**Table 1. Methods for detecting DNA methylation changes in colorectal cancer**

| Study | Methods | Principles of technologies |
|---|---|---|
| | **Methylation detection** | |
| Eads et al. [9] | Methylation-specific PCR | Bisulfite conversion and allele-specific PCR |
| Herman et al. [10] | MethyLight | Bisulfite conversion and real-time PCR |
| Clark et al. [11] | Bisulfite sequencing | Bisulfite conversion, PCR, and sequencing |
| Xiong and Laird [12] | COBRA | Bisulfite conversion, PCR, and restriction digestion |
| Uhlmann et al. [13] | Bisulfite pyrosequencing | Bisulfite conversion, PCR, and pyrosequencing |
| | **Methylation screening** | |
| Costello et al. [14] | RLGS | Restriction digestion, electrophoresis |
| Ushijima et al. [15] | MS-RDA | Restriction digestion, adaptor ligation, and PCR |
| Toyota et al. [16] | MCA-RDA | Restriction digestion, adaptor ligation, and PCR |
| Estecio et al. [17] | MCA-array | Restriction digestion, adaptor ligation, PCR, and microarray |

COBRA—combined bisulfite restriction analysis; MCA-array—methylated CpG island amplification–array; MCA-RDA—methylated CpG island amplification restriction difference analysis; MS-RDA—methylation-sensitive representational difference analysis; PCR—polymerase chain reaction; RLGS—restriction landmark genomic scanning.

methylation. Restriction enzyme digestion followed by Southern blotting has been used, but this method requires high-quality DNA and is time consuming. However, the development of bisulfate-based methylation analysis has dramatically improved the study of DNA methylation (Table 1). With this approach, allele-specific polymerase chain reaction can be used to detect methylated and unmethylated alleles [9,10], after which the amplified products can be sequenced [11] or digested using restriction enzymes that selectively recognize sites containing a CpG site [12]. By applying pyrosequencing, DNA methylation levels can be determined more precisely [13].

Another factor that has limited the study of DNA methylation in cancer is the lack of markers to study. Several approaches enable differential screening of methylated genes in cancer and normal tissues (Table 1). Restriction landmark genomic scanning is one technique. With more than 1000 genes analyzed in each experiment, restriction landmark genomic scanning can be used to evaluate methylation throughout the genome and can be applied to detect cancer-related changes in DNA methylation [14]. Methylation-sensitive representational analysis is another technique that was developed to detect differences between DNA methylation in cancer and normal tissues [15]. However, a limitation of this approach is that methylated alleles are detected as a loss of signal, reflecting the inability of methylation-sensitive restriction enzymes to digest at CpG sites.

We have developed a method called *methylated CpG island amplification* (MCA). MCA enables us to amplify only methylated sequences. DNA fragments differentially methylated in cancer and normal tissues can be identified using an MCA amplicon as a tester and driver to carry out representational difference analysis [16]. MCA can also be applied to promoter microarrays to identify genes methylated in cancer but not in normal tissue [17].

## Role of DNA Methylation in Tumorigenesis of CRC

Earlier studies of DNA methylation demonstrated that the calcitonin gene is hypermethylated in CRC [18]. It was unclear whether methylation of genes such as calcitonin was really involved in tumorigenesis or whether it was an epiphenomenon. However, recent studies have confirmed that DNA methylation plays an important role in tumorigenesis in the colon. For example, it was shown that DNA methylation affects genes such as *RB*, *VHL*, *APC*, *CDH1*, and *BRCA1*, all of which are involved in familial cancer syndrome [19]. Additionally, DNA methylation serves as one of two hit mechanisms for gene inactivation, and genes infrequently mutated in CRCs are often targets of DNA methylation. For example, the *CDKN2A/p16* gene is rarely mutated or deleted in CRC, but it is inactivated by methylation in 40% of tumors [20]. *CDKN2A/p16* methylation is also seen in colorectal adenomas, indicating that DNA methylation is already present in premalignant lesions.

Genes involved in signal transduction pathways are often targets of DNA methylation, among which we identified the secreted frizzled-related protein (SFRP) gene family as one such target [21]. Their products, SFRPs, antagonize Wnt signaling and are frequently silenced in CRC [22]. Ectopic expression of SFRPs suppresses T-cell factor/β-catenin activity in CRC cells carrying *APC* or β-catenin mutations and other negative regulators of Wnt signaling, such as *DKK* genes, which are also silenced by DNA methylation in CRC [23]. This suggests that not only mutations of *APC*/β-catenin, but

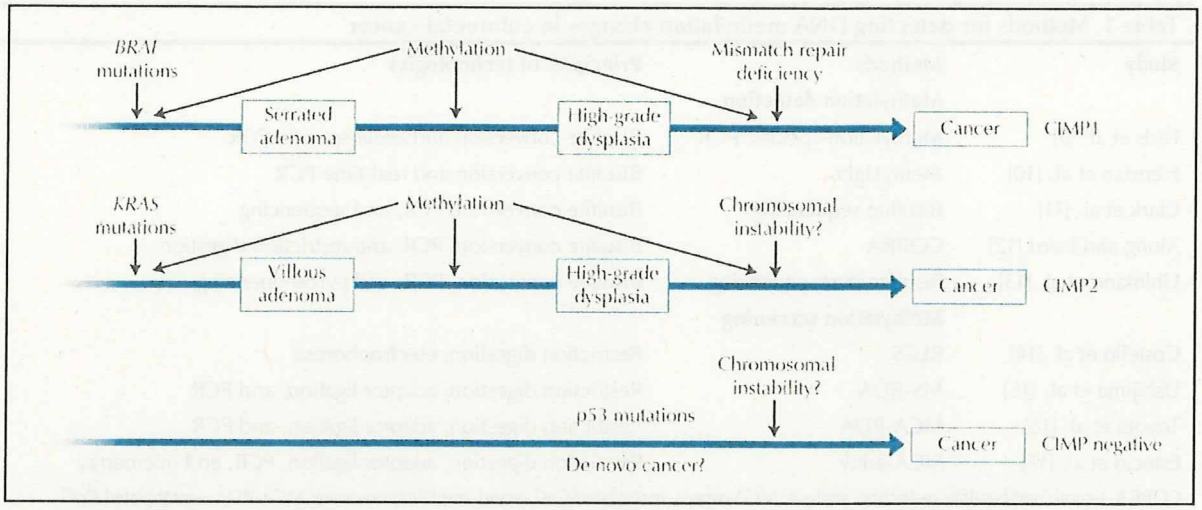


**Figure 1.** Colorectal cancers can be grouped into three distinct groups. CpG island methylator phenotype 1 (CIMP1) tumors, which have a high degree of DNA methylation, frequently show microsatellite instability because of methylation of *hMLH1* and *BRAF* mutation. It has been suggested that CIMP1 tumors arise through the sessile serrated adenoma pathway. CpG island methylator phenotype 2 (CIMP2) tumors frequently show mutation of *KRAS*, but the frequencies of microsatellite instability, *BRAF*, and p53 mutations are low. These tumors have the poorest prognosis. CIMP-negative tumors frequently show p53 mutations.

epigenetic inactivation of negative regulators of Wnt is important for full activation of Wnt signaling during tumorigenesis in CRC.

Mutation of *KRAS* is frequently detected in CRCs and adenomas. In normal cells, activation of *KRAS* induces senescence and apoptosis mediated by negative regulators of Ras [24]. Our group and others have found that two of these negative regulators, Ras association domain family proteins 1 and 2 (RASSF1 and RASSF2), are methylated in CRCs, and ectopic expression of RASSF2 induces morphologic changes in cells and apoptosis [25,26]. Moreover, knockdown of RASSF2 enhances *KRAS*-mediated cellular transformation, indicating RASSF2 has the ability to prevent cellular transformation. Primary CRCs that show *KRAS/BRAF* mutations also frequently show RASSF2 methylation, and the resultant inactivation of RASSF2 enhances *KRAS*-induced oncogenic transformation. DNA methylation also occurs in genes involved in DNA repair and maintaining the integrity of the genome (eg, *MGMT, WRN* [27,28]), inhibition of angiogenesis (eg, *THBS1* [29]), and tumor immunity (eg, *CIITA* [30]).

## CpG Island Methylator Phenotype and Microsatellite Instability

HNPCC shows microsatellite instability (MSI), which leads to alteration of genes containing microsatellite tracts. In HNPCC, MSI is caused by mutations of mismatch repair genes, such as *hMSH2* and *hMLH1* [31]. About 15% to 20% of sporadic CRCs show MSI, although mutation of mismatch repair genes is not frequent, and one mismatch repair gene, *hMLH*, is inactivated by DNA methylation [32]. During the course of our work profiling methylation in CRC, we found that a subset of cancers shows the CpG island methylator phenotype (CIMP), and methylation of *hMLH1* correlates significantly with CIMP, suggesting CIMP causes MSI, which in turn leads to inactivation of *hMLH1* [33].

