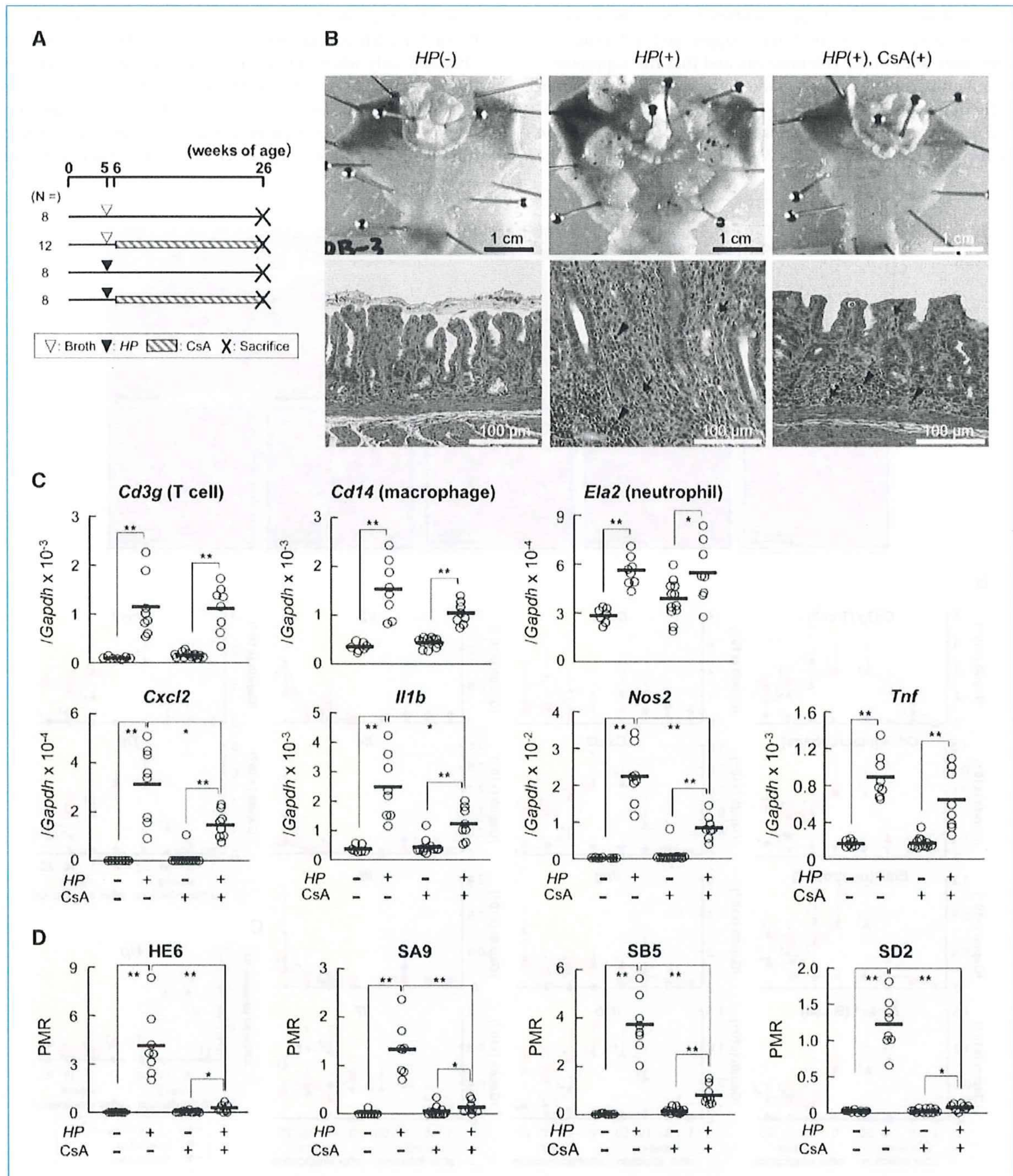

Figure 5. Suppression of inflammation and methylation induction by CsA treatment. A, experimental design for CsA treatment and *HP* infection. B, macroscopic (top) and histologic (bottom) analyses of gastric mucosae. Hyperplastic changes in pyloric area were prominent in *HP*-infected gerbils without 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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POINT OF VIEW

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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

