Cancer Epigenetics

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Abstract

Epigenetics refers to stable alterations in gene expression with no underlying modifications in the genetic sequence and is best exemplified by differentiation, in which multiple cell types diverge physiologically despite a common genetic code. Interest in this area of science has grown over the past decades, especially since it was found to play a major role in physiologic phenomena such as embryogenesis, imprinting, and X chromosome inactivation, and in disease states such as cancer. The latter had been previously thought of as a disease with an exclusive genetic etiology. However, recent data have demonstrated that the complexity of human carcinogenesis cannot be accounted for by genetic alterations alone, but also involves epigenetic changes in processes such as DNA methylation, histone modifications, and microRNA expression. In turn, these molecular alterations lead to permanent changes in the expression of genes that regulate the neoplastic phenotype, such as cellular growth and invasiveness. Targeting epigenetic modifiers has been referred to as epigenetic therapy. The success of this approach in hematopoietic malignancies validates the importance of epigenetic alterations in cancer, not only at the therapeutic level but also with regard to prevention, diagnosis, risk stratification, and prognosis. **CA Cancer J Clin 2010;60:376-392.** **©2010 American Cancer Society, Inc.**

Introduction

The term "epigenetics" refers to variability in gene expression, heritable through mitosis and potentially meiosis, without any underlying modification in the actual genetic sequence. This alteration in gene expression plays a fundamental role in several aspects of natural development, from embryogenesis, in which a resetting of the "epigenetic code" takes place in the very early moments after conception,[1] to the determination of cellular fate and its commitment to a particular lineage. Epigenetics also play a fundamental role in biological diversity such as phenotypic variation among genetically identical individuals.[2] Indeed, epigenetic processes account fully for the differences between queen bees and worker bees in *Apis mellifera* species.[3] Several mechanisms fall under the banner of the epigenetic machinery, the most studied of which are DNA methylation; histone modifications; and small, noncoding RNAs. In this review, we will first describe the general mechanisms through which the epigenetic code is established and then focus on the alterations of the epigenome taking place in cancer, with an emphasis on how these aberrations can potentially be used in the clinical setting.

Epigenetic Mechanisms

DNA Methylation

DNA methylation is a covalent modification of the cytosine ring at the 5′ position of a CpG dinucleotide, whereby a methyl group is deposited on the carbon 5 of that ring using S-adenosyl methionine as a methyl donor. This transfer of methyl group is a replication-dependent reaction catalyzed by DNA methyltransferases (DNMTs), present at the replication fork during the S-phase.[4] CpG dinucleotides, the usual targets of DNA methylation in

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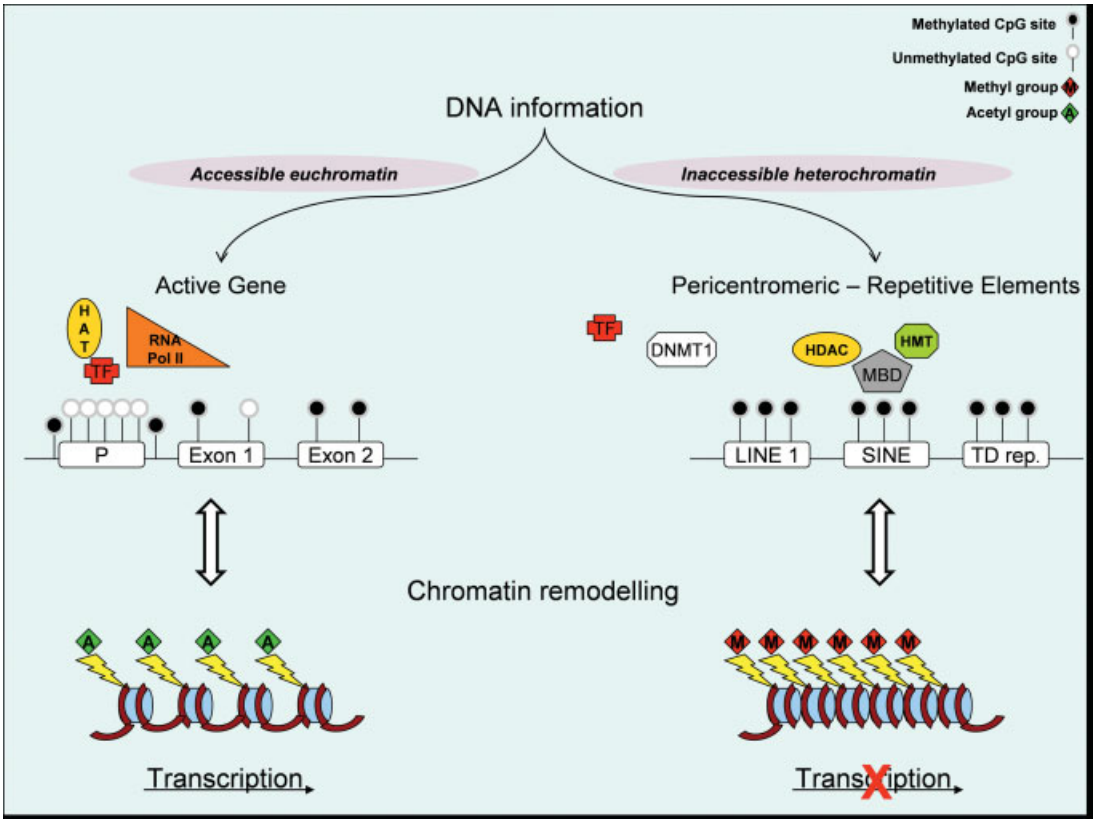

FIGURE 1. Normal Transcriptional Regulation in Higher Eukaryotes Is Shown. DNA is packaged in nucleosomal building blocks in a way that determines its accessibility to the nuclear environment and transcriptional status. (*Left*) Transcriptionally active genes are marked by methylation-free promoters and an open, highly acetylated chromatin configuration that allows access to transcription factors and polymerase II (pol II). (*Right*) Repetitive elements are silenced by high levels of DNA methylation, specific histone lysine methylation, and a closed chromatin state. A switch from active to inactive chromatin characterizes some genes in cancer cells. HAT indicates histone acetyltransferase; TF, transcription factor; RNA pol II, RNA pol II; DNMT1, DNA methyltransferase 1; HDAC, histone deacetylase; MBD, methyl-CpG binding protein; HMT, histone methyltransferase; P, gene promoter; LINE 1, long interspersed nuclear element 1; SINE, short interspersed nuclear element; TD rep, tandem repeats.

mammals, are scattered throughout the genome and present at a lower-than-expected abundance. This has been explained over evolution by the spontaneous deamination of the cytosine in the CpG site into a thymine.[5] However, in certain areas of the genome, a high concentration of these CpG dinucleotides is found, and these are referred to as "CpG islands" (CGIs).[6] These CGIs average 1000 base pairs and can be found at the 5′ promoter region of approximately 50% of genes. In a normal differentiated cell, CpG loci disseminated across the genome are highly methylated, whereas most promoter CGIs are protected from the spreading of methylation inside their boundaries.[6]

