DNA Methylation and Cancer
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ABSTRACT

Abstract

DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. Alterations in DNA methylation are common in a variety of tumors as well as in development. Of all epigenetic modifications, hypermethylation, which represses transcription of the promoter regions of tumor suppressor genes leading to gene silencing, has been most extensively studied. However, global hypomethylation has also been recognized as a cause of oncogenesis. New information concerning the mechanism of methylation and its control has led to the discovery of many regulatory proteins and enzymes. The contribution of dietary folate and methylene tetrahydrofolate reductase polymorphisms to methylation patterns in normal and cancer tissues is under intense investigation. As methylation occurs early and can be detected in body fluids, it may be of potential use in early detection of tumors and for determining the prognosis. Because DNA methylation is reversible, drugs like 5-azacytidine, decitabine, and histone deacetylase inhibitors are being used to treat a variety of tumors. Novel demethylating agents such as antisense DNA methyl transferase and small interference RNA are being developed, making the field of DNA methylation wider and more exciting.


INTRODUCTION

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence. DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target. Epigenetics has evolved as a rapidly developing area of research. Recent studies have shown that epigenetics plays an important role in cancer biology,1,2 viral infections,3 activity of mobile elements,4 somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation.5,6

DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylation occurs in the sequence context 5’CG3’ (also called the CpG dinucleotide), some involves CpA and CpT dinucleotides.7 DNA is made up of four bases, thus there are 16 possible dinucleotide combinations that can occur. Therefore the CpG dinucleotide should occur with a frequency of approximately 6%. However, the actual presence is only 5% to 10% of its predicted frequency.8 This CpG suppression may be related to the hypermutability of methylated cytosine. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions.9 In contrast to the rest of the genome, smaller regions of DNA, called CpG islands, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60% to 70%), have a ratio of CpG to GpC of
at least 0.6, and thus do not show any suppression of the frequency of the dinucleotide CpG. Approximately half of all the genes in humans have CpG islands, and these are present on both housekeeping genes and genes with tissue-specific patterns of expression.

DNA methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3b with its isoforms, and DNMT3L. Methylation can be de novo (when CpG dinucleotides on both DNA strands are unmethylated) or maintenance (when CpG dinucleotides on one strand are methylated). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful de novo methyltransferases. The importance of these enzymes has been shown using several mouse experiments in which the mouse deficient in the gene dies early in development or immediately after birth.

In addition to the DNMTs, the other machinery of methylation includes demethylases, methylation centers triggering DNA methylation, and methylation protection centers. DNA methylation patterns are established early in embryogenesis and are very finely controlled during development. The enzymes that actively demethylate DNA include 5-methylcytosine glycosylase, which removes the methylated cytosine from DNA, leaving the deoxyribose in nucleotide form, and MBD2b, which refers to an isoform that results from initiation of translation at the second methionine codon of the gene encoding methyl-CpG binding domain 2 (MBD2) protein. MBD2b lacks glycosylase or nuclease activity and is thought to cause demethylation by hydrolyzing 5-methylcytosine to cytosine and methanol. However, two independent laboratories have not been able to reproduce these results in mammalian and Xenopus systems.

Earlier it was thought that normal cells become progressively transformed to malignant cells as a consequence of damage to the genome, which could be a gain, loss, or mutation of the genetic information. These events cause critical loss of gene activity and thereby predispose to cancer. DNA methylation can modify the gene activity without changing the gene sequence and has been proposed as one of the two hits in Knudson’s two hits hypothesis for oncogenic transformation. Methylation changes have been implicated in tumorigenesis. Genetic disruption of both DNMT1 and DNMT3b in a colorectal cell line reduced DNA methylation and resulted in the loss of insulin-like growth factor II imprinting, abrogation of silencing of the tumor suppressor gene p16INK4a, and growth suppression.

Multiple intestinal neoplasia mice carry a germ-line mutation of the Dnmt1 gene and treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine could reduce polypt multiplicity in ApcMin−/− mice. Mice deficient in MLH1 (one of the mismatch repair proteins) carrying the hypomorphic Dnmt1 mutation have a reduced incidence of adenomas, whereas the risk of lymphoma in these mice is increased. A less severe disruption of Dnmt1 expression in mice carrying a hypomorphic DNA methyltransferase 1 (Dnmt1) allele reduced Dnmt1 expression to 10% of wild-type levels. This resulted in substantial genome-wide hypomethylation in all tissues, and these mice developed aggressive T-cell lymphomas that displayed a high frequency of chromosome 15 trisomy. Therefore, genomic demethylation may protect against some cancers, such as intestinal tumors in the ApcMin mouse model, but may promote genomic instability and loss of heterozygosity and increase the risk of cancer in other tissues, as seen in hypomethylated mutant mice.