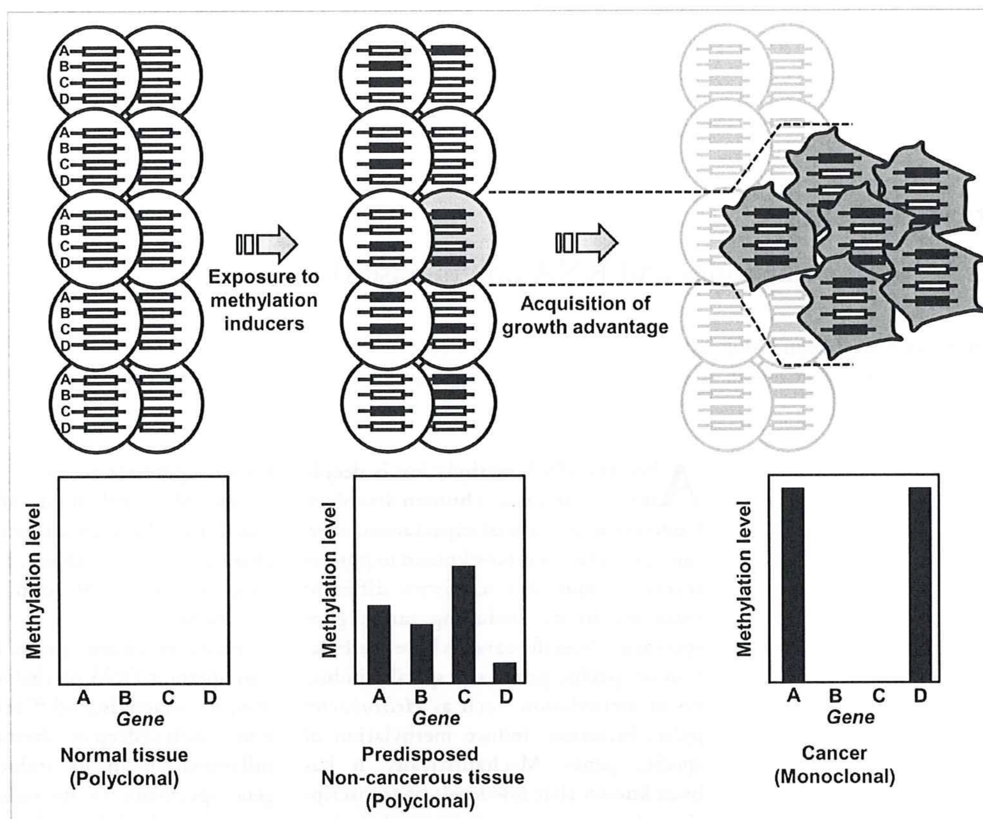

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Figure 1. Different meaning of aberrant DNA methylation in non-cancerous and cancer tissues. Cells in an entirely normal tissue contain no aberrant methylation, and, by exposure to methylation inducers, cells come to harbor aberrant methylation of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

