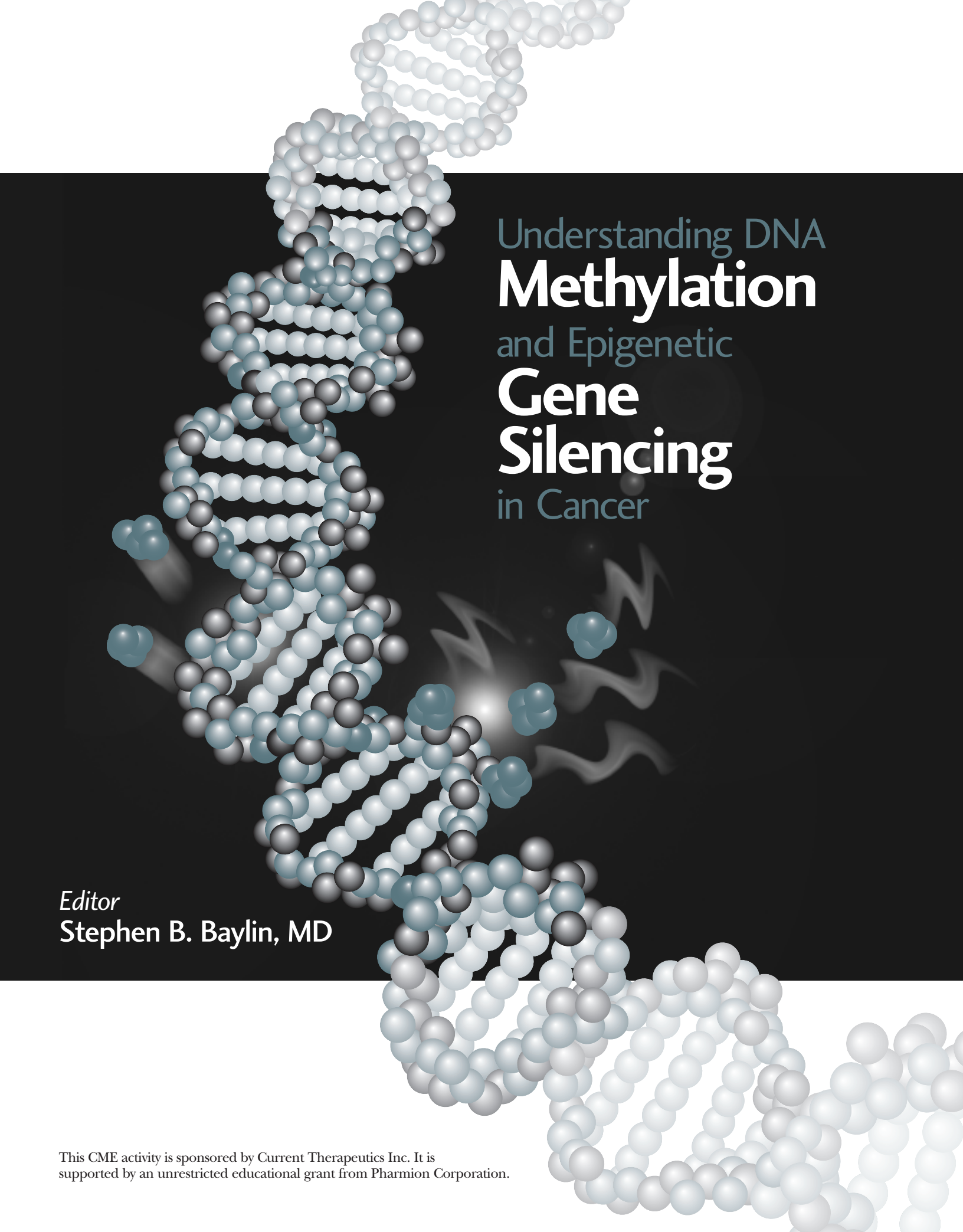

Understanding DNA Methylation and Epigenetic Gene Silencing in Cancer

Editor
Stephen B. Baylin, MD

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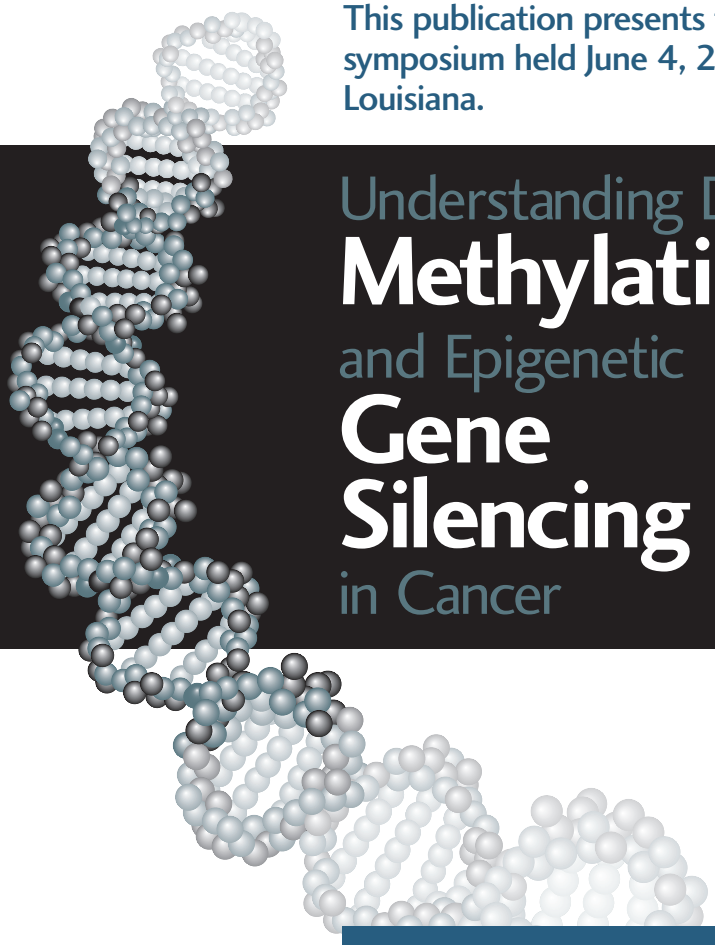

Understanding DNA Methylation and Epigenetic Gene Silencing in Cancer

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The content of this publication is a continuing medical education (CME) activity. To earn CME credit, participants must read the articles, take the self-assessment quiz (page 23), and complete the answer sheet and evaluation form (page 25), earning a score of 70%.
Activity release date: November 2004
Activity expiration date: November 2007

Target Audience

This activity is intended for medical oncology researchers and clinicians.

Educational Objectives

On completion of this educational activity, participants will be able to:

- Recognize the distribution of CpG islands and gene promoters
- Describe the process of DNA methylation
- Explain what mediates DNA methylation
- Describe what couples DNA methylation to gene silencing
- Discuss the relationship between histone modifications and gene expression
- Characterize our current ability to target DNA methylation and other gene silencing processes for therapeutic purposes

Activity Completion Time

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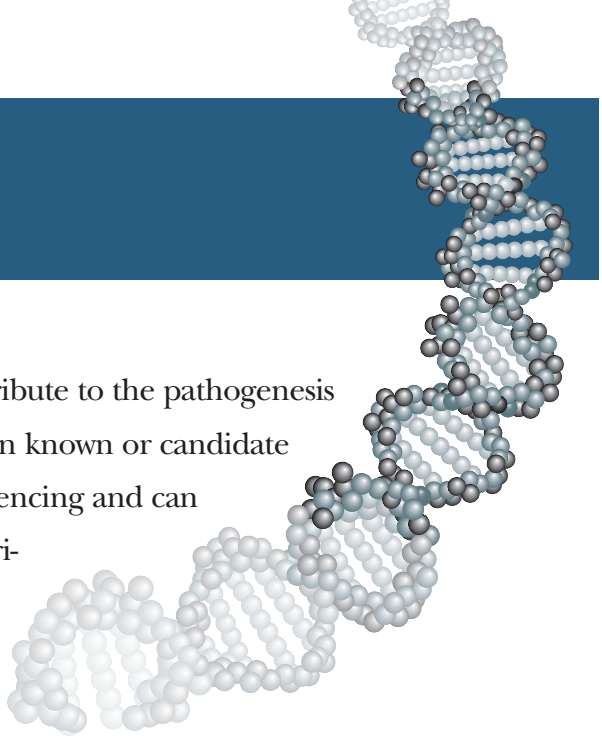
Introduction

Stephen B. Baylin, MD



Epigenetic alterations of gene function are now known to contribute to the pathogenesis of cancer. Specifically, abnormal promoter region methylation in known or candidate tumor suppressor genes contributes to tightly heritable gene silencing and can thereby cause loss of gene function, which contributes to tumorigenesis. Re-expression of abnormally silenced suppressor genes is being researched for potential clinical applications.

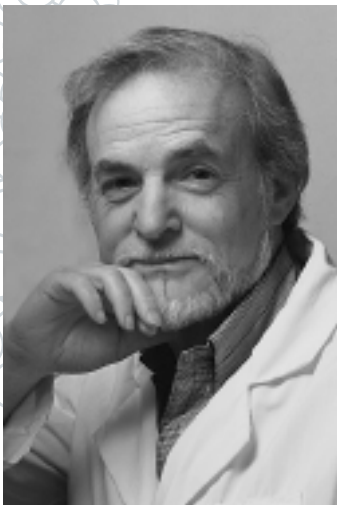
Research into gene silencing has focused on the regions of high CpG content, known as the CpG islands, and these are often located near gene transcription sites, where the transcription of DNA to RNA begins. In normal cells, most promoter-associated CpG islands at transcription sites are unmethylated. The absence of CpG methylation indicates either normal transcription activity, or the fact that a gene can be recruited to express even if it is basally silent. But in cancer cells, promoter region CpG islands are more likely to become methylated, and this can cooperate with other transcriptional silencing chromatin events to tightly prevent the normal transcription of DNA, thereby "silencing" the gene. Loss of tumor suppressor gene function in cancer has been most classically associated with mutations, often in gene coding regions with resultant protein disruption; however, promoter hypermethylation may occur at least as often as such mutational changes as the cause of loss of gene function in known tumor-related genes. Affected genes include those that regulate cell cycle control, cell migration, substratum recognition, differentiation, growth, and apoptosis. Genes known to date to be affected by epigenetic silencing associated with methylation include those that suppress epithelial cancers, such as breast, colon, lung, and prostate cancers, and in lymphomas and leukemias. The list of genes involved is continually growing.

As Dr. Bernard Futscher explains in "Identifying Specific Targets for Epigenetic Reversal in Cancer," additional genes affected by methylation are being discovered as work progresses on characterizing the human genome and the timing of activities associated with the development of cancer. Evidence suggests that many gene hypermethylation changes occur early in the development of tumors. Various tools are being used to produce epigenetic profiles of human cancer cell lines to evaluate the effects of therapeutic agents.

Unlike gene mutation, gene silencing associated with methylation may be reversed, reactivating the tumor-suppression activity of a gene. Two approaches to promoting re-expression of silenced genes have been studied in myelodysplastic syndrome (MDS), a hematologic disorder in which DNA methylation and gene silencing have been demonstrated. Dr. Steven Gore reports the clinical results of these two treatment approaches in "Current and Emerging Therapeutic Applications." The first approach uses methyltransferase inhibitors to stop methylation, allowing normal gene transcription to resume. Compounds that have been shown to inhibit methyltransferase in MDS include 5-azacitidine (azacitidine) and 5-aza-2' deoxycitidine (decitabine). Azacitidine was recently approved by the FDA for treatment of MDS based on clinical trial results demonstrating significant improvement among patients who received the drug, compared with those who received supportive care, including transfusions. Decitabine, a congener of azacitidine, also has been shown in Phase II trials to produce clinically significant results. The second approach, using histone deacetylase (HDAC) inhibitors to re-establish gene transcription, has been used alone and as a sequential therapy following the administration of a methyltransferase inhibitor. For genes with dense promoter methylation in cultured tumor cells, HDAC inhibitors alone generally will not result in reactivation of gene expression but will contribute to such activation if given just after a low dose of azacitidine. In Phase I studies, the HDAC inhibitor sodium phenylbutyrate was shown to produce clinical activity in patients with MDS and acute myelogenous leukemia. The combination of methyltransferase inhibition and HDAC inhibition appears to produce a synergistic effect and has resulted in promising clinical outcomes, including complete remissions in some patients.