The regulation of eukaryotic gene expression is a complex process. Transcription initiation is a highly controlled and integrated event that involves cis-acting and trans-acting factors. The cis-acting elements are DNA sequences that act as the substrate for the trans-acting factors, and the DNA in the vicinity is prepared for transcription. Increased methylation in the promoter region of a gene leads to reduced expression, whereas methylation in the transcription region has a variable effect on gene expression.

Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NFkB, recognize sequences that contain CpG residues, and binding of each has been shown to be inhibited by methylation.

The second mode of repression involves a direct binding of specific transcriptional repressors to methylated DNA. The DNA methylation signals are analyzed by the methyl-CpG–binding proteins, the target being the 5’ methylated CpG sequence. MeCP1 and MeCP2 were the first two protein complexes identified. However, several new proteins have now been identified. They include MBD1, MBD2, MBD4, and Kaiso. MeCP1, MBD1, MBD2, and MBD4 bind to 5mCpG through a motif called the methyl CpG binding domain (MBD). Kaiso, however, is different in mechanism, as it binds through a zinc finger motif. MBD4 is associated with DNA repair, whereas MBD1, MBD2, MeCP2, and Kaiso have been shown to
repress transcription both in vitro and in cell culture assays by interacting with histone deacetylase complexes.26

DNA methylation can also affect histone modifications and chromatin structure, which, in turn, can alter gene expression. The underlying patterns of methylated cytosines are important in guiding histone deacetylation to certain residues.30 At present, there are five known proteins that have the methyl-CpG–binding domain, and four of these (MeCP2, MBD1, MBD2, and MBD3) are implicated in transcriptional repression.26 Three of these (MeCP2, MBD2, and MBD3) are in complexes (MeCP-2, MeCP-1 and Mi-2, respectively) that contain histone deacetylases. Studies of methylated transfected genes containing binding sites for all four of these methyl-binding proteins have shown at least partial abrogation of transcriptional repression by treatment with the histone deacetylase inhibitor, trichostatin A.

Earlier it was suggested that histone modification was secondary to DNA methylation, but recent studies on fungus revealed that histone modification can on its own commence the process of DNA methylation.31

The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation. A zone of deacetylated histone H3 and methylation of histone H3 at lysine 9 surrounds a hypermethylated, silenced hMLH1 promoter, which, when unmethylated and active, is associated with acetylated H3 and methylation of histone H3 at lysine 4 position. Inhibiting DNA methyltransferases, but not histone deacetylases, leads initially to promoter demethylation, followed by gene re-expression, and finally to complete histone code reversal.32

**DNA METHYLATION AND CANCER**

DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns.33 Hypomethylation usually involves repeated DNA sequences, such as long interspersed nuclear elements, whereas hypermethylation involves CpG islands.34

**Hypermethylation in Cancer**

Reports of hypermethylation in cancer far outnumber the reports of hypomethylation in cancer. There are several protective mechanisms that prevent the hypermethylation of the CpG islands. These include active transcription, active demethylation, replication timing, and local chromatin structure preventing access to the DNA methyltransferase.35 To date, numerous genes have been found to undergo hypermethylation in cancer (Table 1). The genes that are susceptible are the genes involved in cell cycle regulation (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4a}, Rb, p14\textsuperscript{ARF}) genes associated with DNA repair (BRCA1, MGMT), apoptosis (DAPK, TMS1), drug resistance, detoxification, differentiation, angiogenesis, and metastasis (Table 1).

Although certain genes such as RASSF1A and p16 are commonly methylated in a variety of cancers, other genes are methylated in specific cancers. One example is the GSTP1 gene, which is hypermethylated in more than 90% of prostate cancers but is largely unmethylated in acute myeloid leukemia.49,55 The mechanisms involved in targeting of methylation to specific genes in cancer remain to be determined. In one report, the leukemia-promoting PML-RAR fusion protein induced gene hypermethylation and silencing by recruiting DNA methyltransferases to target promoters.63 Interestingly, retinoic acid treatment induced promoter demethylation, gene re-expression, and reversion of the transformed phenotype.