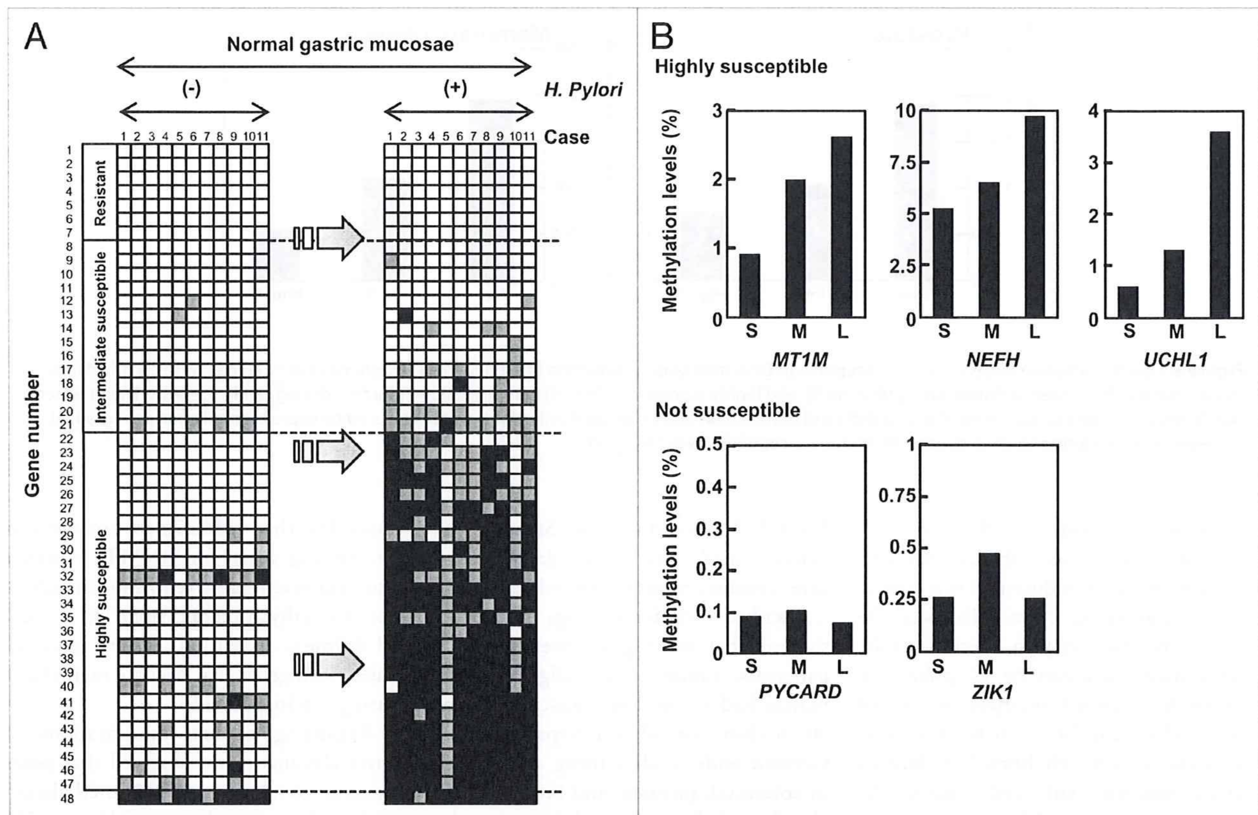

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Figure 2. Target gene specificity in DNA methylation induction in non-cancerous tissues. (A) Methylation profile of the 48 genes in normal gastric mucosae with and without *H. pylori* infection (modified from Nakajima et al.[9]). The methylation status 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]

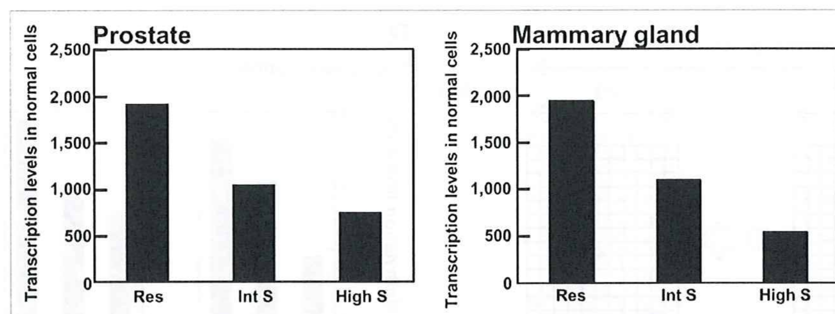

Figure 3. Low transcription levels of genes susceptible to DNA methylation induction (modified from Takeshima et al.[39]). Genes were classified into those resistant (Res), intermediately susceptible (Int S), and highly susceptible (High S) to methylation induction during carcinogenesis. Their transcription 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