Epigenetic Abnormalities and Gene Silencing

Stephen B. Baylin, MD



Although cancer is a disease driven by genetic abnormalities, recent research suggests that epigenetic alterations of gene function are also central to the pathogenesis of these diseases. Epigenetic alterations include heritable changes in gene expression that are not caused by direct alteration of the gene's nucleotide sequence. Abnormal DNA methylation at gene transcription sites can result in epigenetic silencing of genes that protect against tumor formation or that repair DNA.[1] Researchers have only begun to examine how these epigenetic processes contribute to the development of cancer, but initial studies have identified several potential therapeutic strategies to target the molecular mechanisms of epigenetic gene silencing and disrupt molecular pathways that promote tumorigenesis.

DNA Methylation and Gene Silencing

The four nucleotide bases of DNA—cytosine (C), adenine (A), guanine (G), and thymine (T)—form a total of 16 possible dinucleotide pairs. One of these dinucleotides, in which a cytosine is adjacent to a guanosine in the 5' direction (the CpG dinucleotide), occurs at a lower than expected frequency throughout most of the human genome but at a higher than expected frequency in small portions of DNA that are referred to as CpG islands. These CpG islands are often concentrated near gene transcription start sites, the promoter regions where the transcription of DNA to RNA begins.[1]

In the normal cell, most of the CpG dinucleotides at gene promoter regions are unmethylated, whereas CpG islands found at other portions of the genome are generally methylated.[1] The absence of CpG island methylation is a hallmark of an active transcription site that is capable of transcribing DNA to RNA. In cancer cells, this pattern of CpG methylation becomes disrupted: CpG islands in promoter regions of selected genes have an unusually high likelihood of methylation, but CpG dinucleotides that fall outside of promoter regions are less likely than normal to be methylated (Figure 1).[1] The methylation of CpG islands, in association with chromatin modifications that accompany the change, prevents the transcription of the gene's DNA, resulting in transcriptional silencing of the gene. Transcriptional silencing of genes that normally possess antitumor activity results in abnormal cellular events, which contribute to tumor progression.[2] Thus, epigenetic gene silencing is a second mechanism, in addition to gene mutation, by which the production of tumor-suppressing genes is disrupted.[2]

As described in the classic two-hit model of Knudson for loss of tumor suppressor gene function, the genomic disruption of a tumor-suppressor gene will usually not affect cell function unless both alleles of the gene are inactivated.[3] In this model, the loss of function of a tumor-suppressing gene in sporadic cancer occurs when the mutation of one allele (the first hit) is accompanied by the deletion of the portion of the chromosome that contains the second gene copy (the second hit). Loss of tumor suppressor gene function in association with abnormal methylation of promoter CpG islands operates

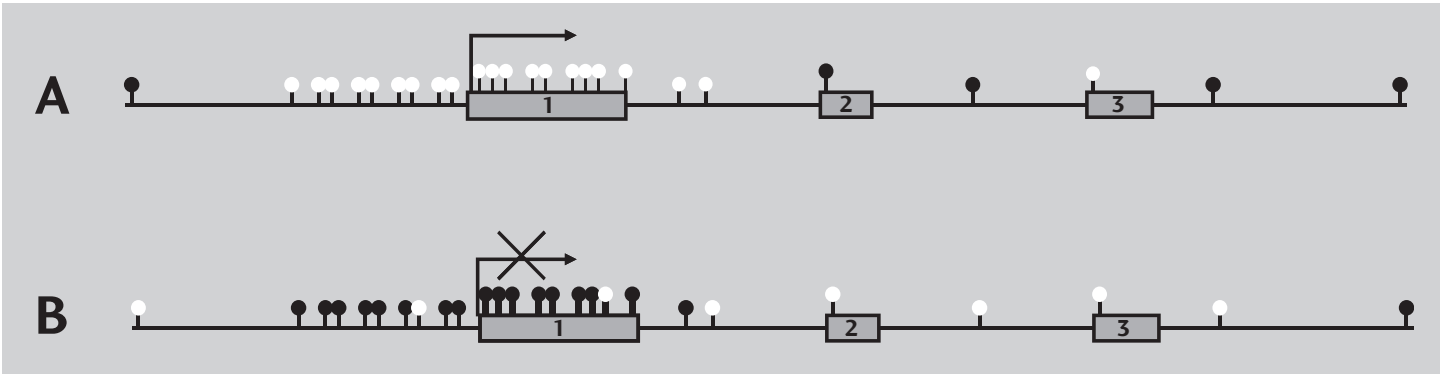


Figure 1. The top illustration shows the pattern of CpG dinucleotide methylation at the gene promoter region of a normal cell (A). Boxes 1, 2, and 3 represent the exons; the lines between the boxes represent the introns. CpG dinucleotides are found in high concentration near the gene promoter region, where they are generally unmethylated (white circles). The absence of CpG methylation at the promoter region of the normal cell is associated with a site of active gene transcription (arrow). These dinucleotides are relatively rare in the remainder of the genome and are generally methylated (black circles). The bottom illustration shows CpG methylation in tumor cells (B). Methylation of the CpGs at the promoter region is associated with suppression of gene transcription (X).

similarly: Where one allele of the gene is methylated, the second allele is deleted. Although the loss of both alleles as the result of mutation is uncommon, both alleles of genes are more often inactivated in association with DNA methylation. The Knudson model also describes the inactivation of a tumor-suppressing gene in inherited cancer when one tumor suppressor gene allele contains a germline mutation, and the loss of the second allele occurs in tumors through a chromosomal deletion. Again, methylation of the CpG-rich promoter region may also provide the second hit in inherited cancers. In this case, DNA methylation is observed on the retained, nonmutated allele only; the mutated allele is not abnormally methylated.

Recent research has identified a growing list of cancer-related genes that develop significant DNA methylation at promoter CpG islands adjacent to transcription start sites (Table 1).[1,2] A review of tumor-related genes across the entire genome identified genes that are deactivated by methylation, by mutation, or by both mechanisms.[2] This analysis found that methylation is at least as common as mutation as a mechanism of gene silencing in currently identified tumor-related genes. It may even be the case that gene silencing by DNA methylation is a much more significant source of gene suppression than mutation. Many of the key gene silencing events occur very early during the premalignant stages of tumor progression, and the process of epigenetic gene silencing continues through the entire progression of human cancer.[1,2,4–6] The genes affected include those that regulate many developmental pathways, controlling processes such as cell migration, cell-substratum recognition, cell differentiation, and the balance between cell growth and apoptosis.[1,2,6]

One class of genes frequently silenced in association with promoter methylation can actually foster carcinogenesis by leading to genetic instability in cells with the resultant accumulation of gene mutations. This effect involves epigenetic silencing of genes that normally act to prevent or correct DNA damage.[2] For example, one of the most commonly suppressed genes in epithelial cancers, lymphomas, and lymphocytic anemias is the tumor suppressing gene $p16^{INK4a}$. Experimental models have shown that the epigenetic suppression of this gene early in the cell growth process results in the bypass of key control mechanisms that ordinarily trigger cell senescence or death,[7] and this then allows for the accrual of chromosomal abnormalities and gene mutations. The loss of the gene *GST-PI*, a nearly universal event in prostate cancer, predisposes cells to oxidative damage, especially at adenines.[8] The loss of a DNA repair gene, *O^6-methylguanine-DNA methyltransferase (MGMT)*, which prevents G to A transitions, occurs early in the course of colon cancer and results in the accumulation of these transitions in important regulatory genes such as *K-RAS* and *p53*.[2] Of the 10% to 15% of patients with colon cancer who have microsatellite instability, approximately 70% to 80% exhibit epigenetic gene silencing of a mismatch repair gene, *MLH1*.[9,10] In addition, methylated cytosine is directly mutagenic, undergoing spontaneous C to T transitions.[11]

Recent research has demonstrated that these epigenetic gene silencing mechanisms can affect cellular processes that are central to the formation of cancers. In some types of cancer such as colorectal cancer, epigenetic gene silencing may be essential to the initiation and early maintenance of tumorigenesis. This has recently been

demonstrated in studies of the Wnt cellular signaling pathway, the overactivity of which appears to be a pivotal early event in the development of colon cancer.[12] Activation of Wnt receptors inhibits the phosphorylation and inactivation of β-catenin, an intracellular signaling molecule. This accumulation of stabilized β-catenin stimulates the transcription of genes in the Wnt signaling pathway. The activation of Wnt signal transduction is partially controlled at the cell membrane by a family of proteins known as secreted frizzled-related proteins (SFRPs), which interact with a family of membrane-bound proteins, frizzled, which are Wnt ligand receptors.[13] The interaction of SFRPs with these Wnt receptors inhibits Wnt pathway activity thus inhibiting the phosphorylation of β-catenin and the transcription of Wnt pathway genes. β-catenin is also regulated within the cell by adenomatous polyposis coli (APC) protein. APC, together with Axin and GSK3β, induce phosphorylation of β-catenin, decreasing the activation of Wnt pathway genes.[13]

Table 1. Familial cancer genes

Methylated	Unmethylated
Rb	*NF1, NF2*
P16^{INK4a}	*MSH2*
VHL	*PTEN*
MLH1	*p53*
E-cadherin	*PTC*
BRCA1	*BRCA2*
APC	*ATM*
PJ (LKB1)	

The conventional view of the role of the Wnt pathway in colon cancer is that Wnt signaling becomes dysregulated as a result of mutations that affect proteins downstream from the interaction of Wnt ligands with cell-surface receptors. For example, one way by which suppression of β-catenin is lost is by the mutation of the gene for APC, resulting in the accumulation of free β-catenin in the nucleus. Gene mutation resulting in the loss of APC function has been recognized as an important contributor to colon cancer for several years.[12] An epigenetic mechanism of gene suppression has also recently been identified in the Wnt pathway, in which the genes encoding the SFRPs are abnormally methylated.[6] Epigenetic silencing of these genes prevents the inhibition of Wnt signaling at the cell membrane, resulting in an increase in β-catenin accumulation. In addition, recent research has found that the restoration of SFRP function reduces Wnt signaling even in cell lines with mutations that inactivate the downstream regulatory protein, APC, or activate β-catenin.[6] This suggests that both epigenetic silencing of SFRP and downstream mutation of regulatory proteins are essential to drive Wnt signaling to its maximum. This methylation of SFRP genes appears to be a very early event in the development of colorectal cancer, occurring in some of the earliest lesions studied, monoclonal aberrant crypt foci.[6]

Reversing DNA Methylation

In contrast with genetic mutation, epigenetic gene silencing is potentially reversible.[2] Experimental evidence suggests that it is possible to reactivate epigenetically silenced genes, providing the basis for strategies to prevent or reverse some types of cancers.[2] As understanding of these epigenetic mechanisms improves, it should be possible to significantly improve current approaches to cancer prevention or therapy.