Many tumors show some kind of hypermethylation of one or more genes (Table 1). One of the most detailed studies was conducted on lung cancer, and more than 40 genes were found to have some degree of alteration in DNA methylation patterns. Of the various genes studied, the commonly hypermethylated ones include RARβ, RASSF1A, CDKN2A, CHD13, and APC.64

Hypermethylation results in loss of expression of a variety of genes critical in the development of breast cancer. These include steroid receptor genes, cell adhesion genes, and inhibitors of matrix metalloproteinases.42 Among the genes commonly hypermethylated in breast cancer are the p16\textsuperscript{INK4a}, estrogen receptor (ER) alpha, the progesterone receptor (PR), BRCA1, GSTP1, TIMP-3, and E-cadherin. The steroid receptor genes, ER and PR, have long been associated with breast cancer. Methylation studies of these have shown that the ER gene has a CpG island in its promoter and first exon areas.47 The ER gene is unmethylated in normal cells and in ER-positive cell lines but shows a high degree of methylation in more than half of primary cancers. The BRCA1 gene, located at chromosome 17q21, is one of the more commonly associated genes in breast cancer, and the protein product is reduced or absent. DNA methylation has been proposed as one of the causes of its inactivation.65

Hypermethylation is associated with many leukemias and other hematologic diseases. Many genes, such as the calcitonin gene, p15\textsuperscript{INK4a}, p21\textsuperscript{Cip1/Waf1}, the ER gene, SDC4, MDR, and so on, were seen to be hypermethylated in a variety of hematologic cancers.66 The calcitonin gene and p15 were hypermethylated in 65% of myelodysplastic syndromes, and it was found that p15 methylation at diagnosis was associated with lower survival and transformation to acute myeloid leukemia.67 Also acquisition of p15 methylation at a later date signaled disease progression.67 These may suggest the role of p15 as a marker of leukemic transformation. Acute myeloid leukemia demonstrated frequent hypermethylation of ER, MYOD1, PITX2, GPR37, and SDC4.68 In addition, in 95% of acute leukemias, the calcitonin gene was found to be hypermethylated.66

The
presence of hypermethylation of the \(p21^{Cip1/Waf1}\) gene, a cyclin-dependent kinase inhibitor, and of the calcitonin gene was associated with many cases of acute lymphoblastic leukemia and was also associated with unfavorable clinical outcome.66

**Hypomethylation in Cancer**

Hypomethylation is the second kind of methylation defect that is observed in a wide variety of malignancies.69,70 It is common in solid tumors such as metastatic hepatocellular cancer,71 in cervical cancer,70 prostate tumors,72 and also in hematologic malignancies such as B-cell chronic lymphocytic leukemia.34 The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive increase with the grade of malignancy.34 The pericentric heterochromatin regions on chromosomes 1 and 16 are heavily hypomethylated in patients with immunodeficiency, centromeric instability, and facial abnormalities and in many cancers. A mutation of \(DNMT3b\) has been found in patients with immunodeficiency, centromeric instability, and facial abnormalities, which causes the instability of the chromatin.73,74 Hypomethylation has been hypothesized to contribute to oncogenesis by activation of oncogenes such as \(c\)MYC and \(H-RAS\) or by activation of latent retrotransposons76,77 or by chromosome instability.78 Long interspersed nuclear elements are the most plentiful mobile DNAs or retrotransposons in the human genome. Hypomethylation of these mobile DNAs causes transcriptional activation and has been found in many types of cancer, such as urinary bladder cancer.79 Hypomethylation of the mobile DNA can also cause disruption of