DNA methylation at gene promoter CGIs has been correlated with permanent expression silencing such as that noted in the inactive X chromosome in women.[4] DNA methylation leads to silencing by direct inhibition of transcription factor binding to their relative sites and by recruitment of methyl-binding domain proteins (MBDs).[4] These MBDs are present in transcription corepressor complexes involving several other members of the epigenetic machinery such as histone deacetylases (HDAC) and histone methyltransferases, resulting in chromatin reconfiguration and gene silencing.[7] One such MBD is MeCP2, the deletion of which causes the neurodevelopmental disorder called Rett syndrome.[8] Throughout evolution, DNA methylation has been used to silence the expression of endogenous repeats and infecting retrotransposons, keeping them from disrupting normal gene expression.[9] An overview of epigenetic regulation in eukaryotic cells is presented in Figure 1. Other physiological phenomena in which DNA methylation in CGIs plays a fundamental role are X chromosome inactivation,[10] genomic imprinting in which one allele is expressed depending on its paternal or maternal origin,[11] and somatic tissue-specific repression of a set of germ cell-specific genes.[12]

Although DNA methylation patterns in adult cells are relatively stable, important changes have been

described in aging tissues. A global decrease in 5-methylcytosine content was reported in cultured human fibroblasts[13] and promoter-specific hypermethylation was observed in epithelial tissues.[14,15] Global profiling using methylated CGI amplification in combination with microarray analysis demonstrated several hundreds of gene promoters to acquire methylation in aging mice intestinal mucosae whereas hundreds of others were found to have a parallel loss of DNA methylation.[16] This linear change of 5-methylcytosine content with aging has a strong tissue specificity and has been shown to be common across mammals. Indeed, both the amount and pattern of DNA methylation have been found to diverge between human monozygotic twins as they age.[17] It is still not clear whether the accumulation of these DNA methylation defects with time is of a random or rather programmed nature, and although their pathophysiologic consequences are unknown, they have been proposed to play a role in aging disorders, including cancer.

DNA methylation is catalyzed by a group of enzymes in mammals called DNMT1, DNMT3a, and DNMT3b. DNMT1, known as the "maintenance methyltransferase," has been shown to have a 10-fold preference for hemimethylated DNA (only 1 of the 2 DNA strands is methylated) compared with an unmethylated strand, and is used mostly by the cell to maintain the DNA methylation status in a stable fashion through cell division.[18] DNMT3a and DNMT3b, known as "de novo" methyltransferases, are used by the mammalian cell to methylate previously unmethylated DNA. It is worth mentioning that DNMT1 demonstrates far higher catalytic activity than DNMT3a and DNMT3b,[19] and all 3 are involved in important cellular functions such as differentiation.[20] The functional importance of these enzymes is highlighted by the fact that DNMT deletion is embryonically lethal in mice.[21]

Post-Translational Histone Modifications

DNA is wrapped around histone proteins to form nucleosomes, in a way that regulates accessibility of the genetic sequence to the nuclear environment.[22] Each nucleosome is comprised of a tetramer of 2 histone 2A (H2A) and 2 histone 2B (H2B) molecules, flanked by H3 and H4 dimers. H3 and H4 have N-terminal tails that, in their deacetylated form, are positively charged, leading to a closed and tight chromatin configuration around the negatively charged deoxyribonucleic acid. The addition of an acetyl group neutralizes the positive charge of the lysine residues in these N-terminal tails, loosening up this tight bond between DNA and histones, resulting in a more open chromatin configuration accessible to being successfully transcribed.[23] Two consecutive nucleosomes are tied together by linker histone H1. Recent studies have shown that the abundance of these linker histones is tightly related to chromatin configuration and might be altered in cancer cells.[24]

Histone modifications comprise a multitude of covalent reactions affecting the histone N-terminal tails, and form a code that fine tunes the way DNA is wrapped around these proteins. These post-translational modifications include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation.[22] These reactions occur in a very targeted and amino acid-specific way, the most studied of which are acetylation and methylation of specific lysine residues on histones H3 and H4. Several enzymes catalyzing these reactions, namely histone acetyltransferases (HAT), HDAC, histone methyltransferases (HMT), and histone demethylases (HDMT), have been identified. These enzymes exert their function in the setting of either transcriptional activator or repressor complexes, depending on the specific substrate residue.

Histone acetylation status results from an intricate cross-talk between HATs and HDACs. HATs are separated according to their cellular location and function into 2 distinct groups: the cytoplasmic B-type HATs and the nuclear A-type HATs.[25] The latter are presumed to have more impact on gene transcription, whereas cytoplasmic HATs can catalyze acetylation of nonhistone proteins. The most studied HAT families are GCN5-related N-acetyltransferase (GNAT), MYST (MOZ, Ybf2/Sas3, Sas2, and Tip60), and p300/CREB-binding protein (CBP), all of which are associated with complexes such as GCN5, PCAF, MOF, and p300/CBP, respectively. These complexes interact with each other and, through both targeted promoter-specific and nontargeted general acetylation reactions, play significant roles in development, differentiation, and cell cycle progression.[26]

HDACs are a class of enzymes catalyzing the opposite action to HATs. They influence a myriad of cellular processes including signal transduction, apoptosis, cell cycle regulation, and cell growth.[27]

HDACs catalyze deacetylation of both histone and nonhistone proteins and, similar to HATs, can be either nuclear or cytoplasmic. This cytoplasmic deacetylase activity can lead to post-translational modifications of transcription factors and chaperone proteins, and can have major effects on several important pathways, such as the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway,[28] the APE1-Ref1 oxidative stress response pathway,[29] and the phosphatase and tensin homolog (PTEN) phosphatase gene.[30] Similarly to HATs, HDACs exert their catalytic activity through an association with protein complexes, such as the sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) protein deacetylase complex.[31]

Histone methylation also plays a major role in gene expression regulation.[32] Histone methylation is associated with transcriptional repression or activation depending on the specific amino acid affected. For example, methylation of histone H3 lysines 4 and 36 is associated with active gene expression, whereas methylation of histone H3 lysines 9 and 27 is associated with gene silencing. Histone methylation is catalyzed by a large number of enzymes, the majority of which contain a specific protein module called SET (su(var)3-9, enhancer-of-zeste, trithorax) domain.[33] Similar to acetylation/deacetylation, histone methylation is reversible and catalyzed by 2 families of HDMTs, namely the lysine-specific demethylase 1 (LSD1) and the Jumonji domain-containing enzymes.[34,35] Histone methylases and HDMTs are usually part of large protein complexes that regulate gene transcription.