DNA methylation can occur at a cytosine that is immediately 5' to a guanosine. The methylation is carried out by one of three DNA methyltransferase enzymes (DNMTs), using S-adenosyl-methionine as the methyl donor.[1] Methylation occurs at carbon 5 of the cytosine molecule, yielding 5-methyl cytosine (Figure 2).[1] The activities of all three DNMTs are blocked by compounds such as azacitidine and decitabine. These agents are incorporated into the DNA of dividing cells, where they irreversibly inhibit the activity of DNMT and prevent hypermethylation of CpG islands.[1]

The functional reversal of DNA methylation and the reactivation of hypermethylated tumor-regulating genes have been demonstrated in model systems that have examined the function of the DNA mismatch repair gene *MLH1* in several cell lines.[10] The RKO cell line is methylated at the promoter region for the *MLH1* gene, resulting in an absence of MLH1 protein. When these cells were treated with azacitidine, the production of MLH1 protein by the cells was restored. Similar findings were noted for a second cell line, SW48, which is also methylated at the CpG promoter region of *MLH1*. This re-expression of MLH1 protein was also shown to have functional consequences. The ability of extracts from the cell lines to carry out DNA mismatch repairs was examined, as shown in Figure 3. HeLa cells, which have an unmethylated *MLH1* gene promoter region, were able to repair the 3 different types of DNA mismatch errors studied. The RKO cell line, which is methylated at *MLH1*, was completely unable to repair these DNA errors. After exposure of the RKO cells to azacitidine, all three of the DNA repair measures were markedly increased, and were generally similar to the repair ability of the HeLa cells. A similar pattern was also noted with the SW48 cell line.

Molecular Mechanisms of Epigenetic Silencing

Truly effective targeting of gene silencing may require a full understanding of the molecular events by which DNA methylation suppresses gene transcription. Some of these details have been identified in recent studies, although

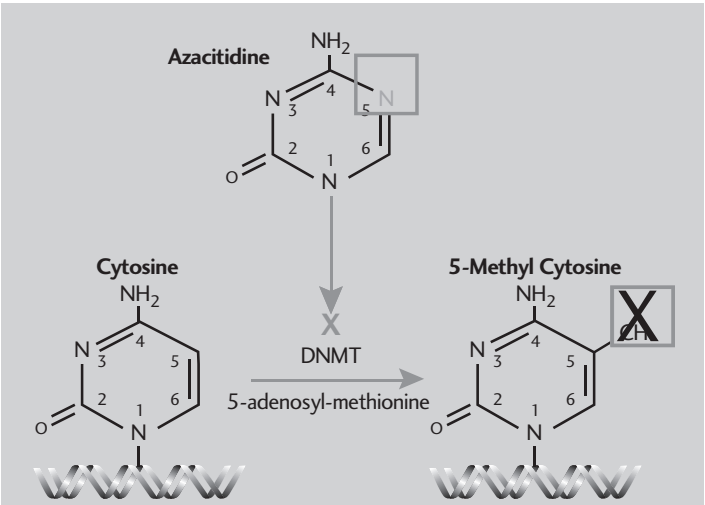


Figure 2. DNA methylation occurs at a cytosine that is immediately 5′ to a guanosine. The cytosine of the CpG dinucleotide is methylated to form 5-methyl cytosine by a family of enzymes known as DNA methyl transferase (DNMTs) using S-adenosyl-methionine as a methyl donor. Methylation inhibitors such as azacitidine or decitabine prevent the methylation of cytosine. Azacitidine and related compounds must first be incorporated into the DNA of the cell, where they irreversibly inhibit DNMTs.

many of the processes remain to be described. The mechanisms of gene silencing depend on a complex series of enzymatic modification of histones (proteins that, together with DNA, form chromatin).[2] It now appears that DNA methylation and a series of modifications of key amino acid residues in histone tails work together to initiate and maintain gene silencing in tumorigenesis. The enzymatic acetylation and methylation of particular amino acid residues of histones result in modifications to the structure of chromatin that transform it from a configuration in which DNA transcription occurs to a configuration in which transcription is repressed.[2] These histone modifications may also promote additional DNA methylation during the progression of cancer.

As noted previously, three DNMTs mediate the process of hypermethylation of gene promoters. DNMTs also appear to act as platforms for a number of other proteins that maintain histones in configurations that suppress the transcriptional capability of chromatin.[2] These include histone deacetylase enzymes (HDACs), which regulate the acetylation and deacetylation of histone residues that increase or decrease gene expression, methyl-CpG binding proteins (MBD1, MBD2) that recognize methylated cytosines and suppress gene transcription, and other transcription-repressing proteins.[2]

The methylation and acetylation of specific histone residues appear to provide a histone code that signals whether the gene is in a transcriptionally active state. One of the most important code marks indicating the transcriptional status of the gene appears to be the acetylation of histone 3 (H3) lysine 9. Acetylation of H3 lysine 9 prevents the subsequent methylation of this lysine residue, which is a transcription silencing mark.[14] Drugs that inhibit HDAC activity (eg, trichostatin [TSA]) prevent the deacetylation of lysine 9 by HDACs, which maintains the chromatin in a configuration of active gene transcription. The acetylation of lysine 9 also increases the likelihood of acetylation of a second important residue, H3 lysine 4. Acetylation at this site is also considered a code mark indicative of chromatin that is transcriptionally active.

These changes in the acetylation and methylation of key histone residues are influenced by the methylation of DNA, as shown in recent experimental studies using cell culture systems. A chromatin precipitation assay of the *MLH1* gene was examined in a colon cancer cell line in which the *MLH1* gene is methylated and silenced, in comparison with a cell line that was not methylated and in which *MLH1* gene is transcribed normally.[15] In cell lines with methylated CpG promoters, H3 lysine 9 was methylated and not acetylated. In the actively transcribed cell line, the opposite pattern was observed: lysine 9 was acetylated and not methylated.

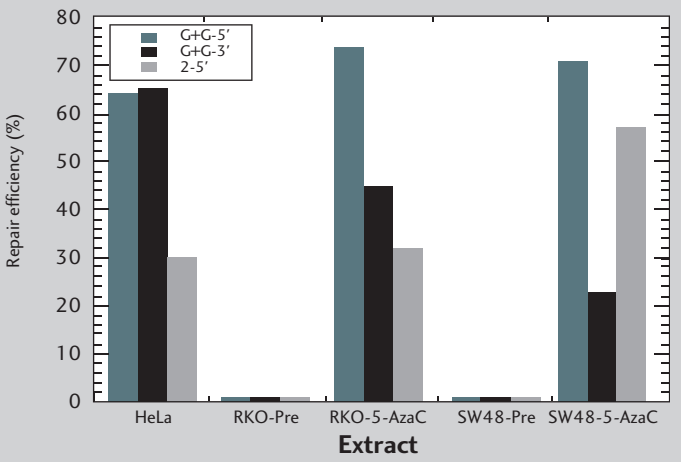


Figure 3. The investigators evaluated the ability of the cells to repair three different DNA mismatches: a G-G mismatch with a nick either 5′ or 3′ to the mismatch (G+G 5′; G+G 3′) or a substrate containing 2 extra bases with a nick 5′ to the unpaired bases. DNA mismatch repair in HeLa cells was used as a control. The DNA repair gene *MLH1* was inactivated in two cell lines (RKO, SW48) by CpG hypermethylation, resulting in the inability of the cells to perform any of the DNA mismatch repairs (RKO-Pre, SW48-Pre). After exposure of the cells to azacitidine, both cell lines were able to perform all 3 DNA mismatch repairs.

How do these chromatin changes regulate gene transcription? Most DNA in the human genome is found to be heavily methylated chromatin in which deacetylated histones form compact nucleosomes.[1] These chromatin regions are transcriptionally silent. The histones are maintained in a deacetylated, compact, and transcriptionally silent state by HDACs.[1] Chromatin in which transcription occurs is characterized by acetylated histones and widely spaced nucleosomes, which permit access of transcription proteins to the promoter (Figure 4).[1] DNA methylation permits the binding of proteins that maintain the chromatin in a transcriptionally silent state, including methyl cytosine binding proteins, HDACs, and transcriptional corepressors. The methylation of CpG islands at gene promoter regions and histone deacetylation, which occurs during tumorigenesis, result in conformational changes that physically obstruct transcription enzymes from reaching the promoter region.

This model suggests the possibility of different therapeutic approaches to restore the expression of suppressed genes. Agents such as azacitidine and decitabine inhibit DNA methyltransferases, and HDAC inhibitors such as TSA inhibit HDACs.[2] Some evidence, however, suggests that DNA methylation appears to dominate the process of maintaining gene suppression, and that HDAC inhibition is not sufficient to restore the function of silenced genes. This has been shown in cell lines in which *MLH1* genes were epigenetically silenced.[16] Exposing the cells to the HDAC inhibitor TSA did not result in the reacetylation of H3 lysine 9 or lysine 4 and did not produce re-expression of the *MLH1* gene. Exposure of the cells to azacitidine, however, resulted in loss of lysine 9 and a return of acetylation at lysine 9 and lysine 4.[15] Thus, azacitidine reverses the CpG methylation and allows formation of the chromatin marks that are associated with gene expression.

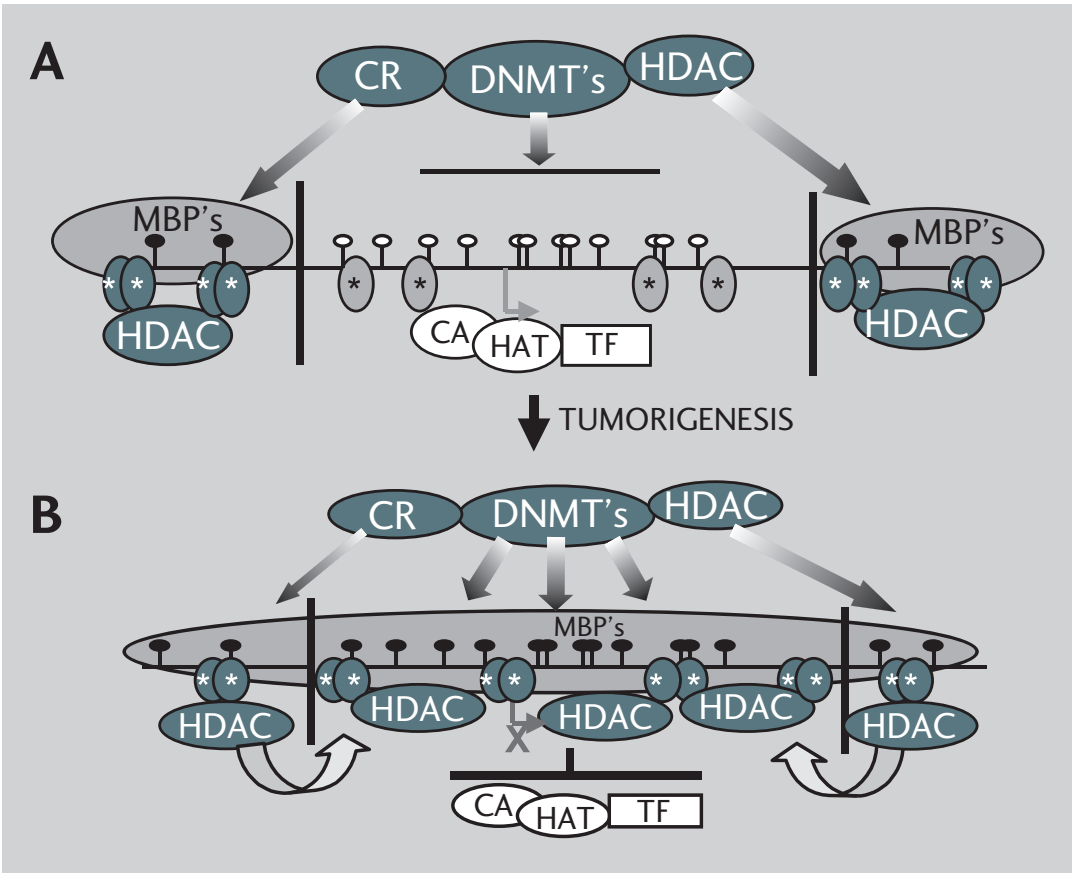


Figure 4. Gene transcription is inhibited as a result of changes to chromatin structure during tumorigenesis. In the normal cell (A), a transcriptionally active, unmethylated gene (white circles) is accessible by enzymes that are important in gene transcription and in maintaining the DNA in a transcriptionally active configuration, including transcriptional coactivators (CA), histone acetyl transferases (HAT), and primary transcription factors (TF). The nucleosomes are widely spaced, with acetylation of key histone H3 residues (light ovals). In the tumor cell (B), the chromatin is transformed to a transcriptionally repressive configuration that is characterized by methylated CpGs, nucleosomes that are compact and closely spaced (dark ovals), with deacetylated histones and methylated H3 lysine 9 residues. The promoter region is now inaccessible to DNA transcription proteins but is accessible to DNMTs and histone deacetylases.