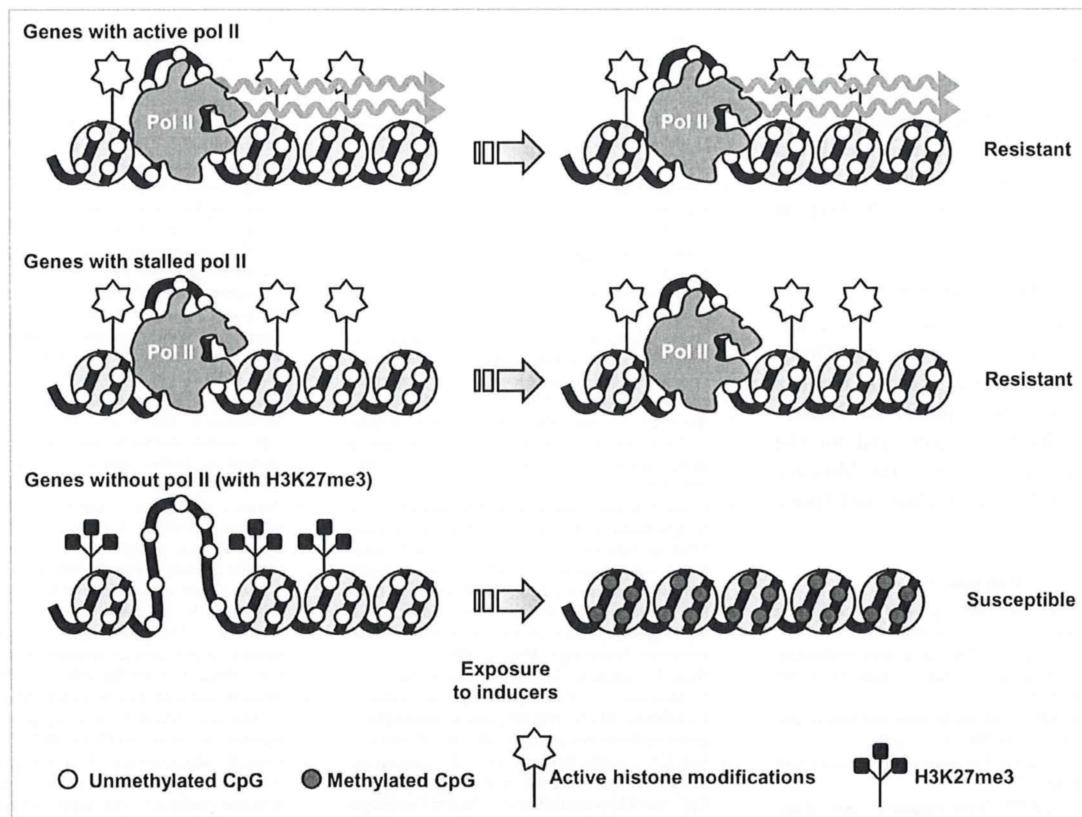

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Figure 4. A scheme of the instructive mechanism of aberrant DNA methylation induction. Both genes with active Pol II and genes with stalled Pol II are resistant to aberrant methylation induction. In contrast, genes without Pol II but with H3K27me3 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[23,31–33] 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.

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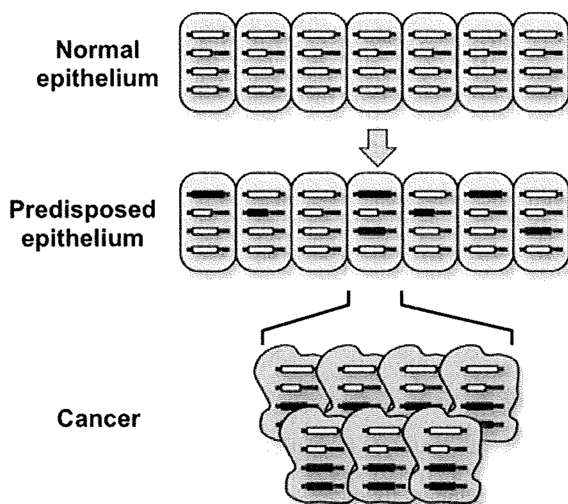

Fig. 1. Epigenetic field for cancerization and clonal selection in cancer. Normal epithelium consists of cells with little aberrant methylation. By exposure to inducers of methylation, specific genes are methylated in minor fractions of cells. A cancer develops from one of the cells that has already 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.

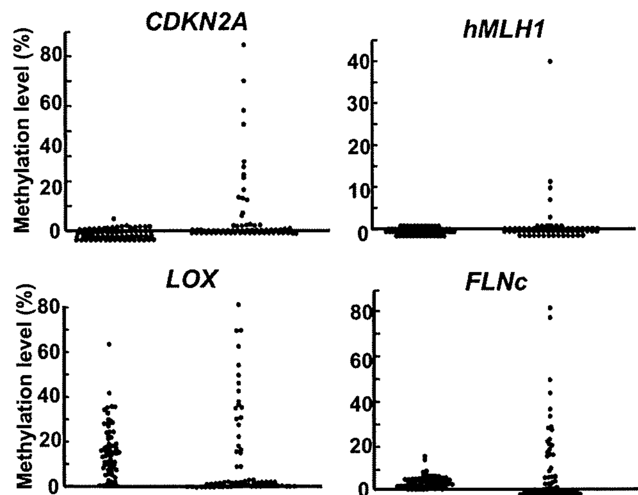

Fig. 2. Distribution patterns of methylation in non-cancerous and cancer tissues. Methylation levels, which reflect fractions of cells with the methylation, were quantified in 66 paired samples of non-cancerous and cancer tissues of gastric cancer patients (modified from Enomoto *et al.*[39]). They showed 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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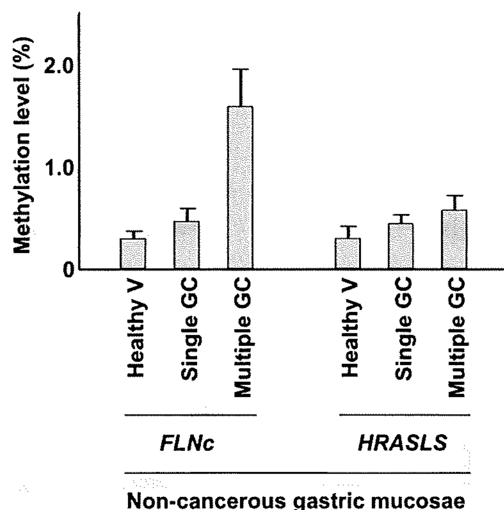