## Distinct Genetic and Epigenetic Alterations in Three Types of CRC

Until recently, the existence of CIMP was controversial because there were no optimal markers to define it. This changed when Weisenberger et al. [34] were able to identify CIMP in CRC using five markers detected using MethyLight. However, these investigators likely focused solely on CIMP1 cases and thus underestimated the CIMP2 group. Recently, Shen et al. [35] analyzed mutations of *BRAF, KRAS*, and p53 and methylation of 27 loci in 97 CRCs; they found that the tumors could be grouped based on the genetic and epigenetic alterations. Unsupervised hierarchical clustering of the DNA methylation data identified three distinct groups of colon cancers: CIMP1, CIMP2, and CIMP negative (Fig. 1). CIMP1 cases showed a high frequency of MSI and *BRAF* mutations (80% and 53%, respectively) but few instances of *KRAS* and/or p53 mutations (16% and 11%, respectively). Conversely, CIMP2 cases were associated with a high frequency of *KRAS* mutations (92%), but MSI and *BRAF* mutations rarely occurred (0% and 4%, respectively), and there was only a low rate of p53 mutations (31%). CIMP-negative cases had a higher rate of p53 mutations (71%) and a lower rate of MSI (12%) and

mutations of *BRAF* (2%) and *KRAS* (33%). The existence of three groups of CRCs was confirmed in a larger study by Barault et al. [36]; they assessed 582 cases. Additionally, there was an inverse correlation between chromosomal instability and the presence of CIMP, indicating that CIMP-positive CRCs have distinct genetic and epigenetic features. Most importantly, CIMP affects the prognosis of patients with CRC. CIMP1 patients have a relatively good prognosis, as MSI-positive CRC patients generally have a better prognosis. By contrast, patients with CIMP2 or CIMP-low microsatellite-stable tumors have a poor prognosis [36,37]. However, the existence of CIMP-high tumors without *BRAF* mutation or MSI indicates there is still more to learn about CIMP and its classification.

## Epigenetic Alteration of MicroRNA Expression

MicroRNAs (miRNAs) are a group of small noncoding RNAs that negatively regulate the translation and stability of partially complementary target mRNAs [38]. Downregulation of a subset of miRNAs is a commonly observed feature of cancers, suggesting these molecules may act as tumor suppressors [39,40]. By identifying miRNAs whose expression was upregulated in CRC cells in which DNMT1 and DNMT3B were knocked out, we were able to identify miRNAs that were epigenetically silenced [41]. Among the 37 miRNAs upregulated by DNMT inhibition, we focused on *miR-34b/c* because recent studies have shown that *miR-34* family members (*miR-34a*, *miR-34b*, and *miR-34c*) are direct targets of p53 [40,42,43]. We found that downregulation of *miR-34b/c* expression was strongly associated with methylation of its neighboring CpG island, which harbors bidirectional promoter activity and also regulates expression of another candidate tumor suppressor gene, B-cell translocation gene 4 (*BTG4*). In addition, ectopic expression of the *miR-34b/c* precursor in CRC cells resulted in downregulation of CDK6, a cyclin-dependent kinase involved in cell growth, and MET, a receptor tyrosine kinase involved in cell growth and metastasis. Epigenetic inactivation of *miR-34b/c* may then attenuate p53 function through dysregulation of the cell cycle and cell growth.

Lujambio et al. [44] showed that *miR-124a*, another regulator of CDK6, is targeted for epigenetic inactivation by methylation in 75% of CRCs. Transfection of *miR-124a* into colon cancer cell lines suppressed levels of CDK6 protein, which led to reduced phosphorylation of ribose in residues 807 and 811. More recently, Lujambio et al. [45] extended their analysis to identify *miR-9*, *miR-34b/c*, and *miR-148a* as targets of epigenetic inactivation in metastatic CRC, which suggests that epigenetic inactivation plays a key role in the silencing of protein-coding genes and noncoding RNA.

## Implications of DNA Methylation in Diagnosis and Therapy

The tumor specificity of DNA methylation profiles suggests it should be possible to diagnose cancers on that basis. Consistent with that idea, DNA methylation can be detected in the fecal DNA of colon cancer patients [46] and the serum of CRC patients [47]. Moreover, the fact that methylation of certain genes (eg, *SFRP1*) can be detected in early lesions, such as aberrant crypt foci, suggests methylation levels may be used to assess cancer risk. Thus, DNA methylation of specific genes may serve as useful molecular markers for the diagnosis of CRC and prediction of patient outcome.

DNA methylation also can be used as a molecular marker to predict response to therapy. For example, CRCs with MSI are less aggressive than others, but they do not respond to 5-fluorouracil (5-FU) because of methylation of *hMLH1* [48]. However, they become responsive upon exposure to 5-aza-2′-deoxycytidine (5-aza-dC) [49]. Thus, methylation of *hMLH1* appears to be a predictive molecular marker of the sensitivity of CRCs to 5-FU. Similarly, methylation of *WRN* correlates with the sensitivity of CRC cells to topoisomerase inhibitors [27], whereas methylation of *MGMT* and *p73* correlates with sensitivity to alkylating agents [50••], and methylation of *CHFR* (a mitotic checkpoint gene) correlates with sensitivity to microtubule inhibitors [51].

Because epigenetic changes are reversible, DNA methylation and histone modification can be reversed using DNA methyltransferase inhibitors or histone deacetylase inhibitors, and DNA methyltransferase inhibitors (azacytidine and decitabine) have been used to treat patients with myelodysplastic syndrome [52]. Although such epigenetic therapy has not yet proved effective in the treatment of CRC, it can be combined with chemotherapeutic agents to enhance the effects of those drugs. For instance, CRCs escape the immune system through inactivation of the tumor antigen presentation system. Treating cancer cells with DNA methyltransferase inhibitors or histone deacetylase inhibitors restores tumor antigen, thereby stimulating immune responses.

## Conclusions

We anticipate that a great deal of new information about the role of the epigenome in CRC will become available in the near future. Understanding the epigenome will be an important step toward a fuller understanding of the molecular mechanisms underlying CRC and may provide the basis for the development of novel diagnostic methods and approaches to therapy.

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## Disclosure

No potential conflicts of interest relevant to this article were reported.

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# Integrated analysis of genetic and epigenetic alterations in cancer

A proposed genetic model describing the transition from normal colonic epithelium to malignant cancer involves mutation of a number of key oncogenes and tumor suppressor genes. However, only subsets of colorectal cancers contain such mutations. Moreover, the heterogeneous pattern of tumor mutations suggests there are multiple alternative pathways leading to colonic tumorigenesis. These alternative pathways involve epigenetic alterations such as the methylation of multiple CpG islands, termed the CpG island methylator phenotype, and cancers with CpG island methylator phenotype show distinct genetic and clinicopathological features. The causes of these epigenetic alterations are still not fully understood, but exogenous pathogens such as *Helicobacter pylori* and Epstein–Barr virus, and the chromosomal translocations seen in leukemia, have all been shown to induce epigenetic alterations of genes.

**KEYWORDS: CpG island methylator phenotype colorectal cancer DNA methylation epigenetics histone tumor suppressor gene**

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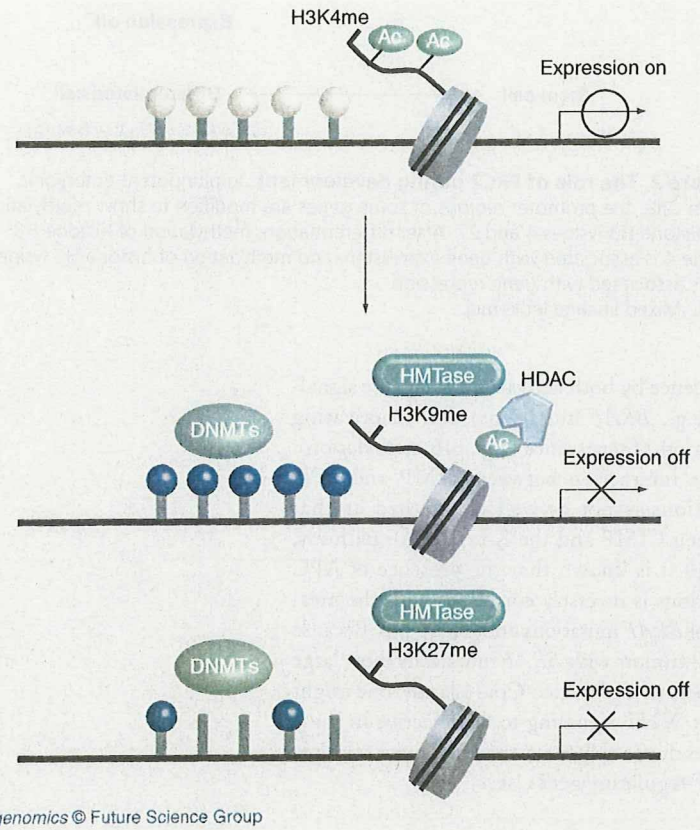
It is now well established that both genetic and epigenetic alterations accumulate during carcinogenesis. Among the epigenetic changes identified to date, DNA methylation, particularly methylation of the 5′ CpG islands of genes, is the best characterized. Under normal physiological conditions, DNA methylation catalyzed by three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) is involved in regulating genome imprinting, X-chromosome inactivation and inactivation of repetitive sequences [1]. In addition to DNA methylation, acetylation and methylation of lysine residues in histones H3 and H4 also play key roles in gene regulation [2]. In general, increases in histone acetylation are associated with increases in transcriptional activity, while decreases in acetylation are associated with gene repression (Figure 1). The acetylation status of histones reflects the balance between the activities of histone acetyltransferase and histone deacetylase. Histones H3 and H4 are also modified by methylation: methylation of H3 lysine 4 is associated with active transcription, while methylation of H3 lysines 9 and 27 is associated with gene repression (Figure 1). Histone modification is also mediated by the polycomb group proteins, which are negative regulators of transcription that act by forming multiple polycomb-repressive complexes (PRCs). Among the PRCs, PRC2, which contains three core components, EZH2, SUZ12 and EED, plays a key role in gene silencing in cancer. EZH2 exhibits histone methyltransferase activity, and SUZ12 and EED are required for that activity (Figure 2). Analysis of genes targeted by PRC2 in pluripotent embryonic stem cells showed that the patterns of polycomb-based repression in cancer are closely associated with the targets of DNA methylation, suggesting that there is a cross-talk between DNA methylation and the targeting of PRC2 [3,4]. Notably, DNA methylation affects a number of tumor suppressor genes, including *RB*, *VHL*, *BRCA1* and *CDH1* [2], as well as genes involved in cell-cycle checkpoints, apoptosis and inhibition of angiogenesis [5]. In the current review, we focus on the association between genetic and epigenetic alterations in human cancer. We also discuss recent progress in genome-wide analysis of DNA methylation.

