TABLE 2 Association between methylation status of CGIs No. 5 and No. 21 and clinicopathological characteristics

Feature	CGI No. 5			CGI No. 21		
	Methylated	Unmethylated	*P* value*	Methylated	Unmethylated	*P* value*
Histological differentiation			0.69			0.27
Well	8	10		7	11	
Moderately	19	23		25	17	
Poorly	13	23		22	14	
T category			0.78			0.29
pT1	2	5		5	2	
pT2	6	9		7	8	
pT3	28	39		36	31	
pT4	4	3		6	1	
Lymphatic invasion			0.30			0.0061
Negative	20	19		15	24	
Positive	22	35		39	18	
Vascular invasion			0.84			0.065
Negative	22	29		24	27	
Positive	18	27		30	15	
Lymph node metastasis			0.0015			0.0035
Negative	12	3		3	12	
Positive	28	53		51	30	

* Statistically significant values: $P < 0.05$

TABLE 3 Multivariate logistic regression analysis to identify factors that influence the presence of lymph node metastasis

	No. of cases	Odds ratio	95% CI	*P* value*
CGI No. 5 (unmethylated vs methylated)	56/40	15.691	3.009–81.821	0.001
CGI No. 21 (methylated vs unmethylated)	54/42	10.392	2.103–51.353	0.004
T category (pT3 + pT4 vs pT1 + pT2)	74/22	4.410	0.958–20.298	0.057
Vascular invasion (positive vs negative)	45/51	2.193	0.504–9.539	0.295
Age (>63 vs ≤63)	48/48	1.519	0.361–6.384	0.568
Gender (female vs male)	17/79	0.634	0.115–3.498	0.601

95% CI 95% confidence interval

* Statistically significant values: $P < 0.05$

Methylation in Noncancerous Esophageal Tissues and Metastatic Lymph Nodes

Methylation levels of CGI No. 5 and No. 21 in noncancerous esophageal tissues of esophageal cancer patients were 2.8% ± 2.3% and 1.1% ± 0.7%, respectively (mean ± SD) and not correlated with those in cancers (correlation coefficients: −0.21 and 0.11, respectively). This result suggested that the methylation in primary cancer tissues was not influenced by methylation in noncancerous esophageal tissues. In addition, methylation of CGI No. 21 was analyzed in 3 metastatic lymph nodes of 3 patients. Its hypermethylation was detected in all of the three lymph nodes, but only in 2 of 3 corresponding primary cancer tissues. This result suggested that cancer cells with methylation of CGI No. 21 had advantage in metastasis or growth in metastatic foci.

Correlation among Methylation Levels of CGIs

To evaluate correlations among methylation levels of CGI No. 5, 21, and the other 23 CGIs, a correlation coefficient was calculated for a given pair of CGIs. The correlation coefficients between the methylation level of CGI No. 21 and those of 6 CGIs (No. 9, 12, 16, 18, 22, 24) were more than 0.4. In contrast, the correlation coefficients between the methylation level of CGI No. 5 and those of the other CGIs were less than 0.2. This suggested that methylation of CGI No. 21 and that of No. 5 are independent events.

DISCUSSION

In the present study, by genome-wide methylation analysis between ESCCs with and without lymph node

