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Review

Epigenetic Field for Cancerization

Toshikazu Ushijima
Carcinogenesis Division, National Cancer Center Research, Institute 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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Epigenetic alterations, represented by aberrant DNA methylation, are deeply involved in human cancers. In gastric cancers, tumor-suppressor genes are inactivated more frequently by promoter methylation than by mutations. We recently showed that *H. pylori* infection, a potent gastric carcinogenic factor, induces methylation of specific genes in the gastric mucosae. When the methylation levels were analyzed in the gastric mucosae of healthy volunteers, cases with a single gastric cancer, and cases with multiple gastric cancers, who have increasing levels of risks for gastric cancers, there was a significant increasing trend in the methylation levels among the individuals without current *H. pylori* infection. This finding unequivocally showed the presence of an epigenetic field for cancerization. The degree of the field defect was measured more conveniently using methylation levels of marker genes than using those of tumor-suppressor genes. The presence of an epigenetic field for cancerization has been indicated for liver, colon, Barrett's esophageal, lung, breast, and renal cancers. Since decreased transcription is involved in the specificity of methylated genes, it is likely that specific genes are methylated according to carcinogenic factors. These findings emphasize the usefulness of DNA methylation as a marker for past exposure to carcinogens and future risk of cancer development.

Keywords: Cancer, DNA methylation, Epigenetics, Field cancerization, Field defect

Introduction

An innovation brings up a new challenge. Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) techniques were recently introduced into clinical practice to treat early gastric cancers saving a large area of the stomach (Gotoda *et al.*, 2006). Now, a high incidence of second primary gastric cancers in the remaining stomach, reaching as high as 2.0% per year, is recognized (Nakajima *et al.*, 2006a). The incidence is extremely high compared with the incidence (0.14% per year) in the general Japanese population (Lee *et al.*, 2006). This contrast clearly shows that at least some gastric cancer cases have gastric mucosae that do not have any tumors but are already predisposed to developing gastric cancers.

The presence of mucosae that are predisposed to cancer development was initially described for oral cancers by Slaughter *et al.*, using the term "field cancerization" (Slaughter *et al.*, 1953). Although the predisposed mucosae can display some histological changes, such as atrophic gastritis and intestinal metaplasia in the stomach, they are essentially made of epithelial cells of polyclonal origins and have few monoclonal lesions. Nevertheless, the predisposed mucosae develop multiple cancers, and this phenomenon was denoted as "field cancerization" or the presence of "field defect" (Braakhuis *et al.*, 2003). Field cancerization has been described for various organs, including the stomach (Nakajima *et al.*, 2006a; Nakajima *et al.*, 2006b), oral cavity (Slaughter *et al.*, 1953; Partridge *et al.*, 2000), the upper aerodigestive tract of smokers (Copper *et al.*, 1993; Sozzi *et al.*, 1995; Wistuba *et al.*, 1997), the esophagus with Barrett change (Eads *et al.*, 2000) or of heavy drinkers or smokers (Miyazaki *et al.*, 2002), and the bladder (Hafner *et al.*, 2002).

Most of the field cancerization has been explained by the presence of cells with genetic alterations (Sozzi *et al.*, 1995; Wistuba *et al.*, 1997; Partridge *et al.*, 2000; Hafner *et al.*, 2002; Braakhuis *et al.*, 2003). However, involvement of epigenetic alterations in field cancerization is shown by our findings in the stomach (Maekita *et al.*, 2006; Nakajima *et al.*, 2006b), in addition to the reports in the liver (Kondo *et al.*, 2000), colon (Hsieh *et al.*, 1998; Issa *et al.*, 2001; Shen *et al.*, 2005), Barrett's esophagus (Eads *et al.*, 2000), lungs (Guo *et al.*, 2004), breasts (Yan *et al.*, 2006), and kidneys (Arai *et al.*, 2006).

In this review, after making a brief introduction to cancer epigenetics, I will focus on an epigenetic field defect for gastric cancers. Its presence has been documented by

*To whom correspondence should be addressed.
Tel: +81 3 3542 2511 ext. 4520; Fax: +81 3 5565 1753
E-mail: tushijim@ncc.go.jp

quantitative analysis of samples with established defect and those without, and its inducer is also evident. Then, I will describe the nature of field defects, including those for other cancers. Finally, I will discuss clinical applications of field defects.

Epigenetics and epigenetic alterations in cancers

Epigenetic information is defined as information other than the DNA sequence that is faithfully replicated upon somatic cell replication. It is carried by DNA methylation at CpG sites, histone modifications, and polycomb complex formation (Baylin and Ohm, 2006). Especially, DNA methylation is known to be replicated with a high fidelity in mammalian cells (Ushijima *et al.*, 2003; Riggs and Xiong, 2004; Laird *et al.*, 2004), and serves as a long-term memory of cells (Li, 2002). DNA methylation in promoter CpG islands very consistently represses transcription of their downstream genes (Fig. 1) (Ushijima, 2005; Baylin and Ohm, 2006), mainly by inducing changes in histone modifications, such as deacetylation of histones and methylation of lysine 9 of histone H3 (Richards and Elgin, 2002). Methylation in gene bodies does not block transcription, and is sometimes associated with active transcription (Miyamoto *et al.*, 2003; Baylin and Ohm, 2006). Even when methylation of a gene body is associated with decreased transcription, such association has many exceptions, and does not have a causal role in gene silencing (Ushijima, 2005).

In cancer cells, "genome-overall hypomethylation and regional hypermethylation" are present. The "genome-overall" hypomethylation is almost always observed in cancers, and is mainly due to hypomethylation of repetitive sequences, which comprise more than 40% of the human genome and are normally heavily methylated (Kaneda *et al.*, 2004a). The hypomethylation can lead to genomic instability and is considered to be involved in tumor progression (Eden *et al.*, 2003). Genome-overall hypomethylation can also involve normally methylated CpG islands, which can induce aberrant transcription of their downstream genes, such as melanoma antigen genes (MAGEs) (de Smet *et al.*, 1999).

Regional hypermethylation has been extensively analyzed in various cancers because methylation of promoter CpG islands of various tumor-suppressor genes can cause their inactivation (Baylin and Ohm, 2006; Ushijima, 2005). At the same time, methylation of CpG islands outside promoter regions is also present in cancers, and it is still unclear whether or not such methylation has any biological consequences. For example, in gastric cancers, *CDKN2A* (*p16*), *CDH1* (*E-cadherin*), *hMLH1*, and *RUNX3* can be inactivated by promoter methylation (Ushijima and Sasako, 2004; Li *et al.*, 2002). In colorectal cancers, *CDKN2A*, *hMLH1*, *HIC1*, *SFRP1*, and many other genes can be inactivated (Baylin and Ohm, 2006). Notably, methylation of some tumor-suppressor gene, such as *SFRP1*, whose inactivation enhances Wnt signaling, was observed in very early lesions of colon carcinogenesis, aberrant crypt foci.

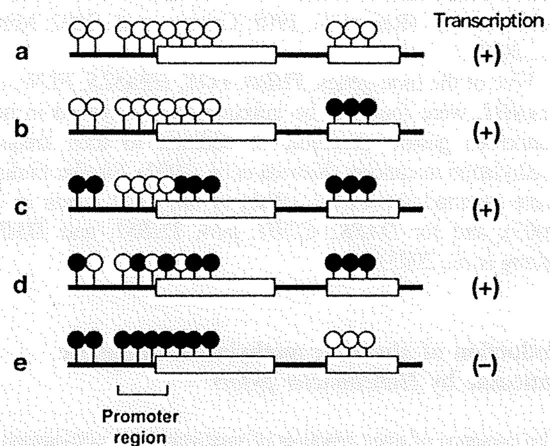

Fig. 1. Methylation of a gene region and its effect on gene transcription. Open circles, unmethylated CpG sites; and closed circles, methylated CpG sites. Methylation of exons (b, c) does not block gene transcription. Mosaic methylation of promoter CpG island also does not block transcription (d). However, dense methylation of promoter CpG islands completely blocks transcription, and is often associated with hypomethylation of downstream regions (e).

In the early 1990's, methylation of promoter CpG islands of tumor suppressor genes was discovered (Ohtani-Fujita *et al.*, 1993; Baylin and Ohm, 2006). Since only a limited number of genes other than tumor-suppressor genes were analyzed, many investigators felt that most genes methylated in cancers were tumor-suppressor genes. However, as more genes were found to be silenced in various cancers by use of genome-wide screening techniques, it is now recognized that promoter CpG islands of many genes are methylated in cancers and only a fraction of them are tumor-suppressor genes (Ushijima, 2005). An extreme example of a gastric cancer cell line has as many as 421 silenced genes, and most of them cannot be tumor-suppressor genes (Yamashita *et al.*, 2006).

The presence of aberrant DNA methylation in non-cancerous gastric mucosae

In gastric cancers, inactivation of *CDKN2A*, *CDH1*, *hMLH1*, and *RUNX3* due to their promoter methylation is more frequently observed than their inactivation due to mutations (Ushijima and Sasako, 2004). We applied a genome-wide screening method for differences in DNA methylation, methylation-sensitive-representational difference analysis (MS-RDA) (Ushijima *et al.*, 1997; Kaneda *et al.*, 2003), to gastric cancers, and identified nine silenced genes (Kaneda *et al.*, 2002). One of the nine genes, Lysyl Oxidase (*LOX*), was later shown to possess a tumor-suppressive function in gastric

cancer cells (Kaneda *et al.*, 2004b), as in prostate, colon, and breast cancers (Ren *et al.*, 1998; Csiszar *et al.*, 2002; Min *et al.*, 2007).

