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REVIEW

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DNA methylation aberrancies as a guide for surveillance and treatment of human cancers

Gangning Liang[a] and Daniel J. Weisenberger[b]

[a]Department of Urology, University of Southern California, USC Norris Comprehensive Cancer Center, Los Angeles, CA, USA; [b]Department of Biochemistry and Molecular Medicine, University of Southern California, USC Norris Comprehensive Cancer Center, Los Angeles, CA, USA

ABSTRACT

DNA methylation aberrancies are hallmarks of human cancers and are characterized by global DNA hypomethylation of repetitive elements and non-CpG rich regions concomitant with locus-specific DNA hypermethylation. DNA methylation changes may result in altered gene expression profiles, most notably the silencing of tumor suppressors, microRNAs, endogenous retorviruses and tumor antigens due to promoter DNA hypermethylation, as well as oncogene upregulation due to gene-body DNA hypermethylation. Here, we review DNA methylation aberrancies in human cancers, their use in cancer surveillance and the interplay between DNA methylation and histone modifications in gene regulation. We also summarize DNA methylation inhibitors and their therapeutic effects in cancer treatment. In this context, we describe the integration of DNA methylation inhibitors with conventional chemotherapies, DNA repair inhibitors and immune-based therapies, to bring the epigenome closer to its normal state and increase sensitivity to other therapeutic agents to improve patient outcome and survival.

Challenges to cancer therapy

According to the US National Cancer Institute (www.cancer.gov), over 100 forms of human cancer have been identified, spanning all tissues in the human body. Multiple tumor subtypes can develop from each tissue; however, the mechanisms of tumorigenesis are not identical between tumor types. This is compounded by the fact that even though human cancer is a result of genetic, epigenetic, and transcriptomic alterations, there is no uniform cancer genomic profile for each primary tumor tissue type or even between tumors within individual subgroups. This has necessitated the use of precision medicine and personalized approaches for targeting the specific genomic signatures for treatment of individual patients or patient subgroups.

The Cancer Genome Atlas (TCGA) took the early steps in addressing this conundrum by characterizing genetic, epigenetic, transcriptomic, and protein alterations in over 11,000 primary tumors spanning nearly 30 tumor types with the ultimate goal of identifying novel therapeutic targets (cancergenome.nih.gov). TCGA and other efforts (summarized in ref.1) have advanced our understanding of the genomic driver mutations, copy number alterations (CNAs), gene expression changes, and the contributions of DNA methylation aberrancies toward the cancer genome. TCGA has verified the majority of known driver somatic mutations in nearly 20 major forms of human cancers, and has characterized additional alterations and low frequency mutations thanks to advancements in next-generation

sequencing and microarray technologies. However, these findings have only recently been described, and as a result, have not yet resulted in transforming cancer patient treatment.

This is especially relevant due to the generally low frequencies of genetic alterations in most types of cancers.[2,3] On the contrary, epigenetic alterations, most notably DNA methylation changes, are substantially more prevalent than genetic alterations in virtually every cancer type, further highlighting their importance as diagnostic tools and therapeutic targets.[4,5] Our understanding of cancer epigenetics, namely DNA methylation and chromatin modification changes, has been tremendously improved due to efforts from multi-institutional, international consortia including TCGA, the Encyclopedia of DNA Elements (ENCODE, www.encodeproject.org), the Roadmap Epigenetics Project (www.roadmapepigenomics.org), Therapeutically Applicable Research to Generate Effective Treatments (TARGET, https://ocg.cancer.gov/programs/target) for pediatric cancers, and others (reviewed in ref.1), as well as from technological advancements in sample throughput and the ability to profile increased genomic content. With these data, DNA methylation, histone modifications, and chromatin structure have become better understood, as are the mechanistic details of DNA methylation inhibition. This review focuses on DNA methylation changes in human cancers, their use as biomarkers of surveillance, attributes of DNA methylation inhibition, and therapeutic strategies using DNA methylation inhibitors with other treatment regimens.

CONTACT Daniel J. Weisenberger ✉ dan.weisenberger@med.usc.edu ✉ Department of Biochemistry and Molecular Medicine, University of Southern California, USC Norris Comprehensive Cancer Center, 1450 Biggy St., NRT G511D, Los Angeles, CA 90033 USA; Gangning Liang ✉ gliang@usc.edu ✉ Department of Urology, University of Southern California, USC Norris Comprehensive Cancer Center, 1441 Eastlake Ave, NOR 7344, Los Angeles, CA 90033 USA.