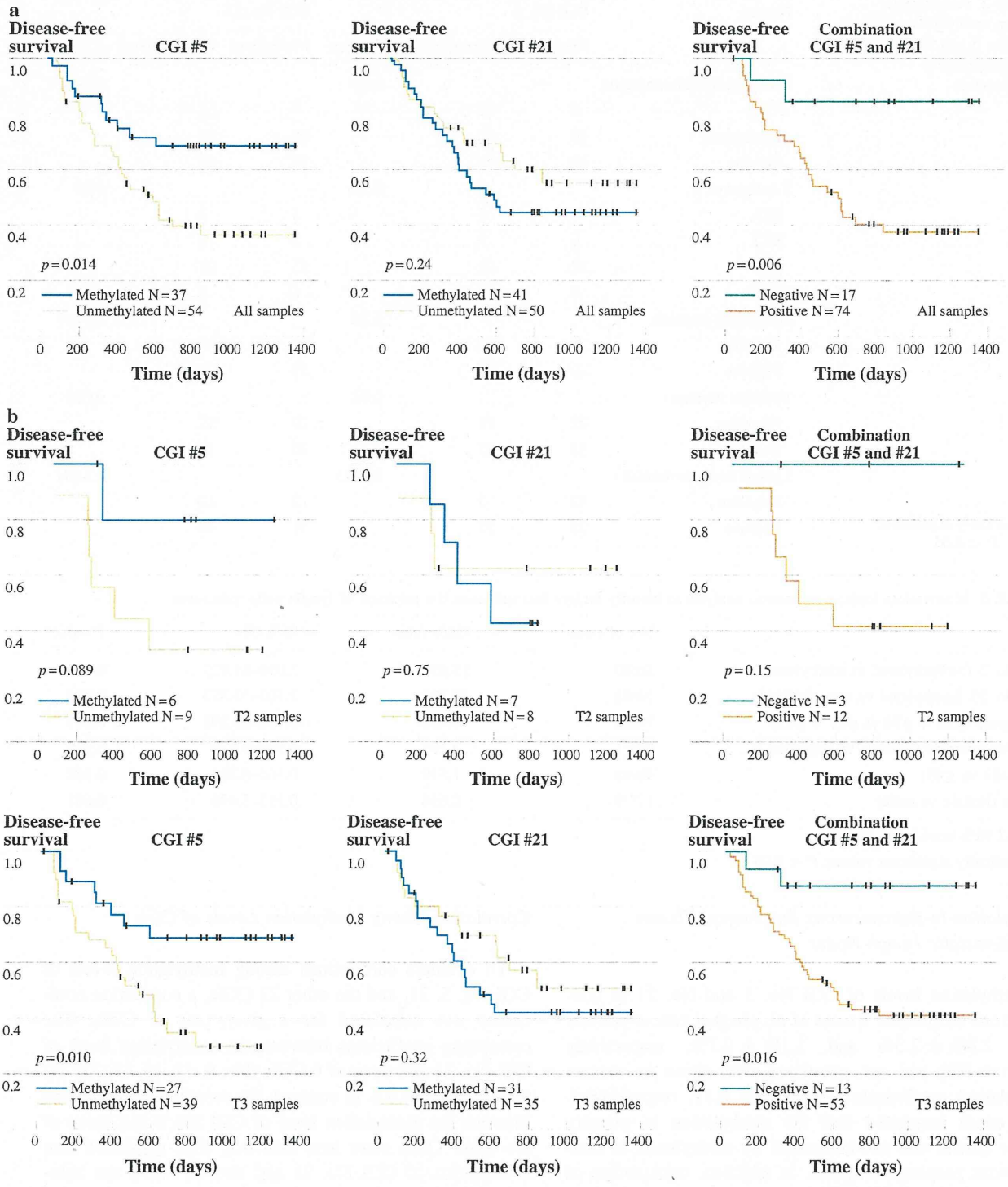

FIG. 3 Influence of methylation statuses of CGI No. 5, No. 21, and their combination on patient survival. **a** Disease-free survival according to methylation statuses of CGI No. 5, No. 21, and their combination. The data for all patients were analyzed by the Kaplan–Meier method, and significant association was observed for CGI No. 5 and the combination. **b** Disease-free survival according to methylation statuses of CGI No. 5, No. 21, and their combination within individuals in T2 or T3 categories. Significant association was confirmed for patients in the T3 category, and the tendency was observed in the T2 category

metastasis and between ESCCs without lymph node metastasis and metastatic lymph nodes, we were able to identify 7 candidate CGIs whose methylation statuses were associated with the presence of lymph node metastasis and to validate the usefulness for 2 of them. Generally, markers isolated by genome-wide screening cannot be brought to clinics due to the overfitting issue caused by multiple testing, and validation is requisite in genome-wide studies.[30] Indeed, even in our study, 5 of the 7 candidates were excluded by the analysis of the validation set despite having small *P* values (0.006 for CGI No. 2) in the analysis of the screening set. Therefore, these 2 validated methylation markers are considered to be promising.

Methylation statuses of the 2 CGIs, No. 5 and No. 21, showed high sensitivity (73% and 59%, respectively) and high specificity (71% and 86%, respectively), and their combination has a further higher sensitivity (93%) and moderate specificity (57%). If cut-off methylation levels were further optimized in the validation set, sensitivity of CGIs No. 5, 21, and their combination will reach 81%, 68%, and 95%, respectively, while specificity will remain at 71%, 71%, and 57%, respectively. Naturally, these must be validated again in a different set of samples. On the other hand, conventional imaging modalities are reported to have only low sensitivity, but high specificity.[4–7] In our study, however, sensitivity was high (91%), but specificity was extremely low (0%). This was considered to be because a low threshold value for the size of metastatic lymph nodes was adopted for high sensitivity in this study, and most patients were in T3/T4 stages (74 of 96).

Methylation statuses of the 2 CGIs were likely to influence the presence of lymph node metastasis independently of the T category by multivariate analysis, which is important for markers to predict presence. It should be noted that most patients in this study were in T3/T4 stages (22 in T1/T2 and 74 in T3/T4 stages), and usefulness in early-stage diseases needs to be established with a larger number of patients in early stages. Furthermore, lymph node metastasis is one of the strongest determinants of prognosis in ESCCs, and it is expected that a marker to predict lymph node metastasis should predict patient prognosis.[31] As expected, the methylation status of CGI No. 5 and the combination of the 2 CGIs were significantly associated with disease-free survival. It is worth conducting a prospective clinical study on the 2 CGIs to prove them as novel markers to predict lymph node metastasis.

