induction and how cell proliferation is involved in it.

As an animal model for methylation induction, we recently demonstrated that inflammation triggered by *H.pylori* infection induces aberrant methylation in the stomach of Mongolian gerbils (*Meriones unguiculatus*) (14). In the gerbil stomach, *H.pylori* with a bacterial virulence factor, cytotoxin-associated gene A (CagA), which is associated with a high risk of human gastric cancers (15), can induce more severe inflammation than that without (16). *Helicobacter felis*, which does not possess CagA (17), can induce chronic gastritis without direct damage of epithelial cells (18,19). High concentrations of ethanol (EtOH) and sodium chloride (NaCl) can induce gastric erosion associated with inflammation (20–22). Their repeated administration can induce persistent inflammation with cell proliferation without transition of inflammatory cell types. In contrast, little inflammation is induced by *N*-methyl-*N*-nitrosourea (MNU), a mutagenic gastric carcinogen (23).

Regarding inflammation-related genes, high expression of *IFNG*, *IL1B*, *TNF*, *NOS2* and *COX2* has been reported in human gastritis induced by *H.pylori* infection (24,25). Also in gerbils, high expression of *Ifng*, *Il1b*, *Cox2* and *Nos2* has been observed (26,27). Our previous time-course study after *H.pylori* infection and eradication in gerbils showed that expression levels of *Cxcl2*, *Il1b*, *Nos2* and *Tnf* were correlated with methylation levels in gastric epithelial cells (GECs) (14). In humans, a polymorphism of *IL1B* is associated with gastric cancer risk (28) and with methylation of multiple genes in gastric cancers (29).

In this study, using five inducers of inflammation (*H.pylori* with CagA, *H.pylori* without CagA, *H.felis*, EtOH and NaCl) and a carcinogen control (MNU), we aimed to clarify the roles of transition of inflammatory cell types, induction of cell proliferation and specific inflammation-related genes in methylation induction.

Materials and methods

Preparation of Helicobacter strains

Helicobacter pylori with CagA (ATCC 43504, also known as NCTC 11637) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *Helicobacter pylori* without CagA, SS1, was kindly provided by Professor Takashi Joh at Nagoya City University (30). *Helicobacter felis* (ATCC 49179) was also obtained from ATCC. Each strain was inoculated in Brucella broth (Becton Dickson, Cockeysville, MD) with 7% vol/vol heat-inactivated fetal bovine serum and incubated at 37°C under microaerobic conditions using an AnaeroPack Campylo (Mitsubishi Gas Chemical, Tokyo, Japan) for 24 h. For the culture of *H.felis*, 0.1% wt/vol of BactoAgar (Becton Dickson) was supplemented. Before harvesting bacteria, their mobility and shape were confirmed under phase contrast microscopy.

Animal experiments and sample preparation

Five-week-old male Mongolian gerbils (MGS/Sea; Kyudo, Tosu, Japan) were randomly assigned to seven groups of eight animals each. Gerbils in groups for *Helicobacter* treatment were inoculated with $\sim 10^8$ CFU/gerbil of *H.pylori* ATCC 43504 (ATCC group), *H.pylori* SS1 (SS1 group) or *H.felis* (HF group) and were kept without further treatment. Gerbils in groups of EtOH and NaCl treatment were administered with 5 ml/kg body wt of 50% EtOH group and saturated NaCl group, respectively, by gavage twice a week from 5 to 25 weeks of age. Gerbils in the group of MNU treatment (MNU group) were administered with 20 p.p.m. of MNU (Sigma–Aldrich, St Louis, MO) in drinking water from 5 to 25 weeks of age. A control group was kept without any treatment.

Abbreviations: CagA, cytotoxin-associated gene A; CGI, CpG island; Dnmt, DNA methyltransferase; EtOH, ethanol; GEC, gastric epithelial cell; MNU, *N*-methyl-*N*-nitrosourea; NaCl, sodium chloride; PCR, polymerase chain reaction; qRT–PCR, quantitative reverse transcriptase–polymerase chain reaction.

At age 25 weeks, all the animals were killed, and their stomachs were resected. From the posterior wall of the pyloric region, GECs were isolated by the gland isolation technique (31) for DNA and RNA extraction. The anterior wall of the pyloric region was further cut into two pieces: one for RNA extraction from the mucosal and submucosal layers and the other for histological analysis. DNA and RNA were extracted as described previously (14). As controls in immunohistochemistry of DNA methyltransferases (Dnmts), adult male mice (C57BL/6N, 11 weeks of age; CLEA Japan, Tokyo, Japan) were purchased and stomachs were resected. The animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation.

Histological analysis

After fixation with 10% neutral formalin, tissues were embedded in paraffin and sections at 3 μm thickness were prepared. For histological analysis, hematoxylin and eosin staining was performed by a routine method. The degrees of infiltration of mononuclear and polymorphonuclear cells, intestinal metaplasia and heterotopic proliferative glands were graded on a four-point scale (0–3; 0, no or faint; 1, mild; 2, moderate and 3, marked) as described previously (32). For immunohistochemical analysis, a rabbit anti-human Ki-67 (Clone SP6; Thermo Fisher Scientific, Fremont, CA) antibody was purchased. Rabbit anti-mouse Dnmt1 (33), Dnmt3a (34) and Dnmt3b (34) antibodies were kindly provided by Professor Shoji Tajima at Osaka University. Rehydrated sections were incubated in HistoVT one (Nacalai Tesque, Kyoto, Japan) at 80°C for 40 min to unmask the antigen. After blocking with 0.5% bovine serum albumin in phosphate-buffered saline, sections were incubated with each primary antibody overnight, and the immune complex was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Microscopic images were captured using the BZ-9000 microscope system (Keyence, Osaka, Japan). To analyze the number of the positive cells, more than five gastric glands in at least three different optic fields were counted, and the labeling index was calculated as a percentage of the positive cells relative to the total counted cells.

Human clinical samples

Human gastric mucosae were obtained by endoscopic biopsy from 7 *H.pylori*-negative (4 men and 3 women; average age 70, ranging from 44 to 83) and 18 *H.pylori*-positive (8 men and 10 women; average age 64, ranging from 46 to 81) persons with informed consents and approval of Institutional Review Boards. Their *H.pylori* infection statuses were determined by the serum anti-*H.pylori* IgG test (SBS, Kanazawa, Japan). Endoscopic superficial gastritis was observed in six of the seven *H.pylori*-negative persons and atrophic gastritis was observed in 14 of the 18 *H.pylori*-positive cases. RNA was extracted with ISOGEN (Wako, Osaka, Japan).

Gene expression analysis

The number of complementary DNA molecules was quantified by quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) as described previously (14). The number of complementary DNA molecules obtained by gene-specific primers (supplementary Table 1 is available at *Carcinogenesis* Online) was normalized to *Gapdh* (*GAPDH*) expression.