Five of the nine genes, *THBD*, *LOX*, *HRASLS*, *FLNc*, and *HAND1*, were found to be infrequently methylated in non-cancerous gastric mucosae, in addition to their frequent methylation in cancers (Kaneda *et al.*, 2002). Similar findings were reported for *CDH1* (Waki *et al.*, 2002; Chan *et al.*, 2003), and for *DAPK*, *CDH1*, *p14*, *THBS1*, and *TIMP-1* (Kang *et al.*, 2003).

Induction of aberrant methylation in gastric mucosae by *Helicobacter pylori*

The presence of trace amounts of methylation in non-cancerous gastric mucosae suggested that some gastric carcinogens could have induced the methylation, and that the degree of methylation could be associated with gastric cancer risk. The most important gastric carcinogenic factor is *Helicobacter pylori* (*H. pylori*) infection, which increases the risk of developing gastric cancers by 2.2- to 21-fold (Uemura *et al.*, 2001; Ekstrom *et al.*, 2001). The presence of *CDH1* methylation was associated with *H. pylori* infection (Chan *et al.*, 2003) while the number of methylated genes was not associated in the other study (Kang *et al.*, 2003). All these studies, including ours, were performed using methylation-specific PCR (MSP), which can potentially overestimate methylation of small amounts of DNA molecules depending upon experimental conditions. The meaning of the methylated DNA molecules in the non-cancerous gastric mucosae could be different, depending upon the quantity of methylated DNA molecules. They could have originated from neoplastic lesions contaminated in "non-cancerous" samples, or from gastric mucosae that constituted the majority of the DNA molecules.

Therefore, we quantified the fraction of methylated DNA molecules in the gastric mucosae of healthy volunteers with (n = 98) and without (n = 56) current *H. pylori* infection by the quantitative methylation-specific PCR (quantitative MSP) method (Maekita *et al.*, 2006). We also analyzed gastric mucosae of gastric cancer cases with (n = 43) and without (n = 29) *H. pylori* infection. The fraction of methylated DNA molecules was considered to reflect the fraction of cells with methylation of individual genes. Since inactivation of tumor-suppressor genes could lead to formation of neoplastic lesions, both tumor-suppressor genes (*CDKN2A* and *LOX*) and genes without evident tumor-suppressor function (*THBD*, *HRASLS*, *FLNc*, and *HAND1*) were analyzed. We also analyzed CpG islands outside promoter regions (exon 1 of *CDKN2A* and exon 8 of *p41ARC*) that were known to be susceptible to DNA methylation (Ushijima *et al.*, 2003; Ushijima, 2005).

It was unequivocally shown that *H. pylori* infection potently induced aberrant methylation in gastric mucosae because methylation levels in *H. pylori*-positive healthy volunteers were 5.4- to 303-fold higher than those in *H. pylori*-negative healthy volunteers (Fig. 2) (Maekita *et al.*, 2006). Although

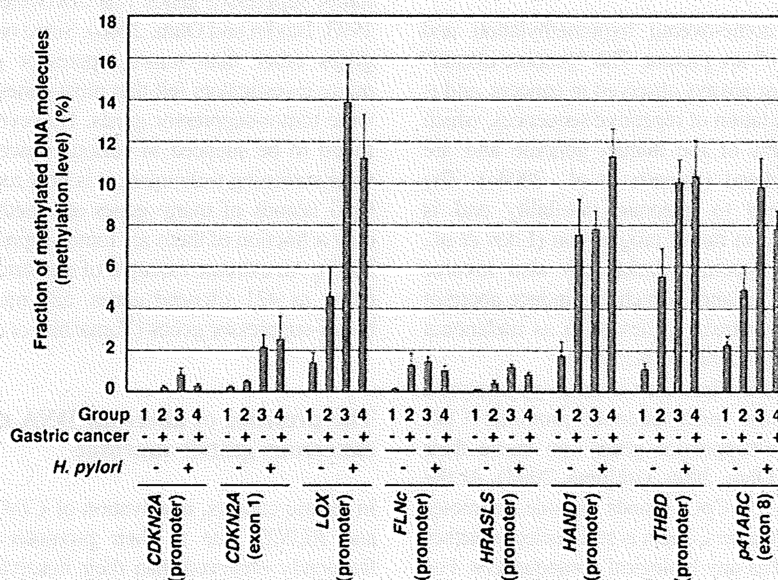

Fig. 2. Methylation levels in the non-cancerous gastric mucosae of healthy volunteers (gastric cancer: -) and gastric cancer cases (+) with and without *H. pylori* infection. Methylation levels were measured for eight regions of seven genes using DNA obtained from antral non-cancerous gastric mucosae. No or low methylation was observed in *H. pylori*-negative healthy volunteers (group 1), and high methylation levels were present in *H. pylori*-positive healthy volunteers (group 3) and cancer cases (group 4). In *H. pylori*-negative cancer cases (group 2), most of whom were considered to have past *H. pylori* infection, methylation levels were lower than individuals with current *H. pylori* infection. Error bars: standard errors. Adopted from Ushijima *et al.*, 2006.

the absolute levels of methylation were also different depending upon a gene region, the same tendency was observed for all the eight regions analyzed. It was also noted that methylation levels of some genes, such as *LOX*, *THBD*, and *HAND1*, reached as high as 20-40% in healthy volunteers with *H. pylori* infection. These high fractions of cells with methylation in many healthy volunteers could never be due to the presence of neoplastic lesions in their gastric mucosae. Rather, it was shown that methylation of preferential genes can be induced in a significant fraction of gastric epithelial cells in a specific condition, such as in the presence of *H. pylori* infection.

Association between methylation levels in gastric mucosae and gastric cancer risks

Next, we compared methylation levels in the gastric mucosae of healthy volunteers and those in the non-cancerous gastric mucosae of cases with differentiated-type gastric cancers (Fig. 2). Since cases with gastric cancers are known to have higher risks of developing second primary gastric cancers (Nakajima *et al.*, 2006a), the gastric mucosae of the cancer cases were considered to have higher risks of developing gastric cancers. Among the *H. pylori*-negative individuals, the cancer cases had 2.2- to 32-fold higher methylation levels than the healthy volunteers (Maekita *et al.*, 2006). When methylation levels were analyzed in healthy volunteers, cases with a single gastric cancer, and cases with multiple gastric cancers, there was a significant increasing trend in the methylation levels (Nakajima *et al.*, 2006b). These two studies demonstrated that the methylation levels in the gastric mucosae correlated with the risks of developing gastric cancers among individuals without current *H. pylori* infection. In contrast, among the *H. pylori*-positive individuals, methylation levels were almost the same in the cancer cases and healthy volunteers, and higher than or equal to those in the gastric mucosae of *H. pylori*-negative cancer cases.

All or the vast majority of gastric cancer cases are known to be associated with *H. pylori* infection (Uemura *et al.*, 2001; Ekstrom *et al.*, 2001). This indicates that the cancer cases without *H. pylori* infection at the time of analysis had past *H. pylori* infection. This was also supported by the presence of gastric atrophy in most of the *H. pylori*-negative cancer cases. Therefore, it was considered that the methylation levels in the gastric mucosae are zero or very low without *H. pylori* infection (*H. pylori*-negative healthy volunteers), increase to very high levels with current, or active, *H. pylori* infection (*H. pylori*-positive healthy volunteers and cancer cases), and decrease to certain levels after eradication or extinction of *H. pylori* infection (*H. pylori*-negative cancer cases) (Fig. 3). This "up-and-down" course was also supported by a recent study showing that CDH1 methylation can be reversed by *H. pylori* eradication by MSP (Chan *et al.*, 2006).

As for the mechanism for the decrease, cell turnover was considered to be the major mechanism since DNA demethylase

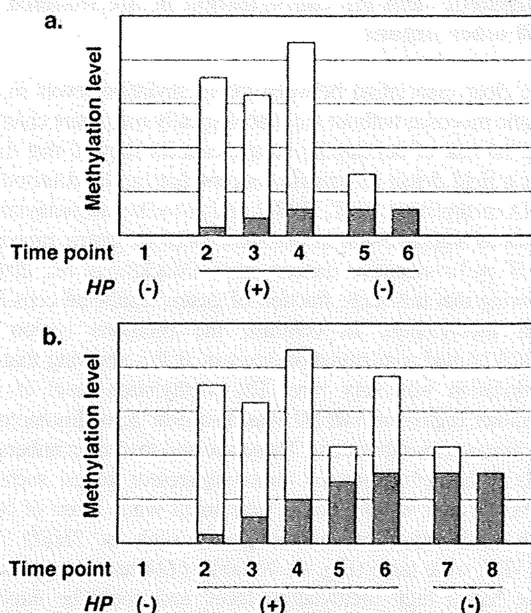

Fig. 3. A time course model of methylation levels in gastric mucosae with *H. pylori* infection. It was shown that individuals who have never had *H. pylori* infection have almost no methylation (point #1). During *H. pylori* infection, both temporary (white column) and permanent (gray column) components of methylation are induced. Although the temporary component fluctuates during the infection (points #2-4 in panel a, and #2-6 in b), the permanent component gradually increases. When *H. pylori* infection discontinues, the temporary component will disappear, leaving only the permanent component (points #5-6 in panel a, and #7-8 in b). The remaining permanent components (point #6 in panel a, and #8 in b) correlate with the risk of developing gastric cancers.

has not been established. The cell turnover was likely to be occurring within epithelial cells. Peripheral lymphocytes of *H. pylori*-positive individuals did not have methylation (Nakajima *et al.*, 2006b), and gastric epithelial cells isolated from Mongolian gerbils infected with *H. pylori* by the gland isolation technique had methylation of specific regions (Niwa *et al.*, unpublished results). We currently hypothesize two types of methylation, one being temporary methylation induced in progenitor or differentiated cells and the other being permanent methylation induced in stem cells (Fig. 3) (Ushijima *et al.*, 2006). The former disappears as new cells are supplied from unmethylated stem cells while the latter does not. By assuming that *H. pylori* infection induces both the temporary and permanent methylation, the decrease in methylation levels after discontinued *H. pylori* infection can be explained. In *H. pylori*-negative individuals, only the permanent methylation remains, and their methylation levels are expected to be proportional to the fraction of stem cells with methylation, and thus to gastric cancer risks.