Fig. 3. Correlation between methylation level and cancer risk. Methylation levels of two marker genes (*FLNc* and *HRASLS*) were quantified in gastric mucosae of healthy individuals (healthy V), non-cancerous gastric mucosae of patients with a single gastric cancer (single GC), and non-cancerous gastric 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

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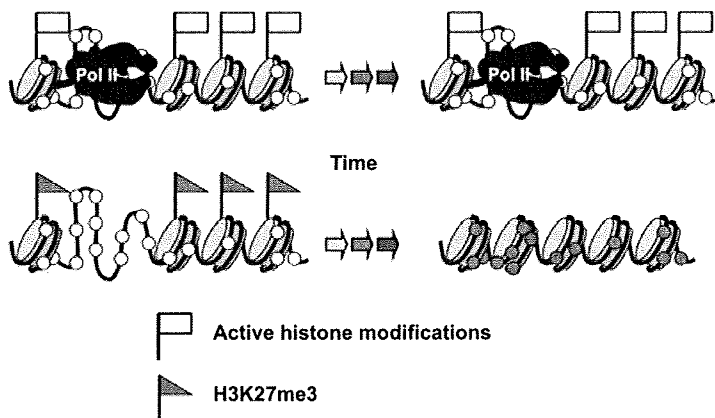

Fig. 4. Determinants of methylation destiny. Genes with RNA polymerase II (pol II), active or stalled, are resistant to DNA methylation, and genes with H3K27me3 are susceptible to DNA methylation. The presence of pol II is associated with the presence of active histone modifications, even if a gene is 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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The presence of a methylation fingerprint of *Helicobacter pylori* infection in human gastric mucosae

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Aberrant DNA methylation is deeply involved in human cancers, but its inducers and targets are still mostly unclear. *Helicobacter pylori* infection was recently shown to induce aberrant methylation in gastric mucosae, and produce a predisposed field for cancerization. Here, we analyzed the presence of target genes in methylation induction by *H. pylori* and the mechanism for the gene specificity. Noncancerous gastric mucosae were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group), and methylation of promoter CpG islands of 48 genes that can be methylated in gastric cancer cell lines was analyzed by methylation-specific PCR. In total, 26 genes were consistently methylated in individuals with current or past infection by *H. pylori*, whereas 7 genes were not methylated at all. In addition, 14 genes were randomly or intermediately methylated in individuals with gastric cancers and the remaining 1 gene was methylated in all the cases. The methylation-susceptible genes had significantly lower mRNA expression levels than the methylation-resistant genes. *H. pylori* infection did not induce mRNA and protein expression of DNA methyltransferases; *DNMT1*, *DNMT3A* or *DNMT3B*. Gene specificity was present in the induction of aberrant DNA methylation by *H. pylori* infection, and low mRNA expression, which could precede methylation, was one of the mechanisms for the gene specificity. These findings open up the possibility that a methylation fingerprint can be used as a novel marker for past exposure to a specific carcinogenic factor.
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Key words: DNA methylation; epigenetic; fingerprint; *Helicobacter pylori*; molecular epidemiology

Aberrant DNA methylation is deeply involved in human cancer development and progression.[1] In some cancer types, such as gastric cancers, tumor-suppressor genes are more frequently inactivated by aberrant DNA methylation than by mutations.[2] Nevertheless, only limited information is available for inducers of aberrant DNA methylation, which include aging, viral infection and ulcerative colitis.[3,4] Also, almost no information is available for gene specificity in methylation induction by a specific factor. Using cancer tissues, it is very difficult to clarify an association between a specific inducer and methylation of a gene. Aberrant methylation of a gene can be present in cancer tissues because its methylation conferred a growth advantage although it was a rare and random event, or because its methylation was carried over from a precursor tissue to a cancer tissue since it was frequently induced in the precursor tissue. In contrast, using a noncancerous tissue, one can assess an effect of a methylation inducer by the fraction of cells with methylation in the polyclonal tissue.