Methylation analysis

Methylation levels of gerbil CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) were analyzed by quantitative methylation-specific polymerase chain reaction (PCR) and were expressed as a percentage of methylated reference as described previously (14). Bisulfite sequencing was conducted after cloning of PCR products after bisulfite modification as described previously (14).

Statistic analysis

To evaluate significant difference between two independent groups of sample data, the Mann–Whitney *U*-test was employed.

Results

Characterization of five kinds of inflammation triggered by the inducers

Gerbils were treated with five kinds of inflammation inducers (*H.pylori* ATCC 43504, *H.pylori* SS1, *H.felis*, EtOH and saturated NaCl solution) and also with MNU (Figure 1A). By histological examination of the pyloric area, the ATCC group had marked infiltration of mononuclear and polymorphonuclear cells into mucosae and submucosae and glands with intestinal metaplasia and heterotopic proliferative glands were occasionally observed (Figure 1B and Table I). The SS1 and HF groups showed milder infiltration of polymorphonuclear and mononuclear cells, less heterotopic proliferative glands and no intestinal metaplasia. The EtOH group showed infiltration of almost only polymorphonuclear cells. The NaCl group showed no or little infiltration of inflammatory cells but had thickened lamina propria. The MNU group showed no histological inflammatory changes but also had thickened lamina propria.

The kinds of infiltrating inflammatory cells were also assessed by qRT–PCR analysis [*Cd3g* (T cell), *Emr1* (macrophage), *Ela2* (neutrophil) and *Ms4a1* (B cell)] of gastric tissues containing both mucosal and submucosal layers (Figure 1C). In the ATCC, SS1 and HF groups, expression of all the four inflammatory cell markers was markedly elevated and met the typical features of chronic inflammation, such as infiltration of mononuclear cells. The macrophage and neutrophil markers were very high in the ATCC group. In the EtOH and NaCl groups, the neutrophil marker was in the same range as in the three *Helicobacter* groups, the macrophage marker was half, and the T- and B-cell markers were almost absent, showing that the inflammation in these groups was persistent acute inflammation. In the MNU group, none of the four markers were significantly elevated. These expression data were in accordance with the histological data, except for the polymorphonuclear infiltration in the NaCl group.

Induction of DNA methylation by the three Helicobacter strains but not by EtOH and NaCl

To assess methylation in GECs (not in infiltrating leukocytes), we used eight of the 10 CGIs known to be methylated in gerbil GECs as markers because these eight CGIs (HE6, HG2, SA9, SC3, SD2, SE3, SF12 and SH6) have been shown not to be methylated in peripheral blood cells (14). First, methylation levels of these CGIs were measured by quantitative methylation-specific PCR in GECs isolated by the gland isolation technique in each group (Figure 2A). The ATCC group had high methylation levels (significant in all the eight CGIs). The SS1 and HF groups also had high methylation levels (significant in six CGIs; HE6, HG2, SA9, SD2, SF12 and SH6) but lower than the ATCC group. The EtOH, NaCl and MNU groups had no increases of methylation in any CGIs.

To confirm the presence of densely methylated DNA molecules, bisulfite sequencing of HE6 was performed in one gerbil in each group (Figure 2B). Gerbils in the ATCC, SS1 and HF groups had densely methylated DNA molecule(s), and their fractions (3, 1–2, 1 of 24, respectively) were in accordance with the methylation level obtained by quantitative methylation-specific PCR. Gerbils in the EtOH, NaCl and MNU groups had no densely methylated molecules. These data showed that aberrant methylation of these CGIs was induced only by inflammation triggered by the three *Helicobacter* strains, most potently by *H.pylori* ATCC 43504-induced inflammation but not by EtOH- or NaCl-induced inflammation.

Insufficient role of cell proliferation in methylation induction

Cell proliferation was analyzed by immunohistochemistry of Ki-67 in gastric mucosae (Figure 3A) and counting the Ki-67 labeling indices (Figure 3B). All the treatment groups showed significant increases in Ki-67 labeling indices. The three *Helicobacter*-infected groups and the NaCl-treated group showed very high Ki-67 labeling indices. The NaCl-treated group, especially which did not show increased methylation levels, showed the highest Ki-67 labeling index. This result showed that induction of cell proliferation is not sufficient to induce DNA methylation.

Inflammation-related genes associated with methylation induction

To dissect inflammation components responsible for methylation induction, qRT–PCR analysis of 10 inflammation-related genes [*Cox2*, *Cxcl2* (*MIP-2*), *Ifng*, *Il1b*, *Il2*, *Il4*, *Il6*, *Il7*, *Nos2* (*iNos*) and *Tnf* (Tnf-α)] was performed using RNA collected from gastric tissues that contained both GECs and inflammatory cells (Figure 4A). In the three *Helicobacter*-infected groups, *Il1b*, *Nos2* and *Tnf* were significantly upregulated. *Ifng*, *Il2*, *Il4* and *Il6* were significantly upregulated in the