## Genetic & epigenetic interactions in colorectal cancer

Cancer-related genetic and epigenetic alterations are perhaps best characterized in colorectal cancer. A genetic model describing the transition from normal colonic epithelium to increasingly dysplastic adenoma and then to malignant cancer has been proposed in which a number of key oncogenes and tumor suppressor genes are identified [6]. Among these is *APC*, which was first identified as a gene responsible for familial adenomatous polyposis [7]. Inactivation of *APC* leads to activation of WNT signaling through translocation of β-catenin to the nucleus [8]. In addition, *K-ras* mutation, which has been shown to be an early event in carcinogenesis and to result in the constitutive activation of Ras signaling, is detected in 30–50% of colorectal cancers.

Finally, *p53*, which regulates a variety of genes involved in cell-cycle checkpoints, apoptosis, angiogenesis inhibition and immune responses, is one of the most frequently altered tumor suppressor genes in human cancers. However, several studies have suggested that the classic model of colorectal cancer evolution will only rationalize tumor formation in a small fraction of colorectal tumors. For instance, Smith *et al.* reported that only 6% of colorectal cancers contain mutations of *APC*, *K-ras* and *p53* [9], and the heterogeneous pattern of tumor mutations suggests there are likely multiple alternative pathways leading to colorectal cancer. A subset of familial colorectal cancers, called hereditary nonpolyposis colorectal cancer, results from germline mutations in mismatch repair (MMR) genes [6]. Microsatellite sequences, such as mono- and di-nucleotide repeats, are frequently mutated because of this MMR deficiency. In addition, approximately 15–20% of sporadic colorectal cancers show microsatellite instability (MSI), which is caused by the lack of MMR gene expression. MMR mutations are rarely found in these tumors, however [10].




*Epigenomics* © Future Science Group

**Figure 1. The role of DNA methylation and histone modification in gene expression.** In the active promoter, CpG sites are unmethylated, most of the lysine residues in the histone tail are acetylated (Ac), and histone H3 lysine 4 is methylated. In a repressed promoter, DNMTs methylate CpG sites, HDAC removes acetyl groups, and histone H3 lysines 9 and 27 are modified by HMTase. Gray circles: Unmethylated CpG sites; blue circles: Methylated CpG sites. DNMT: DNA methyltransferase; HDAC: Histone deacetylase; HMTase: Histone methyltransferase.

Although one early study showed that hypomethylation of DNA is a hallmark of colorectal cancer [11], changes in DNA methylation in cancer were not very well studied until methylation of the cell-cycle checkpoint gene *CDKN2A/p16* was reported [12]. Subsequent studies simultaneously showed that the angiogenesis inhibitor *TSP1/THBS1* [13] and the DNA repair genes *hMLH1* [14] and *MGMT* [15] are all inactivated by DNA methylation. In the early days of epigenetic research it was thought that DNA methylation likely accumulated as a result of selection, but DNA methylation often silences oncogenes such as *COX2*, *TERT* and *EGFR* [16–18]. As such epigenetic silencing of oncogenes could confer a growth disadvantage to cancer cells, a simple Darwinian selection model may not be applicable to changes in DNA methylation in cancer. In addition, sporadic colorectal cancer with MSI showed methylation of multiple CpG islands [13], which suggests that CpG island methylation is not a random occurrence.

The development of techniques with which to screen for DNA methylation – for example, methylated CpG island amplification (MCA) – has enabled us to analyze the methylation of multiple genes [19]. Using markers identified by MCA, we found a subset of colorectal cancers that showed methylation of multiple CpG islands – that is, the CpG island methylator phenotype (CIMP) [20]. Furthermore, there was a significant association between hMLH1 methylation and CIMP, suggesting that MSI-positive colorectal cancer may be caused by CIMP [20]. To investigate the interaction between genetic and epigenetic factors in colorectal cancer, we first examined the rates of *K-ras* and *p53* mutation in tumors with and without CIMP. We found that CIMP cancers were characterized by a high frequency of *K-ras* mutations and a low frequency of *p53* mutations [21]. Moreover, the interactions between CIMP and *K-ras* and *p53* mutations were preserved in colorectal adenomas, suggesting that they occur early during carcinogenesis [21]. Recently, Shen *et al.* reported that colorectal cancers can be divided into three distinct subgroups based on differences in their CIMP status (Figure 3, [22]): CIMP1 cases have a high frequency of MSI and *BRAF* mutations; CIMP2 cases have with a high frequency of *K-ras* mutations, but not

MSI or *BRAF* mutations; and CIMP-negative cases have a higher frequency of p53 mutations, but not MSI, *K-ras* or *BRAF* mutations.

A growing number of studies have now confirmed the existence of CIMP and described its unique disease characteristics [23,24]. CIMP-positive cancers tend to occur in older patients, in proximal locations and in female patients [23]. Colorectal cancers with different CIMP statuses appear to have distinct precancerous lesions, including hyperplastic polyps and serrated adenomas for CIMP1 and villous adenomas for CIMP2 [25,26]. CIMP status also affects patient survival. For example, patients with CIMP1 have a good prognosis because their disease consists mostly of MSI-H cancers. On the other hand, Barault *et al.* showed that CIMP status was an independent predictor of a poor prognosis in microsatellite stable colorectal cancer [27]. The unique characteristics of CIMP-positive tumors have also been reported in other malignancies. For example, Terada *et al.* reported that breast cancers with CIMP show a significantly higher rate of HER2 amplification [28], while gastric cancers with high levels of methylation rarely show mutations of *K-ras* or *p53* [29]. In general, methylation of multiple CpG islands is associated with a poor prognosis in patients with multiple malignancies – for example, acute leukemia with lung cancer and prostate cancer with esophageal cancer [30].

There is a tight link between CIMP and *K-ras/BRAF* mutations, which makes it reasonable to speculate that mutations in the K-ras/BRAF pathway may induce aberrant methylation in cancer. In fact, Ras signaling has been shown to activate effectors mediating epigenetic silencing, including DNMT1, which plays a key role in cellular transformation [31,32]. This suggests that *K-ras/BRAF* activation may be involved in the CIMP phenotype through activation of the DNA methylation machinery. Alternatively, genes affected by CIMP may be involved in the cellular transformation associated with K-ras/BRAF activation. Activation of the K-ras/BRAF pathway induces senescence in cultured primary human cells. In addition, although *K-ras/BRAF* mutations are frequently seen in early lesions of the colon, these tumors undergo senescence. It has therefore been suggested that genes involved in the induction of senescence by K-ras/BRAF are altered during the progression of tumors [33]. For example, several genes involved in Ras-mediated senescence are inactivated by DNA methylation. Thus, colorectal cancers with CIMP may escape

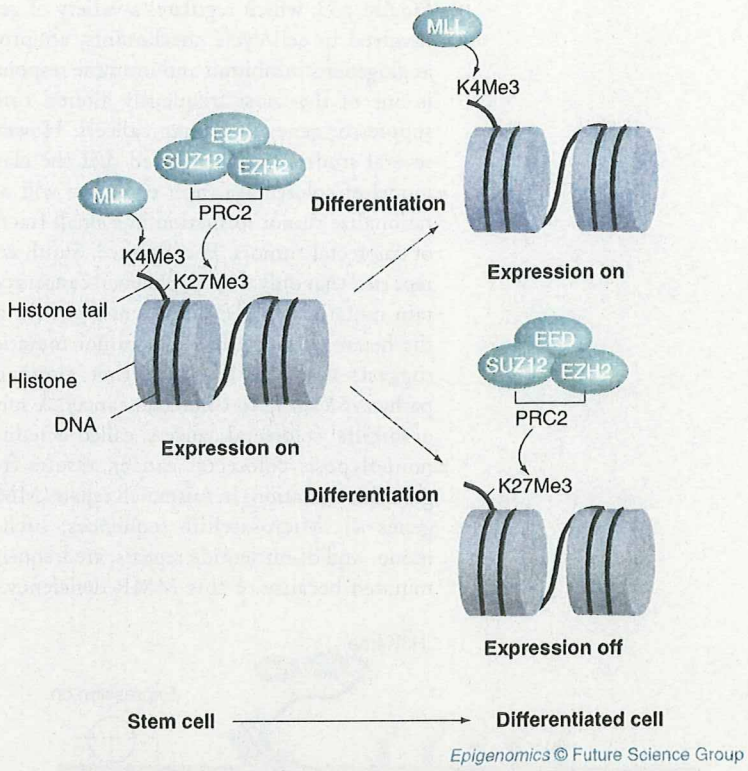



**Figure 2. The role of PRC2 during development.** In pluripotent embryonic stem cells, the promoter regions of some genes are modified to show methylation of histone H3 lysines 4 and 27. After differentiation, methylation of histone H3 lysine 4 is associated with gene expression, and methylation of histone H3 lysine 27 is associated with gene repression.
MLL: Mixed lineage leukemia.

senescence by both activating oncogenic signaling (e.g., *BRAF* mutations) and inactivating regulators of senescence (e.g., p16 methylation).