Gastric mucosa infected by *Helicobacter pylori* is a useful model to examine the possible presence of gene specificity in methylation induction by a specific factor since *H. pylori* infection was recently shown to induce aberrant DNA methylation potently in gastric mucosae.[5] Moreover, the fraction of DNA molecules with aberrant methylation (methylation level) in gastric mucosae of individuals without current *H. pylori* infection was correlated with gastric cancer risk,[5,6] indicating that methylation in noncancerous tissues is related to gastric carcinogenesis. So far, 6 CpG islands in gene promoter regions methylated in gastric cancers[7] were analyzed, and all were methylated in gastric mucosae with current and past infection with *H. pylori*. However, it is unknown whether these 6 genes are preferentially methylated by *H. pylori* infection or *H. pylori* infection induces methylation of random genes.

In this study to analyze the presence of gene specificity for methylation induction, firstly we examined the methylation status of 48 promoter CpG islands in the noncancerous gastric mucosae of 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection). The 48 genes were selected as genes that can be methylation-silenced in gastric cancer cell lines[8] because the vast majority of CpG islands in gene promoter regions are not methylated at all in noncancerous tissues, and we had to newly select genes that have better chances to be methylated in noncancerous tissues. Secondly, we analyzed an association between susceptibility to methylation induction and mRNA expression levels in normal tissue without and with *H. pylori* infection.

Material and methods

Tissue samples and DNA/RNA extraction

For methylation analysis, (noncancerous) gastric mucosa samples were collected from 4 groups of individuals (with and without a gastric cancer, and with and without current *H. pylori* infection; $N = 11$ for each group, average age $= 60.8 \pm 13.8$ years). For analysis of mRNA expression that determines gene specificity of methylation induction, we need to analyze the mRNA expression level in gastric mucosae free of methylation, which, once induced, will cause decreased gene transcription to avoid confusion between cause and consequence. Therefore, samples were collected from 11 healthy volunteers, who were considered to have less chance for methylation induction by *H. pylori* than elderly individuals (7 males and 4 females; 6 with *H. pylori* infection and 5 without; average age $= 34.8 \pm 3.1$ years). Biopsy specimens were taken from one standard site of the stomach (antral regions in the lesser curvature) using sterilized biopsy forceps (Olympus, Tokyo, Japan). *H. pylori* infection status was analyzed by culture test (Eiken, Tokyo, Japan) and rapid urease test (Otsuka, Tokushima, Japan). All the materials were obtained with written informed consents, and the procedures were approved by the institutional review board. High molecular weight DNA was extracted by the standard phenol/chloroform method and total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and an RNeasy Mini kit (Qiagen, Valencia, CA).

Additional Supporting Information may be found in the online version of this article.

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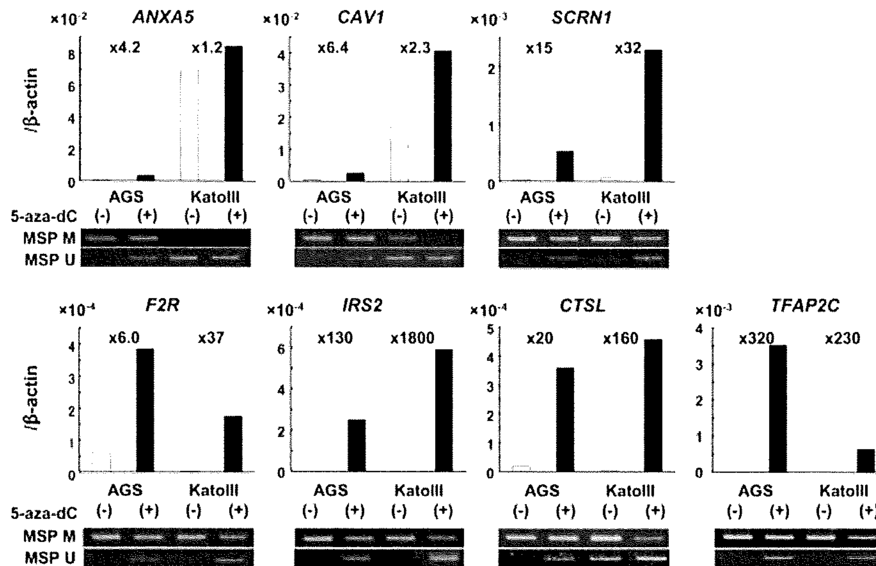

FIGURE 1 – Gene silencing due to methylation of the regions analyzed. mRNA expression and methylation were analyzed by real-time RT-PCR and MSP, respectively, in gastric cancer cell lines (AGS and KATO-III) before and after 5-aza-dC treatment. The fold increases after 5-aza-dC treatment is shown for each cell line. No or little mRNA expression in a cell line(s) without unmethylated DNA molecules and upregulation by the 5-aza-dC treatment was confirmed for the 7 genes randomly selected from the 48 genes.