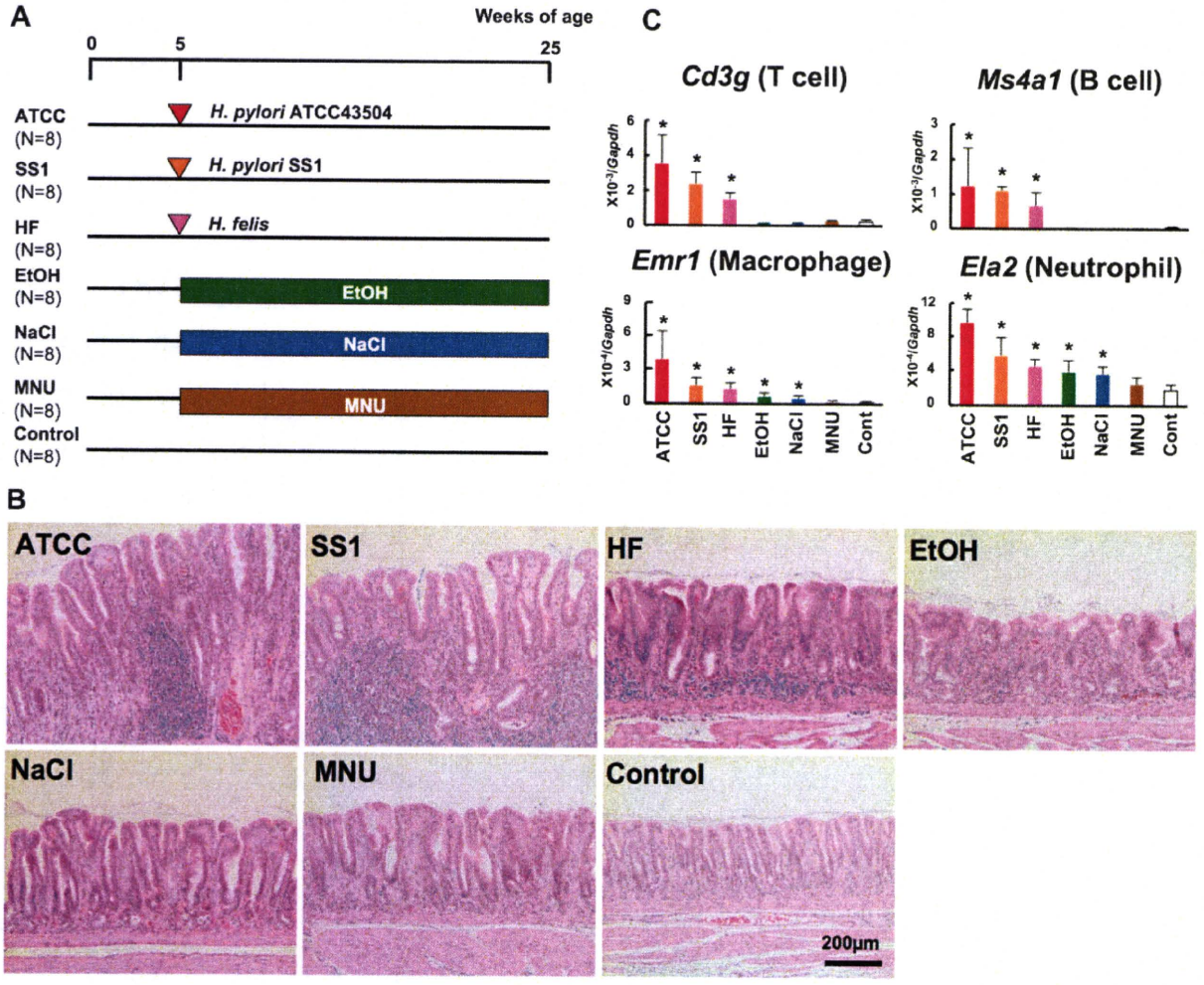

Fig. 1. Treatment of Mongolian gerbils by five inflammation inducers and MNU. (**A**) Experimental design. (**B**) Histology of gastric mucosa after treatment for 20 weeks. Transition of inflammatory cells was observed in the three *Helicobacter* groups. (**C**) Expression levels of inflammatory cell markers. Infiltration of T and B cells was prominent in the three *Helicobacter* groups. Values are shown as mean + SD. $^*P < 0.05$ compared with the control group.

Table I. Histological changes induced by the five inflammation inducers and MNU

Group	Infiltration of mononuclear cells	Infiltration of polymorphonuclear cells	Intestinal metaplasia	Heterotopic proliferative glands
ATCC	2.8 ± 0.5*	2.3 ± 0.7*	0.9 ± 0.6*	1.4 ± 0.9*
SS1	1.6 ± 0.5*	1.1 ± 0.7*	0.0 ± 0.0	0.3 ± 0.5
HF	1.6 ± 0.8*	0.7 ± 0.5*	0.0 ± 0.0	0.4 ± 0.8
EtOH	0.0 ± 0.0	0.9 ± 0.3*	0.0 ± 0.0	0.1 ± 0.3
NaCl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MNU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Values are shown as mean ± SD.
$^*P < 0.01$ compared with control group.

SS1, HF, EtOH and NaCl groups but not in the ATCC group. Expression levels of these genes tended to be higher in the EtOH and NaCl groups than in the SS1 and HF groups. The MNU group did not show any significant changes compared with the control group. These results suggested that upregulation of *Il1b*, *Nos2* and *Tnf* was associated with methylation induction.

Expression of Dnmts

Dnmts are the final effectors that methylate DNA (35). To analyze the relation between expression of Dnmts and aberrant methylation induction, we conducted immunohistochemistry of Dnmts. Antibodies against mouse Dnmt1, Dnmt3a and Dnmt3b were tested in gerbils, and those against Dnmt1 and Dnmt3a were confirmed to have high sensitivity and specificity (supplementary Figure 1 is available at *Carcinogenesis* Online).

Dnmt1 protein was localized in the nuclei of GECs around the proliferative zone of gastric glands (supplementary Figures 1 and 2 are available at *Carcinogenesis* Online). In the ATCC, SS1, HF and NaCl groups, the number of GECs expressing Dnmt1 protein was markedly increased and the highest labeling index was observed in the NaCl group (Figure 4B). The profile of Dnmt1 expression was the same as that of Ki-67 (Figure 3B), indicating that Dnmt1 expression was elevated in association with increased cell proliferation. Dnmt3a protein was localized in the nuclei of most GECs except in some cells in the bottom of the glands. Although GECs expressing Dnmt3a protein significantly decreased in the ATCC, EtOH and MNU groups, the degree of decrease was small (Figure 4B and supplementary Figures 1 and 3 are available at *Carcinogenesis* Online). These results showed that the fractions of GECs expressing Dnmt1 and Dnmt3a in gastric glands were not associated with methylation induction.