The biological bases why unmethylated status of CGI No. 5 and methylated status of CGI No. 21 are associated with the presence of lymph node metastasis are still unknown. Hypomethylation of CGI No. 5 and hypermethylation of CGI No. 21 were not associated, and it was suggested that their methylation was independently associated with lymph node metastasis. Methylation of CGI No. 5 was not associated with loss or increase of *PAX6* expression. In addition, *DKFZp686K1684* hypothetical gene supported by BX648962 is proposed to be located close to CGI No. 5 (Fig. 1), but no expression of the hypothetical gene was detected in ESCC cell lines and those after 5-aza-2′-deoxycitidine treatment (data not shown).[32] Recent genome-wide studies demonstrated that DNA methylation of a nucleosome-free region, a 200–300 bp region upstream to a TSS, is associated with gene silencing, and that methylation of exons tends to be associated with increased expression.[22,33,34] Since CGI No. 5 was not located in these genomic structures, it was reasonable that its methylation status was not associated with gene expression. CGIs No. 5 and No. 21 might be located in promoter regions of unknown genes, including microRNA genes, or in enhancer regions whose methylation is critical for regulation of gene expression levels.

Unmethylated CGI No. 5 was associated with the presence of lymph node metastasis, and this CGI was isolated by comparison between N0 and N1a pools and comparison between N0 and N1b pools. Methylated CGI No. 21 was associated with the presence, and this CGI was isolated by comparison between N0 and LN pools. By conducting 2 ways of screening, we were able to isolate CGIs whose characteristics in detection of lymph node metastasis were different.

In conclusion, we identified 2 CGIs whose methylation statuses in primary ESCCs were associated with the presence of lymph node metastasis. These 2 CGIs are promising candidate markers to predict lymph node metastasis of ESCCs.

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Alu and Satα hypomethylation in *Helicobacter pylori*-infected gastric mucosae

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Global hypomethylation and regional hypermethylation are supposed to be hallmarks of cancer cells. During gastric carcinogenesis, in which *Helicobacter pylori* infection is causally involved, aberrant hypermethylation is already present in *H. pylori*-infected gastric mucosae. In contrast, little is known about global hypomethylation, which can be caused by hypomethylation of individual repetitive elements and other sequences. We, therefore, investigated hypomethylation of individual repetitive elements and the global 5-methylcytosine content in four groups of gastric mucosal samples that represented the time course of *H. pylori* infection and gastric carcinogenesis [gastric mucosae of *H. pylori*-negative healthy volunteers (G1, n = 34), *H. pylori*-positive healthy volunteers (G2, n = 42), *H. pylori*-positive gastric cancer patients (G3, n = 34) and *H. pylori*-negative gastric cancer patients (G4, n = 20)] and 52 primary gastric cancers. Major variants of Alu, LINE1 and Satα were identified, and their methylation levels were quantified by bisulfite pyrosequencing. Compared with G1, the Alu methylation level was decreased in G2, G3, G4 and cancers (89.2–97.1% of that in G1, $p < 0.05$). The Satα methylation level was decreased in G2 (91.6%, $p < 0.05$) and G3 (94.3%, $p = 0.08$) but not in G4 and cancers. The LINE1 methylation level was decreased only in cancers. The 5-methylcytosine content was at similar levels in G2, G3 and G4 and highly variable in cancers. These results showed that Alu and Satα hypomethylation is induced in gastric mucosae by *H. pylori* infection during gastric carcinogenesis, possibly in different target cells, and that global hypomethylation is not always present in human gastric cancers.

Carcinogenesis

Global hypomethylation and regional hypermethylation are supposed to be hallmarks of cancer cells.[1] Global hypomethylation, defined as the content of 5-methylcytosine in the genome,[2] is considered to be due to hypomethylation of repetitive elements, which are normally heavily methylated,[3] and other sequences. Global hypomethylation is known to cause chromatin decondensation that results in chromosomal instability and cancer development.[4–7] In addition, hypomethylation of repetitive elements is associated with its elevated transcription,[8] and that of normally methylated promoter CpG islands can lead to elevated expression of tumor antigens and possible oncogenes.[8–10] On the other hand, hypermethylation is observed in normally unmethylated promoter CpG islands and silences downstream genes, including tumor suppressor and other passenger genes.[11]

Hypermethylation of CpG islands can be present not only in cancers but also in noncancerous tissues.[12] Especially in gastric mucosae, aberrant DNA hypermethylation is induced by *Helicobacter pylori* infection, a major cause of gastric cancers.[13,14] The methylation levels of CpG islands are very low in gastric mucosae of *H. pylori*-negative healthy individuals (G1; incidence of gastric cancers = 0.03% per year or less[15]). They are very high in gastric mucosae of *H. pylori*-positive healthy individuals (G2; incidence = 0.14%[16]) and in noncancerous gastric mucosae of *H. pylori*-positive gastric cancer patients (G3; incidence of secondary gastric cancer = 4.1%[17]). They are high but lower than in G2 and G3 in noncancerous gastric mucosae of *H. pylori*-negative gastric cancer patients (G4; incidence = 6.2%[18]). *H. pylori* infection is known to disappear when severe gastric atrophy is induced as a result of chronic *H. pylori* infection,[19–22] and the four groups, G1–G4, are considered to represent the natural history of *H. pylori* infection. Methylation levels correlate with gastric cancer risk only in *H. pylori*-negative individuals,[13,14] suggesting that methylation levels in these individuals reflect the degree of epigenetic damage in stem cells.[23,24]