Histones can also be targeted by other post-translational modifications such as phosphorylation, ADP-ribosylation, and ubiquitination. These affect a limited number of residues but could play an important role in gene regulation. For example, serine 10 phosphorylation is inversely correlated with lysine methylation, and this methylation/phosphorylation module is conserved across different proteins.[36]

Histone modifications (and DNA methylation) ultimately affect gene expression in part by influencing nucleosome positioning. Active genes demonstrate a lack of nucleosomes at their transcription start site, whereas epigenetically silenced genes have a nucleosome positioned critically at the start of transcription.[37] Thus, nucleosome positioning can be involved in either the activation or repression of gene transcription.[38] The Swi/Snf protein complexes play a major role in this process.[39] Through their targeting to specific gene promoters, these complexes can activate or repress transcription via 3 biochemical processes: nucleosome remodelling, nucleosome sliding, and octamer transfer.[40] It is still unknown whether nucleosome formation and positioning is mainly determined by underlying proximal genetic sequences ("cis effect") or by other mechanisms operated by ATP-dependent nucleosome remodelling complexes in a sequence-independent manner ("trans effect"). Recent studies have suggested that the answer is more likely to be a mixture of the 2, in some type of a nucleosome positioning code governing histone-DNA interactions.[37]

Noncoding RNAs

Small noncoding RNAs refer to a family of RNAs that, by complementarity to the 3′ untranslated region of messenger RNAs, lead to their degradation and subsequent inhibition of gene expression.[41] Part of this family of noncoding RNAs are 20- to 22-nucleotide microRNAs (miRNAs), resulting from the sequential splicing of primary then pre-RNAs. These oligonucleotides are first synthesized as long, noncoding RNAs that are processed by the RNA cleaving enzyme DROSHA in the nucleus, transported into the cytoplasm in the form of short hairpin RNAs, and further cleaved by the enzyme DICER into their final configuration of double-stranded miRNAs.[41] miRNAs are then incorporated in the RNA-induced silencing complex and transported back in the nucleus, where they exert their biological effect. Through Watson-Crick base pairing, miRNAs bind to complementary sequences of mRNAs and induce either degradation or translational silencing of the target mRNAs.[41] It is interesting to note that miRNAs are also themselves epigenetically regulated at their promoter level, and target many genes that play important roles in such processes as cell cycle progression, apoptosis, and differentiation.[42] A single miRNA can have hundreds of target mRNAs, highlighting the implication of this gene regulation system in cellular functions.[43] The study of miRNAs has become the subject of intense interest, especially after the discovery of the fundamental role of these small, noncoding RNAs in a myriad of cellular and biological processes ranging from development to disease states.[44]

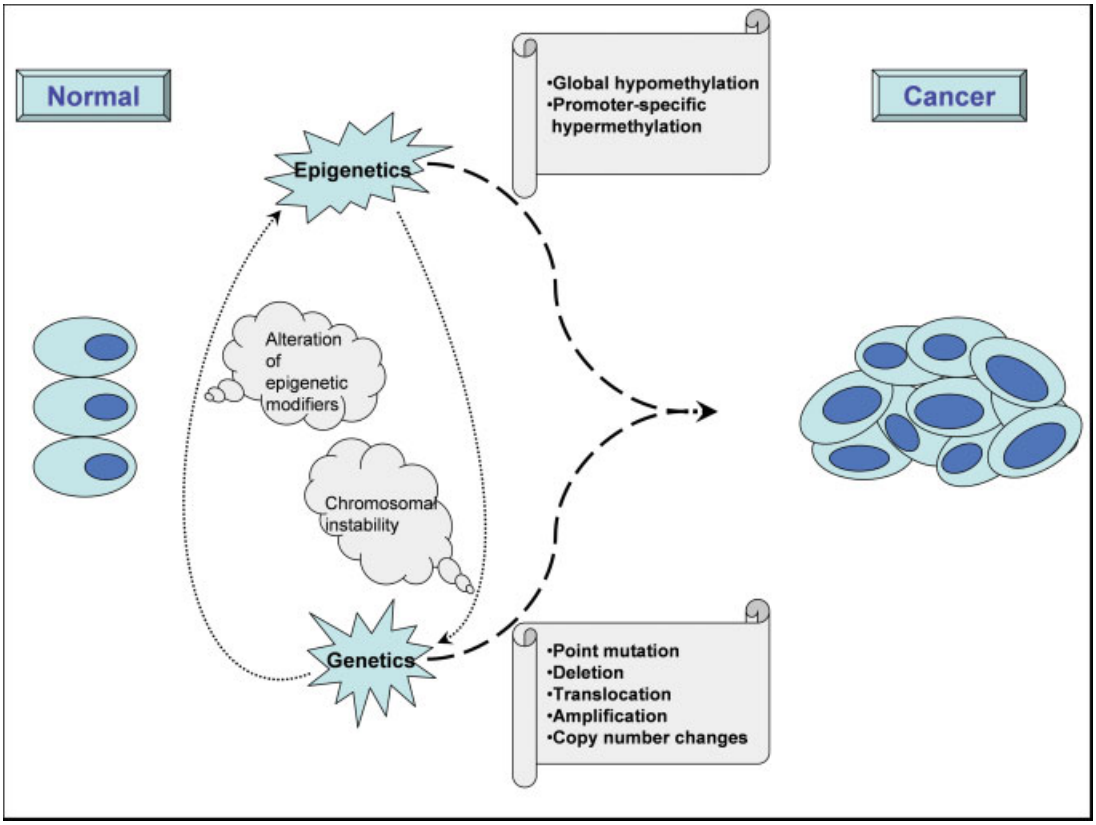

FIGURE 2. Tumorigenic Mechanisms in Mammalian Cells Are Shown. Both genetic and epigenetic aberrations are involved in neoplastic transformation. These 2 alternate pathways of tumorigenesis are linked by an intricate cross-talk and can, either individually or in synergy, lead to the development of the malignant phenotype.

Epigenetic Changes in Cancer

Cancer cells have genome-wide aberrations at the epigenetic level, including global hypomethylation, promoter-specific hypermethylation, histone deacetylation, global down-regulation of miRNAs, and up-regulation of certain actors of the epigenetic machinery such as EZH2. These aberrations confer a selective growth advantage to neoplastic cells, leading to apoptotic deficiency, uninhibited cellular proliferation, and tumorigenicity (Fig. 2). In the following sections, we will describe these different layers of epigenetic regulation and their aberrant functioning in cancer cells.

DNA Methylation in Cancer

Tumorigenesis is a result of the activation of oncogenic and/or inactivation of proapoptotic or tumor suppressor pathways. Initially, these were believed to result exclusively from genetic events such as mutations, amplifications, gene rearrangements, or deletions.[45] We now understand that DNA methylation is an alternate way of silencing tumor suppressor genes, in a manner equivalent to genetic mutations.[46] Examples of this mechanism of tumorigenesis are numerous, notably methylation of the mismatch repair gene human mutL homolog 1 (*MLH1*) in colorectal cancer, the DNA repair gene O-6-methylguanine-DNA methyltransferase (*MGMT*) in gliomas and colorectal cancer, and the cell cycle regulator *p16 (*cyclin-dependent kinase inhibitor 2A [*CDKN2A*]*)* in colorectal and other malignancies.[47] A "cross-talk" has been shown to exist between these mechanisms and genetic ones in a cell. This is exemplified in colorectal cancer, in which CGI promoter hypermethylation has been shown to be present only in the wild-type allele of silenced genes.[48] In addition, aberrant DNA methylation was more frequent than copy number changes when studied on a whole-genome level in malignant gliomas.[49] This is the case in colorectal cancer as well, in which individual tumors are found to harbor more hypermethylated genes than genetic mutations, and within individual genes, hypermethylation was found to be more frequent than genetic changes.[50] DNA methylation effects on pathway alterations can be either direct, by affecting promoters of tumor suppressor genes, or indirect, by silencing known inhibitors of oncogenes, such as the silencing of the secreted

frizzled-related protein (SFRP) family of genes, leading to the activation of the Wnt pathway in colorectal carcinogenesis.[51] Similar to mutations, silencing of tumor suppressor genes confers a selective proliferative advantage to corresponding cells, mediates invasiveness, and facilitates metastasis.