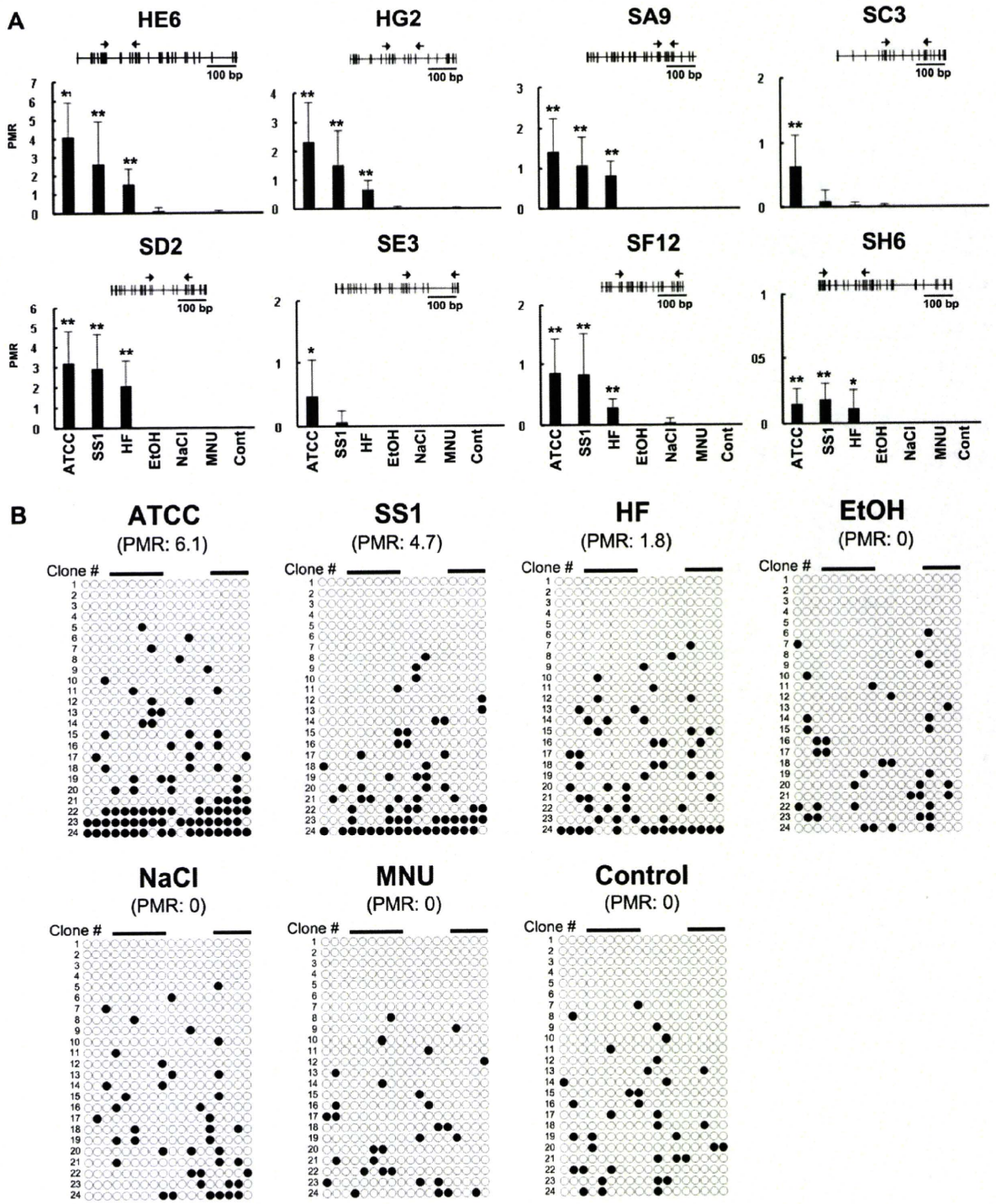

Fig. 2. Methylation induction in GECs by the three *Helicobacter*-induced inflammation but not by EtOH- or NaCl-induced inflammation. (**A**) Methylation levels of eight CGIs assessed by quantitative methylation-specific PCR. Upper panels show CpG maps, and lower panels show methylation levels in percentage of methylated reference. In the upper panel, vertical lines and arrows show individual CpG sites and positions of methylation-specific PCR primers, respectively. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. (**B**) Bisulfite sequencing of HE6 in GECs. Numbers in parentheses indicate percentage of methylated reference of the sample assessed by quantitative methylation-specific PCR. Bars, CpG sites on quantitative methylation-specific PCR primers.

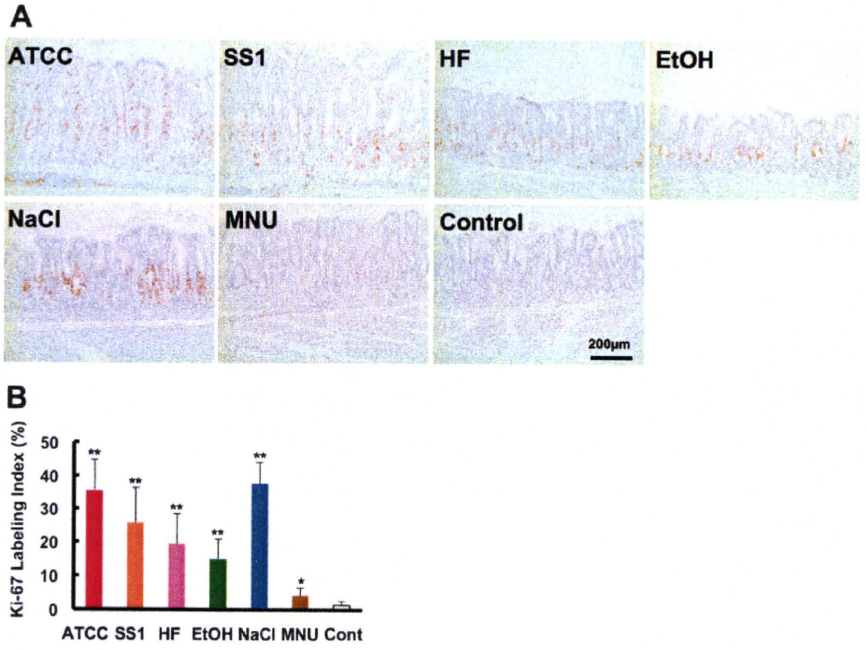

Fig. 3. Cell proliferation of gerbil GECs after the treatment. (**A**) Representative microscopic appearance of Ki-67 immunohistochemistry. (**B**) Ki-67 labeling index. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group. The NaCl group showed a marked increase of cell proliferation.

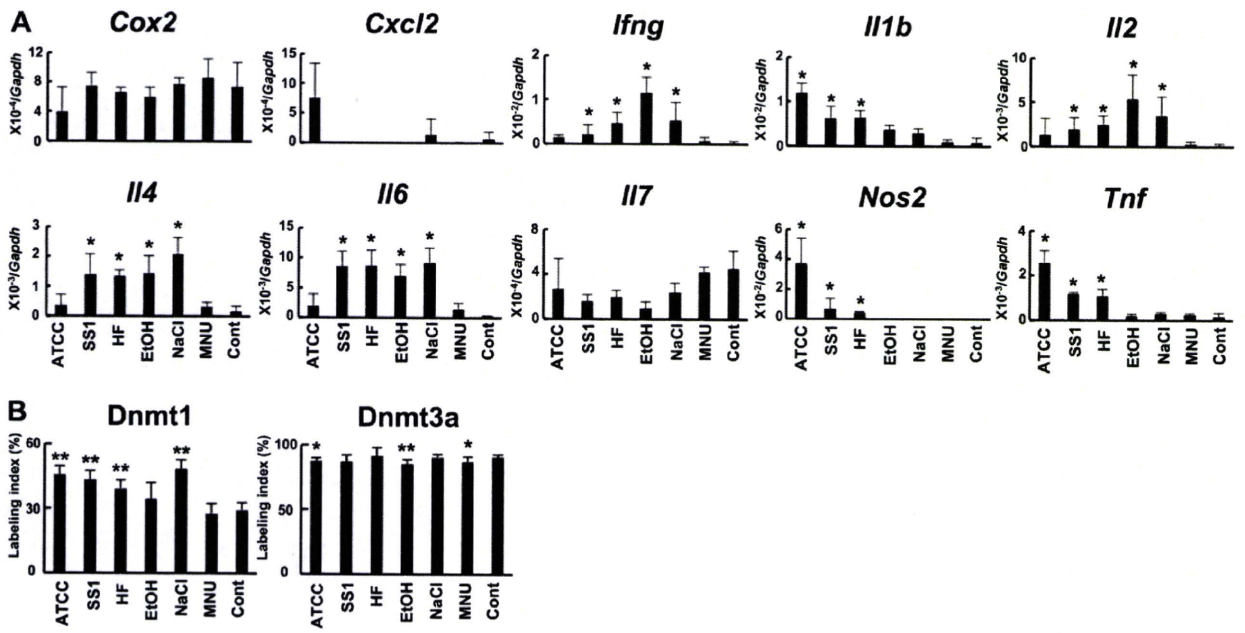

Fig. 4. Expression of inflammation-related genes and Dnmts in the gerbil stomach. (**A**) messenger RNA levels of inflammation-related genes in gerbil gastric tissues containing both mucosal and submucosal layers. Expression levels of *Il1b*, *Nos2* and *Tnf* were elevated only in the three *Helicobacter* groups. (**B**) The fractions of GECs expressing Dnmt proteins in gastric glands by immunohistochemistry. Values are shown as mean + SD. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group.