The interaction between CIMP and APC mutations is not as well established as that between CIMP and the K-ras/BRAF pathway, though it is known that the presence of APC mutations is inversely correlated with the presence of *BRAF* mutations and CIMP [34]. Because CIMP tumors with *BRAF* mutations show large numbers of methylated CpG islands, one might expect WNT signaling to be defective in these tumors due to epigenetic inactivation of multiple WNT-regulating genes [35,36].

## Role of DNA methylation in chromosomal instability

Changes in genome integrity result in large chromosomal gains or losses, a phenomenon termed chromosomal instability (CIN). The integrity of chromosomes is maintained by mitotic checkpoints, which are an important cell cycle control mechanism that protects

against chromosome missegregation and cellular aneuploidy. Such chromosomal aberrations are caused by genetic alterations of genes involved in mitotic checkpoints, including hBub1 and hCDC4 [37,38]. However, mutation of these genes is not frequently seen in cancer, so the causes of CIN remain largely unknown. Recently, an inverse relationship between CIMP and CIN was reported [39,40]. Using a DNA chip that covered entire chromosomes at high resolution, Cheng *et al.* examined the association between CIN and CIMP [40]. They found that CIMP-positive tumors generally possess fewer chromosomal aberrations. In addition, Derks *et al.* reported that DNA methylation of multiple genes is correlated with amplification of 8q23-qter, indicating a linkage between genes located in this region (e.g., c-myc) and CIMP [41]. The majority of cancers have only one genetic instability, MSI or CIN. Most sporadic colorectal cancers with MSI overlap with CIMP, and the inverse relation between CIMP and CIN may reflect this association between MSI and CIMP. Alternatively, CIN may correlate with low levels of methylation. It was recently reported that levels of LINE1 methylation are significantly reduced in colorectal cancer without MSI [42], and that genome-wide demethylation is linked to chromosomal instability in colorectal cancer [43]. This is consistent with the findings that a global loss of methylated cytosines is one of the earliest epigenetic alterations in colorectal cancer [11], and that low levels of DNMT1 induce a loss of heterozygosity at the Apc locus in mice [44]. Further study will be necessary to clarify the relation between DNA methylation and chromosomal abnormalities in cancer.

## Involvement of pathogens in epigenetic alterations in gastric cancer

The causes of aberrant DNA methylation in cancer remain largely unknown. Cancers related to viral infections, including hepatocellular cancer [45], cervical cancer [46] and adult T-cell leukemia [47], all show methylation of multiple CpG islands. However, among the pathogen-associated cancers, the link between infection and epigenetic alterations is best characterized in gastric cancer [29,48–50]. In gastric cancer, mutations in

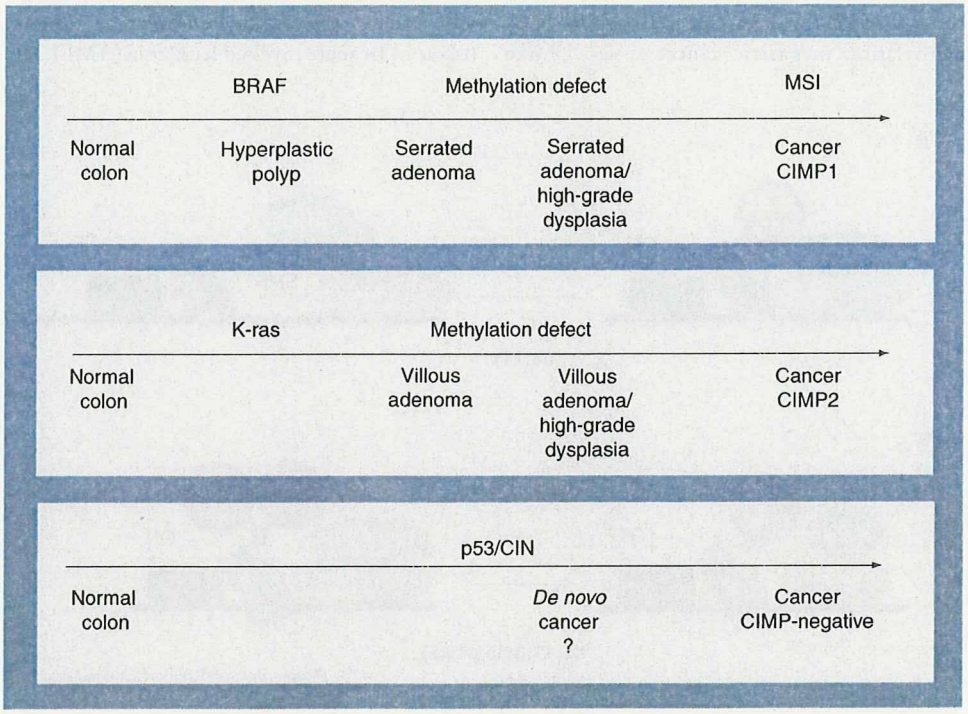



**Figure 3. Three pathways mediating colorectal tumorigenesis.** Based on the CIMP status, the development of colorectal cancer can proceed along three distinct pathways: CIMP1 tumors show a high degree of DNA methylation, microsatellite instability due to methylation of *hMLH1* and *BRAF* gene mutations; CIMP2 tumors show heterogeneous DNA methylation and frequent *K-ras* mutations; and CIMP-negative tumors show frequent *p53* mutations. These three types of tumors have distinct precursors: serrated adenoma for CIMP1, villous adenoma for CIMP2 and tubular adenoma for CIMP-negative.
CIMP: CpG island methylator phenotype; CIN: Chromosomal instability; MSI: Microsatellite instability.

oncogenes and tumor suppressor genes are rarer than in colorectal cancer, although DNA methylation leads to the silencing of numerous genes. Infectious pathogens such as *Helicobacter pylori* and Epstein–Barr virus (EBV) have been implicated in the altered methylation seen in gastric cancer. For instance, methylation levels in the gastric mucosa of individuals with a history of *H. pylori* infection correlate with gastric cancer risk [49]. In addition, enlarged-fold gastritis, which is caused by *H. pylori*, is associated with an increased risk for gastric cancer, and the gastric mucosa in these patients show high levels of promoter methylation and hypomethylation of LINE1 [50]. The molecular mechanisms by which *H. pylori* and other pathogens induce DNA methylation remain unknown. It is known, however, that the chronic gastritis caused by *H. pylori* is associated with mucosal cell proliferation and cytokine expression. On the other hand, the expression levels of three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) are unaffected [51], indicating that other factors are likely involved in the methylation changes seen in gastric cancer.

It was also recently reported that there is a link between EBV infection and changes in DNA methylation in gastric cancer [29,48]. Unlike colorectal cancer with CIMP, gastric cancers with EBV infection rarely show MSI [29]. Gastric cancers with EBV also do not show mutations of *K-ras* or *p53*, but they do show methylation of multiple genes [29]. Similar EBV-associated aberrant methylation of multiple genes is also seen in nasopharyngeal cancers [52]. Several lines of evidence suggest that EBV induces LMP1-mediated expression of DNMT1 [53]. In nasopharyngeal carcinoma, for example, induction of DNMT1 by LMP1 is caused by activation of c-Jun NH2-terminal kinase (JNK)-activator protein (AP)-1 signaling [54]. EBV-associated aberrant methylation in cancer thus appears to be a good model with which to gain insight into the molecular mechanisms underlying the altered DNA methylation in cancer, and may facilitate the development of new therapies.