DNA methylation

DNA methylation in mammalian organisms is largely restricted to the addition of a methyl group to the C-5 position of cytosine in a 5′-CG-3′ (CpG) sequence context, with non-CpG DNA methylation generally found in embryonic stem (ES) cell populations (reviewed in ref.6). Methylated CpGs are more prone to spontaneous deamination to thymine than the rate at which unmethylated CpGs deaminate to uracil; therefore, the CpG content in the human genome is only 20% of what is expected by sequence context alone (reviewed in ref.6). Indeed, the human genome is CpG depleted, and approximately 70% of all CpGs are methylated,[6,7] with exact levels dependent on the tissue type, mostly in transposable elements (*LINE, SINE*, and *ERV*) and intergenic regions of the human genome. However, there are regions of the genome, called CpG islands, that contain their expected CpG content, are unmethylated in normal somatic tissues, and are enriched (> 50%) in gene promoter regions.[6]

Promoter CpG islands are generally not enriched in TATA boxes and other core promoter sequence motifs for focused transcription initiation, but rather show transcriptional permissivity in which transcription factor and RNA polymerase II binding to promoter CpG islands can result in switching between active and inactive transcriptional states (reviewed in ref.8). CpG islands do not have consensus sequences or fixed sizes, but are sources of transcription factor (TF) binding.[8] Several transcription factors, including Sp1, Nrf-1, E2F, and ETS, contain CpG dinucleotides in their recognition and binding sites.

Cancer-specific DNA methylation alterations

DNA methylation changes are hallmarks of every cancer type, and are highlighted by global DNA hypomethylation of repetitive elements and CpG-poor regions concomitant with gene-specific DNA hypermethylation.[9-11] DNA methylation alterations may result in gene expression changes, namely gene silencing due to CpG island (or CpG rich) promoter DNA hypermethylation and gene activation due to DNA hypomethylation of CpG-poor gene promoters. DNA hypomethylated genes can remain silenced by the presence of H3K27me3 repressive mark or the lack of required transcription factors. A recent study also demonstrated that DNA hypermethylation of gene bodies or transcribed regions is associated with oncogene overexpression,[12] showing that these gene expression consequences help propel tumorigenesis. In addition, the frequent occurrence of cancer-linked DNA hypermethylation and DNA hypomethylation is associated with tumor progression and tumor formation. Because of these attributes, DNA methylation alterations are thought to be early events in human tumorigenesis.

De Carvalho et al. performed genome-scale DNA methylation analyses of HCT116 colon cancer cells deficient for one or more DNA methyltransferases (DNMTs) to identify epigenetic driver genes that require DNA methylation for cancer cell survival.[13] These include, A disintegrin and metallopeptidase domain 2 (*ADAM2*), *BCHE, CDO1, ESX1*, interleukin-1 receptor-associated kinase 3 (*IRAK3*), *P2RY14*, and synaptonemal complex protein 3 (*SYCP3*), all of which are epigenetically silenced in human cancers and their DNA demethylation results in cell death. For example, *IRAK3* negatively regulates the anti-apoptotic gene *SURVIVIN* and inhibits MAPK, NF-kB, and STAT3 signaling pathways, which are activated in numerous cancer types. In addition, DNA demethylation and re-expression of *ADAM2* and *SYCP3* can trigger apoptotic cell death.

In light of the epigenetic-based expression changes in human cancers, however, not all DNA methylation changes drive gene expression consequences. In fact, the majority of DNA methylation aberrancies are simply passenger events.[14-16] However, these markings do serve as biomarkers of disease, and have been used in translational studies with diagnostic and prognostic utility to correlate with patient survival, response to chemotherapy, and other clinical co-variates (reviewed in ref.17). In addition, cancer-specific methylated DNA biomarkers can be identified in circulating tumor cells (CTCs) and tumor-derived, cell-free DNA (cfDNA) in blood, urine, or other bodily fluids, as sensitive (early) detection protocols (reviewed in refs. 17,18).