Human relevance of inflammation-related gene expression

To address whether upregulation of specific inflammation-related genes are common in the human stomach, we conducted qRT–PCR of *COX2*, *IFNG*, *IL1B*, *IL6*, *NOS2* and *TNF* using human gastric mucosa samples with and without *H.pylori* infection. Expression levels of *NOS2* and *TNF* were markedly upregulated (27- and 3-fold,

respectively) also in human gastric mucosae (Figure 5). However, *IL1B* expression tended to be lower in gastric mucosae of *H.pylori*-infected individuals.

Discussion

Among the five groups with inflammation, aberrant methylation was induced only in the three *Helicobacter* groups, which showed inflammation with infiltration of mononuclear cells, increased expression of *Il1b*, *Nos2* and *Tnf* and increased cell proliferation. In the EtOH and NaCl groups, these agents were administered repeatedly for 20 weeks, and increased cell proliferation was present at the end of the experiment. The increased proliferation was considered to have persisted for this period because thickening of lamina propria was observed in these two groups. Nevertheless, aberrant methylation was not induced, at least in the CGIs analyzed here. This showed that cell proliferation alone is not sufficient for methylation induction and suggested that both specific types of inflammation and increased cell proliferation are necessary for induction of aberrant methylation.

The inflammation induced in the *Helicobacter* groups was characterized by infiltration of mononuclear cells (lymphocytes and macrophages). In our previous study, suppression of T-cell activation by cyclosporin A remarkably repressed inflammatory response and methylation induction triggered by *H.pylori* infection (14), showing that T-cell activation is involved in methylation induction in this system. However, our recent study in mouse colon demonstrated that aberrant methylation can be induced even in severe combined immunodeficiency mice, which lack functional T and B cells, by dextran sulfate sodium-induced colitis (Katsurano *et al.*, submitted for publication). It is known that, even in severe combined immunodeficiency mice, colitis with macrophage infiltration can be induced (36). If a common mechanism for methylation induction is present in *H.pylori*-infected gastric mucosae and dextran sulfate sodium-treated colonic mucosae, infiltration of macrophages is a candidate for the proximate effector that transmits signal for methylation induction to epithelial cells. It can be considered that, in *H.pylori*-infected gastric mucosae, activation of T cells is required only for the initiation or maintenance of inflammation capable of inducing aberrant DNA methylation.

Among the inflammation-related genes, *Il1b*, *Nos2* and *Tnf* were specifically upregulated in the three *Helicobacter* groups. These three genes are reported to be overexpressed also in human chronic inflammation associated with cancers, such as ulcerative colitis and hepatitis (37–40). *IL1B* promoter polymorphism is associated with risk of human gastric cancers (28) and aberrant methylation of multiple genes in gastric cancers (29). The lack of its upregulation in human gastric mucosae infected with *H.pylori* could be because most of them had superficial gastritis and had already increased *IL1B* expression. *NOS2*, which encodes nitric oxide synthase, was upregulated *in vitro* by administration of IL1B and nitric oxide donors induced methylation of *FMR1* and *HPRT* (41). These suggest that IL1B and NOS2 might be involved in methylation induction. On the other hand, *Ifng*, *Il2*, *Il4* and *Il6* were upregulated mainly in the EtOH and NaCl groups, in which no methylation was induced, and also in the SS1 and HF groups, in which methylation induction levels were lower than in the ATCC group. This suggested a possibility that some (one) of the genes could suppress methylation induction.

SS1 and *H.felis*, which lack CagA, were capable of inducing aberrant methylation although the capacity was weaker than the CagA-positive strain (*H.pylori* ATCC 43504). CagA-positive *H.pylori* strains are known to induce severe gastritis in Mongolian gerbils (16) as confirmed in this study, and this explains their stronger capacity to induce methylation. The three inflammation-related genes associated with methylation induction (*Il1b*, *Nos2* and *Tnf*) had the highest expression in the ATCC group among the three *Helicobacter* groups. CagA-positive *H.pylori* seems to promote methylation induction by maximizing expression of such genes and minimizing expression of genes that suppress methylation induction.

Dnmts are the final effectors to methylate DNA, and their overexpression was observed in various human cancers (35). Immunohistochemical analyses here revealed that Dnmt1 was upregulated in gastric mucosae of gerbils in the three *Helicobacter*-infected groups and the NaCl-treated group. However, the highest expression was observed in the NaCl group, where methylation was not induced. This result indicated that expression of Dnmt1 was not associated with methylation induction but with cell proliferation. Expression of Dnmt3a was significantly but slightly decreased in the ATCC group and this also suggested that the expression itself is not involved in aberrant methylation induction. However, due to the lack of an appropriate antibody, we were not able to exclude the possibility that upregulation of Dnmt3b is involved in methylation induction. Therefore, disturbance in the local balance between Dnmts and factors that protect DNA from aberrant methylation, such as the presence of RNA

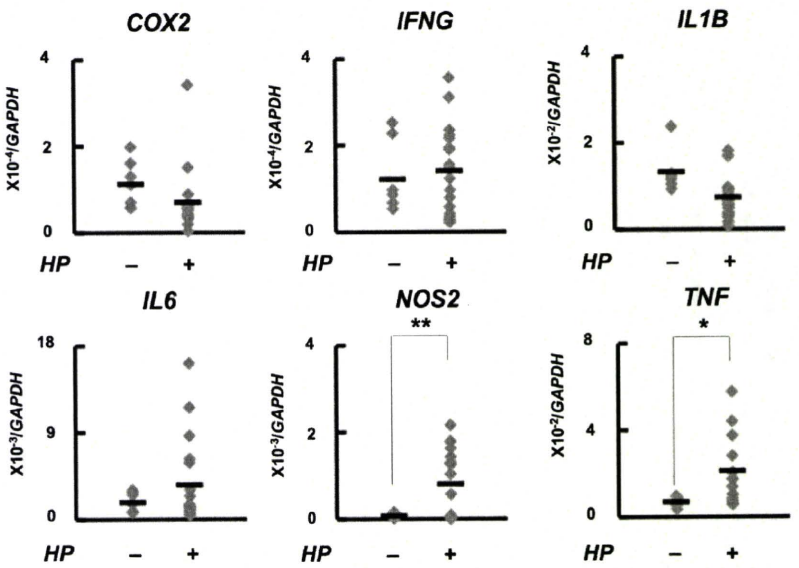

Fig. 5. Human relevance of expression changes in the gerbil stomach. Expression levels of inflammation-related genes were quantified in gastric mucosae of individuals without and with *H.pylori* infection. Bold horizontal bar, the mean expression level; $^{*}P < 0.05$ and $^{**}P < 0.01$.

polymerase II (42) and/or possible overexpression of Dnmt3b might be involved in methylation induction.