In contrast, global hypomethylation during gastric carcinogenesis remains unclear, not only when but also where in the genome it takes place. Major normally methylated repetitive elements consist of Alu, LINE1 and Satα, which

Key words: hypomethylation, repeat sequence, gastric cancer, *Helicobacter pylori*, carcinogenesis
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constitute 10, 17 and 4% of the genome, respectively,[25–27] and collectively cover over 30% of the total CpG sites in the genome.[28,29] Alu and LINE1 belong to interspersed elements,[25] and Satα is a tandem repeat element[30,31] confined to the centromeres.[32] Hypomethylation of Satα is known to be induced by loss-of-function mutations of DNA methyltransferase 3B.[33] As a fundamental basis to understand gastric carcinogenesis, we have to clarify whether or not hypomethylation is present in *H. pylori*-infected gastric mucosae and, if present, which repetitive elements or global 5-methylcytosine content are mainly affected.

In this study, we aimed to clarify these issues. To this end, we first identified major variants of Alu, LINE1 and Satα, and then measured their methylation levels by bisulfite pyrosequencing of DNA from gastric mucosal samples of G1, G2, G3 and G4 and gastric cancer tissues.

Material and Methods

Tissue samples

Gastric mucosae were collected by endoscopic biopsy of the antral region in 34 *H. pylori*-negative (G1: 16 male and 18 female; average age = 51 years, range = 25–91 years) and 42 *H. pylori*-positive healthy volunteers (G2: 21 male and 21 female; average age = 57 years, range = 23–86 years; 19 with gastric atrophy and 23 without; nine with gastric ulcers, eight with duodenal ulcers and three with hyperplastic polyps) and noncancerous gastric mucosae from 34 *H. pylori*-positive (G3: 26 male and 8 female; average age = 68 years, range = 39–87 years; 23 early cancers and 11 advanced cancers) and 20 *H. pylori*-negative gastric cancer patients (G4: 15 male and 5 female; average age = 69 years, range = 38–84 years; 17 early cancers and three advanced cancers). Gastric cancer tissues were obtained from 52 gastric cancer patients (cancers: 52 male; average age = 60 years, range = 29–84 years) who underwent gastrectomy. Informed consents were obtained from all the individuals. Gastric mucosae, noncancerous gastric mucosae and cancer tissues were frozen in liquid nitrogen immediately after biopsy or resection and stored at −80°C until extraction of genomic DNA.

All cancer tissues were histologically diagnosed according to the Japanese classification of gastric carcinoma[34] and were classified according to the Lauren classification system (11 intestinal and 41 diffuse types).[35] *H. pylori* infection status was detected by use of a serum anti-*H. pylori* IgG antibody test (SRL, Tokyo, Japan).

Cell lines and DNA extraction

Six gastric cancer cell lines, AGS, KATOIII, MKN28, MKN45, MKN74 and NUGC3, were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and the American Type Culture Collection (Manassas, VA). Three gastric cancer cell lines, HSC39, HSC44 and HSC57, were gifted by Dr. K. Yanagihara, National Cancer Center Research Institute, Tokyo, Japan. TMK1 was gifted by Dr. W. Yasui, Hiroshima University, Hiroshima, Japan. High molecular weight DNA was extracted by the phenol/chloroform method.

Sequencing analysis of repetitive DNA elements

Genomic DNA of a human gastric cancer cell line (AGS) was amplified by PCR with the primers for the three repetitive DNA elements (Supporting Information Table 1): Alu (AluSp from the database of the Genetic Information Research Institute: http://www.girinst.org/), LINE1 (X58075) and Satα (M38468). The PCR products were cloned into pGEM-T Easy vector (Promega, Madison, WI), and 12–41 clones for each repetitive DNA element were cycle sequenced. Sequencing was performed using a DYEnamic ET Terminator (GE Healthcare, Buckinghamshire, United Kingdom) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Sodium bisulfite modification and bisulfite pyrosequencing

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described.[36] The modified DNA was suspended in 40 μl of Tris-EDTA buffer, and an aliquot of 1 μl was used for bisulfite pyrosequencing. An annealing temperature that could amplify both unmethylated and methylated DNAs was determined by comparing amplification of DNA from peripheral leukocytes (mixture of unmethylated and methylated DNA) and DNA that was fully methylated by *Sss*I methylase (New England Biolabs, Beverly, MA) (Supporting Information Table 2). The PCR product was annealed to 0.2 μM pyrosequencing primers, and pyrosequencing was carried out using the PSQ 96 Pyrosequencing System (Qiagen, Valencia, CA). A methylation level was obtained using PSQ Assay Design software (Qiagen). Two CpG sites (ALU1 and ALU2) were measured for Alu, three for LINE1 (LINE1-1, LINE1-2 and LINE1-3) and one for Satα (SATα).