CpG island methylator phenotypes in human cancers

DNA methylation alterations substantially outnumber somatic mutations in human cancers,[19] and individual tumor types can be stratified into subgroups based on DNA methylation profiles. In 1999, Toyota et al. first identified a unique subset of colorectal tumors positive for a CpG Island Methylator Phenotype (CIMP) that display extensive DNA hypermethylation at a unique set of CpG islands that remained unmethylated in other colorectal tumors and normal tissues.[20] Follow up experiments showed that CIMP tumors are preferentially located in the proximal (right) colon, are enriched in women of older age, and harbor the *BRAF* V600E (*BRAF^{V600E}*) point mutation as well as *MLH1* epigenetic silencing due to promoter DNA hypermethylation and microsatellite instability (MSI).[21] Colorectal CIMP is associated with poor patient outcome; however, this is not completely understood since outcome is also affected by *BRAF* and MSI status.[22-27] A comprehensive, pooled analysis of 33 published reports describing the correlation between CIMP status and patient outcome in 10,635 patients [28] showed that CIMP status is associated with shorter disease free survival (DFS) and overall survival (OS), irrespective of MSI status. In addition, colon cancer patients with microsatellite stable (MSS) disease were significantly associated with CIMP and reduced OS. CIMP status may confer a predictive DFS advantage for patients receiving the conventional 5-fluorouracil (5-FU) based adjuvant therapies.[28] These reports suggest that CIMP-specific DNA methylation may be used for predictive and prognostic biomarkers; however, the efficacy of DNA methylation inhibitors in improving CIMP patient survival and outcome remains to be determined.

CIMPs have been characterized for cancers derived from brain,[29] breast,[30,31] endometrium,[32,33] and stomach.[34-38] However, tumor types that do not contain CIMP-based subgroups also display DNA methylation alterations that correlate with tumor invasiveness and clinical outcome. For example, high frequencies of DNA methylation-derived gene expression

alterations are present in renal cell carcinomas (RCCs).[16] Analysis of TCGA clear cell RCC (ccRCC) data indicated that DNA methylation alterations positively correlate with tumor aggressiveness,[39] and DNA methylation markers associated with ccRCC aggressiveness were characterized in a recent report.[40]

DNA methyltransferases

Cytosine DNA methylation marks are placed by the enzymatic activities of DNMTs using *S*-adenosylmethionine (SAM) as a co-factor. As first modeled by Santi et al., DNMTs catalyze methyl-transfer by first forming a covalent intermediate of the DNMT active site amino acid cysteine residue bound to the C-6 position of the targeted cytosine base.[41] In this first step, the C-5/C-6 double bond becomes a single bond as the enzyme-DNA covalent intermediate is formed, providing the impetus for forming a bond between the C-5 position and the methyl group, thereby releasing SAM. The C-5/C-6 double bond reforms and the enzyme then releases for catalyzing additional methyl-transfer events.

DNMT1 is thought to be responsible for maintenance DNA methylation to copy DNA methylation patterns from parental DNA onto daughter DNA strands soon after cellular DNA replication (reviewed in ref.42). DNMT3A and DNMT3B are referred to as *de novo* DNMTs in that they create new DNA methylation marks at CpG sites that were originally unmethylated and play important roles during embryonic development and tumorigenesis.[42] DNMT2 is a tRNA-methyltransferase, while DNMT3L is a structural protein that is essential in coordinating DNMT3A and DNMT3B-associated DNA methylation during embryonic development.[42] DNMT3C was recently discovered by Barau et al.[43] and is essential for fertility via *de novo* DNA methylation of evolutionarily young retrotransposon promoters of the male germline. Experiments involving HCT116 colon cancer cells harboring hypomorphic knockdown of DNMT1 (*DNMT1*$^{\Delta 2-5}$) and/or knockout of DNMT3B (*DNMT3B*$^{-/-}$) showed that downregulating DNMT1 or DNMT3B alone did not substantially alter global DNA methylation levels; however DNMT1/DNMT3B double knockout (DKO, *DNMT1*$^{-/-}$ *DNMT3B*$^{-/-}$) cells showed nearly complete (95%) DNA demethylation, suggesting that DNMT1 and DNMT3B work in concert to maintain DNA methylation marks.[44-46]