## Epigenetic alterations caused by translocation in leukemia

The proteins involved in the epigenetic regulation of gene expression are often impaired in cancer. For example, the chromosomal translocations seen in leukemia often lead to epigenetic alterations of genes, although such alterations are rarely found in common solid tumors (Figure 4). In acute myeloid leukemia (AML), for

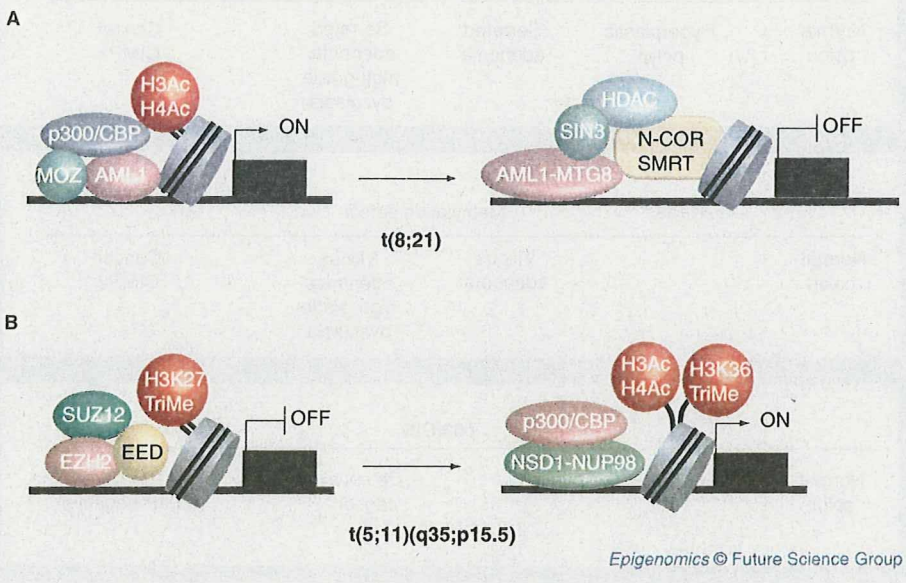



**Figure 4. Model of aberrant transcription caused by translocations in leukemia.**
**(A)** AML–MTG8 fusion protein in AML with t(8;21). AML-MTG8 lacks the p300 binding domain of AML1, which causes HAT activity to be replaced by HDAC activity within the complex. This in turn leads to silencing of genes involved in the differentiation of hematopoietic cells. **(B)** NUP98–NSD1 fusion protein in AML with t(5;11)(q35;p15.5). Histone H3K9 methylation by EZH2 prevents expression of genes such as HOXA9 in differentiated hematopoietic cells. NUP98-NSD1 transactivates HOXA9 via methylation of histone H3K36 and acetylation of histones H3 and H4.
HDAC: Histone deacetylase.

example, a protein complex that induces differentiation of hematopoietic cells is disrupted by translocations such as t(8;21), which generates AML1 (RUNX1)-MTG8 fusion [t(3;21)], leading to the expression of AML1-MTG8 fusion proteins [55]. These fusion proteins then act as dominant negative forms of the core transcription complex. AML1-MTG8 represses genes usually activated by AML1, including *FMS*, *p14ARF* and *C/EBPα* through recruitment of corepressor complexes, including HDAC [56–58]. AML1-MTG8 can also recruit DNMT1, thereby prolonging epigenetic silencing of target genes [59].

In normal hematopoiesis, expression of *HOXA7*, *HOXA9* and *HOXA10* promotes self-renewal. The downregulation of these genes coincides with terminal differentiation, and overexpression of HOXA loci is frequently observed in AML. Chromosomal translocations involve mixed lineage leukemia (MLL), a H3K4 histone methyltransferase that positively regulates gene expression. Leukemogenic MLL fusion proteins delete MLL SET domain lysine 4 methyltransferase activity and fuse MLL to a variety of translocation partners. For example, the MLL-CBP fusion protein affects genes by directly targeting histone acetyltransferase [60], while MLL-AF10 recruits hDotL1 histone methyltransferase to targets [61]. These fusion proteins also play key roles in the activation of HOXA clusters. In addition, approximately 5% of AMLs harbor the t(5;11)(q35;p15.5) translocation, which generates the NUP98–NSD1 fusion protein [62]. NUP98–NSD1 upregulates expression of the HOXA cluster through the methylation of H3K36 and the prevention of H3K27 methylation by EZH2 [63]. Thus, many of the translocations seen in leukemia lead to the creation of fusion proteins that aberrantly modify the histone tail, leading to dysregulation of gene expression. This suggests that these histone-modifying enzymes could be effective targets of therapy.

## Genome-wide methylation analysis

Recent progress in microarray-based techniques has enabled analysis of gene methylation on a genome-wide scale. Two basic approaches are used to prepare microarray probes: methylation-sensitive enzyme digestion followed by adaptor ligation and PCR amplification [64], and immunoprecipitation of methylated DNA using an antibody specific for methylcytosine or methyl-CpG binding protein [65,66]. With microarray-based methylation analysis, the methylation profiles of thousands of genes become available. For instance, by applying a MCA microarray (MCAM) to hepatocellular cancer (HCC), Gao *et al.* assessed the methylation status of approximately 6500 CpG islands in HCC and liver cirrhosis [67]. They found that HCCs arising from liver cirrhosis had significantly more methylation than those arising from chronic hepatitis. In addition, Kuang *et al.* used MCAM to examine the methylation profile in acute lymphocytic leukemia. They found that 404 genes were hypermethylated in acute lymphocytic leukemia and that patients with methylation of multiple CpG islands had a poorer overall survival rate [68]. MCA can also be applied to bacterial artificial chromosome (BAC)-arrays (BAC array-based MCA [BAMCA]). For instance, Arai *et al.* examined the methylation profiles of precancerous regions and clear-cell renal cancers using BAMCA [69]. They found that clinicopathologically aggressive clear-cell renal cancers show methylation profiles that are distinct from less aggressive cancers. They suggested that alteration of DNA methylation during a precancerous stage may generate more malignant clear cell renal cancers and determine patient outcome. In addition, Deng *et al.* performed a massive methylation analysis using DNA prepared using a padlock probe [70]. By using 30,000 probes to examine 66,000 CpG sites in 2020 CpG islands, they assessed the methylation status of human fibroblasts and induced pluripotent stem cells. They found that 288 regions were differentially methylated in the two cell types. We anticipate that the next generation of sequencers will significantly accelerate the exploration of genome-wide methylation.

Large-scale sequencing of cancer genomes has revealed that large numbers of genes undergo somatic mutations in human cancer [71,72]. However, concurrent mutational and methylation studies have been carried out on only a limited scale. Chan *et al.* examined the methylation status of 189 CAN genes, which are mutated in colorectal and breast cancers [73]. By analyzing 36 common targets of mutation in colorectal and breast cancer, they found that 18 genes were silenced by DNA methylation in primary cancers. They also showed that a subset of genes targeted by both genetic and epigenetic events are useful predictors of a poor prognosis.

## Future perspective

Recent studies have shown that there are multiple levels of genetic alterations (e.g., those affecting nucleotides or chromosomes) and epigenetic

alterations (e.g., DNA methylation, histone modification or alteration of chromatin structure) in cancer [74]. Moreover, the heterogeneity of these changes represents a major obstacle to full understanding of the mechanisms underlying cancer development. In this regard, there are three key questions that should be addressed in the future:

- What is the meaning of similar epigenetic alterations in different genome systems?
- What is the relationship between methylation of a specific gene and overall genome dynamics?
- Can individual cells within heterogeneous cell populations be studied for their epigenetic profile?

In addition, there are several potential clinical applications for the integrated analysis of genetic and epigenetic alterations in cancer. Genetic and epigenetic alterations in cancer can provide information useful for selecting appropriate treatments for patients diagnosed with cancer. Moreover, gene mutations and DNA methylation reportedly influence the sensitivity to chemotherapeutic drugs and could serve as molecular markers for predicting the responsiveness of tumors to chemotherapy. However, comprehensive analysis of the pharmacoepigenomics awaits the advent of genome-wide analysis of DNA methylation using microarrays and next-generation sequencers.

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## Executive summary

***CpG island methylator phenotype***

- Cancer with simultaneous methylation of multiple CpG islands.

***Genetic & epigenetic interactions in cancer***

- Colorectal cancers with CpG island methylator phenotype show distinct genetic alterations, including microsatellite instability, BRAF and K-ras mutations and infrequent p53 mutations.

***Chromosomal translocations & histone modifications***

- Chromosomal translocations found in leukemia often cause aberrant histone modification by creating fusion genes that abolish normal histone-modifying activity.