Analysis of the global 5-methylcytosine content

Genomic DNA (2.5 μg) was incubated with five units of DNase I (Sigma, St. Louis, MO) and 4 mM $MgCl_2$ at 37°C for 18 hr. The sample was further treated with three units of nuclease P1 in 10 mM NaAc (pH 5.2) and 50 μg/ml $ZnSO_4$ at 37°C for 7 hr and then with 2.5 units of *Escherichia coli* alkaline phosphatase in 0.1 M NH_4HCO_3 at 37°C for 16 hr. After purification, the samples were subjected to liquid chromatography equipped with a photodiode array detector and an electrospray ionization time-of-flight mass spectrometry (LCMS; LCT premier XE, Waters). Peaks of the four deoxyribonucleotides (2′-deoxyguansine, 2′-deoxyadenosine, 2′-deoxycytidine (dC) and thymidine) were monitored with UV 260 nm, whereas that of 5-methyl-2′-deoxycytidine (5mdC) was detected by a molecular ion of 242 [M+1], retention times of which were compared with that of the authentic sample. Global 5-methylcytosine content was quantified as the fraction of 5mdC quantity in the total 5mdC and dC quantity. The LCMS analysis was performed three times for each sample, and the mean coefficient of variation was confirmed to be less than 3%. Eight of the samples were also subjected to high-performance liquid chromatography (HPLC)-UV.

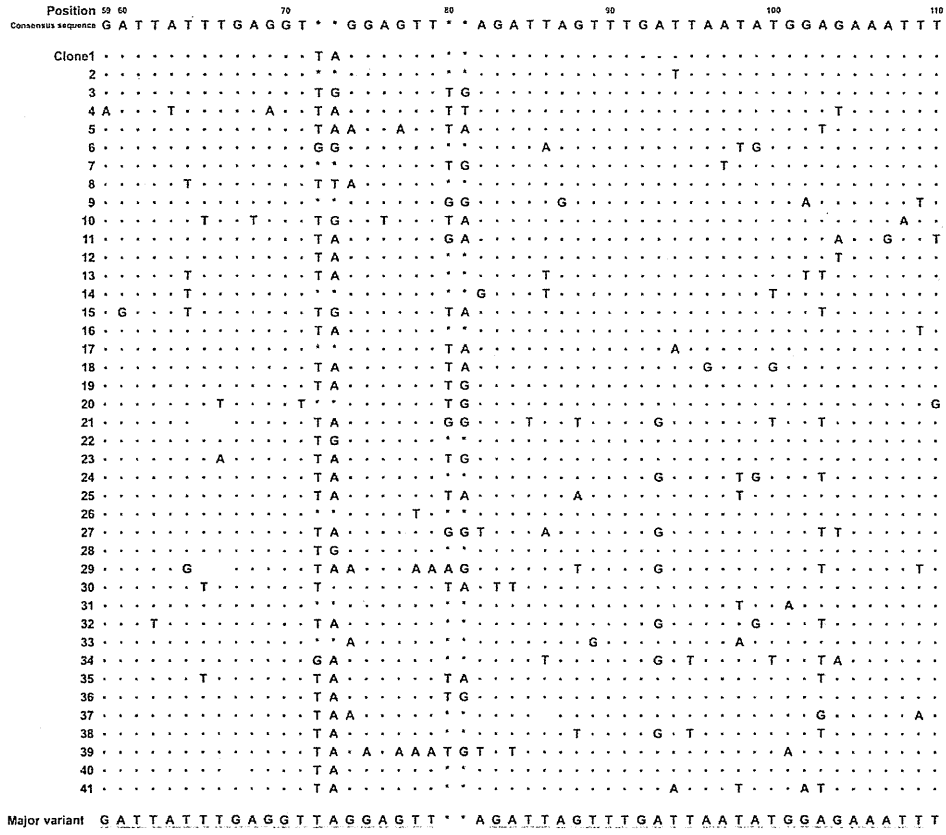

Figure 1. The most frequent Alu sequences after bisulfite conversion and location of CpG sites analyzed by pyrosequencing. The sequence obtained by virtual bisulfite conversion of a consensus sequence in the database is shown at the top. The 41 sequences are obtained by virtual bisulfite treatment of 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.