Two DNMT3A isoforms have been identified,[47] described as DNMT3A and DNMT3A2. DNMT3A is expressed at low levels across all cell types and developmental stages and is localized to heterochromatin; however, DNMT3A2 is mainly expressed in ES cells and embryonic carcinomas and is thought to be the main DNMT3A isoform responsible for *de novo* DNA methylation in ES cells.[47] Moreover, DNMT3A and DNMT3A2 each connect to nucleosomes via interaction with DNMT3L.[48,49] With respect to human cancers, *DNMT3A* somatic mutations have been identified in acute myeloid leukemia (AML) and other blood-based malignancies.[50,51] TCGA identified *DNMT3A* mutations in over 50% of AML tumors, and showed correlation with *NPM1* mutations. Interestingly, AML tumors with mutations in *DNMT3A, NPM1*, and *FLT3* showed substantial DNA hypomethylation compared with apparently normal CD34+/CD34- white blood cells.[51]

Over 30 DNMT3B splice-variant isoforms have been described, some possessing catalytic activity (DNMT3B1, DNMT3B2, DNMT3B6), while others are not catalytically active (DNMT3B3, DNMT3B4, DNMT3B5, DNMT3B7).[52-58] DNMT3B is important for gene-body DNA methylation in a manner irrespective of the isoforms' catalytic activity.[58] The catalytically inactive DNMT3B isoforms, most notably DNMT3B3, are expressed in human cancer cells and may serve as a beacon to recruit DNMT3A when DNMT3L is absent. DNMT3B may act as an accessory protein, much like DNMT3L, to recruit DNMT3A in establishing *de novo* DNA methylation patterns, especially in somatic cells that do not express DNMT3L.[58,59] DNMT3B is mainly involved in maintaining or restoring DNA methylation of CpGs located in gene bodies or transcribed regions by recognition of H3K36me3 marks.[12,58]

DNA demethylation

Since the discovery and characterization of DNMTs, efforts to identify DNA demethylases, enzymes that remove DNA methylation marks, have been difficult and met with controversy. A mammalian DNA demethylase was reported in 1999 that was later identified as MBD2,[60,61] a member of the DNA methylation binding (MBD) protein family;[62,63] however, the DNA demethylase activity of MBD2 was not validated in other laboratories.[63] Several years later, enzymes belonging to the Ten Eleven Translocase (TET) family (TET1, TET2, TET3) were identified and were shown to function by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) using ascorbic acid (vitamin C) as an enhancing cofactor.[64,65] *TET1* was first described as leukemia-associated protein with a CXXC domain (*LCX*) by Ono et al., who sought to characterize novel fusion genes located on chromosome 10q22 that partner with *MLL* in AML patients with chromosome 10 and 11 translocations.[66] TET enzymes have homology to trypanosome base J binding proteins (JBP1 and JBP2) and contain 2-oxoglutatrate and iron-dependent dioxygenase activities similar those found in histone lysine demethylases.[64,67] The CXXC domain is also found in MBD proteins, implicating TET enzymes as possessing methylated DNA binding capabilities.

Subsequent studies showed TET enzymes further convert 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC),[68,69] with both 5fC and 5caC recognized by the DNA glycosylase-mediated base excision repair pathway that ultimately results in the substitution of 5mC with an unmethylated cytosine residue. Unlike DNA methylation catalysis in which methyl marks are directly placed onto unmethylated cytosines, DNA demethylation can occur: 1) passively, by the inability of the DNA methyltransferase machinery to place methyl marks on nascent DNA strands; or 2) actively, by a multi-step process of TET-mediated oxidative conversion and removal by DNA repair mechanisms. The 5hmC marks are found at reduced levels (~1%) compared with 5mC levels (4–5%) in the human genome; however, much like 5mC alterations, 5hmC alterations are present across several forms of human cancers.[70] Levels of 5mC and 5hmC and TET enzyme activities are paramount in understanding the functions and mechanisms of the cancer methylome. Interestingly, vitamin C plasma levels are generally

low in cancer patients,[71] and the strategy of restoring vitamin C levels to physiologic levels may improve sensitivity and efficacy of TET-based DNA demethylation as an effective anti-cancer therapy.