Although these findings suggest that it may be possible to restore chromatin structure to a transcriptionally active state, it has also been noted that cells in which gene expression has been reactivated by demethylating agents may eventually revert to the gene-silenced state after treatment is discontinued. The mechanism by which this return of transcriptional silencing occurs may not be exactly the same as the initial histone and DNA modifications that produced gene silencing, however. This was illustrated in a cell culture study using a cell line in which a tumor-suppressing gene ($p16^{INK4a}$) is silenced as a result of gene methylation.[17] The use of recombinant techniques to inactivate two of the three

DNMTs resulted in a slowing of cell growth, as methylation of $p16^{INK4a}$ gene was disrupted; however, the rate of growth began to increase after the cells had grown through several cycles. This increased growth rate was accompanied by H3 lysine 9 methylation at the $p16^{INK4a}$ promoter, although in this case, the H3 lysine methylation occurred *before* DNA methylation, rather than after it. After additional cell growth cycles, the growth rate of the cells was similar to the growth rate of the wild-type cells and DNA methylation at the $p16^{INK4a}$ had been restored. Thus, despite the elimination of most DNMT activity, the chromatin histone modifications eventually returned, followed by subsequent DNA methylation.

Conclusions

It has long been known that mutations that inactivate cancer-suppressing genes are important in the pathogenesis of cancer. A growing body of research suggests that epigenetic gene silencing, in which tumor-suppressing genes are inactivated as the result of abnormal DNA methylation, may also be a pivotal event in the development of many cancer types. Although the mechanisms by which epigenetic gene silencing occur are not completely understood, it appears that methylation of CpG dinucleotides in gene promoter regions, accompanied by deacetylation and methylation of certain histone residues, prevent the transcriptional machinery of the cell from transcribing DNA to RNA. Agents such as azacitidine or decitabine reverse the abnormal DNA methylation, which permits HDAC inhibitors to restore chromatin to a transcriptionally active configuration. The molecular events that underlie this combination treatment effect are just being established, but this synergy presents a possible clinical paradigm to use HDAC inhibitors and azacitidine together for the prevention or treatment of a wide variety of cancer types.

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Identifying Specific Targets for Epigenetic Reversal in Cancer

Bernard W. Futscher, PhD

Identification of the epigenetic changes that play a role in tumorigenesis is requisite to understanding gene silencing and provides a foundation for the development of new therapeutic strategies targeting the reversal of these changes. The speed with which knowledge about the epigenetic landscape of cancer can be applied toward the design of novel therapies, however, depends in part on how quickly the implicated genes can be identified. The establishment of academic consortia and corporate initiatives to create new information and tools for epigenetic research reflects the high priority assigned to this area. That the human genome contains approximately 25,000 to 40,000 genes with anywhere from 12,000 to 20,000 of these genes being active at a given time in any given cell speaks to the challenge of the task.

Growing List of Genes

The list of oncogenic-related genes known to display CpG methylation continues to grow and includes not only a number of tumor suppressor genes but also genes involved in DNA repair and metastasis inhibition. Available evidence indicates the methylation changes are tumor type-specific and may occur as early events in tumor progression.[1,2] Described histone modifications include acetylation, phosphorylation, methylation, ADP ribosylation, and ubiquitination. Research to date has identified modifications on multiple lysine, arginine, and serine residues of each of the four core histones.[3–6]

The discovery of cancer-related genes silenced by abnormal methylation is further complicated by the heterogeneous distribution of methylated and unmethylated regions throughout the genome. CpG islands are found in more than half of all human genes. While most are located in promoter regions, many are not, although it is likely that they play as yet undefined roles in transcriptional regulation. While most promoter-related CpG islands are normally unmethylated, there are exceptions. Many of the CpG islands of imprinted genes and X-linked genes are methylated,[7,8] such as CpG regions in satellite DNA sequences of pericentromeric heterochromatin. Some genes exhibit CpG island DNA methylation in a cell type-specific manner; for example, the CpG island of the gene may be methylated in mesenchymal cells but not in epithelial cells.[9,10]

A number of experimental approaches are available to facilitate identification of epigenetic targets characterized by DNA methylation or histone modification and to study their mechanisms of oncogenesis. Tumor profiling is one technique. In the first genome-wide analysis of human tumors, Costello et al used restriction landmark genomic scanning (RLGS) to identify methylation-specific profiles associated with individual tumor type.[11] In addition, comprehensive analyses of gene expression patterns following pharmacologic manipulation with DNA methylation inhibitors to reverse methylation have been used successfully to identify targets of epigenetic inactivation.[12] As described elsewhere in this supplement, methylation inhibitors, such as azacitidine, can be used in combination with histone deacetylase (HDAC) inhibitors,

such as phenylbutyrate and all-trans retinoic acid (ATRA). Cell lines or animal models can be genetically engineered so that they are deficient in DNA methyltransferases that mediate CpG island methylation. All of these methods have advantages and disadvantages, and all are particularly useful in some situations but less so in others. It is helpful to have a spectrum of approaches from which to choose for epigenetic target research.

Analysis Techniques

Several different molecular analysis tools can detect epigenetic changes (Table 1). Bisulfite sequencing, methylation-specific PCR (MSP), and MethyLight are excellent assays for analyzing the methylation status of CpG islands in specific DNA sequences, but are limited in their ability to discover new targets.[13–15]

Table 1. Tools for epigenetic/epigenomic research

Bisulfite sequencing
MSP (methylation-specific PCR)
MethyLight
RLGS (restriction landmark genomic scanning)
Gene expression arrays
Promoter arrays
CpG island microarrays
Chromatin immunoprecipitation

Bisulfite sequencing is a particularly powerful tool that has revolutionized the detection of DNA methylation (Figure 1).[16] It involves reacting single-stranded DNA with sodium bisulfite, which selectively deaminates cytosine to uracil but does not react with methylcytosine. The modified DNA sequence produced in the bisulfite reaction is amplified by PCR, and then the amplified DNA is ligated into a plasmid vector for cloning and sequencing. When the DNA is sequenced, only the intact methylated cytosine residues are amplified as cytosine.[17]

Bisulfite sequencing can be performed using DNA isolated from fewer than 100 cells, which is one of the major advantages of this tool, because tumor specimens are typically very small. Other benefits of bisulfite sequencing include its ability to analyze long stretches of the genome to determine very clear patterns of methylation in the DNA, and it yields a quantitative positive display of 5-methylcytosine residues. Bisulfite sequencing, however, requires DNA sequencing, cloning, and PCR product, which taken together make it a very time-consuming and relatively costly technique (Table 2).

MSP, developed by Herman and Baylin, is a very rapid and sensitive technique for methylation screening,

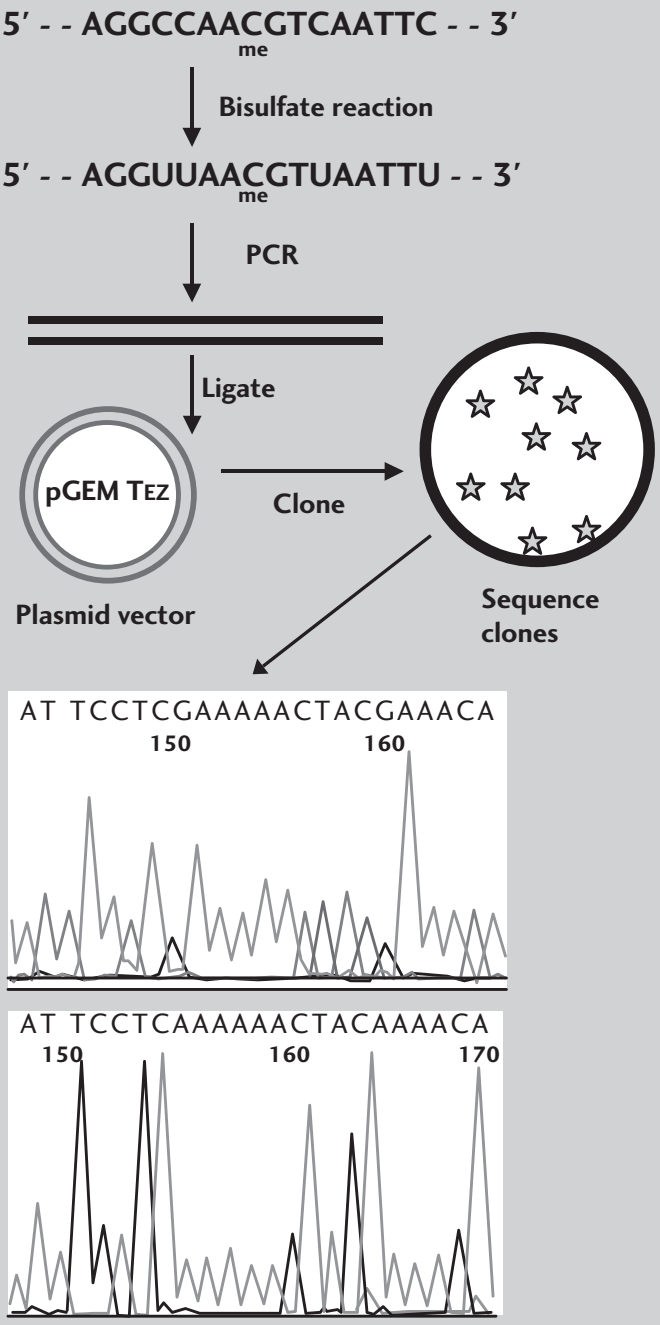

Figure 1. Bisulfite sequencing involves reacting single-stranded DNA with sodium bisulfite, which selectively deaminates cytosine to uracil but does not react with methylcytosine. The modified DNA sequence produced in the bisulfite reaction is amplified by PCR, and then the amplified DNA is ligated into a plasmid vector for cloning and sequencing. When the DNA is sequenced, only the intact methylated cytosine residues are amplified as cytosine.

although it is not highly quantitative (Table 3).[14] MSP is performed using sodium bisulfite to modify the DNA and convert unmethylated cytosines to uracil. Subsequent amplification is performed with primers specific for the

Table 2. Bisulfite sequencing

Advantages
Minimal starting material required (DNA isolated from fewer than 100 cells)
Allows for analysis of patterns of methylation
Yields a quantitative positive display of 5-methylcytosine residues
Disadvantages
Time consuming
Costly

methylated versus unmethylated DNA, and the analysis is performed with simple gel electrophoresis. MethyLight is the next generation of the MSP assay. The work up of the sample and the premise of the assays are identical. The MethyLight approach is an advance: While maintaining the exquisite sensitivity provided by standard MSP, the assay is made more quantitative, and less labor intensive through the incorporation of a real time "TaqMan" PCR format (Table 4).[18]

When the epigenetically silenced genes involved in oncogenesis are not known, alternative methods must be used in a discovery process to scan the genome and identify potential hot spots. Techniques available for screening include RLGS; gene expression arrays, which are used as a surrogate to see what genes are turned on after exposure to DNA methyltransferase inhibitors like azacitidine; promoter arrays; and CpG island microarrays (CGI microarrays).