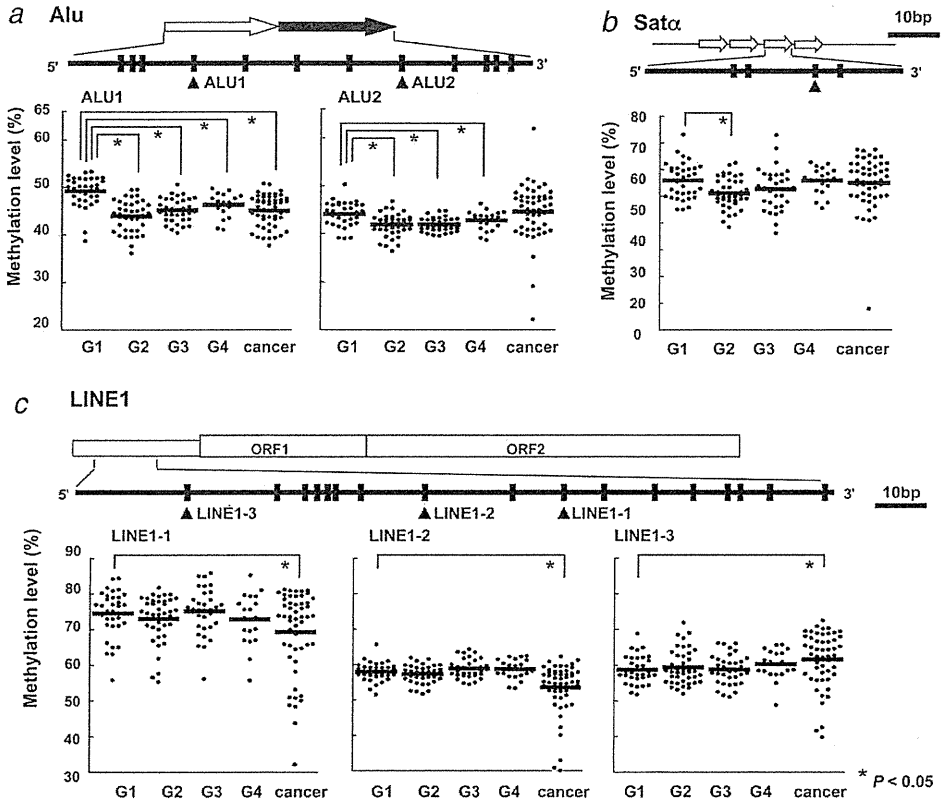

Figure 2. Methylation levels of the three repetitive DNA elements in gastric mucosae of G1-G4 and gastric cancers. Vertical ticks, individual CpG sites; arrowheads, locations of the measured CpG site. A horizontal line in a chart represents a mean methylation level for each group. (*a*) Distribution 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, $p < 0.05$).

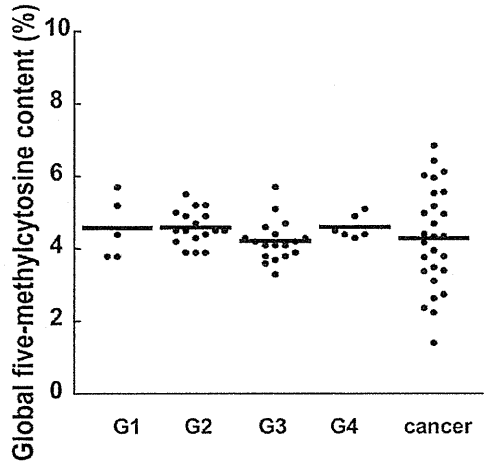

Figure 3. Distribution of global 5-methylcytosine content in G1-G4 and cancers. A horizontal line represents the mean global 5-methylcytosine content for each group. The global 5-methylcytosine content was at a similar level among G1, G2, G3 and G4. In cancers, the global 5-methylcytosine content was 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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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.

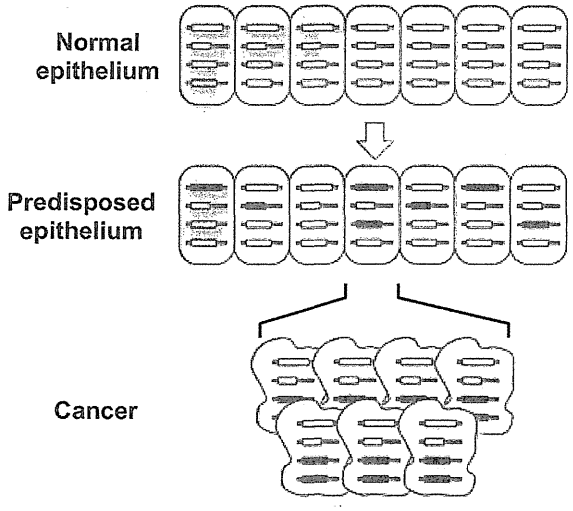

Fig. 1. Epigenetic field for cancerization and clonal selection in cancer. Normal epithelium consists of cells with little aberrant methylation. By exposure to inducers of methylation, specific genes are methylated in minor fractions of cells. A cancer develops from one of the cells that has already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