Epigenetic field for cancerization in the stomach and other organs

The clear association between the methylation levels in the gastric mucosae without any histologically malignant changes and the risk of developing gastric cancers showed that there was a field defect for gastric cancers that can be detected by DNA methylation. *LOX*, *THBD*, and *HAND1* had methylation levels as high as 5-8% in the non-cancerous gastric mucosae of *H. pylori*-negative cancer cases (Maekita *et al*., 2006), showing that this large fraction of gastric epithelial cells had their methylation. In contrast, the promoter region of *CDKN2A* had a methylation level of 0.2%, showing that its methylation was very rare. The methylation level of the promoter region of *hMLH1* was also near zero (Enomoto *et al*., manuscript submitted). These showed that the numbers of cells with methylation of tumor-suppressor genes, such as *CDKN2A* and *hMLH1*, were very small while those of cells with methylation of marker genes, such as *THBD* and *HAND1*, were large (Fig. 4). Notably, *LOX* tumor-suppressor gene had a high methylation level, and could be directly involved in the formation of field defect. Since the methylation levels of the tumor-suppressor genes correlate with those of the marker genes, the degree of field defect can be measured using marker genes whose methylation levels can be accurately measured.

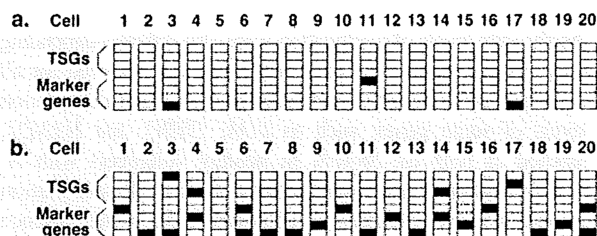

Fig. 4. Methylation of tumor-suppressor genes (TSGs) and marker genes. (a) A tissue without field defect. (b) A tissue with field defect. Open boxes, unmethylated genes; closed boxes, methylated genes. A tissue with field defect has low methylation levels of tumor-suppressor genes, and high levels of marker genes. Although methylation of marker genes is not directly involved in carcinogenesis, their methylation levels correlate with those of tumor-suppressor genes, and thus gastric cancer risks. However, since methylation of a marker gene is not requisite for carcinogenesis, a cancer arising from a mocosa with methylation of a marker gene does not necessarily have methylation of the gene.

The fractions of cells with methylation of tumor-suppressor genes, such as *CDKN2A* and *hMLH1*, were very small, but were considered to be much larger than the fraction of cells with mutations of specific genes. Although information on such a fraction of cells in the gastric mucosae is not available,

Table 1. Studies on epigenetic field for cancerization

Cancer	Inducing factor	Detection method and analysis way	Genes analyzed	Non-predisposed samples/ methylation in defect vs non-predisposed	Author
Liver cancer	HBV and HCV	COBRA/Incidence	*CDKN2A*, *hMLH1*, *THBS-1*, and five MINT loci	8 normal livers 4/5 vs 0/8	Kondo, 2000
Colorectal cancer (UC-associated)	UC	MSP/Incidence	*CDKN2A*	Not available	Hsieh, 1998
	UC	COBRA/ Quantitative	*ER*, *MYOD*, *CKDN2A*, and *CSPG2*	5 non-UC patients not significant	Issa, 2001
Barrett's cancer	Reflux esophagitis?	MethyLight/ Incidence	*APC*, *CDKN2A*, and *ESR1*	Not available	Eads, 2000
Lung cancer	Smoking?	MSP/Incidence	*CDKN2A*, *MGMT*, *DAPK*, *SOCS1*, *RASSF1A*, *COX2*, and *RARβ*	Not available	Guo, 2004
Colorectal cancer (sporadic)	Unknown	COBRA/ Quantitative	*MGMT*	33 healthy subjects 8.8% vs 2% 22/44 vs 4/33	Shen, 2005
Gastric cancer	*H. pylori*	qMSP /Quantitative	*CDKN2A*, *LOX*, *THBD*, *HRASLS*, *FLNc*, *HAND1*, and *p41ARC*	98 healthy subjects 2.2-to 32-fold increase in methylation levels	Maekita, 2006
Breast cancer	Unknown	qMSP/Incidence	*CYP26A1*	25 samples from reduction mammoplasty 4/5 vs 0/16	Yan, 2006
Renal cancer	Unknown	MSP/Incidence	*CDKN2A*, *hMLH1*, *THBS-1*, and five MINT loci	9 samples without renal cancers 44/60 vs 1/9 etc.	Arai, 2006

"Methylation in defect vs non-predisposed" describes the incidence (or methylation level) in histologically non-malignant, but predisposed area vs that in non-predisposed area. COBRA, combined bisulfite restriction analysis; qMSP, quantitative MSP; UC, ulcerative colitis; HBV, hepatitis B virus; and HCV, hepatitis C virus.

fractions of cells with mutations of a marker gene were in the range of $10\text{-}10^3/10^6$ in animal models exposed to carcinogens (Nagao *et al.*, 2001). Therefore, it is suggested that the number of epigenetically predisposed cells is much larger than genetically predisposed cells in the gastric mucosae after *H. pylori* infection. It was considered that the chance of suffering the next genetic/epigenetic alterations is much higher in epigenetically predisposed cells, and that the degree of field defect can be measured using DNA methylation as a marker.

Looking at cancers of other organs (Table 1), the presence of an epigenetic field for cancerization (field defect) was first suggested by the increased incidence of aberrant methylation in the non-cancerous liver tissues of cases with hepatocellular carcinomas (Kondo *et al.*, 2000). Similar findings were obtained in the colonic mucosae of cases with colorectal cancers developed from ulcerative colitis (Issa *et al.*, 2001), in Barrett's esophagus (Eads *et al.*, 2000), and in the bronchial epithelium of lung cancer cases (Guo *et al.*, 2004). It is critically important to compare predisposed and non-predisposed mucosae for demonstration of the field defect. Therefore, Shen *et al.* quantified MGMT methylation levels in the colonic mucosae of colorectal cancer cases and healthy individuals, and unequivocally showed the presence of epigenetic field defect (Shen *et al.*, 2005). Our studies adopted a concept of marker genes and utilized an accurate method of quantitative MSP. Most notably, these demonstrated the presence of an inducer of the field defect, *H. pylori* (Maekita *et al.*, 2006; Nakajima *et al.*, 2006b). Recently, the presence of epigenetic field defect was indicated in breast cancers (Yan *et al.*, 2006) and renal cancers (Arai *et al.*, 2006). These multiple studies on epigenetic field defect underscore its reality and importance.

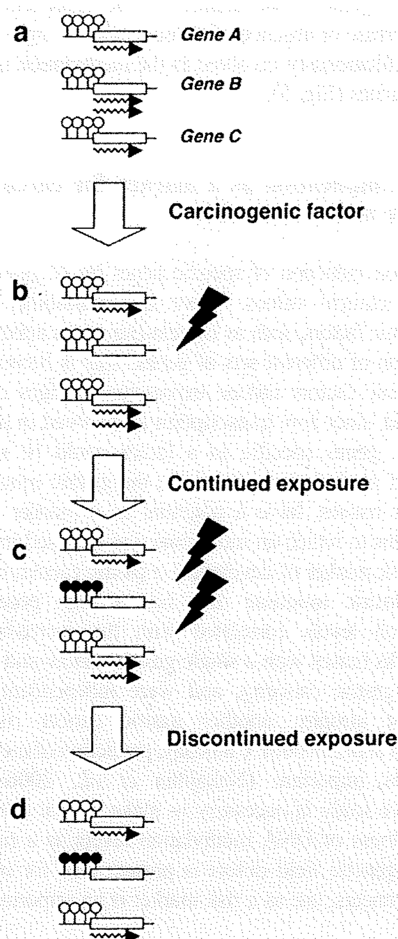

Fig. 5. A model for methylation induction in CpG islands of preferential genes. (a) Physiological transcription statuses (shown by wavy arrows) of three hypothetical genes (genes A, B, and C). (b) When a carcinogenic factor with epigenetic mechanisms is present, changes in the transcription levels (decreased gene B and increased gene C transcriptions) and abnormalities in epigenetic regulatory machineries (shown by lightning) are induced. (c) When the exposure continues, methylation of a gene (gene B) with no or low transcription can be induced. (d) The methylation alteration continues even after carcinogen exposure discontinued.

Inducing factors of methylation and target specificity

Inducing factors, except for gastric cancers, of methylation are still unclear. Although aging is well-known as an inducing factor of methylation (Issa *et al.*, 1994), field defect due to aging is unknown. Rather, methylation induction by ulcerative colitis (Hsieh *et al.*, 1998; Issa *et al.*, 2001) and chronic hepatitis (Kondo *et al.*, 2000) is likely to be involved in the formation of field defect. Ulcerative colitis, chronic hepatitis, and *H. pylori* infection all involve chronic inflammation, and at least some types of inflammation seem to lead to abnormalities in the epigenetic regulation. Actually, a proinflammatory allele of interleukin 1β, is associated with an increased risk of gastric cancers, especially when *H. pylori* infection is present (El-Omar *et al.*, 2000; Lee *et al.*, 2004).