Cell lines and 5-aza-dC treatment

Gastric cancer cell lines, AGS and KATO-III, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). For treatment with a demethylating agent, 5-aza-2′-deoxycitidine (5-aza-dC, Sigma, St. Louis, MO), cells were seeded on day 0, media containing 0.3 μM 5-aza-dC was freshly added on days 1 and 3, and cells were harvested on day 5. Genomic DNA and total RNA were isolated in the same way as the primary samples.

Bisulfite treatment and methylation-specific PCR

Bisulfite treatment was performed as previously described.[9] Briefly, DNA samples (1 μg each) digested by *Bam*HI were denatured in 0.3 N NaOH at 37°C for 15 min. The samples underwent 15 cycles of 30-sec denaturation at 95°C and 15-min incubation at 50°C in 3.1 N sodium bisulfite (pH 5.0) and 0.5 mM hydroquinone. The samples were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), and desulfonated in 0.3 N NaOH. DNA was ethanol precipitated and dissolved in 40 μL of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), respectively,[8] using 2 μL of the sodium bisulfite-treated DNA. A region upstream of a putative transcriptional start site (200 bp or less) was analyzed, and CpG maps of all the genes are shown in the Supporting Information Figure 1. DNA methylated with *Sss*I methylase was used to determine a specific condition of PCR for the M set, and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Sciences) was used for the U set. A number of PCR cycles that would yield a minimal visible band was determined using these fully methylated DNA (for M primers) and fully unmethylated DNA (for U primers), and a further 4 cycles were added for actual analysis of test samples. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that for unmethylated DNA, respectively.

Quantitative reverse transcription PCR

cDNA was synthesized from 1 μg of total RNA using a Superscript II kit (Life Technologies, Rockville, MD) with a random primer. Real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME). The number of molecules of a specific gene in a sample was measured by comparing its amplification with that of standard samples, which contained 10^1–10^7 copies of the gene. The standard samples were produced by PCR amplification and purification using Zymo-Spin I™ Columns (Zymo Research, Orange, CA). The amount of the standard samples was measured by OD 260 nm and also by quantification of band intensities after electrophoresis. The mRNA quantity of each gene was normalized to that of β-actin. The primers and PCR conditions are shown in the Supporting Information Table 1. The difference of mRNA expression levels between 2 groups of genes was analyzed by the Welch *t*-test method (both sided).

Western blot analysis

Each 100 μg whole-cell lysate sample was subjected to SDS-PAGE (10% acrylamide gel) and blotted to PVDF membrane. DNMT1 and DNMT3A were detected using rabbit polyclonal antibody against human DNMT1 (NEB, Beverly, MA), human DNMT3A (Cell Signaling Technology, Danvers, MA), respectively at 1/1,000 dilution. DNMT3B was detected using goat polyclonal antibody against human DNMT3B (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/500 dilution. Horse radish peroxidase-conjugated secondary antibody (antirabbit; Cell Signaling Technology, antigoat; Santa Cruz Biotechnology) was used at 1/5,000 dilution.

Results

Confirmation of gene silencing due to promoter CpG islands

The 48 genes consisted of 32 randomly and 16 arbitrarily selected genes from 421 genes that had been identified as methylation-silenced genes in a gastric cancer cell line using microarray analysis of cells treated with 5-aza-dC, and MSP analysis.[8] First,