The CGI microarray technique, first developed by Tim Huang, permits simultaneous assessment of thousands of potential targets of DNA methylation on a single chip.[19] It involves arraying of CpG island clones on glass slides, preparation of target sample amplicons, and hybridization of the amplicons onto the CGI microarrays (Figure 2).

In a similar fashion, we obtained a human CpG island library from the UK Human Genome Mapping Project, and then arrayed and DNA sequence-validated 6,800 genomic clones from this library.[20] Database construction began with crude chromatograms. After duplicates were removed, all 6,800 elements were subjected to Basic Local Alignment Search Tool (BLAST) analysis against the University of California, Santa Cruz, High Throughput Genomic and nr DNA-sequence databases and were sequenced. In the CpG island database, each clone is assigned an identification number and its characteristics are listed, including such information as its chromosome location, whether it is a promoter or found in the 5' flanking region, its GC content, and what restriction sites exist in the clone. The latter information is useful for determining the utility of restriction enzyme analysis as well as the actual sequence.

Figure 3 depicts the steps in CGI microarray methylation analysis. It is performed using tumor-derived genomic DNA samples that are obtained using a restriction enzyme (Mse1) able to cut immediately outside of the CpG island. Next, catch linkers bearing PCR primer sequences are added to the Mse1 fragments. The sample is split into a reference portion, which serves as a denominator to determine how well the genome amplification worked, and into a test portion that is digested with McrBC, a methylation sensitive restriction enzyme that only digests DNA if the region is methylated. The two portions are amplified by PCR, and then the DNA is direct labeled with Cy5 red (test) or Cy3 green (reference) fluorescent dyes. DNA fragments not digested by McrBC in the test sample produce Cy5-labeled PCR product while no labeled PCR product is produced if there were methylated fragments digested by McrBC. The labeled test and reference PCR products are mixed and spotted onto the glass slide. The hybridized slides are scanned and the acquired images analyzed to identify methylated signals.

Table 3. Methylation-specific PCR

Advantages
Rapid technique for methylation screening
Most sensitive technique available
Disadvantage
Not highly quantitative
Occasional PCR problems

Table 4. MethyLight

Advantages
Rapid screening of hundreds to thousands of samples
Highly sensitive
Less labor intensive variation of MSP
Real-time PCR format
No further manipulation after PCR step
Disadvantages
Does not yield high resolution methylation information
Cannot accurately determine methylation percentage at a single CpG

Chromatin immunoprecipitation (ChIP) microarrays

ChIP microarrays represent a method for analyzing DNA-protein interactions *in vivo*. The technique for ChIP was first described by Kondo et al (Figure 4).[21] First, the cells are fixed with formaldehyde, which cross links histones to DNA, and then they are washed, resuspended, and sonicated before the lysate is divided into two fractions. The first fraction is treated with antibodies to the proteins of interest so that the DNA associated with those proteins can be selectively recovered. Those antibodies can be directed against the acetylated or methylated histones or to proteins such as p53. The second fraction serves as an input control. The samples are incubated overnight to allow immunoprecipitation of the chromatin complexes, which are subsequently collected and further processed so that the excess protein is digested away to yield acetyl-histone or methyl-histone enriched DNA. Next, the DNA is tagged with a fluorescent label and analyzed with the CGI microarrays.

Research Applications

We have used these various tools of epigenetic/epigenomic research in our laboratory in a series of studies to produce epigenetic profiles of several human cancer cell lines and to study the epigenomic response to pharmacologic agents capable of altering the epigenetic landscape.

Bisulfite sequencing

Based on the use of bisulfite sequencing, we demonstrated that loss of *MASPIN* expression in breast cancer cells *in vivo* was often linked to aberrant methylation of the *MASPIN* promoter and that the aberrant methylation occurred as an early event.[1] *MASPIN* is a potent tumor suppressor gene first identified as a candidate gene in breast cancer through use of a gene survey approach that demonstrated absence of its mRNA in tumor cells compared with normal cells.[22] *MASPIN* is also downregulated in some other cancers, including prostate cancer,[23] and has been shown to inhibit mammary and prostatic tumor cell invasion and motility, as well as angiogenesis and metastasis.[22,24–28] *In vitro* studies using breast cancer cells showed a tight link between loss of *MASPIN* expression and aberrant cytosine methylation and histone deacetylation of the gene promoter.[29–31] Examining archival breast tissue from normal controls and a small number of patients with various stages of breast cancer, Maass and colleagues found loss of *MASPIN* expression was a feature of early breast cancer.[32]

We used immunohistochemistry staining to confirm the latter finding in a larger sample and performed bisulfite sequencing to demonstrate that *MASPIN* silencing *in vivo* is often due to aberrant cytosine methylation in the promoter region.[1] Our study examined 30 archival ductal carcinoma *in situ* (DCIS) specimens obtained during lumpectomy or mastectomy and used healthy tissue removed during reduction mammoplasty in two women as a control. Immunohistochemical staining for MASPIN protein expression was performed with a *MASPIN* antibody, and DNA for the bisulfite sequence analysis was extracted from normal and neoplastic ductal epithelial cells obtained by laser capture microdissection.

The immunohistochemical studies showed the ductal epithelial cells from the controls stained positive for *MASPIN* whereas 17 (57%) of the neoplastic ductal epithelial cell specimens were negative for *MASPIN* immunoreactivity. Even in specimens where *MASPIN* expression was absent in the tumor cells, the myoepithelial cells surrounding the transformed ductal cells and the epithelial cells from adjacent normal ducts were *MASPIN*-positive.

Using the results of the bisulfite sequencing analysis for DNA methylation, we found the *MASPIN* promoter was completely unmethylated in ductal epithelial cells derived from the reduction mammoplasty specimens and from normal ducts in the DCIS specimens. Of 11 tumor

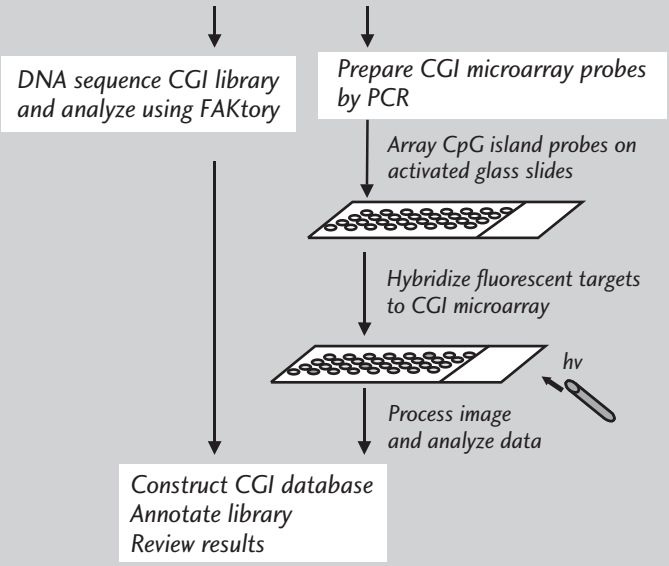

Figure 2. The CGI microarray technique permits simultaneous assessment of thousands of potential targets of DNA methylation on a single chip. It involves arraying of CpG island clones on glass slides, preparation of target sample amplicons, and hybridization of the amplicons onto the CGI microarrays. In the CpG island database, each clone is assigned an identification number and its characteristics are listed, including such information as its chromosome location, whether it is a promoter or found in the 5′ flanking region, its GC content, and what restriction sites exist in the clone. The latter information is useful for determining the utility of restriction enzyme analysis as well as the actual sequence.

specimens analyzed that had demonstrated loss of *MASPIN* expression by immunohistochemistry, 6 (55%) were found to have aberrant methylation of the *MASPIN* promoter, indicating that epigenetic dysfunction precedes malignant transformation of the breast.

CGI microarray analysis

We have used CGI microarray technology to analyze cytosine methylation and histone acetylation in human acute promyelocytic leukemia cells (NB4) before and after ATRA treatment in order to acquire a better understanding of how those two epigenetic changes to the genome of NB4 cells directly influence gene expression and subsequent phenotypic characteristics.[33] The NB4 cells provide a useful model for monitoring treatment-induced DNA methylation and histone acetylation pattern changes that might suggest new targets of epigenetic dysfunction. They contain the reciprocal translocation [t(15;17)(q22;q21)] that leads to production of the chimeric protein PML-RARα.[34] When retinoid is absent, PML-RARα represses target gene transcription by recruiting HDAC complexes with resultant blockade of granulocytic differentiation and continuous proliferation.[35,36] Treatment with ATRA results in transcriptional reprogramming accompanied by changes in chromatin structure with return of granulocytic differentiation.

Our studies compared NB4 cells before and after ATRA treatment using normal peripheral blood mononuclear cells (PBMCs) as controls. Mitochondrial DNA elements in the CpG island library were used in data normalization; mitochondrial DNA is unmethylated in all cells, and so in CGI microarray analysis, it yields the same amplification product from both the McrBC-digested and reference samples. The CGI microarray cytosine methylation analyses showed that that technique could be used to detect aberrant methylation. We found increased methylation of numerous CpG islands on the untreated NB4 cells relative to the PBMCs and validated those findings using bisulfite sequencing of previously sequenced individual clones. Comparison of clones taken from normal PBMCs and untreated NB4 cells showed, as expected, that *RARβ* was inappropriately methylated in the NB4 cells but not in the PBMCs. Similar studies were performed using other genes, including *HOXA1* and *MARK2*. This study also discovered many previously unknown methylated CpG islands in the leukemic cells and showed that ATRA had no effect on NB4 cytosine methylation.

ChIP with antibodies specific for acetylated histones H4 was used to capture histone-enriched genomic regions, and DNA purified from the chromatin of the PBMCs and the NB4 cells before and after ATRA treatment was assessed for histone acetylation in CGI microarray analysis. Those studies showed that the NB4 cells were generally hypoacetylated, compared with the PBMCs, and ATRA was a potent driver of histone reacetylation in the leukemic cells. Compared with the controls, the ATRA-treated NB4 cells exhibited dramatic increases in acetylation, with about 20% of CpG island promoters

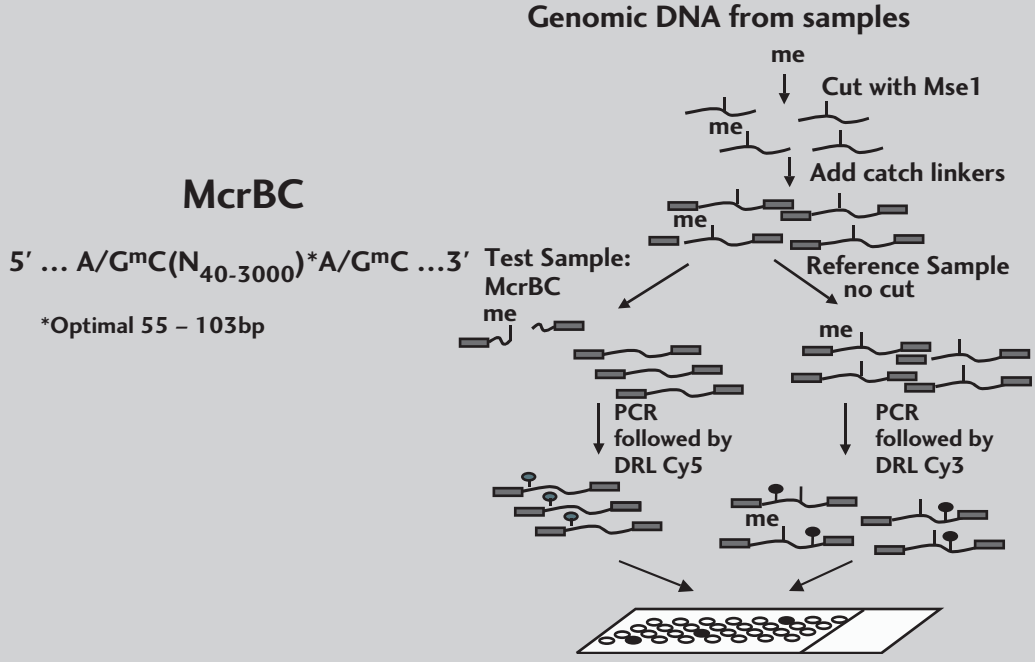

Figure 3. CGI microarray is performed using tumor-derived genomic DNA samples that are obtained using a restriction enzyme (Mse1) able to cut immediately outside of the CpG island. Catch linkers bearing PCR primer sequences are added to the Mse1 fragments. The sample is split into a reference portion and a test portion that is digested with McrBC, a methylation sensitive restriction enzyme that only digests DNA if the region is methylated. The two portions are amplified by PCR, then the DNA is direct labeled with Cy5 red (test) or Cy3 green (reference) fluorescent dyes. DNA fragments not digested by McrBC in the test sample produce Cy5-labeled PCR product while no labeled PCR product is produced if there were methylated fragments digested by McrBC. The labeled test and reference PCR products are mixed and spotted onto the glass slide. The hybridized slides are scanned and the acquired images analyzed to identify methylated signals.