Methylation of preferential genes was induced by *H. pylori* infection (Maekita *et al.*, 2006). Our study using 48 genes (Yamashita *et al.*, 2006) showed that some of these genes were susceptible to methylation induction by *H. pylori* while others were resistant (manuscript in preparation). We believe that a decrease or absence of transcription is deeply involved in the specificity of methylated genes. First, methylation analysis of exogenous or endogenous genes with and without transcription showed that low transcription is a trigger of methylation (Song *et al.*, 2002; de Smet *et al.*, 2004). Second, our extensive analysis on genes methylated in various types of cancers showed that most genes methylated in cancers are those untranscribed in normal counterpart cells (Furuta *et al.*, 2006; Ushijima, 2005). At the same time, it is also true that genes with similar low transcription levels are not affected equally, and there should be some additional mechanisms for the specificity.

Taken together, abnormalities in the epigenetic regulation and a decrease or absence of transcription of target genes seem to be simultaneously involved in the methylation induction of specific genes (Fig. 5).

Clinical implication as a marker for carcinogen exposure and cancer risk

Methylation induction of specific genes by *H. pylori* infection also has clinical values. There is a possibility that other carcinogenic factors, such as Epstein-Barr virus infection, induce methylation of different sets of genes. This is because different carcinogenic factors induce expression changes of different genes, and, since low transcription is involved in methylation induction, genes specific to a factor could be methylated. Therefore, methylation patterns in the gastric mucosae, or in any other tissues, have a potential as a marker to identify carcinogens to which an individual was exposed in the past.

As a risk marker of developing a gastric cancer, methylation in the gastric mucosae also has a high potential. The methylation levels correlated with the increasing gastric cancer risks (cases with a single gastric cancer and those with multiple gastric cancers), and were independent from the degree of atrophy, another gastric cancer risk marker (Nakajima *et al.*, 2006b). Clinically, prediction of metachronous cancers is important (Nakajima *et al.*, 2006a), and a prospective study is necessary to make a final evaluation on the usefulness of DNA methylation levels as a risk marker. Since epigenetic field defect is present also for many other types of cancers, use as a risk marker is an important field.

Epilogue

The presence of an epigenetic field for cancerization is now evident for gastric cancers, and such a field is likely to be present also for liver, colon, Barrett's esophageal, lung, breast, and renal cancers. Aberrant DNA methylation is now shown to be involved not only in cancers but also in disorders with polyclonal origins (Mihara *et al.*, 2006; Robertson, 2005). Epigenetic therapy is now actively being developed (Yoo and Jones, 2006), and its application to field defects has a potential as a preventive method for cancers and possibly other disorders. Research in this field has a strong potential to reveal new diagnostic markers and possibly therapeutic targets.

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Lack of association between CpG island methylator phenotype in human gastric cancers and methylation in their background non-cancerous gastric mucosae

Shotaro Enomoto,[1,2] Takao Maekita,[1,2] Tetsuya Tsukamoto,[3] Takeshi Nakajima,[1] Kazuyuki Nakazawa,[2] Masae Tatematsu,[3] Masao Ichinose[2] and Toshikazu Ushijima[1,3]

[1]Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [2]Second Department of Internal Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-0012; [3]Division of Oncological Pathology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa, Nagoya 464-8681, Japan

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The presence of high levels of aberrant DNA methylation in gastric mucosae correlates with risk of gastric cancer. Some gastric cancers are known to have methylation of multiple CpG islands (CGI), which is referred to as the CGI methylator phenotype (CIMP). In the present study, we aimed to clarify the possible association between the CIMP in cancers and high methylation levels in their background mucosae by accurate quantitative methylation analysis of 14 carefully selected promoter CGI. Methylation levels were measured in 66 cancers and their background mucosae, along with 19 normal mucosae of healthy volunteers. Methylation in cancers was classified as absent (methylation level = 0%) or positive. The number of methylated CGI in a cancer showed a continuous distribution, and cancers were classified as CIMP high (21 cases), CIMP low (30 cases), or CIMP negative (15 cases). CIMP-high gastric cancer patients had significantly better survival rates than CIMP-negative patients. Of the Epstein–Barr virus-positive gastric cancers studied, eight out of nine presented as CIMP high. Methylation in background mucosae showed a unimodal distribution, and was assessed by their degree. The gastric mucosae of cancer patients showed higher levels than normal gastric mucosae of healthy volunteers. Finally, the CIMP-high, CIMP-low, and CIMP-negative statuses in cancers were not associated with methylation levels of individual genes and their means in the background mucosae. These showed that the CIMP statuses in gastric cancers had no association with methylation levels in the background gastric mucosae. (*Cancer Sci* 2007; 98: 1853–1861)

Gastric cancer is one of the major causes of cancer death in Asia and some European countries.[1] Regarding the molecular mechanisms of gastric cancers, inactivation of *p53*, *CDH1*, *CDKN2A*, and *hMLH1* is well known, and the latter three genes are inactivated more frequently by aberrant DNA methylation of their promoter CpG islands (CGI) than by mutations.[2] As an etiological factor for gastric cancers, *Helicobacter pylori* infection is known to elevate gastric cancer risk 2.2–21-fold.[3–5] We have recently shown that *H. pylori* infection potently induces aberrant DNA methylation in gastric mucosae,[6] and that DNA methylation levels in gastric mucosae correlate with risk of gastric cancer in individuals without current *H. pylori* infection.[6,7] These findings explain why aberrant DNA methylation is frequently associated with gastric cancers.

Methylation of multiple CGI in a cancer was first observed in colorectal cancers.[8] The number of methylated CGI showed a bimodal distribution,[8,9] and the phenotype was designated as the CGI methylator phenotype (CIMP). A recent study using accurate and non-biased quantitative methylation analysis showed that a group of colorectal cancers with CIMP was associated with *BRAF* mutations.[10] Analysis of methylation levels in the matched non-cancerous background colonic mucosa showed that high methylation levels of specific genes were associated with the presence of CIMP in colorectal cancers.[11] In contrast and surprisingly, although colonic mucosae of patients with ulcerative colitis show accumulation of aberrant methylation,[12,13] CIMP-positive cancers were less frequent in ulcerative colitis-associated colorectal cancers than in sporadic colorectal cancers.[14]

Unlike colorectal cancers, the number of methylated CGI does not show a bimodal distribution in gastric cancers,[15–19] and these cancers have been classified as CIMP high, CIMP low, and CIMP negative for convenience. However, some studies observed that CIMP-high groups were associated with better prognosis,[17,19,20] or with infection with Epstein–Barr virus (EBV).[19–22] These results indicated that CIMP-positive gastric cancers might consist of several different entities. Although gastric cancers arising from gastric mucosae with high methylation levels are likely to have methylation of multiple CGI, the association has not been demonstrated.

Technical limitations may help to explain the ambiguity of CIMP status in gastric cancers. CIMP in gastric cancers has been analyzed only by qualitative methods, such as conventional methylation-specific polymerase chain reaction (PCR), or by combined bisulfite-restriction analysis, which is limited in the number and location of CpG sites that can be analyzed. In addition, a methylation profile of a cancer is dependent on the CGI used for the analysis.[8,16,23] It is known that CGI in different locations relative to a gene show different susceptibility to DNA methylation,[24] therefore CGI with a uniform location relative to a gene should be used. To avoid selection bias of cells with methylation of a CGI, CGI whose methylation does not confer positive or negative selection should be used.

The aim of the present study was to clarify the presence of CIMP in human gastric cancers by an accurate quantitative methylation analysis of selected CGI, and to analyze the effect of methylation in the background non-cancerous gastric mucosae on the CIMP in cancers. We analyzed promoter CGI of one putative tumor-suppressor gene (*LOX*),[25] and 11 genes that can be methylated not only in gastric cancers but also in non-cancerous gastric mucosae, and are unlikely to cause selection bias.[26] We also analyzed promoter CGI of two tumor-suppressor genes (*CDKN2A* and *hMLH1*).