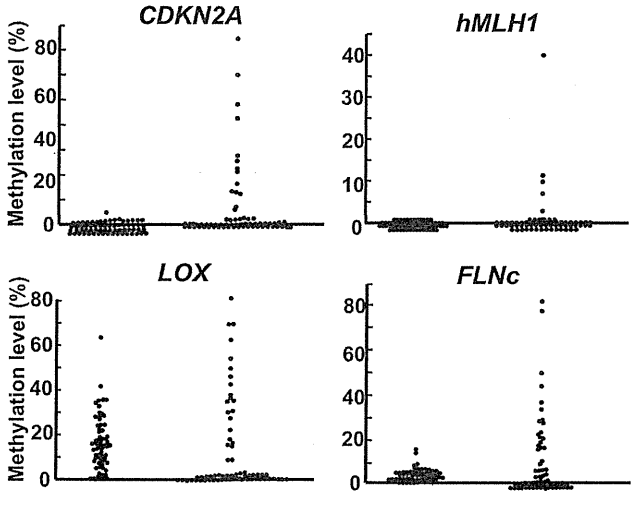

Fig. 2. Distribution patterns of methylation in non-cancerous and cancer tissues. Methylation levels, which reflect fractions of cells with the methylation, were quantified in 66 paired samples of non-cancerous and cancer tissues of gastric cancer patients (modified from Enomoto *et al.*[39]). They showed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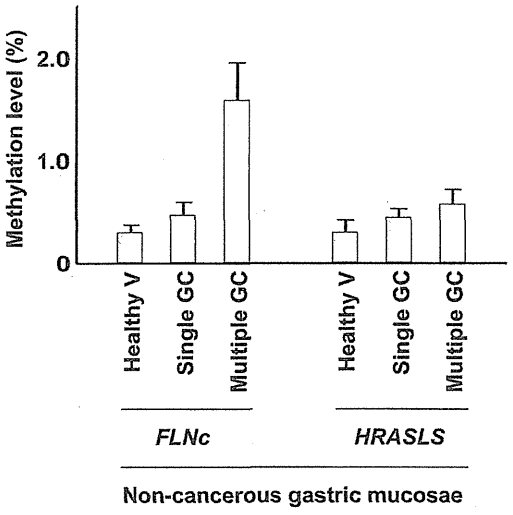

Fig. 3. Correlation between methylation level and cancer risk. Methylation levels of two marker genes (*FLNc* and *HRASLS*) were quantified in gastric mucosae of healthy individuals (healthy V), non-cancerous gastric mucosae of patients with a single gastric cancer (single GC), and non-cancerous gastric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

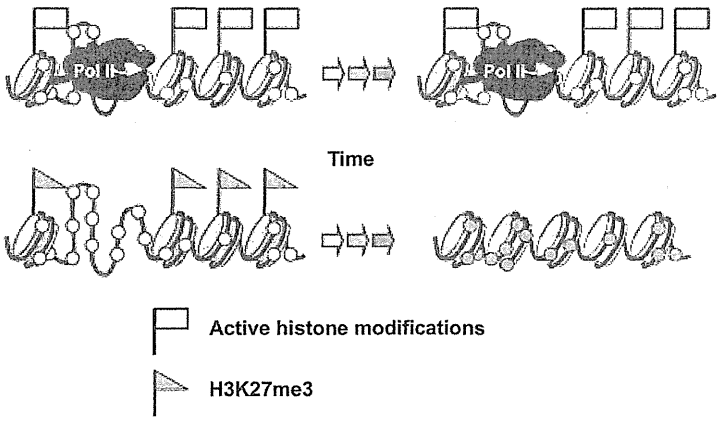

Fig. 4. Determinants of methylation destiny. Genes with RNA polymerase II (pol II), active or stalled, are resistant to DNA methylation, and genes with H3K27me3 are susceptible to DNA methylation. The presence of pol II is associated with the presence of active histone modifications, even if a gene is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