In conclusion, inflammation due to infection of *Helicobacter* strains had a high capacity to induce methylation in GECs, regardless of their CagA status. Increased cell proliferation was not sufficient for methylation induction. Therefore, specific types of inflammation, characterized by infiltration of mononuclear cells and expression of specific inflammation-related genes, along with increased cell proliferation were considered to be necessary for methylation induction.

Supplementary material

Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

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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 carcinogenesis. 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 carcinogenesis. 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[23,31–33] 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.

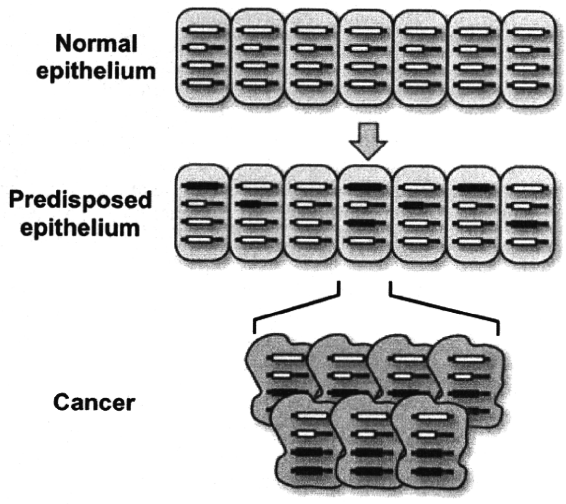

Fig. 1. Epigenetic field for cancerization and clonal selection in cancer. Normal epithelium consists of cells with little aberrant methylation. By exposure to inducers of methylation, specific genes are methylated in minor fractions of cells. A cancer develops from one of the cells that has already accumulated silencing of driver genes. From the viewpoint of assessment of an effect of an inducer, analysis of non-cancerous tissues provides overall information on the genes methylated, and that of a cancer provides information on the genes stochastically methylated in the very precursor cell and driver genes.

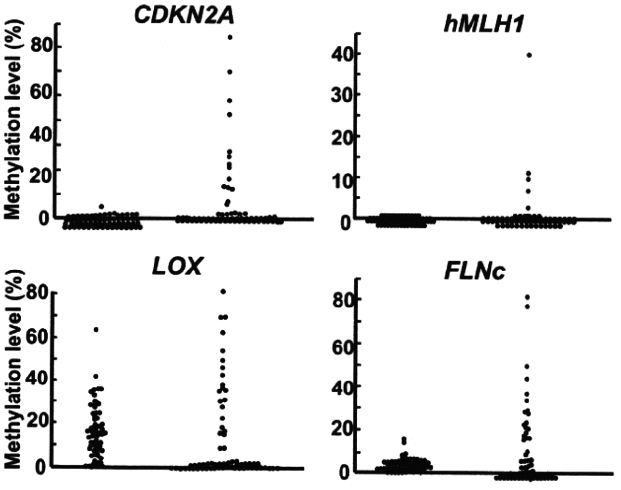

Fig. 2. Distribution patterns of methylation in non-cancerous and cancer tissues. Methylation levels, which reflect fractions of cells with the methylation, were quantified in 66 paired samples of non-cancerous and cancer tissues of gastric cancer patients (modified from Enomoto *et al.*[39]). They showed a unimodal distribution in non-cancerous tissues, and a "bimodal" distribution, namely zero or positive, in cancer tissues. This finding supports the idea that methylation in a non-cancerous tissue reflects events in many cells in the tissue whereas that in a cancer tissue mostly reflects only events in its single precursor cell.

can be affected by the overall exposure level to its inducers and by the susceptibility of individual genes to undergo an alteration. In actual analysis, the proportion of target cells, such as content of epithelial cells in a sample with epithelial and stromal cells, also affects the fraction of cells with an alteration.

In contrast, in cancer tissues, an alteration responsible for clonal growth (driver) is present in all the cancer cells. Even if an alteration is not a driver, if the alteration has taken place before the clonal growth started, it is present in all the cancer cells. In actual analysis, cancer samples contain a large contamination of non-cancer cells, and the fraction of cells with the alteration is mainly determined by the fraction of cancer cells in a sample. If an alteration is induced after initiation of clonal growth, it can be present in a fraction of cancer cells, and its overall fraction is determined by the fraction within cancer cells and by the fraction of cancer cells within a sample.

These theoretical considerations were substantiated by actual measurement of cells with methylation of specific genes in non-cancerous and cancer tissues of gastric cancer patients (Fig. 2) and esophageal cancer patients.[39,40] The methylation level, which reflects the fraction of DNA molecules with methylation and thus the fraction of cells with the methylation, shows a unimodal distribution in non-cancerous tissues, especially for the weak tumor-suppressor gene *LOX* and the marker gene *FLNc*.[41] It shows a "bimodal" distribution, namely zero or positive, in cancer tissues, especially for the tumor-suppressor genes *CDKN2A* and *MLH1*.

Rare presence of mutations in non-cancerous tissues. Adjacent non-cancerous tissues are often used as a control for cancer tissues, and are regarded not to have detectable levels of mutations. To detect accurately such low levels of mutations in non-cancerous tissues, transgenic animals in which rare mutations can be quantified by selectable mutations of a marker gene have been developed.[42,43] Using these transgenic animals and various carcinogenic factors, mutation frequencies of a specific marker gene in non-cancerous tissues have been shown to be $\sim 10^{-5}$/cell, and to be 10^{-3}/cell, even in a tissue heavily exposed to a mutagenic compound.[44] This very low frequency of mutations in non-cancerous tissues gives a rationale for the routine use of such tissues as a control.