Materials and Methods

Patients and tissue samples. Sixty-six gastric cancer tissue specimens and background non-cancerous gastric mucosae were

[3]To whom correspondence should be addressed. E-mail: tushijim@ncc.go.jp

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Table 1. Primers for real-time methylation-specific polymerase chain reaction

Gene	Methylation status	Primer sequences		Length (bp)	Annealing temperature (°C)
		Forward (5′→3′)	Reverse (5′→3′)		
CDKN2A	M	TTGGTAGTTAGGAAGGTTGTATCGC	TCCCTACTCCCAACCGCG	126	66
	U	GGTAGTTAGGAAGGTTGTATTGT	TCCCTACTCCCAACCACA	124	60
hMLH1	M	CGTTAAGTATTTTTTTCGTTTTGC	TCCGCTCTTCCTATTAATTCG	136	59
	U	AGTGTTAAGTATTTTTTTTGTTTTGT	CTATCCACTCTTCCTATTAATTCA	141	56
LOX	M	ATAAATAGTTGAGGGGCGGTC	CGACAATCCCGAAAAACG	120	61
	U	ATAAATAGTTGAGGGGTGGTT	ACAACAATCCCAAAAAACA	121	58
FLNc	M	GAGAGAGAGTTAGAGAGCGGTCGAGC	GACCACGAAACTCGCTACGCTACG	121	70
	U	GAGAGAGAGTTAGAGAGTGGTTGAGT	AACCACAAAACTCACTACACTACA	121	63
HRASLS	M	GTGTATTTATGATGGGTGTATTC	ACCAAACGCTATACTAAACG	88	59
	U	GTATTTATGATGGGTGTATTT	CACCAAACACTATACTAAACA	87	57
HAND1	M	ATAGTTTAGGGCGTTGGTC	CTACTCTACGAACTTAAAAAAACG	100	57
	U	AATAGTTTAGGGTGTTGGTT	CTACTCTACAAACTTAAAAAAACA	101	55
THBD	M	CGTTCGTTTTTATTCGGCGTC	GCCAAACCCCATCTCATCG	118	60
	U	ATGTGTTTGTTTTTATTTGGTGTT	CAAACCCCATCTCATCAAA	119	56
F2R	M	TTAGGAGGGTCGAGACGGTCGC	TCCTCTAAACACCGTTAATTCG	113	61
	U	TTTTAGGAGGGTTGAGATGGTTGT	TTCCTCTAAACACCATTAATTCACA	116	61
NT5E	M	AGTCGATAGTCGCGTTAGGGTC	GAACAACTAAAACCGAAACTCG	169	55
	U	TAGTTGATAGTTGTGTTAGGGTT	AACTAAAACCAAAACTCAATACC	166	57
GREM1	M	CGTCGGTATTTAAACGGGAGAC	GAAACTCGACGCGAAATCAACG	121	59
	U	TGTTGGTATTTAAATGGGAGAT	CAAAACTCAACACAAAATCAACA	122	57
ZNF177	M	GTAGGAGTATTTGCGATGTTTC	AAAATAACGAAACGACGAACG	128	63
	U	GTTTTTAAGTTTTTAGGGTGAATTT	AAACAACAAACACCCACTTCCA	97	56
CLDN3	M	AGGTTTTGGAGAGCGCGGTTTC	ACCCTAAACTAAAACCGATACG	86	59
	U†	GGTGGTAGGGGTGGAGTTGT	CCTACCCCAACATTATAAACCACA	125	64
PAX6	M	CGGGATTTATCGGCGGAGTC	AACCTCGCGCCAACCG	104	63
	U	GTAATATTTTGTGTGAGAGTGAGT	TCCTCCTACACCTAAACCAAAACA	115	61
CTSL	M	GATTTTATTTTGCGTCGTTTC	ACGCTACGATTAACTATACCG	163	59
	U	GTTTGATTTTATTTTGTGTTGTTTT	ACTACACTACAATTAACTATACCA	170	59

†Primer set designed on the bottom strand; M, specific to methylated DNA; U, specific to unmethylated DNA.

obtained from 66 patients (48 men and 18 women; average age 61.9 years, ranging from 35 to 81 years) who underwent gastrectomy due to gastric cancers. Normal gastric mucosae were also obtained from 19 *H. pylori*-negative healthy volunteers (5 men and 14 women; average age 59.1 years, ranging from 29 to 91 years) who underwent endoscopy for gastric cancer screening. Informed consent was obtained from all of the patients and healthy volunteers before collection of the samples. Cancers and background non-cancerous mucosae were frozen in liquid nitrogen immediately after biopsy, and stored at –80°C until extraction of genomic DNA. High-molecular weight DNA was extracted using the phenol–chloroform method. All of the cancers were diagnosed histologically according to the Japanese classification of gastric carcinoma,[27] and classified according to the Lauren classification system.[28]

Epstein–Barr virus-positive gastric cancers were determined by the presence of *EBER1* in gastric cancer tissues by *in situ* hybridization using formalin-fixed and paraffin-embedded specimens.[29] The presence of *H. pylori* infection in gastric cancer patients was analyzed by detecting genomic DNA of *H. pylori* in gastric mucosae using the PCR method. The *H. pylori*-specific primers were: forward primer, 5′-AAC CCC CTT TCT TAG TTG CT-3′; and reverse primer, 5′-CAT GGC TGA TTT GCG ATT AC-3′. The presence of *H. pylori* infection in healthy volunteers was analyzed using a serum anti-*H. pylori* IgG antibody test (SBS, Kanagawa, Japan).

Sodium bisulfite modification, quantitative methylation-specific PCR, and bisulfite sequencing. Bisulfite modification was carried out using 500 ng *Bam*HI-digested genomic DNA as described previously,[30] and the modified DNA was suspended in 40 µL TE buffer.

For real-time methylation-specific PCR, an aliquot of 2 µL was amplified by PCR using a primer set specific to methylated or unmethylated sequences. Fully unmethylated DNA was prepared by amplifying human genomic DNA without *H. pylori* infection using the GenomiPhi amplification system (Amersham Biosciences, Uppsala, Sweden),[31] and fully methylated DNA was prepared by methylating genomic DNA with *Sss*I methylase (New England Biolabs, Beverly, MA, USA). Using this control DNA, an annealing temperature specific for a primer set was determined. Real-time PCR was carried out using SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Standard DNA was prepared by cloning PCR products into the pGEM-T Easy vector (Promega, Madison, WI, USA) or by purifying their PCR products using the Wizard SV Gel and PCR clean-up system (Promega). The number of molecules in a sample was determined by comparing its amplification with that of standard DNA that contained known numbers of molecules (10–10^6 molecules). Based on the numbers of methylated molecules and unmethylated molecules for a genomic region in a sample, methylation levels were calculated as the fraction of methylated molecules in the total number of DNA molecules (no. methylated molecules + no. unmethylated molecules). The primer sequences and PCR conditions are shown in Table 1.

For bisulfite sequencing of *LOX*, 1 µL of the sodium bisulfite-treated DNA was amplified using 5′-AAG TTA GTG TGT TTT AGG ATG TGT GT-3′ and 5′-CTT CCC TTT CCC CTT TCT CAA T-3′,[25] which were common to the methylated and unmethylated DNA sequences. PCR products were cloned into a pGEM-T Easy vector, and 10 clones or more were cycle sequenced for each sample.

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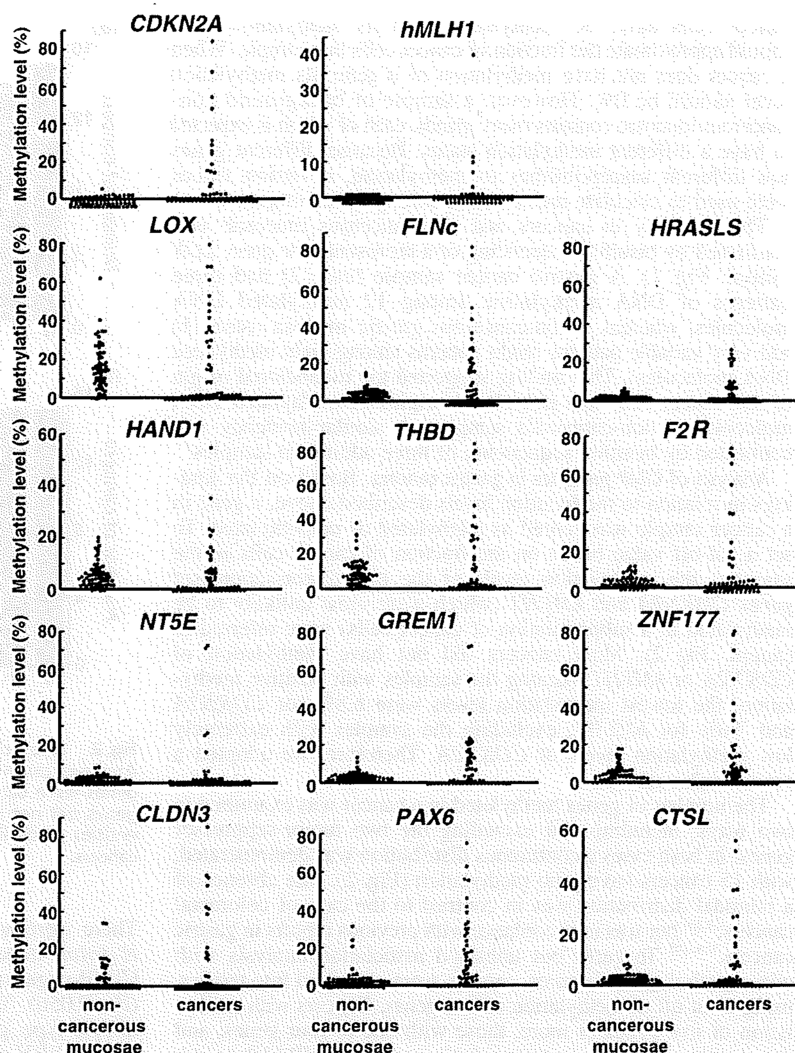

Fig. 1. Methylation levels of the 14 promoter CpG islands (CGI) in 66 gastric cancers and their background non-cancerous gastric mucosae. Methylation levels were analyzed by the quantitative methylation-specific polymerase chain reaction method. In the cancers, the methylation levels were classified as absent (methylation level = 0%) or positive. In the background non-cancerous mucosae, methylation levels showed unimodal distributions, especially for *LOX, FLNc, HAND1, THBD, F2R*, and *ZNF177*. Very low levels of and no methylation were detected for *CDKN2A* (except for one case with 7.1% methylation) and *hMLH1*, respectively.

Calculation of deviation values and statistical analysis. For each gene, the deviation value of a case was calculated as:

$$\text{methylation level of the case (\%)} - \text{mean methylation level (\%)/SD} \times 10 + 50.$$

The 'average of the deviation values' of a case was calculated as the average of the deviation values of the 12 genes in the case.