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- •• of considerable interest

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# Sensitive and Specific Detection of Early Gastric Cancer with DNA Methylation Analysis of Gastric Washes

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***Background & Aims:*** **Aberrant DNA methylation is an early and frequent process in gastric carcinogenesis and could be useful for detection of gastric neoplasia. We hypothesized that methylation analysis of DNA recovered from gastric washes could be used to detect gastric cancer.** ***Methods:*** **We studied 51 candidate genes in 7 gastric cancer cell lines and 24 samples (training set) and identified 6 for further studies. We examined the methylation status of these genes in a test set consisting of 131 gastric neoplasias at various stages. Finally, we validated the 6 candidate genes in a different population of 40 primary gastric cancer samples and 113 nonneoplastic gastric mucosa samples.** ***Results:*** **Six genes (*MINT25, RORA, GDNF, ADAM23, PRDM5, MLF1*) showed frequent differential methylation between gastric cancer and normal mucosa in the training, test, and validation sets. GDNF and MINT25 were most sensitive molecular markers of early stage gastric cancer, whereas PRDM5 and MLF1 were markers of a field defect. There was a close correlation ($r = 0.5–0.9$, $P = .03–.001$) between methylation levels in tumor biopsy and gastric washes. MINT25 methylation had the best sensitivity (90%), specificity (96%), and area under the receiver operating characteristic curve (0.961) in terms of tumor detection in gastric washes.** ***Conclusions:*** **These findings suggest MINT25 is a sensitive and specific marker for screening in gastric cancer. Additionally, we have developed a new method for gastric cancer detection by DNA methylation in gastric washes.**

Gastric cancer is the second-leading cause of cancer death in the world. Its prognosis is determined by clinical stage at diagnosis and treatment.[1–3] Diagnostic tools such as gastrointestinal (GI) endoscopy followed by pathologic analysis or fluoroscopy or both have proven useful; however, the mortality rate has remained high throughout the world. The sensitivity and specificity of GI endoscopy is high, but its diagnostic power depends on the technical skill of the endoscopist. Endoscopic biopsy is a topical procedure whereby only a small portion of abnormal tissue is removed. It can be difficult to determine which tissue layer to remove which occasionally leads to a misdiagnosis. Moreover, GI endoscopy is neither comfortable nor risk free for patients, and it is associated with frequent morbidity. Furthermore, gastric cancer is more prevalent among elderly patients, who are likely to be taking medications such as antiplatelet or anticoagulant drugs, which further complicates the procedure.

The need for less-invasive and more-efficient diagnostic tools has led to a search for gastric cancer antigens.[4,5] However, we now know that common biomarkers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are not found frequently enough to yield high specificity or sensitivity assays. Molecular markers that distinguish benign from clinically silent malignant disease are needed to reduce the number of unnecessary endoscopic biopsies and to increase power for early-stage detection of gastric dysplasia and early gastric cancer.

Cytosine DNA methylation is an important epigenetic change which leads to the recruitment of transcription repressors and chromatin changes. During the development and progression of gastric cancer, many genes are silenced by aberrant methylation of cytosine-phosphate-guanosine (CpG) islands (CGIs), which are CpG dinucleotide-rich areas located within the promoters of approximately 60% of human genes.[6] Aberrant DNA methylation occurs more frequently than mutations in gastric cancer.[7–13] Studies have detected cancer-specific DNA methylation in stool, blood plasma, urine, and pancreatic juice in several different cancers.[14,15] Furthermore, concordant promoter hyper-

*Abbreviations used in this paper:* 5-aza-dC, 5-aza-2′-deoxycytidine; ADJ, normal tissue adjacent to tumors; ANOVA, analysis of variance; bp, base pair; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CGI, CpG island; CpG, cytosine-phosphate-guanosine; EN, stomach mucosal tissue in endoscopically normal patient; EW, stomach wash sample in endoscopically normal patient; GI, gastrointestinal; PCR, polymerase chain reaction; ROC, receiver operating characteristic; T, tumor tissue in patient with gastric cancer; W, stomach wash sample in patient with gastric cancer.


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methylation of multiple genes, which is described as the "CpG island methylator phenotype," has been found in both gastric and colorectal carcinomas.[16-21] Therefore, these epigenetic methylation markers could be useful for detecting gastric cancer. It has also been proposed that DNA methylation analysis could be useful to detect field cancerization in this disease.[22-24]

Because many mucosal cells can be found in stomach juice, the detection of molecular markers in stomach juice is a possible noninvasive approach to screening for gastric cancer. However, because of the secretion of hydrochloric acid from parietal cells in gastric mucosa, stomach juice is strongly acidic, with a pH $< 3$. It is difficult to use cells from gastric juice for molecular studies because of the DNA damage caused by this acidity.[25] One alternative to stomach juice is the use of gastric washes for molecular analysis. Endoscopists obtain washes for analysis by washing with a saline solution around the stomach mucosa during routine endoscopic examination. Moreover, given that cells exfoliate abundantly into the gastric washes and that undamaged DNA recovered from the washes can be assayed with sensitive and quantitative techniques, there is a strong biologic rationale to pursue this emerging technology.

Here, we identified 6 methylation markers for the detection of early gastric neoplasia. Applying this to DNA from gastric washes, we found a high sensitivity and specificity in detecting gastric cancer.

## Materials and Methods

### *Cell Lines*

Seven gastric cancer cell lines (Snu1, Snu5, AGS, MKN7, MKN74, MKN45, KatoIII) were obtained from the American Type Culture Collection (Manassas, VA) or the Japanese Collection of Research Bioresources (Tokyo, Japan). To analyze restoration of each gene expression, cell lines were incubated for 96 hours with 1 $\mu$mol/L 5-aza-2′-deoxycytidine (5-aza-dC), after which they were harvested and their RNA was extracted for further analysis.

### *Patient Characteristics and Sample Collection*

Training and test sets of gastric tumor tissues were collected from Korean patients who underwent endoscopic or surgical resection of gastric dysplasia or cancer at Yonsei University Wonju Christian Hospital (Wonju, Korea) from January 2000 to December 2004. A total of 131 tumor tissue samples from 131 patients (Table 1*A*) were randomly collected and examined; 40 gastric dysplasias, 48 early gastric cancers, and 43 advanced gastric cancers. Among 78 surgically resected cancer patients, 64 matched adjacent gastric samples found not to contain cancer cells and $\geq 2$ cm distant from the lesion were also used for methylation analysis. In addition to the tumor samples, nonneoplastic gastric mucosal samples from 22 age-matched patients who underwent surgery for peptic ulcer disease during the same period were also obtained for the analysis. Serial sections from paraffin-embedded tissue blocks were obtained and used for both histologic diagnosis and tissue DNA extraction in all cases. For all cases, the cancer epithelial compartments were needle microdissected from 10-$\mu$m thick sections. Microdissected areas were assessed by an expert pathologist (M-Y.C.) to estimate the percentage of gastric tumor cells in each preparation, and the microdissected areas of the gastric tumors were estimated to contain $> 70\%$ cellularity.

For the validation set (Table 1*B*), we collected tumor tissues (T), adjacent normal tissues (ADJ), and stomach wash samples (W) from October 2005 to September 2006 in a different population of patients (Japanese) with localized gastric cancer. All patients had never been treated for their cancer at the time of sample collection at St. Marianna University School of Medicine Hospital (Kanagawa, Japan). In addition, we collected stomach mucosal tissues (EN) and stomach wash samples (EW) from endoscopically normal patients who were undergoing endoscopy at the same hospital. No significant differences in age and sex were seen between the patients with gastric cancer and the endoscopically normal subjects. This study protocol was approved by the institutional review board of both Yonsei University Wonju Christian Hospital and St. Marianna University School of Medicine, and informed consent was obtained from each patient.

To obtain gastric washes, patients were required to swallow a liquid solution (100 mL of water containing 80 mg of dimethylpolysiloxane [Gascon: Kissei Pharmaceutical Co, Ltd, Matsumoto, Japan], 1 g of sodium bicarbonate, and 20,000 units of pronase [Pronase MS; Kaken Pharmaceutical Co, Ltd, Tokyo, Japan]) approximately 10 minutes before endoscopic examination. After local anesthesia had been administered, the endoscope was inserted into the stomach through the esophagus, and the effect of premedication with pronase on the visualization of the gastric mucosal wall was assessed. During endoscopic examination, the endoscopist washed the stomach wall with a washing solution of 5% Gascon in water. Wash solution was applied to the entire stomach wall, with no exclusive focus on areas that appeared abnormal. Gastric washes were aspirated through the suction channel of the endoscope into specimen collection containers (No. 16200BZZ00045; Nippon Sherwood, Tokyo, Japan). The specimen collection container was directly connected to the endoscope modulator, and the washes were vacuumed manually. The samples were immediately centrifuged, and the pellets were frozen at $-80°$C. DNA was extracted with the use of the standard phenol-chloroform method.