### Table 1. Genes Commonly Methylated in Human Cancer and Their Role in Tumor Development

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role in Tumor Development</th>
<th>Site of Tumor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Deranged regulation of cell proliferation, cell migration, cell adhesion, cytoskeletal reorganization, and chromosomal stability</td>
<td>Breast</td>
<td>36</td>
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<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Esophageal</td>
<td></td>
</tr>
<tr>
<td>BRCA1</td>
<td>Implicated in DNA repair and transcription activation</td>
<td>Breast</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian</td>
<td>39</td>
</tr>
<tr>
<td>CDKN2A/p16</td>
<td>Cyclin-dependent kinase inhibitor</td>
<td>GIT</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Head and neck</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NHL</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>40</td>
</tr>
<tr>
<td>DAPK1</td>
<td>Calcium/calmodulin-dependent enzyme that phosphorylates serine/threonine residues on proteins; Suppression of apoptosis</td>
<td>Lung</td>
<td>43</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Increasing proliferation, invasion, and/or metastasis</td>
<td>Breast</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thyroid</td>
<td>45</td>
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<tr>
<td></td>
<td></td>
<td>Gastric</td>
<td>46</td>
</tr>
<tr>
<td>ER</td>
<td>Hormone resistance</td>
<td>Breast</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate</td>
<td>48</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Loss of detoxification of active metabolites of several carcinogens</td>
<td>Prostate</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal</td>
<td>50</td>
</tr>
<tr>
<td>hMLH1</td>
<td>Defective DNA mismatch repair and gene mutations</td>
<td>Colon</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gastric</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endometrium</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian</td>
<td>53</td>
</tr>
<tr>
<td>MGMT</td>
<td>p53-related gene involved in DNA repair and drug resistance</td>
<td>Lung</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>54</td>
</tr>
<tr>
<td>p15</td>
<td>Unrestrained entry of cells into activation and proliferation</td>
<td>Leukemia</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymphoma</td>
<td>56, 57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squamous cell carcinoma, lung</td>
<td></td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Loss of negative regulator control of cell proliferation through inhibition of G1/S-phase progression</td>
<td>Lung</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ovarian</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nasopharyngeal</td>
<td>60</td>
</tr>
<tr>
<td>Rb</td>
<td>Failure to repress the transcription of cellular genes required for DNA replication and cell division</td>
<td>Retinoblastoma</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oligodendroglioma</td>
<td>62</td>
</tr>
<tr>
<td>VHL</td>
<td>Altered RNA stability through and erroneous degradation of RNA-bound proteins</td>
<td>Renal cell cancer</td>
<td>59</td>
</tr>
</tbody>
</table>

*Abbreviations:* APC, adenomatous polyposis coli; BRCA1, breast cancer 1; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; ER, estrogen receptor; GSTP1, glutathione S-transferase Pi 1; hMLH1, Mut L homologue 1; MGMT, O-6 methylguanine-DNA methyltransferase; RASSF1A, Ras association domain family member 1; Rb, retinoblastoma; VHL, von Hippel-Lindau; GIT, gastrointestinal tract; NHL, non-Hodgkin’s lymphoma.
expression of the adjacent gene as well. The L1 mutational insertions have been found to disrupt the APC gene and the CMYC gene in colon and breast cancers, respectively.4

**Folic Acid, Gene Polymorphism, DNA Methylation, and Cancer**

The role of nutrients in affecting gene expression through interaction with genetic polymorphism and modulation of DNA methylation has received considerable attention recently. The disruption of homeostasis in the vitamin-dependent, one-carbon (methyl group) metabolism affects the risk of heart disease, neural tube defects, and cancer. Such disruption can occur as the result of deficiencies of the two essential micronutrients involved in this metabolism: folate and cobalamin (vitamin B12).

One-carbon metabolism is divided into two main branches: one branch consists of reactions involving purine and thymidine synthesis and the other involves synthesis of methionine and s-adenosylmethionine for protein and polyamine synthesis and methylation reactions. An enzyme that shunts methyl groups from the first of these branches to the second is methylenetetrahydrofolate reductase (MTHFR; Fig 1). MTHFR irreversibly converts 5,10-methylenetetrahydrofolate (5,10-CH$_2$-THF) to 5-methyl-THF, which then donates a methyl group to homocysteine to produce methionine (Fig 1).

Individuals who are severely deficient in MTHFR activity because of a germline mutation have excessive amounts of homocysteine in the blood and urine and develop severe mental retardation and thrombo-occlusive vascular disease.80 Interestingly, there exist two common low-function polymorphic variants of MTHFR: the T variant at nucleotide 677 (MTHFR C677T) and the C variant at nucleotide 1298 (MTHFR A1298C). The first of these variants, C677T, has been shown to be associated with higher baseline homocysteine levels in the serum and is associated with an increased risk of vascular disease and neural tube defects.81 This polymorphism is linked to increased risk for certain cancers, such as endometrial cancer, breast cancer, ovarian cancer,82 esophageal cancer,83 and gastric cancer,84 and decreased risk for others, such as leukemia85 and colorectal cancer.86 The cancer risk associated with MTHFR polymorphisms may be modulated by folate intake.