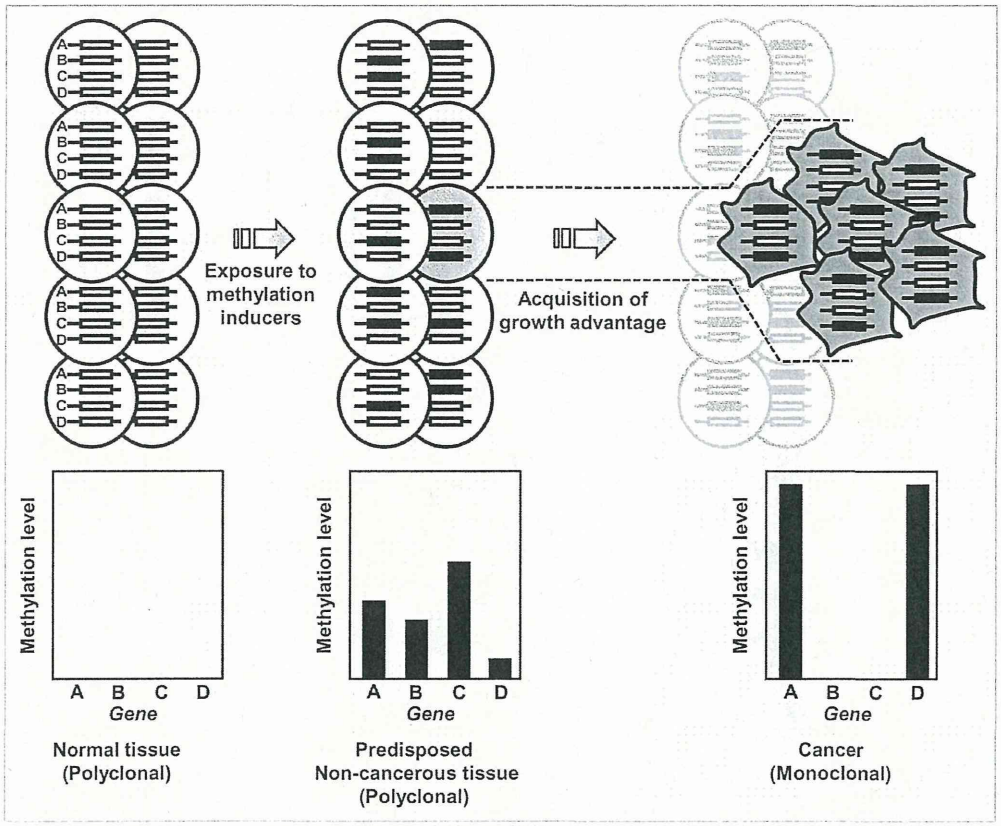

Figure 1. Different meaning of aberrant DNA methylation in non-cancerous and cancer tissues. Cells in an entirely normal tissue contain no aberrant methylation, and, by exposure to methylation inducers, cells come to harbor aberrant methylation of specific genes. A cancer, consisting of many cancer cells,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

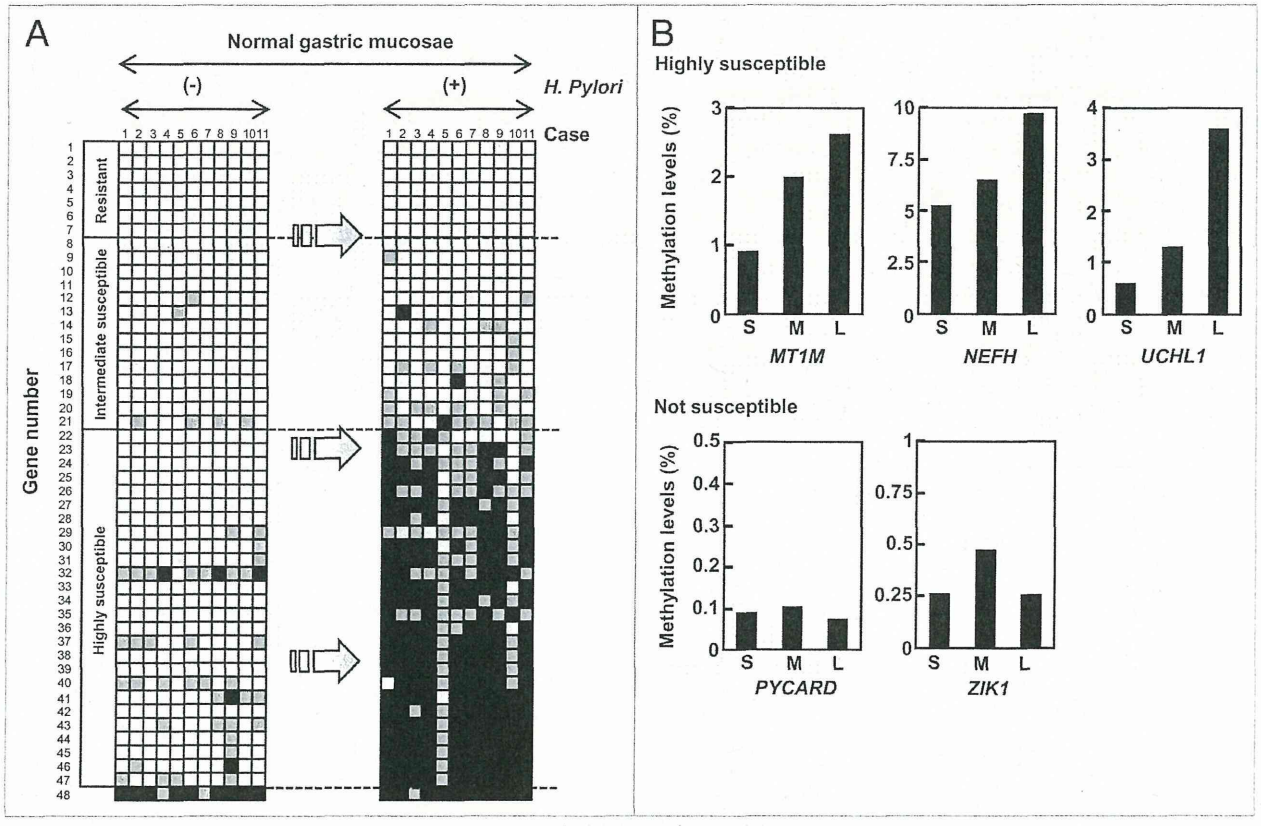

Figure 2. Target gene specificity in DNA methylation induction in non-cancerous tissues. (A) Methylation profile of the 48 genes in normal gastric mucosae with and without *H. pylori* infection (modified from Nakajima et al.[9]). The methylation status of each gene is represented as unmethylated (white),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