DNA hypermethylation is an early event in tumorigenesis, most likely playing a major role in tumor initiation and progression, and creating a fertile ground for the accumulation of a multitude of simultaneous genetic and epigenetic aberrations.[52] This is supported by the finding of a "field defect," in which normal tissue adjacent to a tumor is found to harbor several "epi-mutations" as well, most notably in colorectal cancers[53] but also in gastric cancer and liver cancer. Another example is *MGMT* hypermethylation, which plays a direct role in the accumulation of G-to-A mutations in the *KRAS* gene in colorectal tumors.[54] These data led to a new thinking regarding the mechanisms behind tumor initiation and progression, even at the earliest stages of carcinogenesis.

Aberrant patterns of DNA methylation in cancer have significant interneoplastic and interindividual variability, accounting not only for tumor type specificity but also personal variability.[55] The latter is best represented by the presence of a subgroup of patients demonstrating high levels of simultaneous gene promoter methylation, defining a phenomenon now known as CGI methylator phenotype or CIMP.[56] The best studied subgroup of CIMP-positive patients was described in colon cancer, in which these tumors were reported to comprise 20% to 40% of cases and were found to be associated with microsatellite instability (MSI), a defective human MutL homolog (MLH1) function, a location mostly in the ascending colon, an older patient age, and female predominance.[57] These CIMP-positive tumors often are clinically distinct from those in the rest of the patient population for the tumor type in question, which suggests that DNA methylation could be used for personalized cancer treatment in the clinical oncology setting.

On the other end of the spectrum, we find global DNA hypomethylation, the first epigenetic alteration noted in cancer cells.[58] In various cancers, 5 methylcytosine content was found to decrease by an average of 10%.[59] This affects both repetitive elements such as LINE1 and Alu[60] and specific gene promoters.[61] One potential consequence of profound hypomethylation is genomic instability, predisposing patients to mutations, deletions, amplifications, inversions, and translocations.[62] This may occur in part through reactivation of mobile elements. Indeed, hypomethylation correlates with a higher rate of chromosomal changes in patients with colon cancer[63] and is associated with a poor prognosis.[64] Another potential consequence of DNA hypomethylation is the reactivation of normally silenced genes.[65] This could lead to the disruption of normal gene expression and potential activation of growth-promoting and antiapoptotic pathways. Furthermore, promoter hypomethylation can lead to reactivation of miRNAs embedded in the coding regions of certain genes, resulting in silencing or aberrant expression of the corresponding protein.[42] Hypomethylation by genetic disruption of DNMT1 is protective against carcinogenesis in some models,[66] but can also promote tumor formation in others.[67]

Histone Modifications in Cancer

There is limited information regarding global histone modification profiling in cancer cell lines and primary tumors. Recent studies have demonstrated a global loss of histone H4 lysine 16 monoacetylation and histone H4 lysine 20 trimethylation in cancer.[68] These modifications were found to occur throughout the genome, specifically overlapping with areas of DNA hypomethylation in repetitive sequences. Conversely, loss of histone H3 lysine 9 acetylation and lysine 4 dimethylation or trimethylation and gain of histone H3 lysine 9 dimethylation or trimethylation and lysine 27 trimethylation can be found at specific gene promoters and can contribute to tumorigenesis by silencing critical tumor suppressor genes.[69] One interesting observation is the correlation between genes that are marked by DNA methylation in cancer and those found to be bound to the repressive polycomb group (PcG) proteins in embryonal cells.[70] These 2 groups appear to overlap, implying that certain genes are "poised" for silencing and "predetermined" to be the target of specific repressive histone marks in cancer.

Unlike DNA methylation, in which a bona fide DNA demethylase has not yet been identified, post-translational histone modifications are well characterized as a 2-way street governed by a balance of catalytic enzymes.[71] Shifting of this balance in cancer can occur through altered expression or function of epigenetic

modifiers, and this has been found to play a role in both murine and human neoplasia. For example, the histone methyltransferase PcG protein EZH2 catalyzes H3K27 trimethylation.[72] Its overexpression was found to promote tumor growth both in vitro and in vivo,[73] and is present in several cancers in the clinical setting, such as melanomas, lymphomas, and prostate and breast cancers.[74] EZH2 has also been found to be useful as a potential biomarker to distinguish aggressive prostate and breast tumors from more indolent ones.[73,75] In prostate cancer, EZH2 expression has been correlated with aberrant H3K27 trimethylation affecting potential tumor suppressor genes.[76] Recently, mutations of EZH2 were found in lymphomas,[77] but their functional significance there remains to be clarified. The H3K27 repressive methylation mark can also be over-represented in cancer through an alternative mechanism, inactivation of a specific H3K27 demethylase, *UTX*.[78] The latter has been shown to be somatically mutated in several tumor types, such as multiple myeloma, esophageal squamous cell carcinoma, and renal cell carcinoma.[78] Reintroduction of UTX in cancer cells presenting with an inactivating mutation of this gene led to a reversion of the malignant phenotype.[78] Another histone methylase, multiple myeloma SET domain (*MMSET*), is genetically altered by a common chromosomal translocation in multiple myeloma, resulting in altered expression of target genes.[79] In addition, the histone H3 lysine 9 methyltransferase *SUV39H* may play a role in carcinogenic initiation and progression.[80] Its deletion in mice was found to lead to chromosomal instability and increased tumor formation.[80] Perhaps one of the most relevant clinical entities highlighting the importance of HMTs in cancer is the 11q23 translocation in leukemias.[81] These have rearrangements giving rise to a multitude of fusion proteins involving the mixed lineage leukemia (MLL1) H3 lysine 4 HMT. MLL1 fusion proteins act as constitutively active chimeric transcription factors and lead to up-regulation of downstream homeobox (*HOX*) genes and activation of several leukemogenic pathways such as RAS and fms-related tyrosine kinase 3 (FLT3). MLL leukemias appear to have a unique transcriptional signature[82] and a poor prognosis overall.[81]

In addition to alterations in histone methylases/HDMTs in cancer, numerous changes in gene-specific histone acetylation have also been described. These can be primary or secondary to aberrant recruitment. For example, the chimeric oncoprotein promyelocytic leukemia-retinoic acid receptor α (PML-RARα) produced by the t(15:17) translocation in acute promyelocytic leukemia targets specific promoters through the aberrant recruitment of HDACs and HMTs, leading to silencing of gene expression.[83,84] In addition, DNA hypermethylation can lead to aberrant HDAC and HMT recruitment to specific promoters.[4] Conversely, direct primary changes in HATs/HDACs can also occur in cancer. Several studies have demonstrated a direct effect of p300/CBP HAT on cellular proliferation.[85] There is an interesting interaction reported between p300/CBP and the viral oncogenic protein E1A.[86] This association disrupts the interaction between the p300/CBP complex and other HATs, in turn leading to increased tumorigenesis. This mimics the effect of E1A on the retinoblastoma (Rb) tumor suppressor gene.[87] Mutations of p300/CBP are also found in Rubinstein-Taybi syndrome, a developmental disorder associated with an increased risk of solid tumors, leukemias, and lymphomas.[86] p300 mutations have also been noted in several human malignancies, including glioblastomas and breast and colorectal cancers.[86]

