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 mucosa. 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., 2006b; Nakajima et al., 2006c), oral cavity (Slaughter et al., 1953; Partridge et al., 2000), the upper aerodigestive tract of smokers (Copper et al., 1993; Sozzi et al., 1995; Wisztuba 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 (Hafer et al., 2002).

Most of the field cancerization has been explained by the presence of cells with genetic alterations (Sozzi et al., 1995; Wisztuba et al., 1997; Partridge et al., 2000; Hafer 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
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). This 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 Sasaki, 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.

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 mucosa

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 Sasaki, 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 mucosa, 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 mucosa by Helicobacter pylori**

The presence of trace amounts of methylation in non-cancerous gastric mucosa 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 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 mucosa 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 mucosa that constituted the majority of the DNA molecules.

Therefore, we quantified the fraction of methylated DNA molecules in the gastric mucosa 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 mucosa 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 mucosa 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](image)

**Fig. 2.** Methylation levels in the non-cancerous gastric mucosa 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 mucosa. 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; Eksurom 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 demethyllase 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 mucosa 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 mucosa 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.

Table 1. Studies on epigenetic field for cancerization

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Inducing factor</th>
<th>Detection method and analysis way</th>
<th>Genes analyzed</th>
<th>Non-predisposed samples/methylation in defect vs non-predisposed</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver cancer</td>
<td>HBV and HCV</td>
<td>COBRA/Incidence</td>
<td>CDKN2A, hMLH1, THBS-1, and five MINT loci</td>
<td>8 normal livers 4/5 vs 0/8</td>
<td>Kondo, 2000</td>
</tr>
<tr>
<td>Colorectal cancer (UC-associated)</td>
<td>UC</td>
<td>MSP/Incidence</td>
<td>CDKN2A</td>
<td>Not available</td>
<td>Hsieh, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>COBRA/Quantitative</td>
<td>ER, MYOD, CDKN2A, and CSPG2</td>
<td>5 non-UC patients not significant</td>
<td>Issa, 2001</td>
</tr>
<tr>
<td>Barrett’s cancer</td>
<td>Reflux esophagitis?</td>
<td>MethylLight/Incidence</td>
<td>APC, CDKN2A, and ESR1</td>
<td>Not available</td>
<td>Eads, 2000</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Smoking?</td>
<td>MSP/Incidence</td>
<td>CDKN2A, MGMT, DAPK, SOCS1, RASSF1A, COX2, and RARβ</td>
<td>Not available</td>
<td>Guo, 2004</td>
</tr>
<tr>
<td>Colorectal cancer (sporadic)</td>
<td>Unknown</td>
<td>COBRA/Quantitative</td>
<td>MGMT</td>
<td>33 healthy subjects 8.8% vs 2% 22/44 vs 4/33</td>
<td>Shen, 2005</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td><em>H. pylori</em></td>
<td>qMSP/Quantitative</td>
<td>CDKN2A, LOX, THBD, HRAS1S, FLNC, HAND1, and p41ARC</td>
<td>98 healthy subjects 2.2-to 32-fold increase in methylation levels 25 samples from reduction mammoplasty 4/5 vs 0/16</td>
<td>Maekita, 2006</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Unknown</td>
<td>qMSP/Incidence</td>
<td>CYP26A1</td>
<td>9 samples without renal cancers 44/60 vs 1/9 etc.</td>
<td>Yan, 2006</td>
</tr>
<tr>
<td>Renal cancer</td>
<td>Unknown</td>
<td>MSP/Incidence</td>
<td>CDKN2A, hMLH1, THBS-1, and five MINT loci</td>
<td>9 samples without renal cancers 44/60 vs 1/9 etc.</td>
<td>Arai, 2006</td>
</tr>
</tbody>
</table>

"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 $10^{-10}$ 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 mucosa 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 mucosa 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 mucosa 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.

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 mucosa, 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 mucosa 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., 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 esophagus, 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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