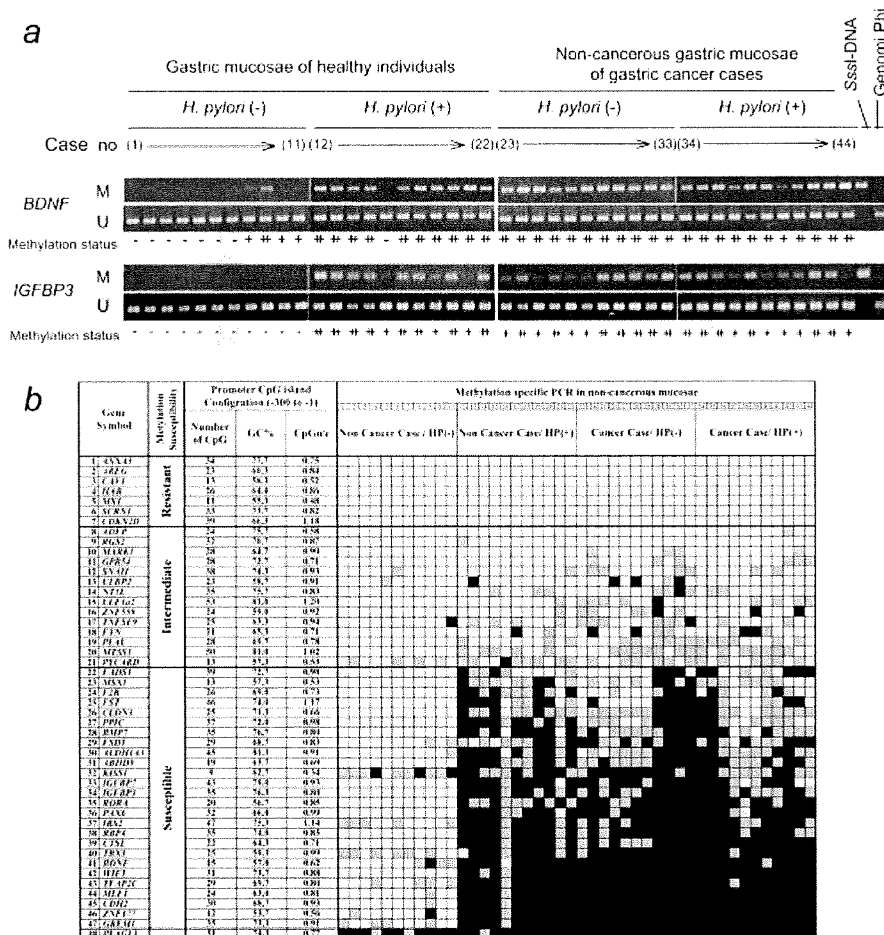

FIGURE 2 – Methylation profile of the 48 genes in noncancerous gastric mucosae. (*a*) Representative results of MSP. Samples 1–11, gastric mucosae of healthy individuals without *H. pylori* infection; 12–22, those with *H. pylori* infection; 23–33, noncancerous gastric mucosae of gastric cancer cases without *H. pylori* infection; and 34–44, those with *H. pylori* infection. Methylation levels were classified as none (−), low (+), high (++) according to the intensity of the band for methylated DNA molecules compared with that of fully methylated control DNA. (*b*) Overview of the results of all the 48 genes. The genes were aligned in the order of increasing numbers of individuals with methylation. Closed, hatched, and open boxes represent the methylation levels of high (++), low (+), and none (−), respectively. Rows 1–7, the 7 genes completely resistant to methylation induction in any cases; rows 8–21, genes methylated randomly or more frequently in individuals with cancers; and rows 22–47, genes susceptible to methylation induction by *H. pylori* infection. CpG island configuration (number of CpG sites, G+C content, and CpG score) in 300 bp upstream regions from transcription start sites is also shown. The presence of methylation-resistant and methylation-susceptible genes was clearly revealed. No clear difference in the CpG island configuration was observed between the 2 groups.

we analyzed mRNA expression of 7 of the 48 genes before and after 5-aza-dC treatment using real-time RT-PCR (Fig. 1). It was confirmed that no or little mRNA expression was present in cell lines without unmethylated DNA molecules and that mRNA expression was upregulated by the 5-aza-dC treatment.

Gene specificity in methylation induction by H. pylori infection in gastric mucosae

We then analyzed the methylation status of the promoter CpG islands of the 48 genes in the (noncancerous) gastric mucosae of 4 groups of individuals; those with and without *H. pylori* infection and with and without a gastric cancer. Since MSP can produce inconsistent results if inappropriately performed, we carefully selected a PCR cycle for each primer set so that false positive and negative results were not produced. We scored the methylation status as negative, weakly positive or positive by comparing the band density with that of a fully methylated control (representative results in Fig. 2*a*).

When all the genes were aligned in the order of number of samples with methylation (Fig. 2*b*), the 48 genes were divided into 3 groups: (*i*) 7 genes that were completely unmethylated in any of the 4 groups (genes 1–7 in Fig. 2*b*; methylation-resistant genes), (*ii*) 14 genes that were methylated randomly or more frequently in individuals with cancers (genes 9–21; intermediate genes); and (*iii*) 26 genes that were consistently methylated in the individuals with *H. pylori* infection or with a gastric cancer (genes 22–47; methylation-susceptible genes). The remaining 1 gene, *PLAGL1*, was methylated in all the individuals. This demonstrated that some genes are resistant to methylation induction by *H. pylori* infection while others are susceptible, namely the presence of gene specificity in methylation induction.

Lack of association between CpG island configuration and methylation susceptibility

The 48 genes analyzed here all had CpG islands in their promoter regions. However, based on recent reports,[10] there was a