One of the limitations of studying histone modifications in cancer is the requirement for a relatively large number of fresh or fresh frozen cells. This has limited the study of these modifications in clinical tissue samples, although some data are beginning to accumulate in leukemias.[88] Advances in technology to analyze histone modifications are needed to improve our understanding of various tumors.

miRNAs in Cancer

The first studies that suggested a link between miRNA deregulation and cancer were focusing on observations made in *Caenorhabditis elegans* and later in *Drosophila*, with the discovery of *lin-4* and *let-7* miRNAs in the former[89,90] and the *Bantam* miRNA in the latter.[91] Knockout of *lin-4* or *let-7* in *C. elegans* led to abnormal differentiation,[92] whereas *Bantam* up-regulation in *Drosophila* led to cellular growth and the inhibition of apoptosis.[93] Mice studies confirmed the previous findings, and *Dicer* knockout led to a defective miRNA production and impaired cellular differentiation.[94] These observations suggested that miRNAs might play a role in human neoplasia. Indeed, microarray studies have shown that there are global alterations in miRNA expression in cancer,[95]

with many miRNAs that are down-regulated by genetic or epigenetic events, and some that are up-regulated. For example, the *let-7* family of miRNAs is aberrantly down-regulated in breast and lung tumors, leading to RAS pathway oncogenic activation.[96] Another example is the down-regulation of miR-15 and miR-16 in chronic lymphocytic leukemia (CLL) and resultant activation of the BCL2 proto-oncogene.[97] Overexpressed miRNAs include the miR-17-92 cluster, which plays a role in the development of lung and breast cancers as well as chronic myeloid leukemia through targeting of the transcription factor E2F1, a major cell cycle regulator.[98] miR-17-92 cluster amplification has also been shown to frequently play a role in the development of B-cell lymphoma.[99] Its overexpression led to increased disease aggressiveness in mouse models.[100] This cluster of miRNAs has also been shown to be activated by the oncogene *c-myc*,[101] highlighting its importance in tumorigenesis.

An interesting question relates to mechanisms of miRNA deregulation in cancer. Many miRNAs are transcriptionally regulated in a similar way as protein-coding genes and can be overexpressed by genetic mechanisms (eg, amplification) or suppressed by genetic (eg, deletion) or epigenetic (eg, hypermethylation) ones. Recently, *DICER* and *DROSHA* expressions were also found to be altered in some cancers.[102]

Clinical Applications: Epigenetic Tumor Markers

The rationale for the use of aberrant DNA methylation of a particular gene or a set of selected genes for clinical assessment comes from its frequency, stability, and variability between patients, which may indicate clinical usefulness. As mentioned earlier, DNA methylation is a stable and clonally propagated mark. Furthermore, DNA is less prone to degradation than RNA. Highly sensitive and/or quantitative methylation detection techniques are available, such as bisulfite pyrosequencing,[103] methylation-specific polymerase chain reaction,[104] or bisulfite treatment combined with high-throughput deep sequencing.[105] Moreover, aberrant methylation of some gene promoters is more common and easier to detect than the presence of mutations. This is especially valuable if the cancer cell or the cancer cell-derived free DNA is embedded in non-neoplastic cells or normal DNA molecules. Examples illustrating the potential use of epigenetic biomarkers in a clinical setting are described below and in Table 1.[57,63,106-124]

Aberrant DNA Methylation in Cancer Risk Assessment and Prevention

There are 2 potential ways by which DNA methylation can be used for risk assessment: the detection of constitutional aberrant DNA methylation and the detection of acquired abnormalities that are harbingers of cancer development. The first relates to the transgenerational transmissibility of epigenetic alterations. Although a resetting of epigenetic marks takes place in the germline,[1] making the heritability of epigenetic modifications between parents and their offspring highly improbable, constitutional epigenetic alterations are noted in certain individuals,[125] which could be either inherited or an acquired germline defect. The clinical entity that illustrates this clearly is the autosomal dominant hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, in which affected individuals are highly predisposed to developing colorectal and endometrial cancers at a relatively young age.[126] This syndrome is caused by defects in mismatch repair, leading to MSI. Genes potentially involved are *MLH1*, human mutS homolog 2 (*MSH2*), *MSH6*, and postmeiotic segregation increased 2 (S. cerevisiae) (*PMS2*). It is interesting to note that a few individuals with HNPCC were described in whom no sequence mutation was detected in any of these genes, whereas *MLH1* or *MSH2* promoter methylation was found to be present in normal tissues, including circulating white blood cells.[127] In the case of *MSH2*, this has been traced to a mutation in the tumor-associated calcium signal transducer 1 (*TACSTD1*) gene immediately adjacent to *MSH2*, leading to aberrant transcription through its promoter and associated DNA hypermethylation.[128] No such mutation was detected for *MLH1*, which therefore appears to be a rare germline defect that is occasionally inherited. Constitutional epigenetic changes (epimutations) can also result from genetic variations in the form of single nucleotide polymorphisms occasionally occurring in close proximity to a promoter and resulting in a predisposition toward acquired DNA methylation. This likely occurs via disruption of binding of transacting protective proteins such as Sp1.[129] Thus, the transgenerational heritability of epigenetic modifications can

TABLE 1. Examples of Clinically Relevant Epigenetic Biomarkers

EPIGENETIC BIOMARKER	CLINICAL RELEVANCE	SUPPORTING LITERATURE	SENSITIVITY/SPECIFICITY/OR/HR
Hypermethylation of *GSTP1*	Diagnosis/early detection of prostate cancer	Cairns 2001,[106] Lee 1994,[107] Eilers 2007[122]	Sensitivity/specificity: 92%/86%
Hypermethylation of *DAPK*	Association with early recurrence and pathological stage in bladder cancer	Jarmalaite 2008,[108] Catto 2005[109]	OR, 2.2 (95% CI, 1.04-4.5)
Hypermethylation of *MGMT*	Predictor of response to carmustine and temozolomide in gliomas	Hegi 2005,[110] Esteller 2000[111]	HR for death associated with nonmethylation, 9.5 (95% CI, 3.0-42.7) HR for progression of disease associated with nonmethylation, 10.8 (95% CI, 4.4-30.8)
CIMP	Subtype classification, risk stratification, and prognostic relevance in colorectal cancer, leukemias, MDS, etc.	Issa 2008,[63] Shen 2007,[57] Issa 2005,[112] Issa 2004,[113] Shen 2002,[114] Shen 2010[123]	HR for overall survival in MDS patients, 1.68 (95% CI, 1.0-2.81) HR for progression-free survival in MDS patients, 1.95 (95% CI, 1.18-3.21)
CIMP	Correlation with favorable prognosis in gliomas	Noushmehr 2010[115]	G-CIMP status as an independent predictor of survival ($P < .01$)
CIMP	Determinant of poor prognosis in neuroblastomas	Abe 2005[116]	HR, 22.1 (95% CI, 5.3-93.4)
Promoter methylation of *p16*, *CDH13*, *RASSF1A*, and *APC*	Association with early recurrence in stage I NSCLC	Brock 2008[117]	OR of recurrent cancer, 25.25
Promoter methylation of *p16* and of *MGMT-RASSF1A-DAPK-PAX5α* in plasma and sputum, respectively	Association with smoking and lung cancer risk	Belinsky 2005,[118] Belinsky 2006[124]	OR for cancer development, 6.5 Sensitivity/specificity: 65%/65%
Quantitation of promoter methylation of *p16*, $p14^{ARF}$, *MGMT*, and *GSTP1*	Detection of bladder cancer in urine sediment DNA	Hoque 2006[119]	Sensitivity/specificity: 82%/96%
Global histone modification profiling in primary prostatectomy tissue samples	Correlation with prognosis and risk of recurrence in low-grade prostate cancer	Seligson 2005[120]	HR, 9.2 (95% CI, 1.02-82.2)
microRNA signature	Association with clinical outcome (event-free survival) in cytogenetically normal AML patients with high-risk molecular features	Marcucci 2008[121]	HR for an event, 1.8 (95% CI, 1.0-3.0)