When folate intake is sufficient, individuals carrying the variant MTHFR genotypes may have a decreased risk, because under these conditions, enhanced genomic integrity would be achieved as a result of the greater availability of the MTHFR substrate 5,10-CH$_2$-THF for DNA synthesis because of increased availability of methyl groups for conversion of uracil to thymidine (Fig 1). Inadequate thymidine pools, on the other hand, lead to increased incorporation of uracil into DNA, thereby resulting in strand breaks that are precursors for chromosome translocations and deletions.84

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![Fig 1. An overview of the folic acid pathway, cytosine methylation, and gene silencing. The mechanism of action of demethylating agents, HDAC inhibitors, and other agents is also shown. dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; DHF, dihydrofolate; THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; DNMT, DNA methyltransferase; MBD, methyl Cpg binding domain; TSA, trichostatin A.](image-url)
When folate intake is low, both DNA methylation and DNA synthesis/repair might be impaired in individuals with the polymorphism, which in turn would result in increased risk of carcinogenesis.84 Folate deficiency has been shown to result in both hypo- and hyper–gene-specific methylation. In animal studies, folate deficiency has been shown to result in exon-specific hypomethylation of the \(p53\) gene87 as well as increased DNA methyltransferase activity.88 However, with continued folate deficiency, an increase in both \(p53\) and genome-wide methylation was seen.89

A recent study examined the effect of folate status and the MTHFR C677T polymorphism on genomic DNA methylation in peripheral-blood mononuclear-cell DNA. It was found that the MTHFR C677T polymorphism influences DNA methylation status through an interaction with folate status.90 Another study examined the relationship between plasma folate status and colorectal adenomas with reference to effect modification by the genetic polymorphism (C677T) of the MTHFR gene. When compared with subjects with the CC or CT genotype, those with the TT genotype showed a decreased risk of colorectal adenomas when they had high levels of plasma folate (adjusted odds ratio, 0.58; 95% CI, 0.21 to 1.61) and an increased risk when they had low folate levels (adjusted odds ratio, 2.13; 95% CI, 0.82 to 5.54).91 Further investigations are needed to examine the effect of folic acid levels and MTHFR polymorphisms in relation to cancer risk.

A vast amount of knowledge has been gained in the last 4 years about altered methylation patterns in human cancers. Tumor-specific methylation changes in different genes have been identified and documented. The potential clinical application of this information is in cancer diagnosis, prognosis, and therapeutics.

Recent advances in the techniques for detection of methylation include powerful tools such as sodium bisulfite conversion, cDNA microarray, restriction landmark genomic scanning, and CpG island microarrays.92 The sodium bisulfite method is ideal for mapping the normal and aberrant patterns of methylation. Bisulfite converts unmethylated cytosines to uracil, leaving methylated cytosines unchanged. After bisulfite modification, there are a number of methods available to study CpG island methylation. These include sequencing, methylation-specific polymerase chain reaction, combined bisulfite restriction analyses, methylation-sensitive single nucleotide primer extension, and methylation-sensitive single-strand conformational polymorphism.1,92 Software programs are available to help design primers for bisulfite-treated DNA.93 To be useful as a routine diagnostic tool, the actual methylation detection method has to be sensitive, quick, easy, and reproducible. Of the various techniques available, methylation-specific polymerase chain reaction seems to be most useful at present.

An early diagnosis is critical for the successful treatment of many types of cancer. The traditional methods of diagnosis (such as cytology, histopathology, immunohistochemistry, serology, and so on) are useful, but molecular markers can further subclassify the tumors. The methylation profile can distinguish tumor types and subtypes and perhaps the response to chemotherapeutic agents and survival. Methylation changes often precede apparent malignant changes and thus may be of use in early diagnosis of cancer. Moreover, sensitive detection of cancer cells could be obtained from plasma in many cases5 (Table 2).