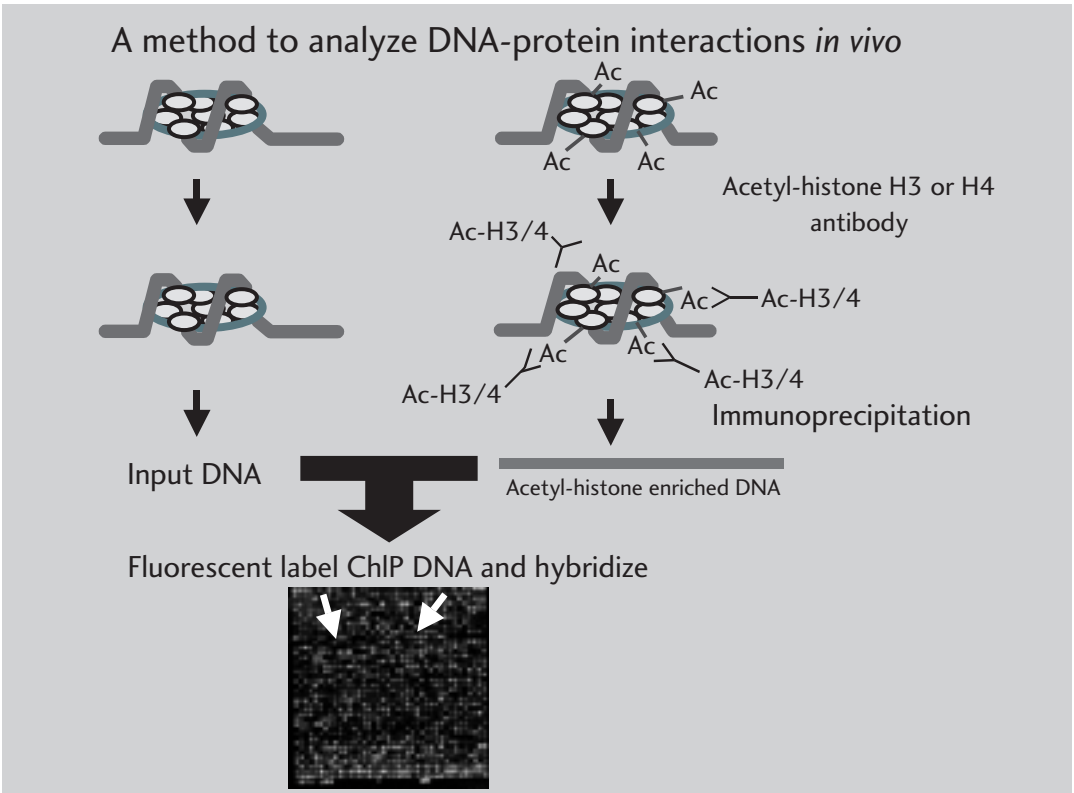

Figure 4. Cells are fixed with formaldehyde, which cross links histones to DNA, and then they are washed, resuspended, and sonicated before the lysate is divided into two fractions. The first fraction is treated with antibodies to the proteins of interest so that the DNA associated with those proteins can be selectively recovered. Those antibodies can be directed against the acetylated or methylated histones or to such proteins as p53. The second fraction serves as an input control. The samples are incubated overnight to allow immunoprecipitation of the chromatin complexes, which are subsequently collected and further processed so that the excess protein is digested away to yield acetyl-histone or methyl-histone enriched DNA. The DNA is then tagged with a fluorescent label and analyzed with the CGI microarrays.

becoming hyperacetylated after treatment with ATRA. Interestingly, small but significant increases in histone acetylation were also seen in about 80% of satellite sequences associated with pericentromeric heterochromatin.

The histone acetylation changes detected by microarray profiling were confirmed using real time PCR analysis of selected clones from the ChIP DNA. *RARβ* was among the genes associated with CpG islands that displayed increased levels of histone acetylation. Again, that finding demonstrated the accuracy of the assay since *RARβ* is a known target of ATRA and serves as a positive control. ATRA induced changes in histone acetylation status for several other genes, however; increases were noted for *HOXA1* and *MARK2*, while a CpG island associated with *MAD1L1* showed decreased histone acetylation. *HOXA1* is of particular interest as a future research target since its CpG island was also found to be aberrantly methylated and because it is a member of the homeobox gene family whose dysregulation has been linked to leukemogenesis.[37–39]

We have also undertaken studies using human breast cancer cell lines (MDA-MB-231 and UACC 1179) to determine if CGI microarrays can be used to identify new epigenetic targets that are selectively affected by epigenetic modifiers. Each of those cell lines has both well defined and unknown aberrantly methylated CpG islands and is known to be sensitive to transcriptional reprogramming induced by azacitidine. Therefore, they provide a good system for determining whether the CGI microarrays can be used as a screening technique to detect changes in the DNA methylation state after treatment with methylation inhibitors.

In one experiment, breast cancer cells were treated with azacitidine in concentrations ranging from 1 to 100 micromolar. The findings are still preliminary, but they have been replicated by two independent researchers and showed encouragingly that azacitidine treatment increased by about 20-fold the number of CpG island clones that are scored as demethylated compared with the untreated controls. Achieving demethylation required exposure to azacitidine while the cells were proliferating so that they were able to incorporate the drug, and importantly, the effect was found to be dose-related and extended to repetitive elements rather than just single copy sequences.

Having validated the ability of the CGI array hybridization technique to identify DNA methylation, we are also interested in using it to try to distinguish normal from disease states and thus potential epigenetic targets. Those studies began with myelodysplastic syndrome (MDS) and have expanded to include acute myeloid leukemia, breast cancer, and ovarian cancer.

Historically, treatment options for MDS have been very limited, and management consists primarily of supportive care. Results from recent clinical studies, however, have been encouraging in suggesting that epigenetic modification using DNA methyltransferase inhibitors (azacitidine,

decitabine) as well as various HDAC inhibitors (depsipeptide, suberoyl anilide hydroxyamic acid, valproate) can lead to transcriptional reprogramming with restoration of maturation programs.[40–47] In our investigation, which analyzed DNA derived from bone marrow of 42 patients with MDS and 10 healthy controls, application of multidimensional scaling to the CGI array results showed a clear separation between the normal and diseased marrow, which suggests the presence of dramatic epigenetic differences between the two cell populations (Figure 5).

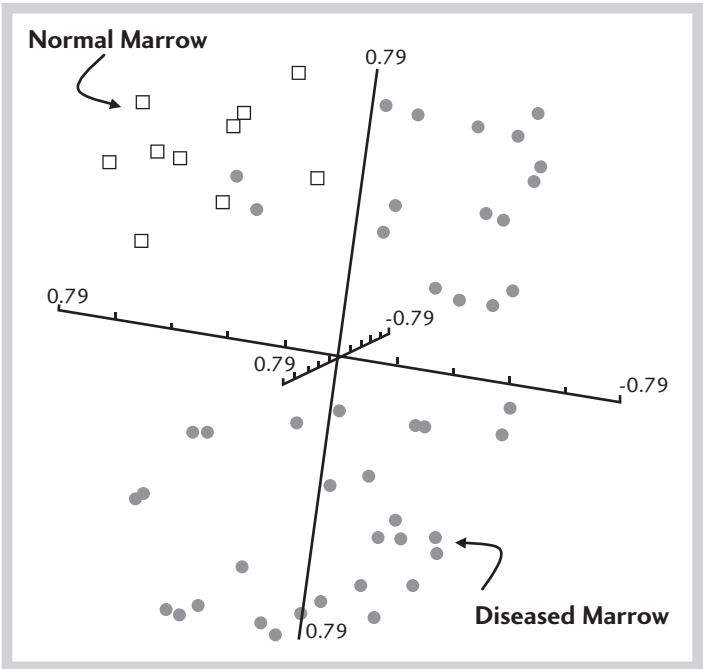

Figure 5. An analysis of DNA derived from bone marrow of 42 patients with MDS and 10 healthy controls, showed a clear separation between the normal and diseased marrow, which suggests the presence of dramatic epigenetic differences between the two cell populations.

Conclusions

The identification of epigenetic signatures of human cancers has the potential to translate into many valuable diagnostic and therapeutic applications. Characterization of the timing of epigenetic changes as events in tumor development and progression may provide molecular screening tools for risk assessment and early detection, as well as for prognostic monitoring. In addition, pharmacologic reversal of inappropriate DNA methylation and histone modification is emerging as a powerful new platform for the development of novel oncology drugs.

Epigenetic/epigenomic analysis is an exploding field. Equipped with a variety of molecular approaches for profiling the epigenetic changes that drive cancer progression, researchers are making significant progress in identifying promising new targets that may serve as the foundation for future advancements in cancer diagnosis, monitoring, and treatment.

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Current and Emerging Therapeutic Applications

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Abnormal methylation of DNA is associated with the development and progression of certain types of cancer. Methylation occurs when DNA methyltransferase links a methyl group to a cytosine base. Methylation of promoter regions of genes results in transcriptional silencing; thus, methylation of tumor suppressor genes may result in unregulated cell division, abnormal cell growth, and the development of cancer.[1,2]

Re-expression of abnormally silenced tumor suppressor genes has become a target for cancer research. DNA methyltransferase inhibitors reverse gene methylation, allowing normal transcription to resume. A second approach to gene re-expression is through inhibition of histone deacetylase (HDAC). Removal of acetyl groups from lysine tails of histones leads to a transcriptionally repressive conformation of chromatin (heterochromatin), impeding transcription of genes packaged within that region of chromatin. Inhibiting HDAC may lead to remodeling of chromatin to a transcriptionally active conformation (euchromatin), resulting in more normal transcription.[2,3]

Methyltransferase inhibitors and HDAC inhibitors have each been studied in myelodysplastic syndromes (MDS), hematologic disorders in which DNA methylation and gene silencing have been demonstrated.[4] Compounds that have been shown to inhibit methyltransferase in MDS include azacitidine and decitabine. Recently, azacitidine was approved as the first drug indicated for the treatment of MDS. The HDAC inhibitor sodium phenylbutyrate also has been studied in MDS.[2,5]

MDS Overview

MDS is characterized by hematopoiesis leading to bone marrow failure and various types of cytopenias including anemia, leukopenia, and thrombocytopenia.[6,7] Up to 40% of MDS cases progress to acute myeloid leukemia (AML). The estimated incidence of MDS in the United States ranges from 15,000 to 20,000 new cases annually.[6]

Symptoms include weakness, fatigue, palpitations, dizziness, hemorrhage, and infection. Until recently, most patients received supportive care consisting of antibiotics and red blood cell (RBC) and platelet transfusions.[8] Various growth factors have been used alone or in combination to stimulate blood cell production. Chemotherapy and allogeneic stem cell transplantation have been successful for some younger patients, but mortality is high.[1,6,8]

Methylation of promoter genes has been identified in 68% of AML samples and in 35% of MDS samples. Methylation density increases in MDS with disease progression.[3] DNA methyltransferase inhibitors have been shown to improve hematologic parameters and delay progression of MDS.[4] The clinical efficacy of demethylating

agents used alone and sequentially with HDAC inhibitors for the treatment of MDS is described in greater detail in this article.