OR indicates odds ratio; HR, hazard ratio; *GSTP1*, glutathione S-transferase-π; *DAPK*, death-associated protein kinase; 95% CI, 95% confidence interval; *MGMT*, O-6-methylguanine-DNA methyltransferase; CIMP, CpG island methylator phenotype; MDS, myelodysplastic syndrome; G-CIMP, glioma CpG island methylator phenotype; *CDH13*, cadherin 13, H-cadherin (heart); *RASSF1A*, RAS association family 1A; *APC*, adenomatous polyposis coli; NSCLC, non-small cell lung cancer; *PAX5α*, paired box gene 5 α; AML, acute myeloid leukemia.

result either from cis-acting events or from epigenetic transmission per se, but a familial cancer predisposition related exclusively to epigenetic phenomena appears to be relatively rare.

The second approach to cancer risk assessment is based on methylation studies of normal or preneoplastic tissues to detect acquired epimutations. For example, in lung cancer, methylation of the *p16* gene was found to be present in preneoplastic lesions in smokers whereas no methylation was detected in never-smokers. Hence, *p16* methylation in conjunction with other genes (such as *p14*, *p15*, *E-cadherin*, and RAS association family 1A [*RASSF1A*]) has been proposed as a biomarker to assess a patient's risk for developing lung cancer, and this is being tested by detecting methylation in sputum.[118] Indeed, in one prospective study of 98 cases and 92 matched controls, promoter methylation of 14 genes in sputum was evaluated for lung cancer risk assessment. Promoter hypermethylation of 6 genes was found to be associated with a greater than 50% risk for subsequently developing lung cancer. The concomitant hypermethylation of 3 or more of these 6 genes was associated with an odds ratio of 6.5 for developing lung cancer, with a sensitivity

and specificity in the range of 65%.[124] It is interesting to note that hypermethylation of *P16* and *MGMT* was detectable in sputum years before the clinical occurrence of lung cancer.[130] Another example is found in colorectal cancer patients, in whom loss of imprinting (LOI) of insulin-like growth factor II (*IGF II*) was found concurrently in cancer and adjacent normal colorectal tissue. LOI of *IGF II* was also found in peripheral blood lymphocytes and its measurement was found to be predictive of the risk of developing colon cancer.[131] Also in the colon, age-related methylation in normal tissues has been proposed to mark a field defect associated with cancer risk, and measurement of this field could be a useful biomarker.[52] These data are relevant to cancer prevention because DNA methylation can be reversed by drug intervention. Therefore, its detection at a preneoplastic stage would open the door to cancer prevention strategies, either passively through close monitoring of the investigated tissue (serial colonoscopies/bronchoscopies, imaging studies, etc.) or actively by the use of hypomethylating drugs and/or chromatin remodelling agents to try and revert the premalignant phenotype.

Aberrant DNA Methylation as a Diagnostic Tool

Aberrant methylation has been tested in the clinical setting as a diagnostic biomarker in biopsy specimens or in bodily fluids such as serum, sputum, bronchoalveolar lavage, saliva, urine, pleural or peritoneal effusions, and stool. For example, glutathione S-transferase-π (*GSTP1*) promoter hypermethylation was found in 100% of human prostatic carcinoma tissue specimens in one study[107] and was able to detect the presence of malignancy in biopsy samples in a study of 86 patients in whom prostate cancer was suspected, with a sensitivity and specificity of 92% and 86%, respectively, and positive and negative predictive values of 82% and 94%, respectively.[122] Similarly, the presence of vimentin methylation in stool samples was found to have a 46% sensitivity (95% confidence interval [95% CI], 35%-56%) and a 90% specificity (95% CI, 85%-94%) in diagnosing colon cancer.[132] A potential lack of specificity of single markers can be remedied by the use of a panel of several aberrantly methylated genes. For example, methylation of a panel of 9 genes in urine sediment DNA from 175 patients and 94 controls was able to predict the presence of bladder cancer with a sensitivity of 82% (95% CI, 75%-87%) and a specificity of 96% (95% CI, 90%-99%).[119] One limitation to the use of DNA methylation as a biomarker for disease diagnosis and assessment is the possibility that aberrant methylation could originate from a precancerous lesion or reflect an age-related phenomenon.[14] Indeed, most studies published to date have suggested that this approach has a low positive predictive value despite relatively good sensitivity and specificity. More sensitive methods are being developed to address this issue.[105]

Aberrant DNA Methylation and Assessment of Prognosis/Response to Therapeutics

Methylation patterns can be useful to assess clinical outcomes or response to chemotherapeutic agents. In general, high levels of DNA methylation are associated with a poor prognosis such as in lung cancer[117] or myelodysplastic syndrome (MDS).[123] In a study of 51 cases with stage I non-small cell lung cancer (NSCLC) who developed an early recurrence after curative surgical resection and 116 controls who were free of disease recurrence after surgery, the promoter methylation status of 7 genes was investigated in tumor and lymph node samples for its association with NSCLC recurrence. Methylation of 4 of those genes (*P16*, cadherin 13 [*CDH13*], *RASSF1A*, and adenomatous polyposis coli [*APC*]) demonstrated an independent association with tumor recurrence, with methylation of *P16* and *CDH13* found to have an odds ratio of recurrent cancer of 15.5 and 25.25, respectively, in the training and combined training-validation cohorts. Similarly, MDS patients with higher levels of methylation, as assessed by studying a panel of 10 genes, were found to have a shorter median overall survival (12.3 months vs 17.5 months, respectively; $P = .04$) and a shorter median progression-free survival (6.4 months vs 14.9 months, respectively; $P = .009$) when compared with patients with lower levels of methylation. However, in some instances, intense hypermethylation defines a distinct subgroup of cancers that may have a favorable prognosis. This is the case in colon cancer, in which simultaneous methylation of multiple genes termed CIMP is associated with *MLH1* methylation, which results in a favorable prognosis.[56] CIMP has also been described recently in glioblastoma multiforme, in which it also was found to be associated with a better outcome; CIMP-positive cases were significantly younger at the time of diagnosis (median age of 36 years vs 59 years, respectively),

TABLE 2. US Food and Drug Administration-Approved Epigenetic-Acting Drugs

EPIGENETIC-ACTING DRUG	CLINICAL INDICATION	MAJOR DATA	SUPPORTING LITERATURE
DNA methyltransferase inhibitors			
5-azacytidine (azacitidine)	Symptomatic MDS	16% overall response rate; 66% hematologic improvement/transfusion independence	Kaminskas 2005[137] Fenaux 2009[138]
5-aza-2′-deoxycytidine (decitabine)	Intermediate and High-risk MDS	73% objective response rate; 34% complete response rate	Kantarjian 2007[139]
Histone deacetylase inhibitors			
Suberoylanilide hydroxamic acid (vorinostat)	Progressive, persistent, or recurrent cutaneous T-cell lymphoma	30% objective response rate	Mann 2007[140]
Romidepsin (depsipeptide)	Progressive, persistent, or recurrent cutaneous T-cell lymphoma	34% overall response rate; 6% complete response rate	Piekarz 2009[141]