### Table 2. Detection of Cancer in Body Fluids Using DNA Methylation as Marker

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Gene</th>
<th>Specimen</th>
<th>Methylation (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancer</td>
<td>GSTP1</td>
<td>Serum/plasma</td>
<td>72</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ejaculates</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Non–small-cell lung</td>
<td>APC</td>
<td>Serum/plasma</td>
<td>47</td>
<td>95</td>
</tr>
<tr>
<td>Cancer</td>
<td>CDKN2A</td>
<td>Serum/plasma</td>
<td>50</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>p16, DAPK, MGMT, GSTP1</td>
<td>Serum/Plasma</td>
<td>73</td>
<td>97</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>p16BNK1a</td>
<td>Serum/plasma</td>
<td>23</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Cyclin D2, RARα, Twist gene</td>
<td>Ductal lavage</td>
<td>85</td>
<td>99</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>p14ARF</td>
<td>Plasma</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>p16</td>
<td>Plasma/serum</td>
<td>38</td>
<td>101</td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td>APC-adenocarcinoma</td>
<td>Serum/Plasma</td>
<td>27</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>APC-SCC</td>
<td>Serum/Plasma</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Liver cancer</td>
<td>p15</td>
<td>Serum/plasma</td>
<td>81</td>
<td>102</td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>p16, DAPK, GSTP1 and MGMT</td>
<td>Serum/plasma</td>
<td>42</td>
<td>41</td>
</tr>
</tbody>
</table>

Abbreviations: GSTP1, glutathione S-transferase Pi 1; APC, adenomatous polyposis coli; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; MGMT, O-6 methylguanine-DNA methyltransferase.
Examples include bladder cancer, breast cancer, colorectal cancer, esophageal cancer, gastric cancer, lung cancer, prostate cancer, head and neck tumors, and liver cancer. In addition, samples obtained by exfoliative cytology, endoscopic brush techniques, and biopsy, as well as urine, saliva, and sputum samples, can be used. These are noninvasive or minimally invasive procedures and often much easier to collect and process. The sensitivity and specificity of DNA methylation markers in cancer diagnosis depends on several factors, including the type of cancer and the gene to be studied, the type of body fluid to be used, and the technique involved. The assay needs to be standardized and shown to be useful in a prospective fashion before it can become clinically useful.

**DNA Methylation as a Prognostic Marker**

Because DNA methylation is closely related to the development of cancer, it would be interesting to know whether its presence or absence affects the prognosis as well. This would help in modifying initial treatment options, monitoring patient response to therapy, and predicting survival. Recently, many studies have shown several methylated genes to be closely related to the prognosis. In colorectal cancers, the promoter region of the CDKN2A gene was found to be hypermethylated in 61.1% of tumor samples, and this was correlated with the traditional prognostic indicators, such as tumor grading and Dukes’ staging. In another study, p16 promoter methylation was detected in 42% of tumors and was seen to be more frequent in Dukes’ stage C and D than in stages A and B. Methylation in lung cancer has also been widely studied. Several genes methylated in lung cancer show promise as a prognostic marker. These include the RASSFIA gene, whose lung cancer showed reduced expression of the HIC-1 gene had a relatively shorter survival period. Prostate cancers with higher Gleason score are correlated with a higher degree of methylation of many genes, such as the RARB, RASSFIA, GSTP1, and CDH13.

The methylation profile may also help in predicting response to a chemotherapeutic agent. Methylation of the promoter region of the DNA repair gene MGMT increased the sensitivity of gliomas to alkylating agents. In another study, it was found that methylation of the hMLH1 gene in colorectal cell lines was associated with increased resistance to the drug fluorouracil.

**DNA METHYLATION AND NOVEL THERAPEUTIC STRATEGIES**

Unlike genetic modifications, epigenetic changes are reversible, making them a therapeutic target. Experiments using cell culture have shown that demethylating drugs can reverse the silencing of genes resulting from methylation. This potential to reverse DNA methylation and re-express the affected critical genes presents an attractive option for exploring clinical use in malignancies. Several clinical trials are presently underway using azacytidine, histone deacetylase inhibitors, and phenylbutyrate to reactivate the silenced genes in hematologic and solid tumors.

The commonly used drugs targeting methylation are azacytidine (5-azacytidine), decitabine (5-aza-2’-deoxycytidine), fazarabine (1-β-D-arabinofuransyl-5-azacytosine), and dihydro-5-azacytidine. These are all derivatives of deoxycytidine with some modification at the fifth position in the pyrimidine ring. Other drugs include zebularine and antisense oligodeoxynucleotides. Histone deacetylase (HDAC) inhibitors are also being tried as potential chemotherapeutic agents.