Clinical Results in MDS

Results from the Cancer and Leukemia Group B (CALGB) trial provided the basis for approval of azacitidine.[9] A total of 191 high-risk MDS patients were randomized to treatment with azacitidine (n = 99) or to supportive care (n = 92) consisting of transfusions and antibiotics. Patients in the active treatment arm received at least four cycles of azacitidine 75 mg/m^2/d injected subcutaneously for 7 days every 28 days. Patients in the supportive care arm were observed for at least 4 months or until disease progression occurred. Supportive care patients whose disease progressed were crossed over to treatment with azacitidine. A complete response was defined by bone marrow that was normal, or that contained <5% blasts, and normal peripheral blood measurements. Partial response was defined as bone marrow with ≤50% of initial bone marrow blasts, ≤50% restitution of initial deficits in peripheral blood measures, and no need for transfusions.

The total response rate among patients who received azacitidine was 60%, including a 7% complete response and a 16% partial response rate (Table 1). There was no meaningful response rate among patients in the supportive care arm, with only 5% showing improvement. Among supportive care patients who crossed over to active treatment, 47% showed some improvement, with 10% demonstrating complete response and 4% showing a partial response. In addition to the complete and partial responses, trilineage response developed among a significant number of patients scored as "hematologic improvement" (22%) but did not occur for any patient in the supportive care arm. The median duration of response among patients who achieved complete response, partial response, or improvement was 15 months (Figure 1). Measures of quality of life (fatigue, physical functioning, dyspnea, psychosocial distress, and positive affect) improved significantly for both the azacitidine arm patients and those in the crossover group who had been stable or worsening on supportive care. Patients who received azacitidine had decreased risk for AML transformation (median time to transformation 12 months in the supportive care arm vs 21 months in the azacitidine arm; $P = 0.007$) with a trend to increased survival in the azacitidine arm.

Table 1. Azacitidine vs Supportive Care Response

	Supportive Care N = 92	Azacitidine N = 99	Cross-over N = 49
Complete response	0 (0%)	7 (7%)*	5 (10%)*
Partial response	0 (0%)	15 (15%)**	2 (4%)
Improved	5 (5%)	38 (38%)**	16 (33%)
Total	5 (5%)	60 (60%)**	23 (47%)

*P <0.01
**P <0.0001

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Myelosuppression was the most common toxicity associated with azacitidine. Toxicity was transient and mild, with patients recovering in time for the next treatment cycle. Nausea and vomiting occurred among 4% of patients.[9]

Analysis of patients treated with azacitidine at Mt. Sinai School of Medicine, New York, revealed important information about cytogenetic responses.[3,10] Among treated patients who had clonal abnormalities, 8% developed a cytogenetic complete response, but 13% developed additional abnormalities. During treatment, clonal abnormalities developed among 36% of patients in the azacitidine treatment arm who initially had no abnormalities. This result is similar to the natural rate of abnormalities that typically develop in untreated MDS patients. Nevertheless, 60% of patients with clonal abnormalities developed hematologic improvement despite persistence of the abnormal clone.[3,10] This suggests that improved hematopoiesis induced by azacitidine reflects clonal remission; that is, more effective hematopoiesis develops from the abnormal clone. These results stand in contrast to usual approaches to cytotoxic therapy in hematologic malignancies in which bone marrow aplasia is induced to repopulate normal stem cells.

A congener of azacitidine, decitabine also has been studied as a treatment for MDS, although it is currently unapproved for this use.[11] In a Phase II study, decitabine was administered intravenously to 66 patients at a rate of 45 mg/m^2/d given in three divided doses for 3 days. This cycle was repeated every 6 weeks for no more than six cycles. Results were clinically significant, with 13 complete remissions, three partial responses, and 16 improvements. Decitabine increased platelet counts substantially even after a single treatment cycle. The most common adverse events included fever, infection, sepsis, neutropenia, and anemia. One patient suffered seizures that may have been treatment-related. The treatment-related mortality rate during this study was 8%, suggesting that the dose administered may have been too high. The median dura-

tion of treatment response was 31 weeks, which likely reflects the six-cycle limitation in treatment.

Results from a Phase III study of decitabine were recently announced in a press release.[12] A total of 170 patients were enrolled and randomized to decitabine or supportive care consisting of antibiotics, growth factors, and transfusions. Data were analyzed after 92 patients reached the primary end point of disease progression to AML or death. The median time to disease progression was 338 days for patients who received decitabine and 263 days for patients who received supportive care. The overall response rate among patients who received decitabine was 22%, with nine complete responders (10%) and 11 partial responders (12%). None of the patients in the supportive care group responded. Adverse events from decitabine included leukopenia, febrile neutropenia, nausea, constipation, diarrhea, vomiting, pneumonia, arthralgia, headache, and insomnia. Mortality rates were 12% for patients who received decitabine and 9% for patients who received supportive care.[12]

Combination Approaches to Re-expression

The most commonly methylated gene in malignant myeloid cells is the cyclin-dependent kinase inhibitor *p15*^{INK4B}, which is methylated in approximately 70% of AML samples and 50% of MDS samples. The incidence of *p15* methylation increases with disease progression, but *p15* methylation has no independent prognostic significance when blast percentages are included as a variable in the analysis.[3] Other methylated genes include *E-cadherin*, *p73*, and *RARβ*. Considering the biologic importance of the methylated genes in MDS, the use of demethylating agents such as azacitidine and decitabine to produce epigenetic changes that reverse gene silencing appears logical. But the relationship of DNA methyltransferase inhibition to the clinical activity of these agents is not fully understood.[3]

Another approach to re-establishing gene transcription is the inhibition of HDAC.[2] Since deacetylated histones are associated with transcriptionally inactivated chromatin, and since methylated DNA recruits HDAC through specific methylated DNA-binding proteins,[13] a better gene re-expression approach to reactivating gene re-expression was proposed using a sequential combination of a methyltransferase inhibitor with an HDAC inhibitor (Figure 2).[14] In fact, the subsequent addition of an HDAC inhibitor to cells treated with a DNA methyl-

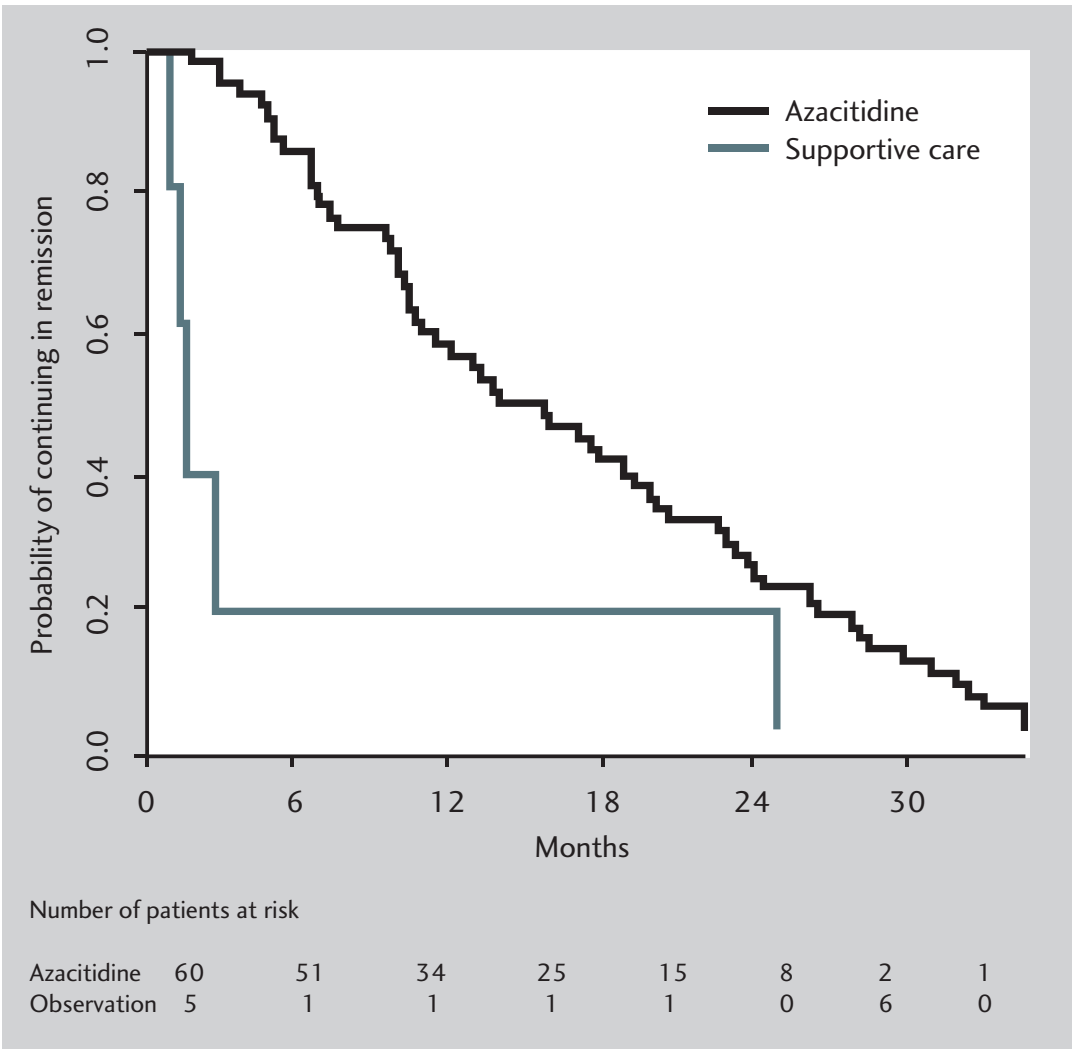

Figure 1. In the CALGB study, 191 high-risk MDS patients were randomized to treatment with azacitidine (n = 99) or to supportive care (n = 92) consisting of transfusions and antibiotics. The median duration of response among patients who achieved complete response, partial response, or improvement was 15 months.

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transferase inhibitor has an additive or synergistic impact on the re-expression of methylated genes in cancer cells.[14] Methylation inhibition must occur first before gene re-expression can be established, so the use of an HDAC inhibitor must follow demethylation.