Pairwise differences in age were analyzed using Student's *t*-test with Bonferroni's correction, and an overall difference in age was analyzed using the Kruskal–Wallis test. Pairwise and overall differences in sex, histology, lymph-node metastasis, *H. pylori*-infection status, and EBV-infection status were analyzed using Fisher's exact test. Correlations between the number of methylated genes in cancers and methylation levels (mean deviation values) were analyzed using Pearson's correlation coefficient. Patient survival was calculated from the date of surgery until the date of death or the last follow up. Survival curves were analyzed using the Kaplan–Meier method and differences in the survival rates were evaluated using the log-rank test. All tests were two-sided, and a *P*-value of less than 0.05 was considered statistically significant. All of the analyses were carried out using SPSS (SPSS, Chicago, IL, USA).

Results

Methylation quantification in cancers and background non-cancerous mucosae. Methylation levels of the promoter CGI were analyzed quantitatively for the 14 promoter CGI in 66 gastric cancers and their background non-cancerous gastric mucosae (Fig. 1), and in 19 normal gastric mucosae of *H. pylori*-negative healthy volunteers. Methylation levels showed different patterns of distribution between cancers and their background non-cancerous mucosae. In cancers, 15–60 (23–90%) of the 66 samples had a methylation level of 0%, whereas the others had methylation levels ranging up to 85%. This indicated that cancer samples could be classified into those without methylation (methylation level = 0%) and those with methylation. In contrast, the methylation levels in the background non-cancerous mucosae followed a unimodal distribution, particularly for *LOX, FLNc, HAND1, THBD, F2R*, and *ZNF177*. Notably, methylation of the *hMLH1* tumor-suppressor gene was not detected at all in the non-cancerous mucosae.

These data supported the suggestion that methylation levels should be interpreted differently in cancers and in non-cancerous tissues, reflecting their monoclonal and polyclonal natures, respectively. When a cancer has methylation of a gene, all of the

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cancer cells have the methylation, and its methylation level should approximate the fraction of cancer cells in a sample. When a cancer does not have methylation of a gene, its methylation level should be 0%. However, a sample of background non-cancerous mucosae contains many glands, each of which is expected to have a different methylation status. Because different genes had different susceptibilities to methylation, deviation values were used to calculate mean values among different genes.

The clonality in cancers and non-cancerous mucosae was confirmed by bisulfite sequencing of a representative gene, *LOX* (Suppl. Fig. 1). A gastric cancer sample (case 2) had three patterns of DNA methylation among 12 methylated DNA molecules, whereas a non-cancerous gastric mucosa (case 11) had very variable patterns (eight patterns among eight methylated DNA molecules). This finding supported the monoclonal origin of the cancer cells and polyclonal origins of methylated DNA molecules in non-cancerous samples. A similar tendency was confirmed by bisulfite sequencing of three additional samples.

Analysis of CIMP presence in gastric cancers. Based on the findings in relation to methylation levels described above, a gene in a cancer sample was scored as methylated or unmethylated. To set a cut-off value based on the fraction of cancer cells in the samples, the methylation levels of the two tumor-suppressor genes *CDKN2A* and *hMLH1* (which were most unlikely to be methylated in a subpopulation of cancer cells) were referred to (Suppl. Fig. 2). Most cancers did not have methylation of *CDKN2A* or *hMLH1*. Among the samples with positive methylation, the lowest methylation levels were 6.8% for *CDKN2A* and 7.0% for *hMLH1*, excluding the samples with extremely low methylation levels of *CDKN2A*. Therefore, we adopted a cut-off value of 6%.

The number of genes methylated in a cancer was examined in two ways, including and excluding the two tumor-suppressor genes. In both cases a continuous distribution was demonstrated, with 15 cancers having no methylation (Fig. 2). The absence of a bimodal distribution was in contrast to the case of colorectal cancers,[8,10] but was in accordance with previous reports in gastric cancers.[15–19] To carry out unbiased association analysis with clinicopathological features, cancers were classified into tertiles, using a cut-off of methylation of five genes. Cancers with methylation of five genes or more, those with one to four genes, and those with no methylation were designated as CIMP high (cases 1–21), CIMP low (cases 22–51), and CIMP negative (cases 52–66), respectively.

Relationship between CIMP in cancers and methylation in background mucosae. To examine the relationship between CIMP in gastric cancers and methylation levels in the background non-cancerous gastric mucosae, all of the samples were sorted according to the number of genes methylated in the cancers, and then according to the average of the deviation values of the 12 genes in the background non-cancerous mucosae (Fig. 3). The methylation levels in the non-cancerous mucosae of cancer patients (except for cases 2, 10, 21, 51, and 66) were higher than normal gastric mucosae of the *H. pylori*-negative healthy volunteers, which was in accordance with our previous findings.[6]

For more detailed analysis, the methylation levels in the background non-cancerous mucosae were compared among the CIMP-high, CIMP-low, and CIMP-negative cancers. However, no significant differences were observed for methylation levels of the 12 genes (Fig. 4a), or for the average of the deviation values of the 12 genes (49.9 ± 7.9, 50.3 ± 5.1, 49.5 ± 3.9, respectively; mean ± SD, $P = 0.650$). When the number of genes methylated in cancers and the mean deviation value in the background mucosae were analyzed, again no correlation was observed (Fig. 4b; $r = -0.036$, $P = 0.774$).

Clinicopathological features of CIMP in gastric cancers. Associations between CIMP status (CIMP high, CIMP low, and CIMP negative) and clinicopathological characteristics were analyzed.

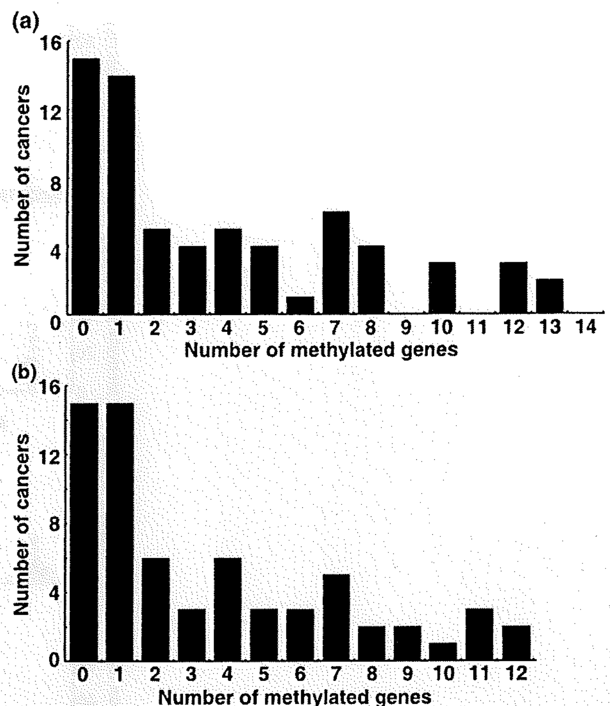

Fig. 2. Distribution of gastric cancers by number of methylated genes. Analyses (a) including and (b) excluding the two tumor-suppressor genes were carried out. In both analyses, the number of methylated genes did not show a bimodal distribution, suggesting that multiple entities underlie the CpG island methylator phenotype in gastric cancers.

There were no differences in sex, lymph-node metastasis, and *H. pylori*-infection status among the three groups (Table 2). The CIMP-negative group was younger than the CIMP-low group ($P = 0.056$). The CIMP-high group had a higher incidence of diffuse-type gastric cancers than the CIMP-low group ($P = 0.048$). EBV-associated gastric cancers mostly displayed CIMP-high status. By Kaplan–Meier survival analysis, the CIMP-high group showed significantly better survival than the CIMP-negative group ($P = 0.026$), and better survival than the CIMP-low group ($P = 0.088$) (Fig. 5).

Finally, the effect of EBV infection on the methylation levels of the background non-cancerous mucosae was analyzed using the average of the deviation values of the 12 genes. Its mean ± SD was 49.5 ± 4.2 and 50.7 ± 9.6 in the CIMP-high groups with and without EBV infection, respectively, and no significant difference was observed ($P = 0.875$). Some cases with EBV infection showed very low methylation levels in the background mucosae (cases 2 and 15; Fig. 3).