MDS indicates myelodysplastic syndrome.

closely associated with *IDH1* somatic mutations, and had a significantly better survival (median survival of 150 weeks vs 42 weeks, respectively) compared with CIMP-negative cases ($P = .0165$).[115]

Methylation can also be useful as a predictive biomarker. For example, methylation of the *MGMT* DNA repair gene reportedly correlates with a good response to temozolomide and better overall clinical outcome in patients with glioblastoma multiforme.[110] Indeed, *MGMT* promoter methylation, present in approximately 45% of cases, was found to be correlated with a significant benefit from temozolomide therapy (median survival of 21.7 months compared with 15.3 months without temozolomide therapy; $P = .007$). In patients without *MGMT* methylation, the effects of temozolomide were less clear (median survival of 12.7 months compared with 11.8 months without temozolomide therapy; $P = .06$). These data suggest that tumor methylation profiling could be useful for risk stratification and making therapeutic decisions.

miRNAs in Cancer Diagnosis, Classification, and Prognosis

miRNA profiling has been shown to be informative both as a diagnostic tool and as a potential prognostic biomarker.[95] For example, miRNA profiling was shown to be useful in a series of tissue samples derived from metastatic sites of unknown primary origins.[133] The prognostic significance of miRNAs in cancer is currently being extensively studied. In CLL, the expression of a panel of 13 miRNAs was shown to correlate with disease aggressiveness as reflected by the time elapsed between diagnosis and first treatment.[134] However, this predictive ability has not been shown to be independent from other CLL prognostic markers.[134] In lung cancer, miR-155 and let-7 miRNA levels were found to be correlated with disease aggressiveness and clinical outcome.[135] Higher let-7 levels were associated with a more indolent disease and better survival after surgical resection. miR-155 has also been shown to be of prognostic value in patients with diffuse large B-cell lymphoma,[136] in whom it is present at significantly higher levels in the activated B-cell phenotype than in the germinal center phenotype. miRNA profiling could also be useful in the future as part of a model integrating multiple prognostic information.[121]

Clinical Applications: Epigenetic Therapy

With the understanding of the mechanisms underlying the silencing of tumor suppressor genes in cancer came the idea of pharmacologically relieving the inhibitory effects of DNA methylation and chromatin remodelling on gene expression. There are 2 classes of drugs that modify epigenetics and have been approved by the US Food and Drug Administration (FDA) for the treatment of cancer: DNA methylation inhibitors and HDAC inhibitors (Table 2).[137-141]

The available DNA methylation inhibitors are nucleoside analogues that exert their demethylating activity through the establishment of an irreversible covalent bond with DNMTs after their incorporation into DNA.[142] Hypomethylation requires that the cells

be proliferating after DNMT inhibition. These DNA methylation inhibitors were first introduced in the clinic several decades ago. At that time, they were used as cytotoxic chemotherapy at relatively high doses[143] and were found to be toxic (at these doses) without great antitumor activity. In the past 10 to 15 years, these drugs were reintroduced at lower doses that promoted the hypomethylating effect, and results of clinical trials indicated that repeated exposure induced DNA demethylation accompanied by a better antineoplastic effect than when used at higher doses.[144] This led to the approval of 5-azacytidine (azacitidine) in 2004[145] and 5-aza-2′-deoxycytidine (decitabine) in 2006[146] by the US FDA for the treatment of patients with MDS. Azacitidine induced an overall response rate in the range of 20% to 60% and significantly improved survival compared with standard of care.[138] Decitabine induced a high response rate at optimal doses[139] (complete response [CR]/pathologic CR rate of 40%) and has been shown to prolong survival when compared with historical controls.[147] The major side effect with these drugs is myelosuppression and the regimens used currently are well tolerated.[144] Some of the shortcomings of these drugs are their relatively short half-life, their instability in aqueous solutions, a lack of specificity inherent to their mechanism of action, and the fact that acquired resistance is nearly universal, without a clear mechanism. This has led to a search for potentially different DNA methylation inhibitors and several were identified such as the cytidine analogue zebularine, the antiarrhythmic procainamide (a weak inhibitor), and SGI-1027, a drug that may inhibit DNA methylation without requiring incorporation.[148]

Another interesting class of epigenetically targeted drugs are HDAC inhibitors.[149] HDAC inhibitors were initially identified through differentiation screens. These drugs target the catalytic domain of HDACs, thus interfering with their substrate recognition. Most HDAC inhibitors affect zinc-dependent HDACs and are divided into several classes depending on their chemical nature. The ones described to date comprise the short-chain fatty acids (such as sodium phenylbutyrate, sodium butyrate, and valproic acid); the hydroxamic acids (such as trichostatin A, vorinostat, and panobinostat); the cyclic peptides (such as romidepsin); and the benzamides, comprised of MGCD-0103 and entinostat. In 2006, suberoylanilide hydroxamic acid (vorinostat) was approved by the US FDA for the treatment of patients with progressive, persistent, or recurrent cutaneous T-cell lymphoma.[140] Recently, depsipeptide (romidepsin) received FDA approval for use in the refractory form of the same disease. Clinical trials of these and other HDAC inhibitors in other malignancies are currently ongoing. Early results suggest activity in other lymphoid malignancies such as Hodgkin lymphoma, but limited activity in solid tumors.[150]

Similar to DNA hypomethylating agents, HDAC inhibitors suffer from non-gene selectivity. The exact mechanism by which these drugs exert their gene expression reactivating effect is still unclear. One straightforward mechanism proposed is the induced hyperacetylation of histone proteins, leading to an open chromatin configuration and transcriptional activation.[149] However, the mechanism of action of these drugs is more complex because they are active both in the nucleus and the cytoplasm, and HDACs catalyze the deacetylation of both histone and nonhistone proteins. In fact, HDAC inhibitors might very well be exerting their antitumor activity through apoptosis or cellular differentiation induction by affecting multiple cellular pathways, some transcriptionally and some post-transcriptionally. Some of these pathways, along with the biological effects epigenetically targeted drugs have on tumor cells, are shown in Figure 3. There is currently interest in developing drugs that target other epigenetic pathways such as histone methylases/HDMT, MBDs, and histone readers.