DNA methylation in non-cancerous tissues and aging. Once the situation goes to DNA methylation, many investigators noticed that trace amounts of DNA with methylation are present in non-cancerous tissues of cancer patients. However, it is usually difficult to distinguish whether such methylation is a simple drift or fluctuation without any biological or pathological meaning or something associated with cancer development. A pioneering work by Issa *et al.* analyzed the correlation between age and levels of methylation, and convincingly showed that aging is one factor that induces DNA methylation.[45]

Association between methylation accumulation and cancer risk: Epigenetic field for cancerization. We systematically collected gastric tissue samples from healthy individuals and gastric cancer patients (non-cancerous part) in an age-matched manner.[46] Methylation levels of eight CGI in various positions against TSS were accurately quantified. Methylation levels in non-cancerous gastric tissues of gastric cancer patients were in the range 0.2–8.2%, and were much higher than those in gastric mucosae of healthy individuals. This showed that very high levels of methylation can be present in non-cancerous tissues, different from mutations. The finding also suggested that accumulation of methylation is related to gastric cancer risk. Subsequently, gastric mucosae of patients with multiple gastric cancers were shown to have higher methylation levels than those of patients with a single gastric cancer (Fig. 3).[47] These discoveries clearly demonstrated that methylation levels in gastric mucosae correlate with gastric cancer risk.

A higher incidence or level of methylation in non-cancerous tissues of cancer patients than that in the corresponding tissues of healthy individuals was also observed for liver,[48] colon,[49] esophageal,[50] and renal[51] cancers. In these types of cancers, accumulation of methylation is likely to be involved in the formation of a field for cancerization (Fig. 1).[52] The gene inactivated by methylation of its promoter CGI in non-cancerous

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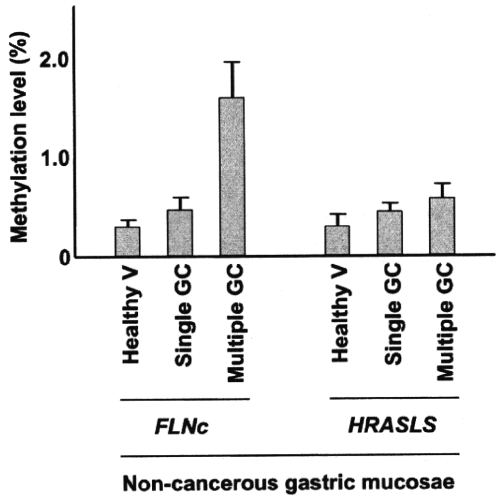

Fig. 3. Correlation between methylation level and cancer risk. Methylation levels of two marker genes (*FLNc* and *HRASLS*) were quantified in gastric mucosae of healthy individuals (healthy V), non-cancerous gastric mucosae of patients with a single gastric cancer (single GC), and non-cancerous gastric mucosae of patients with multiple gastric cancers (multiple GC) (modified from Nakajima *et al.*[47]). This showed that accumulation levels of specific genes in non-cancerous gastric mucosae can correlate with gastric cancer risk. Taken together with the findings in other types of cancers, quantification of methylation levels in normal-appearing tissues is a promising cancer risk marker that reflects one's own life history.

tissues might be a weak tumor-suppressor gene that does not induce cellular transformation by itself, such as *SFRP1*,[53] or might be a passenger that is methylated in parallel with tumor-suppressor genes.

Inducers of methylation in contrast with those of mutations

Epidemiology indicates that cancer is mainly caused by environmental factors,[54] and identification of inducers of aberrant DNA methylation, in addition to those of mutations, is critically important. However, only limited information is available for the inducers of aberrant methylation.[55]

Inducers of mutations. Clarification of inducers of mutations, namely mutagens, constitutes a large field of science, and comprehensive description is beyond the scope of this article. Simplistically, mutations are induced by exogenous mutagenic factors, such as chemicals and radiation, and endogenous factors, such as oxygen radicals.[56] Mutagenic chemicals are contained in diverse sources, including tobacco smoke, overcooked food, and many synthetic chemicals.

Inducers of DNA methylation. To identify inducers of aberrant methylation in humans, analysis of non-cancerous tissues is important because the methylation level in non-cancerous tissues reflects how potently the methylation was induced by a factor (Fig. 1). Aging was the first factor that was identified to promote accumulation of DNA methylation,[45] and quantification of methylation in non-cancerous colonic tissues contributed to the identification.

Afterwards, the presence of methylation in colonic mucosae of patients with ulcerative colitis indicated that chronic inflammation is an important inducer of methylation.[57,58] The importance of chronic inflammation was further supported by the presence of methylation in non-cancerous liver tissue of patients with hepatitis,[48] in inflammatory reflux esophagitis,[59] and in non-cancerous gastric tissue of individuals infected by *Helicobacter pylori*.[46] However, the molecular mechanisms of how chronic inflammation induces aberrant methylation are almost unknown.

There can be chemicals that induce aberrant DNA methylation, but few chemicals are known. If we want to identify a chemical whose primary mode of action is induction of gene silencing, methylation induction in NFR of multiple genes should be demonstrated. Methylation of an exon can be induced as a result of gene expression change, and methylation of a NFR of a specific gene can be induced as a result of loss of its expression, as described below. One of the reasons why methylation-inducing chemicals have not been identified might be the lack of suitable assay systems, and efforts to develop such systems are being made.[55,60]

Gene specificity in methylation induction

Mutations are considered to affect random genes, with some preference for actively transcribed genes.[18,61] Although there is sequence specificity depending on mutagenic factors,[62] there is little gene specificity. Many investigators thought that DNA methylation would have a similar nature in random target genes, but it has now been shown that there is strong target gene specificity in methylation induction.

Presence of target gene specificity in methylation induction. It was initially found that specific CGI are methylated in specific tumor types, and the presence of gene specificity for methylation induction was indicated.[27,37] However, analysis of a cancer tissue reveals only events in its single precursor cell, and the information obtained is very stochastic. Analysis of a panel of cancers can reflect events in the precursor cells of the cancers, but the number of precursor cells analyzed is still limited to the number of cancers analyzed.

In order to avoid selection bias by gene function, and to analyze as many cells as possible, analysis of a non-cancerous tissue is advantageous. We analyzed methylation of a panel of genes in gastric mucosae with and without *H. pylori* infection, and showed that specific genes are methylated in gastric mucosae with *H. pylori* infection.[63] We also analyzed the methylation levels of a panel of genes in esophageal mucosae, and found that specific genes are methylated in correlation with smoking history.[40] These showed that specific inducers of aberrant DNA methylation induce methylation of specific genes. The presence of a "methylation fingerprint" of individual methylation inducers suggests that the fingerprint can be used as a marker for past exposure to specific carcinogenic factors in our lives.