After the collection of gastric washes, biopsies were performed with biopsy forceps (Radial Jaw; Boston Sci-

**Table 1.** Clinicopathologic Characteristics of Patients and Controls Studied

| | A. Test set | | | |
|---|---|---|---|---|
| | Nonneoplastic (n = 22) | Dysplasias (n = 40) | Early cancer (n = 48) | Advanced cancer (n = 43) |
| Age, mean ± SD, y[a] | 61 ± 14 | 65 ± 8 | 63 ± 12 | 60 ± 11 |
| Sex, n[a] | | | | |
| Male | 18 | 31 | 35 | 32 |
| Female | 4 | 9 | 13 | 11 |
| Location, n | | | | |
| Proximal[b] | 1 | 4 | 14 | 22 |
| Distal[b] | 21 | 36 | 34 | 21 |
| Endoscopic findings, n | | | | |
| Protruded | — | 5 | 2 | — |
| Flat | — | 33 | 11 | — |
| Depressed | — | 2 | 35 | — |
| Bormann type 1 | — | — | — | 3 |
| Bormann type 2 | — | — | — | 12 |
| Bormann type 3 | — | — | — | 25 |
| Bormann type 4 | — | — | — | 3 |
| Histologic grades, n | | | | |
| Low-grade dysplasia | — | 19 | — | — |
| High-grade dysplasia | — | 21 | — | — |
| Differentiation, n | | | | |
| Well or moderate | — | — | 25 | 10 |
| Poorly or signet-ring cell type | — | — | 23 | 33 |
| Lauren classification, n | | | | |
| Intestinal | — | — | 40 | 30 |
| Diffuse | — | — | 8 | 13 |

| | B. Validation set (gastric washes) | |
|---|---|---|
| | Nonneoplastic (n = 48) | Gastric cancer (n = 20) |
| Age, mean ± SD, y[b] | 66 ± 20 | 65 ± 18 |
| Sex, n[b] | | |
| Male | 20 | 13 |
| Female | 28 | 7 |
| Location, n | | |
| Proximal[b] | — | 11 |
| Distal[b] | — | 9 |
| Tumor size < 20 mm, n | — | 4 |
| Lauren classification, n | | |
| Intestinal | — | 10 |
| Diffuse | — | 10 |
| UICC clinical stage, n | | |
| Stage I | — | 6 |
| Stage II | — | 4 |
| Stage III | — | 9 |
| Stage IV | — | 1 |

Note: UICC indicates International Union Against Cancer.
[a]Mean age and sex were not different among 4 groups.
[b]Proximal, cardia, fundus, and body; distal, angle, and antrum.

entific Corp, Natick, MA) under endoscopic guidance with a GIF-Q240 endoscope with the use of the EVIS LUCERA system (Olympus, Inc, Tokyo, Japan). Mucosal samples of the gastric body 5 mm in diameter were collected for biopsy. In the endoscopically normal subjects, endoscopic biopsy was performed at the corpus. In patients with gastric cancer, 2 biopsy samples were collected per site of cancer and adjacent tissue.

The concentration and quantity of all DNA extracted from biopsied tissues and gastric washes were measured with the NanoDrop spectrophotometer (ND-1000 Spectrophotometer; Nano Drop Technologies, Wilmington, DE).

### *Endoscopic and Histopathologic Analysis of Gastric Neoplasia*

The endoscopic appearance of gastric dysplasia was classified on the basis of gastroscopic findings, as protruded-type (0-I), flat-type (IIa or IIb) or depressed-type (IIc, IIc + IIa, or III). Early gastric cancer was defined

by a depth of tumor invasion limited to the submucosal layer of the stomach regardless of the presence of lymph node involvement. Advanced gastric cancer was classified endoscopically with the Borrmann classification. All resected gastric neoplasias were diagnosed histologically by a pathologist (M.-Y.C.) according to the World Health Organization classification (Supplementary Figure 1).

### *Bisulfite Polymerase Chain Reaction and Pyrosequencing Analysis of DNA Methylation*

Bisulfite treatment of gDNA was performed with an EpiTect bisulfite kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Bisulfite-treated DNA (1 $\mu$L) was used as a template in subsequent polymerase chain reaction (PCR). All of the primers and PCR conditions used for amplifying promoter CpG DNA fragments of candidate methylation marker genes are listed in Supplementary Table 1*A*. For most assays, we used touchdown PCR. All PCR assays included a denaturation step at 95°C for 30 seconds, followed by an annealing step at various temperatures for 30 seconds, and an extension step at 72°C for 30 seconds. After PCR, the biotinylated strand was captured on streptavidin-coated beads (Amersham Bioscience, Uppsala, Sweden) and incubated with sequencing primers (Supplementary Table 1*B*). Pyrosequencing was performed with PSQ HS 96 Gold single-nucleotide polymorphism reagents on a PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). The protocol for pyrosequencing has been described in detail previously.[26] Pyrosequencing quantitatively measures the methylation status of several CpG sites in a given promoter. These adjacent sites usually show highly concordant methylation. Therefore, the mean percentage of methylation of detected sites was used as a representative value for each gene promoter.

### *K-ras and p53 Mutation Analysis with the Use of DNA from Tumor and Gastric Washes*

Direct sequencing was conducted to identify mutations in codons 12 and 13 of the K-*ras* gene and in exons 2 through 11 of the p53 gene in T and W samples; primer sequences were obtained from a previous report, with minor modifications.[27] PCR products were directly sequenced in the M.D. Anderson Core Sequencing Facility with the use of the same primers.

### *Reverse Transcription PCR*

First-strand cDNA was prepared by reverse transcription of 5-$\mu$g samples of total RNA with the use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative reverse transcription-PCR was carried out with the use of TaqMan Gene Expression Assays (GDNF, Hs00181185_m1; PRDM5, Hs00924598_m1; ADAM23, Hs01046804_m1; MLF1, Hs0023695_m1; RORA, Hs00536545_m1; and glyceraldehyde-3-phosphate dehydrogenase, Hs_00266705_gl; Applied Biosystems) with a 7500 Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions.[28] SDS2.1 software (Applied Biosystems) was used to do comparative $\Delta$-Ct analysis. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

### *Detection of* Helicobacter pylori *with DNA from Gastric Washes*

PCR was used to evaluate the presence of *ureA*, to show *H. pylori* infection, with the use of HPU1 and HPU2 primers.[29] PCR for *cagA* was performed to type the *H. pylori* strains, with the use of cagA1 and cagA2 primers.[30]

### *Selection of Candidate Genes in Gastric Cancer for Methylation Analysis*

We first selected a total of 51 candidate genes. Eight of the 51 genes were identified as hypermethylated in multiple cancers by methylated CGI amplification and microarray[31] or methylated CGI amplification coupled with representational difference analysis (MCA − RDA).[32] In addition, we identified from a literature search 43 genes that were described as potential methylated tumor suppressor genes in gastric cancer cells or tissues.

### *Statistical Analysis*

All statistical analyses were performed with SPSS for Windows, version 12 (SPSS, Inc, Chicago, IL) and PRISM software for Windows, version 4 (GraphPad Prism, Inc, San Diego, CA). Methylation level (in %) was analyzed as a continuous variable for comparison of each gene with clinicopathologic features; means and 95% confidence intervals were calculated. Comparisons of categorical variables were made using the chi-square test and Fisher's exact test when appropriate. Associations between continuous variables or levels of promoter methylation and clinicopathologic variables were evaluated with the use of analysis of variance (ANOVA) and Student's $t$ test. In parallel, we computed the median DNA methylation value and range for each sample, and we defined the receiver operating characteristic (ROC) curve in SPSS software. The $z$ score analysis was used to normalize the methylation levels of several genes in each sample. The $z$ score for each gene was calculated as follows: $z$ score = (methylation level of each sample − mean value of methylation level)/standard deviation of methylation level. In this analysis, a $z$ score $> 0$ means that the methylation level is greater than the mean value for the population. We examined possible correlations between DNA promoter methylation levels and patient age in 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM*, and *MLF1*) found in normal-appearing mucosa, by calculating Spearman's nonparametric correlation coefficients ($r$ and $P$, respectively). All reported $P$ values were 2-sided, and $P < .05$ was considered statistically significant.

**Table 2.** Methylation Status of the 6 Differentially Methylated Markers According to Gastric Neoplastic Progression

| Methylation, % | NGM (n = 22) | ADJ (n = 64) | GD (n = 40) | EGC (n = 48) | AGC (n = 43) | Cutoff[a] | *P* value[b] |
|---|---|---|---|---|---|---|---|
| *MINT25* | | | | | | | |
| Methylation, mean (95% CI) | 5.9 (2.7–9) | 16.7 (7.2–6.3) | 67.4 (57.2–77.5)[c] | 58.6 (45.3–71.8)[c] | 60.3 (49.9–70.7)[c] | 17.2 | < .001 |
| Frequency | 6.7 | 23.3 | 89.7 | 77.8 | 84.4 | | |
| *RORAa* | | | | | | | |
| Methylation, mean (95% CI) | 6.4 (5.3–7.6) | 9.7 (7.4–12.0)[d] | 36.8 (31.7–41.9)[c] | 32.4 (26.1–38.7)[c] | 17.6 (13.0–22.1) | 11.6 | < .001 |
| Frequency | 0 | 23 | 92.5 | 83.3 | 58.1 | | |
| *GDNF* | | | | | | | |
| Methylation, mean (95% CI) | 9.7 (7.9–11.6) | 16.6 (13.1–20.1)[d] | 35.4 (29.2–41.5)[c] | 39.8 (33.8–45.9)[c] | 27 (22.0–32.0)[c] | 17.9 | < .001 |
| Frequency | 4.5 | 31.6 | 76.3 | 84.8 | 74.4 | | |
| *ADAM23* | | | | | | | |
| Methylation, mean (95% CI) | 2.8 (1.2–4.4) | 10.1 (4.9–15.4)[d] | 38.7 (31.0–46.4)[c] | 27.4 (18.0–36.7)[c] | 19.3 (12.0–26.5) | 10.2 | < .001 |
| Frequency | 9.5 | 18.3 | 89.7 | 54.5 | 48.8 | | |
| *PRDM5* | | | | | | | |
| Methylation, mean (95% CI) | 25.1 (18.4–31.7) | 31.8 (24.9–38.8) | 74.3 (64.5–84.1)[c] | 59.4 (48.2–70.6)[c] | 38.8 (29.3–48.2) | 52.6 | < .001 |
| Frequency | 0 | 25.5 | 85.3 | 63.6 | 33.3 | | |
| *MLF1* | | | | | | | |
| Methylation, mean (95% CI) | 11 (8.2–13.7) | 14.2 (11.8–16.6) | 35.8 (29.2–42.3)[c] | 26.5 (19.9–33.0)[c] | 19.4 (14.9–23.9) | 23.5 | < .001 |
| Frequency | 4.5 | 16.7 | 67.5 | 50 | 41.9 | | |

ADJ, normal-appearing gastric mucosa adjacent to gastric cancer; AGC, advanced gastric cancer; EGC, early gastric cancer; GD, gastric dysplasia; NGM, nonneoplastic gastric mucosa.
[a]The cutoff value for the 7 hypermethylated genes was determined according to the mean methylation level of each gene + 2 SDs.
[b]The methylation levels in the 5 groups were compared with ANOVA.
[c]Significantly different from nonneoplastic gastric mucosa in Tukey's multiple comparisons ($P < .05$).
[d]Significantly different from nonneoplastic gastric mucosa in *t* test ($P < .05$).