Sodium Phenylbutyrate for HDAC Inhibition

Sodium phenylbutyrate was initially studied for its potential effects in myeloid malignancies and was found to produce modest clinical activity in high-risk myeloid neoplasms. In Phase I studies, sodium phenylbutyrate was administered *in vitro* in sustained plasma concentrations up to 1 mM.[2] With *in vitro* concentrations of approximately 0.25 mM, HDAC inhibition occurred, suggesting that sodium phenylbutyrate may act as an effective HDAC inhibitor.[15] When used alone, this compound produced some clinical activity in MDS and AML,[5,16] but in combination with azacitidine or decitabine, sodium phenylbutyrate may lead to additive or synergistic effects in producing gene differentiation and re-expression.[3]

These approaches are under clinical investigation and include a recently completed study of sequential azacitidine and sodium phenylbutyrate in patients with MDS and AML. In this study, a variety of azacitidine doses and schedules were administered in an attempt to optimize methylation changes. The azacitidine dose schedule included 25 mg/m^2/d for 14 days, 50 mg/m^2/d for 5, 10, and 14 days, and 75 mg/m^2/d for 5 days. Sodium phenylbutyrate was administered beginning on the final day of azacitidine at its maximum tolerated dose, 375 mg/kg/d, as an intravenous continuous infusion for 7 days. Results are pending. Other combinations of methyltransferase and HDAC inhibitors under investigation include decitabine plus valproic acid (MD Anderson Cancer Center), decitabine plus FK228 (depsipeptide) (National Cancer Institute), and an upcoming trial of azacitidine plus MS-275 (Johns Hopkins, Mt. Sinai, and University of Maryland).

Patient Selection

Studies of azacitidine and decitabine have included a broad range of patients with varying prognoses. Clinical data from the pivotal studies of MDS demonstrated significant benefit for patients of all International Prognostic Scoring System subsets. Patients who are likely to benefit from treatment with a demethylating agent include those who receive frequent transfusions and are at increased risk for iron overload, those who have excess blasts and hematologic instability, and those whose disease is at risk of progression. As studies continue and more is known about toxicity profiles for certain types of patients, the criteria for patient selection may narrow.

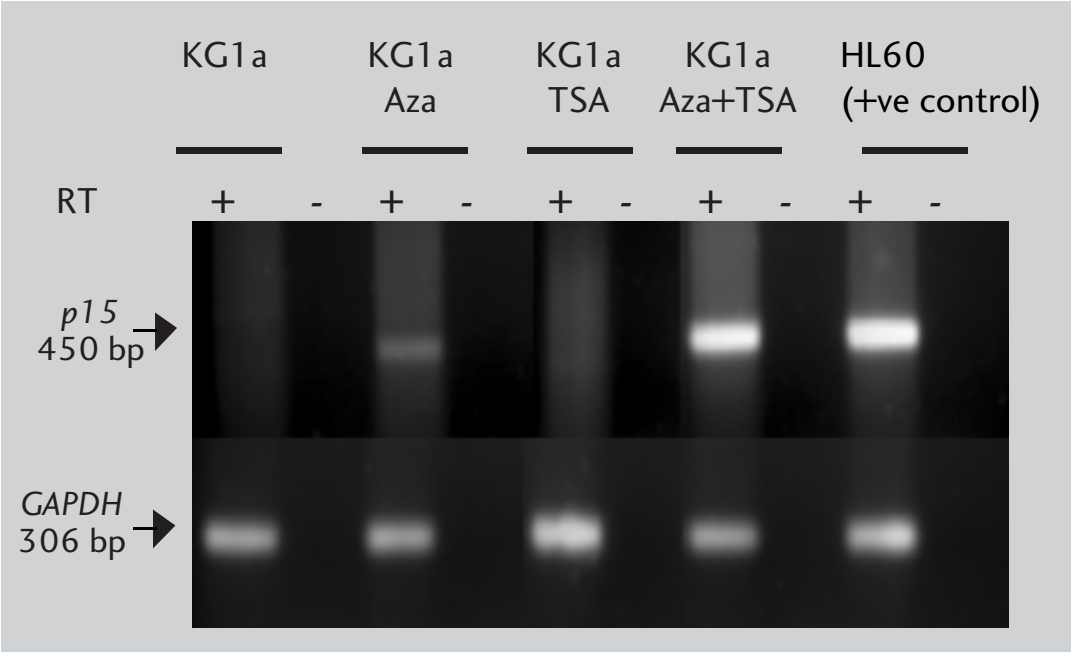

Figure 2. This figure illustrates the re-expression of *p15* on KG1a cells, which are extensively methylated in leukemia, through sequential methyltransferase and HDAC inhibition.

The Future of Epigenetic Targeting

Potential targets for epigenetic therapy include overcoming transcriptional repression induced by leukemia-specific fusion genes and modifying the sensitivity of cells to cytotoxic therapy. Another potential use for demethylating agents is in the treatment of sickle cell disease. Decitabine was administered to patients with sickle cell anemia and produced increases in F cells, fetal hemoglobin, total hemoglobin, and platelet counts, while causing minimal adverse effects. Low-dose studies are suggested as a means to produce clinical benefit while decreasing the risk of cytotoxicity.[17]

Researchers are also particularly interested in whether demethylating agents will work in solid tumors. Several obstacles to studying solid tumors remain, including drug delivery and the differences in the biology of solid tumors. The elements that are epigenetically regulated in leukemia are different from those in solid tumors, but the work that is underway in hematologic diseases is laying the groundwork for the study of solid tumors.

Conclusions

Epigenetic modifications are prime targets for therapeutic modulation in hematologic malignancies and potentially for other types of cancer, as well as sickle cell disease. DNA methyltransferase inhibitors currently are the most active single agents for the treatment of MDS. Clinical data has demonstrated their efficacy in delaying disease progression, and early data suggest a benefit in survival. Additional study is needed to confirm or add to these findings.

The optimal dosing schedule for methyltransferase inhibitors is also still under study. Sequential therapy with methyltransferase inhibitors and HDAC inhibitors may improve outcomes in MDS. Whether the synergistic effects of methyltransferase inhibitors and HDAC inhibitors noted *in vitro* will be reproduced *in vivo* remains to be seen. The development of additional HDAC inhibitors is ongoing and may provide many new combinations for use with azacitidine and decitabine.

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Self-assessment Quiz

1. Epigenetic gene silencing:

A. refers to direct alterations in the gene's nucleotide sequence.

B. is not associated with heritable changes in gene expression.

C. can be due to abnormal DNA methylation in gene promoter regions.

D. occurs as an irreversible, late event in cancer progression.

2. Azacitidine and decitabine:

A. bind to cell surface receptors to induce apoptosis.

B. are irreversible inhibitors of DNA methyltransferase enzymes.

C. cleave methyl groups from cytosine in CpG islands.

D. have activity that is specific for only a single DNA methyltransferase enzyme.

3. These proteins, together with DNA, form chromatin:

A. Trichostatins

B. CpG islands

C. Histones

D. Methyl-CPG binding proteins

4. Acetylation of histone 3 lysine 9:

A. is a code mark indicating transcriptional status of a gene.

B. induces subsequent methylation of the lysine residue.

C. prevents acetylation of histone 3 lysine 4.

D. is reversed by trichostatin.

5. Epigenetic silencing due to hypermethylation affects what gene as an early event in the pathogenesis of colorectal cancer?

A. *SFRP*

B. *APC*

C. *Beta-catenin*

D. *Wnt*

6. DNA methyltransferase enzymes:

A. repair hypermethylated CpG islands.

B. are activated by azacitidine.

C. act as platforms for proteins maintaining histones in configurations that suppress the transcriptional capability of chromatin.

D. represent a family of eight enzymes.

7. Inhibition of HDAC activity that prevents the deacetylation of lysine 9:

A. is achieved by treatment with azacitidine.

B. restores gene expression even in the presence of aberrant cytosine methylation.

C. restores gene expression permanently, even after treatment is discontinued.

D. is achieved by drugs such as trichostatin.

8. Which CpG islands are normally not methylated?

A. Promoter-related islands

B. Islands of imprinted genes

C. Islands of X-linked genes

D. Islands in satellite DNA sequences of pericentromeric heterochromatin

9. Which of the following methods can be used to search for unknown epigenetically silenced genes?

A. Bisulfite sequencing

B. MethyLight

C. Methylation-specific PCR

D. CpG island microarrays

10. Bisulfite sequencing:

A. uses sodium bisulfite to demethylate cytosine.

B. is an efficient technique that avoids the need for DNA sequencing and cloning after PCR.

C. requires DNA from a minimum of 1,000 cells.

D. yields a quantitative positive display of 5-methylcytosine residues.

11. *MASPIN:*

A. is an important pro-oncogenic gene.

B. is expressed only in breast cancer.

C. may be silenced in breast cancer due to aberrant methylation of its promoter region.

D. may be silenced in breast cancer due to histone deamination of its promoter region.

12. Treatment of NB4 human acute promyelocytic leukemia cells with all-trans retinoic acid causes:

A. cytosine demethylation.

B. chromatin structure changes leading to return of granulocytic differentiation.

C. histone deamination.

D. increased expression of *MASPIN*.

13. Epigenetic/epigenomic studies show CpG islands of which gene(s) are inappropriately methylated in leukemic NB4 cells but not in normal peripheral blood lymphocytes?

A. *RARβ*

B. *MAD1L1*

C. *MARK2*

D. All of the above

14. ChIP microarrays:

A. are a technique based on bisulfite sequencing.

B. use antibodies directed against acetylated or methylated histones.

C. use a plasmid vector for cloning and sequencing of amplified DNA.

D. identify expressed proteins in cell specimens using immunohistochemical stains.

15. Which of the following is the first drug approved for the treatment of myelodysplastic syndrome (MDS)?

A. Sodium phenylbutyrate

B. Azacitidine

C. All-trans retinoic acid

D. Decitabine

16. The estimated incidence of MDS in the United States is:

A. less than 10,000 cases per year.

B. 15,000 to 20,000 cases per year.

C. 50,000 to 60,000 cases per year.

D. 100,000 to 110,000 cases per year.

17. The most commonly methylated gene in malignant myeloid cells is:

A. $p15^{INK4B}$.

B. *E-cadherin*.

C. *p73*.

D. *RARβ*.

18. Decitabine, a congener of azacitidine:

A. produced inferior response rates compared with supportive care alone.

B. has been shown to produce an overall response rate of 22%.

C. further decreases platelet counts after a single treatment cycle.

D. has shown no effect on disease progression compared with supportive care.

19. Sodium phenylbutyrate:

A. is a methyltransferase inhibitor.

B. may lead to additive or synergistic effects when used with azacitidine.

C. must be administered concurrently with azacitidine.

D. is administered subcutaneously.

20. MDS patients likely to benefit from treatment with a demethylating agent include individuals who:

A. do not need frequent transfusions.

B. have excess blasts and hematologic instability.

C. are at low risk for progression to AML.

D. are not responding to supportive care.

Answer Sheet/Evaluation Form

Understanding DNA Methylation and Epigenetic Gene Silencing in Cancer

For each question, please circle the letter that corresponds to the correct answer. A score of 70% correct is required to obtain a maximum of 2 category 1 credits toward the AMA Physician's Recognition Award.

1.	a	b	c	d	6.	a	b	c	d	11.	a	b	c	d	16.	a	b	c	d
2.	a	b	c	d	7.	a	b	c	d	12.	a	b	c	d	17.	a	b	c	d
3.	a	b	c	d	8.	a	b	c	d	13.	a	b	c	d	18.	a	b	c	d
4.	a	b	c	d	9.	a	b	c	d	14.	a	b	c	d	19.	a	b	c	d
5.	a	b	c	d	10.	a	b	c	d	15.	a	b	c	d	20.	a	b	c	d

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Please take a few moments to complete the following evaluation of this CME activity so that we may serve you better in the future.

1. CME activities must be free of commercial bias for or against any product. In this regard, how would you rate this activity?

☐ Excellent ☐ Good ☐ Fair ☐ Poor

2. How would you rate the overall educational quality of this activity?

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3. How well did this activity meet its stated learning objectives?

☐ Excellent ☐ Good ☐ Fair ☐ Poor

4. What is the likelihood that you will make gradual or long-term changes in your clinical practice as a result of this activity? Please specify.

☐ Excellent ☐ Good ☐ Fair ☐ Poor _____

5. What suggestions do you have for improving this activity (eg, changes in objectives, educational technique, length, format)?

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