The variants of deoxycytidine act by a number of methods. Decitabine gets incorporated into the DNA after phosphorylation whereas azacytidine gets incorporated preferentially into RNA. After incorporation, they may cause cell death by obstructing DNA synthesis or may induce structural instability and DNA damage by binding to DNMT. Preclinical studies on decitabine have shown that it reverses methylation in a number of cell lines and in cells from human leukemia patients. Clinical trials in solid tumors showed response rates of less than 10%. However, trials on hematologic malignancies have been more successful. A randomized study compared the combination of ansacrine and etoposide with the same two agents plus azacitidine for the therapy of induction-resistant childhood acute myeloid leukemia. The complete response rate was higher with the three-drug regimen. A significant response to decitabine 5-aza-2’-deoxycytidine treatment in chronic myelogenous leukemia and myelodysplastic syndrome has been observed. In responding patients with myelodysplastic syndrome, demethylation of p15 cyclin-dependent kinase inhibitor gene and re-establishment of normal p15 protein expression after treatment with 5-aza-2’-deoxycytidine suggests pharmacologic demethylation as a possible mechanism that results in hematologic response. Among the novel agents of demethylation that are potentially useful are DNMT1 antisense and Si RNA. MG98 is an antisense oligodeoxynucleotide against DNMT1. It hybridizes to the DNMT1 mRNA sequence and causes mRNA degradation. Treatment with MG 98 resulted in demethylation and re-expression of p16 in bladder and colon cancer cell lines. Robert et al. studied the effect of 5-aza-2’-deoxycytidine, antisense, and Si RNA
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on the methylation of tumor suppressor genes and function of DNMTs. In their study on colon cancer cell lines, they found that the main method by which 5-aza-2'-deoxycytidine works is by the inhibition of DNMT1 and not of DNMT3A or DNMT3B. Si RNA could also be used to target a protein that contributes to methylation-mediated gene repression.124

Because DNA methylation represses gene expression in part through histone deacetylation, HDAC inhibitors have been used to activate expression from methylated genes. Although HDAC inhibitors fail to activate expression from densely methylated genes when used alone, they can synergize with a demethylating agent in inducing expression from methylated genes.125 In a phase I trial of depsipeptide, a HDAC inhibitor, three patients with cutaneous T-cell lymphoma had a partial response, and one patient with peripheral T-cell lymphoma had a complete response.126 HDAC inhibitors induced remission in transgenic models of therapy-resistant acute promyelocytic leukemia.127 Clinical trials using a combination of demethylating agent and HDAC inhibitors in cancer are presently underway. Other drugs that have shown effects on methylation-mediated gene repression include the CNS-acting sodium valproate128 and the cardiovascular drugs hydralazine and procainamide.129 5-lipoxygenase is an enzyme that is involved in the synthesis of arachidonic acid and is associated with neurodegeneration.128 Sodium valproate increased the levels of 5-lipoxygenase mRNA in both proliferating and differentiated cells,128 by direct inhibition of histone deacetylases,130 thus valproate could affect brain functioning. Studies on breast cancer cell lines using hydralazine and procainamide showed that these drugs induced demethylation and caused re-expression of ER, RARβ, and p16 in cell cultures.129 Clinical evaluation of these drugs in one case of cervical cancer and one of head and neck cancer showed re-expression of the RARβ and p16 genes, suggesting their use as potential anticancer agents.129 However, further laboratory studies and clinical trials are needed to fully establish the therapeutic efficacy of methylation inhibitors and their use in combination with histone deacetylases and conventional chemotherapeutic agents.

It must also be remembered that widespread use of demethylating drugs can have serious side effects and even promote malignant transformations of genes. The use of methyl transferase inhibitor in one study stimulated the metastatic potential of pancreatic cancer through activation of invasion-promoting genes.131

The combined strategies of anticancer therapy may ultimately offer the most successful approach. The combined approach may lead to reduction in the dose of each drug used and reduce the side effects of each individual agent. How to combine the demethylating agents and histone deacetylase inhibitors with conventional chemotherapy to achieve the best outcome remains to be determined. If the exact methylation profiles of tumors are available and drugs targeting the specific genes are obtainable, then the treatment of cancer could be more focused and rational.

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Authors’ Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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