Molecular mechanisms of target gene specificity. As a molecular mechanism for gene specificity, low transcription was suggested in pioneering studies that used an exogenously introduced gene and endogenous genes demethylated by a demethylating agent.[64,65] Analysis of selected genes in embryonic stem cells, along with normal adult tissue, and cancer cells revealed that genes marked with trimethylation of histone H3 lysine 27 (H3K27me3) in embryonic stem cells are likely to become methylated in cancers.[66–68] The finding was further supported by a genome-wide analysis of genes with H3K27me3 in cancer cells and corresponding normal cells.[19]

In addition to these factors that confer susceptibility to DNA methylation, the presence of RNA polymerase II (pol II), active or stalled, in NFR was shown to confer resistance to DNA methylation.[34] Although the presence of active histone modifications also confers resistance, the effect of active histone modifications was overridden by the presence of pol II in multivariate analysis, suggesting that the presence of pol II is the final effector that protects NFR from DNA methylation. Taken all together, DNA methylation of NFR is protected by the presence of pol II regardless of transcription levels, and promoted by the

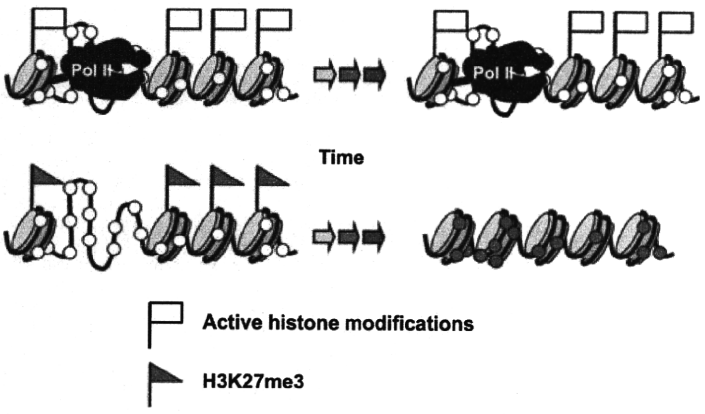

Fig. 4. Determinants of methylation destiny. Genes with RNA polymerase II (pol II), active or stalled, are resistant to DNA methylation, and genes with H3K27me3 are susceptible to DNA methylation. The presence of pol II is associated with the presence of active histone modifications, even if a gene is not actively transcribed. Open and closed circles show unmethylated and methylated CpG sites, respectively.

presence of H3K27me3 (Fig. 4). Once DNA methylation is induced in susceptible NFR, the H3K27me3 mark almost disappears[19] or decreases to a very low level.[69]

Reversibility of alterations

One of the major differences, or most important difference, between mutations and DNA methylation is reversibility. Physiologically, epigenetic modifications undergo dynamic changes during development, differentiation, and reprogramming.[70,71] In somatic cells the demethylating agents 5-azacytidine and 5-aza-2′-deoxycytidine have long been used in the laboratory.[72] Now these agents have come into clinics and are showing very promising effects in hematological malignancies.[73] The detailed pharmacological mechanisms and usage are summarized in the reviews cited above.

Future perspectives

Now, unique characteristics of DNA methylation are clear, but many questions still remain. Are there any chemicals that induce aberrant methylation of NFR directly, not as a result of gene expression changes? How does chronic inflammation induce aberrant DNA methylation? Do we know enough about the determinants of gene specificity?

At the same time, the biomedical application of DNA methylation is becoming more promising. The large number of genes methylated in a cancer increases the chance of successful identification of methylation biomarkers to predict patient prognosis and response to therapeutics. Cancer-specific methylation can be used for detection of cancer cells. The presence of an epigenetic field for cancerization in normal-appearing tissues can be used as a cancer risk marker, which reflects one's own life history. The deep involvement of chronic inflammation in methylation induction indicates that suppression of components involved in the induction can be utilized as a target of cancer prevention. The methylation fingerprint can be used in epigenetic epidemiology.

Mutations have not been considered as a cause of disorders that involve irreversible alteration of cellular functions, such as neurodegenerative disorders, diabetes, immunological disorders, and renal disorders. This was because mutations are rare events and cannot affect as many cells as the function of a tissue is affected as a whole. However, methylation can be induced in many more cells in a tissue, and genes affected are specific. This suggests that a critical gene can be inactivated in a significant fraction of cells, and raises the possibility that aberrant DNA methylation is causally involved in chronic disorders other than cancers.

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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

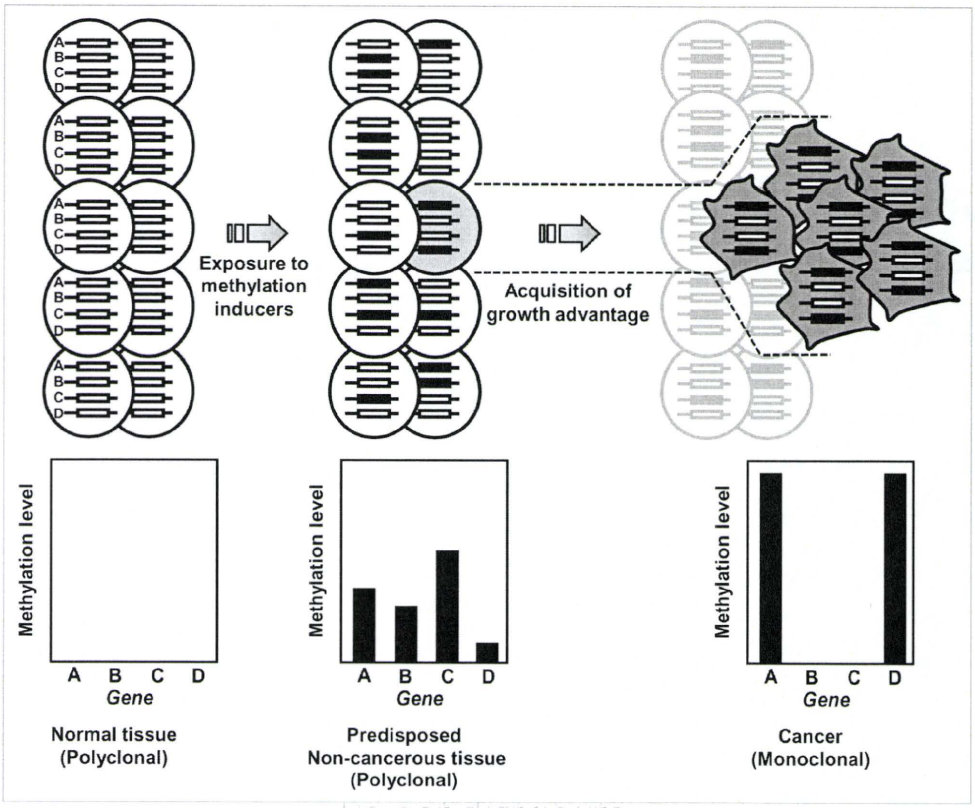

Figure 1. Different meaning of aberrant DNA methylation in non-cancerous and cancer tissues. Cells in an entirely normal tissue contain no aberrant methylation, and, by exposure to methylation inducers, cells come to harbor aberrant methylation of specific genes. A cancer, consisting of many cancer cells, 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]