The lack of specificity of epigenetically targeted drugs raises concerns about their use in clinical practice. Some of these concerns would be the reactivation of normally silenced sequences (such as repetitive elements) or imprinted genes. This reactivation could theoretically lead to allelic imbalance or genomic instability, and other deleterious effects of retrotransposon activation. To date, there are no data supporting these concerns clinically, but it is possible that problems will emerge after several years of therapy. This has led researchers in the field to try and develop new compounds selectively targeting specific genes. One example is the development of a methylated oligonucleotide directed toward the 5′ promoter region of the insulin-like growth factor 2 growth-promoting gene, subsequently leading to the methylation of this promoter and transient silencing of the gene.[151] This line

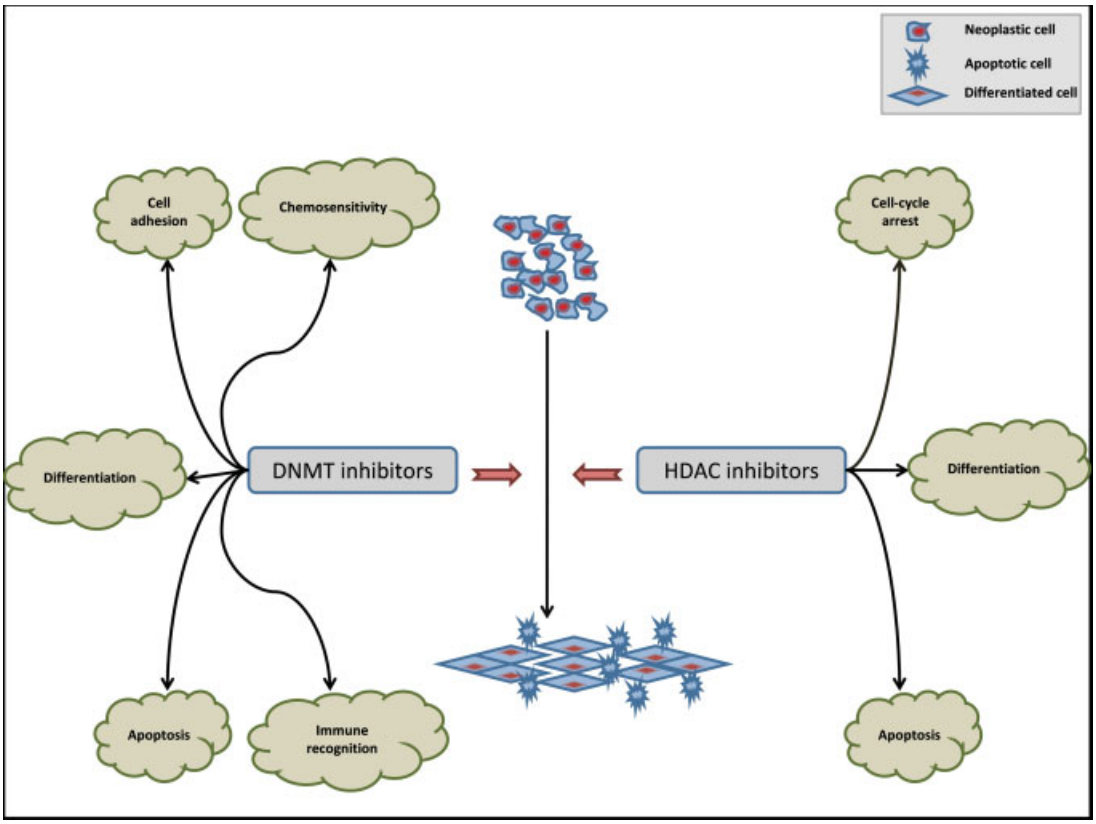

FIGURE 3. Epigenetic Therapy Is Shown. The 2 main families of epigenetically acting drugs, DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, exert their antineoplastic effect via several mechanisms such as cell cycle arrest, apoptosis induction, and immune recognition. These effects eventually result in differentiation or cancer cell death.

of research is still in its infancy and could face significant problems in drug delivery.

It is important to mention that epigenetic drugs are promising not only as single agents but also in combination with other epigenetically targeted drugs or with conventional chemotherapy. Several studies, both in vitro and in vivo, have demonstrated the synergistic effect of sequentially administering DNMT inhibitors (such as decitabine) and HDAC inhibitors (such as vorinostat),[142] and this approach is currently being tested in clinical trials. Furthermore, a synergistic effect was also found when combining epigenetic drugs with conventional chemotherapy,[152] and trials are currently testing these combinations in the clinical setting for several tumor types.

Several studies have tried to link DNA methylation profiles at study entry with response to therapy. To date, these studies of a limited number of genes have been negative.[123] Entire epigenome studies of this issue are currently ongoing. Studies also have tried to correlate global hypomethylation, as assessed by the methylation levels of LINE1 and Alus repetitive elements, at days 5 and 12 after decitabine therapy with clinical response. Results were controversial. Indeed, some studies found a trend toward a positive correlation between global hypomethylation at day 5 and clinical response[144] in patients with leukemia, whereas other studies found an inverse correlation between levels of hypomethylation at day 12 and achievement of CR[153] in patients with chronic myelogenous leukemia that was resistant to imatinib mesylate. The latter finding was hypothesized to be due to a cell death mechanism of response, with the resistant cells capable of sustaining more hypomethylation.

In contrast to DNA methylation markers, gene expression induction has been consistently linked to subsequent response to decitabine. This has been demonstrated for *P15*,[139] *ER*,[154] *P53R2*/ribonucleoside-diphosphate reductase subunit M2 B (*RRM2B*),[155] and miR-29b.[156]

Perhaps one of the major drawbacks of epigenetic therapy is the presence of spontaneous and/or acquired resistance to these drugs, both in vitro and in vivo. Indeed, in a panel of cancer cell lines, resistance to the hypomethylating agent decitabine was manifested by a 1000-fold difference in the half maximal

(50%) inhibitory concentration (IC_{50}) of this drug among the cell lines tested.[157] Resistance mechanisms were hypothesized to be related to variations in the parameters affecting nucleoside analogue metabolism, starting with transport inside the cell (human equilibrative nucleoside transporter 1 [hENT1] and hENT2), initial phosphorylation (deoxycytidine kinase [DCK] for decitabine and uridine-cytidine kinase [UCK] for azacitidine), and finally catabolism by the enzyme cytidine deaminase (CDA). Indeed, in vitro studies demonstrated that low levels of DCK and hENT1 and high levels of CDA were correlated with resistance to hypomethylating agents. In fact, the observed cross-resistance between decitabine and cytarabine (2 nucleoside analogues sharing the same need for phosphorylation by DCK for incorporation into the DNA) and the lack of cross-resistance between decitabine and azacytidine indicate that incorporation into the DNA plays a major role in cancer cell resistance to nucleoside analogues, including decitabine. These observations were found to be relevant in vivo as well, because low levels of DCK/low DCK activity were correlated with poor response to nucleoside analogues in, for example, childhood acute lymphoblastic leukemias[158] and pancreatic cancer.[159]

The full-scale implementation of these molecular markers of sensitivity to hypomethylating agents in the clinical setting faces several challenges. One is the less-than-perfect correlation between the clinical activity of these drugs and their hypomethylating effect. One possibility is that beyond a certain threshold, more hypomethylation does not correlate with a better clinical outcome. In fact, there may be molecular barriers downstream of hypomethylation that prevent adequate gene reactivation. Another possibility is the hypomethylation-independent mechanisms of antineoplastic activity of decitabine and azacitidine. Both drugs can induce DNA damage at relatively high doses, and azacitidine also affects RNA methylation.[160] Both of these effects may also be involved in clinical responses.

Conclusions

Understanding the complexity of the epigenome and of all the actors involved in modulating its interactions with genomic sequences is of fundamental importance in health and disease. This understanding will allow us to reach newer horizons in our search for the mechanisms governing cellular fate. On the tumorigenic spectrum, the time when we switch from untargeted cytotoxicity to reversion of the malignant phenotype is drawing near. ■

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