Discussion

The present study is the first report of concurrent analysis of the methylation levels both in gastric cancers and in their background non-cancerous gastric mucosae. We used quantitative methylation-specific PCR, which is very accurate and can analyze any CpG sites,[6,32] to measure the methylation levels of 14 promoter CGI, consisting of 12 marker genes and two tumor-suppressor genes, in 66 matched cancers and non-cancerous mucosae. As expected from their monoclonal and polyclonal origins, methylation levels in cancers and non-cancerous mucosae showed entirely different patterns. Therefore, methylation in cancers was assessed as positive or negative, and that in

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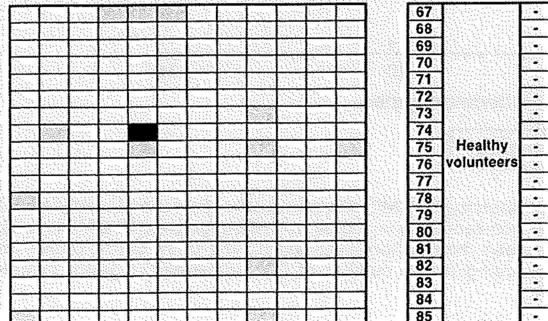
Avarage of the deviation value	Case No.		H.pylori	EBV	No. of methylated loci in 12 genes
51	1	CIMP-high ($n = 21$)	+	+	12
42	2		-	+	12
51	3		+	+	11
49	4		+	-	11
45	5		+	+	11
52	6		+	+	10
46	7		+	-	9
45	8		+	+	9
49	9		+	-	8
42	10		+	-	8
76	11		+	-	7
60	12		+	-	7
49	13		+	-	7
46	14		+	-	7
44	15		+	+	7
54	16		+	-	6
47	17		+	+	6
44	18		-	-	6
57	19		+	-	5
56	20		-	-	5
42	21		+	-	5
63	22	CIMP-low ($n = 30$)	+	-	4
53	23		+	-	4
51	24		+	-	4
50	25		+	-	4
49	26		+	-	4
47	27		+	-	4
52	28		+	+	3
49	29		+	-	3
49	30		+	-	3
55	31		+	-	2
50	32		+	-	2
50	33		-	-	2
48	34		+	-	2
48	35		+	-	2
44	36		+	-	2
59	37		+	-	1
57	38		+	-	1
57	39		+	-	1
56	40		+	-	1
55	41		+	-	1
52	42		+	-	1
49	43		+	-	1
49	44		+	-	1
48	45		+	-	1
48	46		+	-	1
46	47		+	-	1
46	48		+	-	1
45	49		+	-	1
43	50		+	-	1
41	51		+	-	1
54	52	CIMP(-) ($n = 15$)	+	-	0
54	53		+	-	0
53	54		+	-	0
53	55		+	-	0
52	56		+	-	0
52	57		+	-	0
51	58		+	-	0
50	59		+	-	0
50	60		+	-	0
48	61		+	-	0
48	62		+	-	0
47	63		+	-	0
46	64		+	-	0
42	65		-	-	0
41	66		-	-	0



Case No.		H.pylori
67	Healthy volunteers	-
68		-
69		-
70		-
71		-
72		-
73		-
74		-
75		-
76		-
77		-
78		-
79		-
80		-
81		-
82		-
83		-
84		-
85		-

Fig. 3. Relationship between CpG island (CGI) methylator phenotype (CIMP) status in gastric cancers and methylation levels in their background non-cancerous gastric mucosae. The cancer samples (right half) were scored as positive or negative (shown by the black and white boxes, respectively), and the background non-cancerous mucosae (left half) were analyzed by the deviation values of methylation levels (black, deviation value ≥ 65; dark gray, 55 ≤ deviation value < 65; light gray, 45 ≤ deviation value < 55; and white, deviation value < 45 or methylation level < 0.5%). The 66 samples were first sorted by number of methylated CGI, and then by mean deviation value of methylation in the non-cancerous mucosae. Cancers with methylation of five genes or more, those with one to four genes, and those with no methylation were designated as CIMP high (cases 1–21), CIMP low (cases 22–51), and CIMP negative (cases 52–66), respectively. No correlation was observed between CIMP in cancers and methylation levels in non-cancerous mucosae. Methylation levels in the non-cancerous mucosae were higher than those of normal mucosae of *Helicobacter pylori*-negative healthy volunteers. The presence of Epstein–Barr virus and *H. pylori* is indicated by a plus (+) sign.

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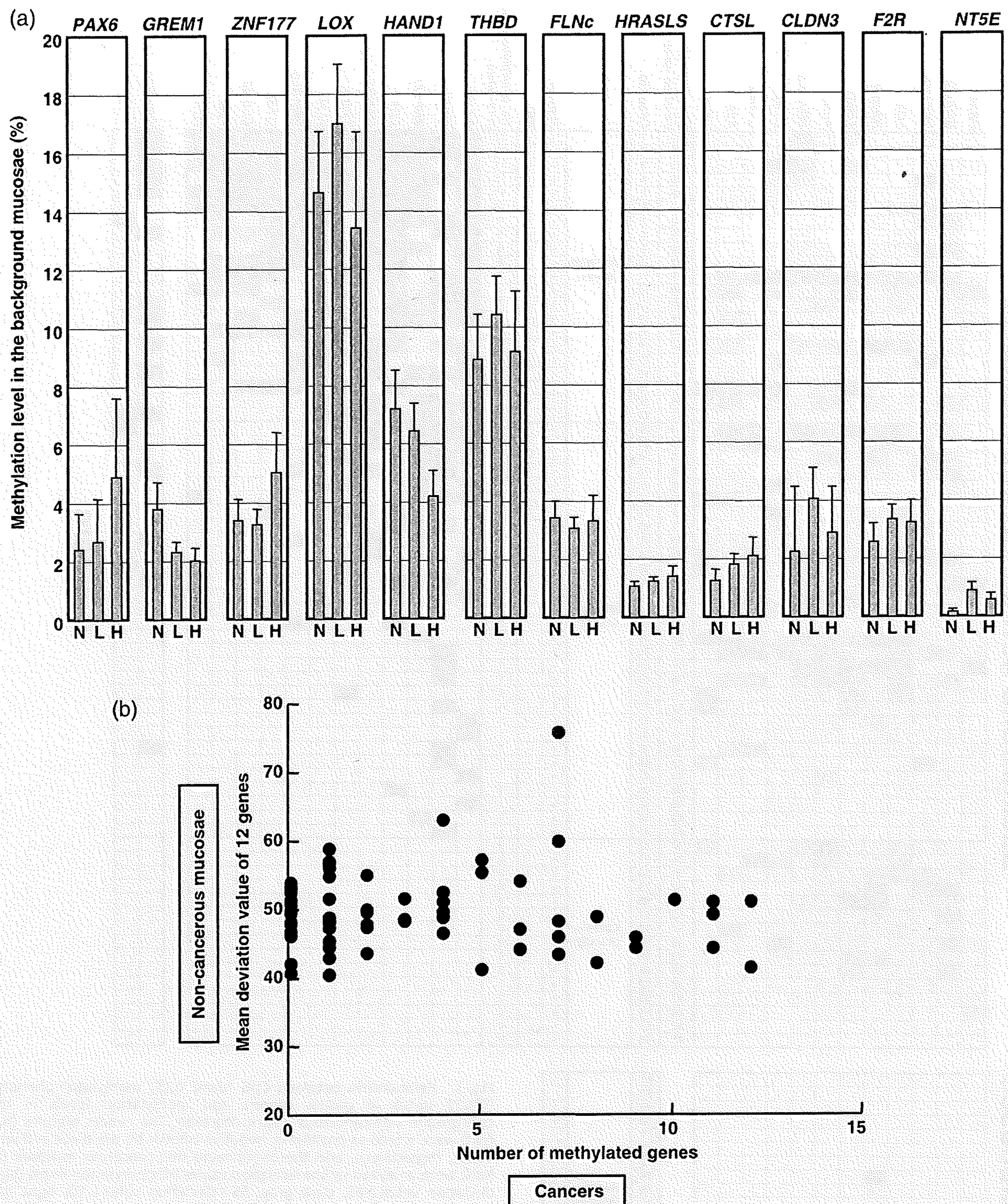

Fig. 4. Detailed analysis of the correlation between CpG island (CGI) methylator phenotype (CIMP) status in gastric cancers and methylation levels in non-cancerous gastric mucosae. (a) Methylation levels of individual genes in non-cancerous mucosae in CIMP-negative (N), CIMP-low (L), and CIMP-high (H) gastric cancers. No significant differences were observed in the mean methylation levels among the three gastric cancer groups. Error bars: standard errors (SE). (b) Mean deviation values of the 12 genes according to the number of CGI methylated in gastric cancers. No correlation was observed (Pearson's correlation coefficient; $r = -0.036$, $P = 0.774$).

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Table 2. Clinicopathological features of the CpG island methylator phenotype (CIMP)-high, CIMP-low and CIMP-negative cancers

Factor	CIMP high (*n* = 21)	CIMP low (*n* = 30)	CIMP negative (*n* = 15)	CIMP-high versus CIMP-low *P*-value	CIMP-high versus CIMP-negative *P*-value	CIMP-low versus CIMP-negative *P*-value	Overall *P*-value
Age (years)	61.1 ± 11.1	65.0 ± 9.0	57.0 ± 12.5	0.612	0.729	0.056	0.092
Sex (male/female)	14/7	23/7	11/4	0.529	0.729	1.000	0.726
Histology (intestinal/diffuse)	2/19	11/19	4/11	0.048	0.214	0.738	0.072
Lymph-node metastasis (positive/negative)	18/3	25/5	12/3	1.000	0.677	1.000	0.916
Helicobacter pylori infection (positive/negative)	18/3	29/1	13/2	0.293	1.000	0.254	0.341
Epstein–Barr virus infection (positive/negative)	8/13	1/29	0/15	0.002	0.011	1.000	0.000

Mean ± SD is shown for age, and number of cases is shown for all other parameters.