## Results

### *Clinicopathologic Characteristics of Patients*

Clinicopathologic characteristics of patients with gastric dysplasia, early gastric cancer, or advanced gastric cancer and age-matched controls without neoplasia in the training set and test set are listed in Table 1*A*. Table 1*B* lists the characteristics of patients in the validation set; in those, samples included Tumor tissue (T), normal-appearing tissue adjacent to tumors (ADJ), stomach mucosal tissue in endoscopically normal patients (EN), stomach wash samples in patients with gastric cancer (W), and stomach wash samples in endoscopically normal patients (EW). We observed no significant differences among these groups.

### *Methylation of Selected Markers in Gastric Neoplastic Progression with the Training and Test Sets*

We determined the levels of methylation of all genes in 7 gastric cancer cell lines (AGS, KatoIII, MKN7, MKN45, MKN74, Snu1, and Snu5) and compared them with normal blood DNA obtained from 2 healthy persons. Methylation was determined by bisulfite-pyrosequencing, a quantitative method that yields percentage of methylated alleles in the studied DNA (examples in Supplementary Figure 2). The first criterion for marker selection among the candidate genes was hypermethylation (>15%) in > 3 of the 7 cancer cell lines but a lack of methylation (≤15%) in normal peripheral blood DNA. We found that 51 genes were hypermethylated in > 3 of the 7 cell lines (data not shown). Next, we compared the levels of methylation of the 51 genes in a test set of 13 gastric cancer tissue samples and 11 age- and sex-matched normal gastric mucosa samples (Supplementary Table 2). On the basis of differential methylation, we selected 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*) as potential markers for the detection of gastric neoplasia.

Next, we tested these 6 potential markers with an independent set of gastric neoplasia samples (test set). The results are summarized in Table 2 and Figure 1. Methylation levels increased significantly in gastric neoplastic progression from nonneoplastic gastric mucosa tissue to normal adjacent tissue to tumors and to early gastric cancer samples for all 6 marker genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*; $P < .001$, according to ANOVA). When considering progressive stages, 2 patterns were evident: (1) type 1 markers, showing consistently high levels of methylation in both gastric dysplasia and cancer (MINT25 and GDNF); (2) type 2 markers, showing high levels of methylation in early gastric cancer and gastric dysplasia but decreased levels in advanced gastric cancer (RORA, ADAM23, PRDM5, and MLF1). Of interest, use of the type 2 markers showed higher methylation levels in gastric dysplasia than in

advanced gastric cancer ($P < .001$), which is consistent with our studies in ulcerative colitis and colon cancer.[33]

### *Epigenetic Silencing of Selected in Gastric Cancer Cell Lines*

To examine the expression profile of the candidate marker genes, we initially carried out real-time PCR with cDNA from normal colon and normal stomach tissues and 7 gastric cancer cells (MKN7, MKN74, MKN45, Snu1, Snu5, AGS, KatoIII). We detected expression of *GDNF*, *PRDM5*, *ADAM23*, *MLF1*, and *RORA* in both normal colon and stomach tissues; conversely, we detected weak expression of these genes in gastric cancer cells and also an inverse correlation of expression with DNA methylation level. MINT25 is an alternatively spliced form of the *CABIN1* gene and was therefore not studied. Treating those 7 cell lines with the DNA methyltransferase inhibitor 5-aza-dC restored silenced gene expression (Supplementary Figure 3).

### *DNA Methylation Levels of the 6 Genes in the Validation Set*

To analyze the potential of these genes in screening for gastric cancer, we tested a validation set that also included gastric washes. We first measured DNA concentrations in T, N, W, EN, and EW samples. Gastric washes consistently yielded more DNA than did tissue biopsy samples from the same patients (Supplementary Figure 4). To test the quality of recovered nucleic acids, we ran the DNA on 1% agarose gels and found higher molecular weight (intact) DNA in gastric washes from some patients with cancer than from healthy controls (Supplementary Figure 5). DNA spectrophotometer analysis also showed better quality of DNA from washes from patients with cancer. Next, we stained gastric washes, and we could see both cancer cells and normal cells in the washes (cancer cells were stained by CEA). Many cells were already breaking down in the gastric washes. Finally, we tested by PCR the quality of DNA and found that it was more difficult to amplify DNA from controls than from patients with cancer, especially with fragments more than 200 base pairs (bp) (Supplementary Figure 5).

We next measured DNA methylation levels of the 6 genes (*MINT25*, *RORA*, *PRDM5*, *MLF1*, *ADAM23*, and *GDNF*) by pyrosequencing analysis in 153 validation set samples: T (20), ADJ (17), W (20), EN (48), and EW (48). The *z* scores of these 6 genes were significantly different in T and EN samples (Figure 1*B*). The difference in methylation densities between T and ADJ ($P < .0001$), as well as between W and EW ($P < .0001$), were highest in MINT25. The methylation levels of the genes tested in T samples did not differ significantly according to sex, age, or tumor stage except that methylation levels of *MLF1* and *ADAM23* showed a positive correlation with age (*MLF1*: $r = 0.5$, $P = .001$; *ADAM23*: $r = 0.3$, $P = .05$).

The DNA methylation densities of *PRDM5* and *MLF1* were significantly different between T and EN ($P < .0001$) and between W and EW ($P < .0001$) samples. However, the results did not show a significant difference between T and ADJ samples ($P = .16$). Moreover, DNA methylation densities in ADJ samples were significantly higher than methylation densities in EN samples ($P < .0001$), suggesting that these 2 genes are potential markers of an epigenetic field defect (Figure 1*B*).

Correlations in methylation levels between biopsy (T) and gastric wash (W) are shown in Figure 2. The methylation levels of all 6 genes were closely correlated by Spearman's analysis (*MINT25*: $r = 0.7$, $P = .001$; *RORA*: $r = 0.5$, $P = .03$; *PRDM5*: $r = 0.7$, $P < .001$; *MLF1*: $r = 0.9$, $P < .001$; *ADAM23*: $r = 0.7$, $P < .001$; *GDNF*: $r = 0.9$, $P < .001$). These results show that gastric washes closely mirrored gastric biopsy results. We therefore analyzed the sensitivity and specificity of the gastric wash methylation assays statistically with the use of single-gene and multigene panels. Each cutoff value was determined with ROC curves (Figure 3), and sensitivity and specificity were calculated. The best results were 90% sensitivity and 96% specificity with MINT25 alone and 95% sensitivity and 92% specificity with a combination of MINT25, ADAM23, and GDNF (Table 3). Six early-stage gastric cancer samples were included in validation sets. Methylation in gastric washes enabled detection of these cancers in 83.3% of cases (5/6) by MINT25, 66.7% (4/6) by GDNF, and 83.3% (5/6) by PRDM5. These results suggest that DNA from gastric washes can be an appropriate alternative to DNA from biopsied tissue for the determination of methylation status in gastric cancer and to screen for this deadly disease.

### *Genetic Analysis with Biopsy and Gastric Washes in Gastric Cancer*

We checked for the presence of mutations in codons 12 and 13 of the K-*ras* gene and in exons 4 through 10 of the p53 gene in T and W samples. No K-*ras* mutations were detected in codon 12 or 13 in any sample. Mutations of p53 were found in 1 (5%) of 20 T samples and in 1 (5%) of 20 W samples. Both mutations were a 1-bp deletion in exon 10 (1006delG, heterozygous), and the 2 samples were from the same patient.

### *Detection of* H. pylori

*H. pylori* requires urease protein to survive in the stomach flora because it needs protection from the acidic solution secreted by parietal cells. Therefore, we used *ureA* gene detection as a positive control for *H. pylori*. Twenty-six gastric wash samples were found to be *H. pylori* positive, and 42 were *H. pylori* negative. Of the 26 *H. pylori*–positive samples, 12 were from patients with cancer (12/20, 60%) and 14 were from cancer-free persons (14/48, 29%). In addition, 9 subjects were *cagA* positive in