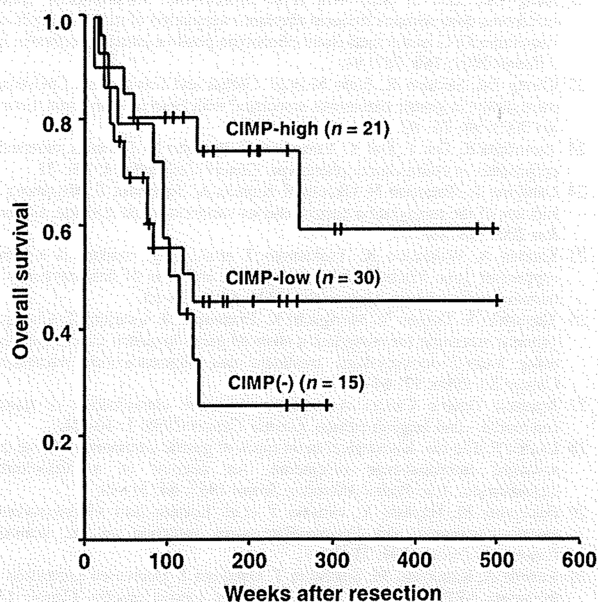

Fig. 5. Kaplan–Meier survival analysis of gastric cancer patients according to CpG island methylator phenotype (CIMP) status. The CIMP-high patients tended to show better survival than the CIMP-low patients ($P = 0.088$). The CIMP-high patients showed significantly better survival than the CIMP-negative patients ($P = 0.026$).

non-cancerous mucosae was assessed by its degree using the methylation levels or the deviation values.

The number of methylated genes in a cancer did not show a bimodal distribution, suggesting that CIMP in gastric cancers consists of multiple entities or even does not exist. To classify gastric cancers without a bias, we chose to put them into tertiles. The first group was CIMP negative (15 cancers without methylation), and the remaining 51 cancers were classified as 30 CIMP-low and 21 CIMP-high cancers with a cut-off of five genes methylated. The fraction of CIMP-high gastric cancers (32%) in this classification was similar to those (24–41%) in previous studies.[15–20] Eight of the nine EBV-associated gastric cancers belonged to the CIMP-high cancers,[19–22] and CIMP-high cancer patients tended to have a better prognosis than CIMP-low and CIMP-negative patients, also as reported.[17,19,20] These findings suggested that, although CIMP consists of multiple entities, CIMP itself does exist in gastric cancers. Even when using a different cut-off value (four genes), the above findings did not change (data not shown).

Initially, we expected that gastric cancers arising from a background gastric mucosa with high methylation levels would display a CIMP-high status. However, contrary to our expectation, the CIMP status in cancers did not correlate with the methylation levels in non-cancerous mucosae. This apparent discrepancy can be explained in two ways. First, most of methylation present in gastric cancers could have been induced after a cancer cell was produced. This idea is supported by the fact that some CIMP-high gastric cancers had unique clinicopathological characteristics, and by our previous finding that some gastric cancer cell lines appeared to have an intrinsic abnormality that increased the rate of methylation events.[33,34] Second, methylation levels in the entire gastric mucosae might not reflect methylation levels in the precursor cells for gastric cancers. Although the precise origin of gastric cancer cells has not been clarified, the number of stem and progenitor cells in a gastric gland is known to be relatively small.[35]

We adopted a cut-off value of 6% to score cancer samples with positive methylation. This value was based on the methylation levels of *CDKN2A* and *hMLH1*, which were considered to reflect the fraction of cancer cell-derived DNA in the samples. Although some cancers showed methylation levels between 1 and 3%, most methylation-positive cancers had methylation levels higher than 6%. Methylation levels of 1–3% in cancers was considered to be present in a subpopulation of cancer cells, as histological analysis of gastric cancers did not support such a small fraction of cancer cells occurring in a tissue sample. The 6% methylation cut-off value used in the present study is comparable with previous reports using MethyLight technology, in which a cut-off value of 4% was used to best discriminate between normal and malignant tissues.[36,37]

Among the 12 marker genes used in the present study, five genes (*LOX*, *HRASLS*, *FLNc*, *HAND1*, and *THBD*) were identified by methylation-sensitive representational difference analysis as methylated in gastric cancers, and were used to analyze gastric cancers for their CIMP statuses in previous studies.[16,19] The remaining seven genes were identified by treating a gastric cancer cell line with a demethylating agent, 5-aza-2′ deoxycytidine, and screening using an oligonucleotide microarray.[26] Because these 12 genes were methylated not only in cancers but also in non-cancerous gastric mucosae, we used these genes as marker genes. Although *LOX* has tumor-suppressive activity in gastric cancers,[25] the remaining 11 genes were unlikely to have such activity and were considered suitable to analyze the effects of factors that induce aberrant DNA methylation in an unbiased manner.

We recently showed that *H. pylori* infection, a potent gastric carcinogenic factor, induces methylation of specific genes in non-cancerous gastric mucosae,[6] and methylation levels increase in the order of healthy volunteers, cases with a single gastric cancer, and cases with multiple gastric cancers.[7] The high methylation levels in the non-cancerous mucosae of cancer cases observed here were considered to reflect their current or past exposure to *H. pylori*, and to be associated with the methylation

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of tumor-suppressor genes. Because the methylation levels of tumor-suppressor genes are very low in non-cancerous gastric mucosae, as observed for *CDKN2A* and *hMLH1* here, currently their accurate measurement is technically very difficult and the use of marker genes has value.

In conclusion, this is the first study that has revealed no correlation between CIMP status in gastric cancers and methylation levels in their background non-cancerous gastric mucosae.

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Supplementary Material

The following supplementary material is available for this article:

Fig S1. (a) The genomic structure of the *LOX* promoter region. Vertical ticks show individual GpC sites (top) and CpG sites (bottom). The regions analyzed by real-time MSP and bisulfite sequencing are shown. (b) The results of bisulfite sequencing in a cancer and non-cancerous mucosa samples. Methylation patterns in the cancer were very limited among the methylated DNA molecules while those in the non-cancerous sample were highly variable. Closed and open circles show methylated and unmethylated CpG sites, respectively.

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Fig S2. Distribution of the methylation levels of the *CDKN2A* and *hMLH1* tumor suppressor genes in cancer tissues. Most cancers had a methylation level of 0%. The lowest methylation level was 6.8% for *CDKN2A* and 7.0% for *hMLH1* among the samples with positive methylation, excluding the samples with extremely low methylation levels.

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Identification of *PRTFDC1* silencing and aberrant promoter methylation of *GPR150*, *ITGA8* and *HOXD11* in ovarian cancers

Li-yi Cai [a,b], Masanobu Abe [a], Shun-ichiro Izumi [b], Masayoshi Imura [a], Toshiharu Yasugi [c], Toshikazu Ushijima [a,*]

[a] *Carcinogenesis Division, National Cancer Center Research Institute, Japan*
[b] *Department of Obstetrics and Gynecology, Tokai University School of Medicine, Japan*
[c] *Department of Obstetrics and Gynecology, The University of Tokyo Graduate School of Medicine, Japan*

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Abstract

Methylated promoter CpG islands (CGIs) can be used to find novel tumor-suppressor genes and disease markers. In this study, to identify promoter CGIs aberrantly methylated in human ovarian cancers, we performed a genome-wide screening for differentially methylated DNA fragments using methylation-sensitive-representational difference analysis (MS-RDA). MS-RDA isolated 185 DNA fragments specifically methylated in an ovarian cancer cell line (ES-2), compared with a normal human ovarian surface epithelial cell line (HOSE6-3), and 33 of them were derived from putative promoter CGIs. Ten ovarian cancer cell lines were analyzed by methylation-specific PCR, and seven (*GPR150*, *LOC222171*, *PRTFDC1*, *LOC339210*, *ITGA8*, *C9orf64* and *HOXD11*) of the 33 CGIs were methylated in one or more of the cell lines. Their downstream genes were barely expressed in cell lines without unmethylated DNA molecules by quantitative reverse-transcription-PCR. Demethylation of methylated cell lines with 5-aza-2′-deoxycytidine restored expression of two genes (*PRTFDC1* and *C9orf64*). In primary ovarian cancers, CGIs of *GPR150* (in 4 of 15 cancers), *ITGA8* (2/15), *PRTFDC1* (1/15), and *HOXD11* (1/15) were methylated. Silencing of *PRTFDC1* was revealed here for the first time, and aberrant methylation of *GPR150*, *ITGA8* and *HOXD11* could be candidate tumor markers.
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Keywords: Ovarian cancer; CpG island; DNA methylation; Epigenetics

Introduction

Aberrant methylation of CpG islands (CGIs) in gene promoter regions is known to silence their downstream genes (Herman and Baylin, 2003; Jones, 2005). In ovarian cancers, gene silencing due to promoter methylation is reported for *CDKN2A/p16* (0–40%) (Kawauchi et al., 2004; Shih et al., 1997; Katsaros et al., 2004), *BRCA1* (15%) (Esteller et al., 2000; Baldwin et al., 2000), *hMLH1* (48%) (Geisler et al., 2003) and *RASSF1A* (10–40%) (Yoon et al., 2001; Agathanggelou et al., 2001). In addition to analysis of known tumor-suppressor genes, genome-wide screenings for aberrantly methylated CGIs have been successfully used to identify novel tumor-suppressor genes in colorectal, hepatocellular, gastric and other cancers (Ushijima, 2005). Since genetic and epigenetic alterations known in ovarian cancers are still limited (Li and Karlan, 2001; Imura et al., 2006), genome-wide screening procedures are expected to be useful to identify novel tumor-suppressor genes in ovarian cancers.

Silencing of a specific gene and methylation profiles of a set of genes can also be used as clinical biomarkers to predict drug response, prognosis, and other clinically useful information (Laird, 2003; Miyamoto and Ushijima, 2005). In ovarian cancers, novel prognostic marker genes were successfully identified by use of a genome-wide screening of aberrant methylation (Wei et al., 2002, 2006). Furthermore, DNA methylation itself can be used to detect cancer cells or cancer-derived DNA, taking advantage of techniques that detect

* Corresponding author. 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Tel.: +81 3 3547 5240; fax: +81 3 5565 1753.
E-mail address: tushijim@ncc.go.jp (T. Ushijima).

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