IDH1 *mutations drive CIMP in human gliomas*

As mentioned above, CIMP subgroups exist in multiple human cancer types, in which subsets of tumors show cancer-specific DNA methylation at unique sets of CpG islands. A glioma-specific CIMP (G-CIMP) was reported by Noushmehr et al. in 2010, in which approximately 15% of the glioblastomas (grade IV gliomas) analyzed showed extensive DNA hypermethylation. G-CIMP patients show improved survival and younger age, as well as unique genomic profiles, including the presence of *TP53* mutations and a lack of copy number alterations.[29] Among these, a heterozygous point mutation in isocitrate dehydrogenase 1 (*IDH1*), corresponding to the R132 amino acid (IDH1^{R132H}), which was first identified by Parsons et al.[72] IDH1 functions as a dimer in the citric acid (TCA) cycle by converting isocitrate to α-ketoglutarate (α-KG).[73] IDH1^{R132H} further converts α-KG to D-2-hydroxyglutarate (2-HG).[74] The latter is an inhibitor of TET activity[75] and subsequent TET-based DNA demethylation, which results in the hypermethylated DNA feature of these tumors. The introduction of IDH1^{R132H} into cancer cells is sufficient to drive G-CIMP DNA hypermethylation, thus providing evidence of the driving effects of this mutation in cancer cells.[76,77]

Extensive tumor genome characterization has also unveiled *TET* mutations in AML patients [51,78-83] and low frequency *IDH* mutations in colorectal cancers and cutaneous melanomas.[84,85] However, the presence of CIMPs in colon, breast, stomach and endometrial tumors does not correlate with any significant frequencies of *IDH* or *TET* mutations, suggesting that CIMP DNA methylation profiles may be generated by distinct mechanisms unique to each tumor type.

Chromatin structure and histone modifications

Chromatin modifications also shape the epigenome in regulating gene expression profiles in both normal and cancer cells. DNA of 146 bp in length is wrapped twice around histone octamers containing dimers of histones H2A, H2B, H3, and H4. Modifications of the N-terminal tail amino acids of Histones H3 and H4 are associated with euchromatic (open) and heterochromatic (closed) chromatin states (reviewed in [86]). These are commonly referred to as writers (adding marks), erasers (removing markings), and readers (scanning and binding to specific chromatin markings for transcriptional activation or silencing).

The histone code[87] describes how gene expression activity (off/on) is correlated with specific histone modifications of lysine and arginine amino acids of histone N-terminal tails. These modifications include methylation (me), acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and others. Namely, histone H3 lysine acetylation at positions 9 (H3K9Ac) and 14 (H3K14Ac) is associated with open chromatin at promoter gene regions and active gene expression, as are histone H3 lysine 4 trimethylation (H3K4me3) markings at gene promoter regions that also block DNA methylation.[88,89] H3K4 monomethylation (HeK4me1) is a marking of enhancer regions of the genome, and H3K27 acetylation is a marking of active enhancer activity when accompanied by K3K4me1 marks.[89,90] H3K36me3 marks are found in gene-body regions of actively transcribed genes via the catalytic activity of the SETD2 histone methyltransferase.[89] SETD2 is thought to interact with DNMT3B in maintaining DNA methylation of most transcribed gene-body regions, thus providing additional evidence linking chromatin modifications (H3K36me3) and DNA methylation (5mC) marks in the human genome.[12,58]

The repressive histone H3 lysine 27 trimethylation (H3K27me3) marks are placed by the EZH2 component of the Polycomb Repressive Complex 2 (PRC2),[86] while H3K9 methylation (H3K9me2 and H3K9me3) is linked to repression of repetitive elements and the pericentric chromosomal regions. Genomic regions marked by K3K9me and H3K27me may also harbor DNA methylation to provide an additional level of gene regulation;[91] however, H3K9me3 or H3K27me3 occupancy may be independent of DNA methylation within a specific genomic region. Gene regions with co-localization of H3K27me3 and H3K4me3 marks are referred to as bivalent chromatin, as they contain both active (H3K4me3) and repressive (H3K27me3) marks. These are of particular importance in ES cells, in which the presence of both marks at specific gene regions, mostly transcription factors and other developmental regulators, correlates with their being poised for potential gene activity when required during cellular differentiation and development.[92] H3K27me3 is retained upon differentiation to a state where gene silencing is required, while H3K4me3 is retained in differentiated cells in which gene activity is required.

Gene promoter regions marked by H3K27me3 occupancy are prone to cancer-specific DNA hypermethylation,[93-95] suggestive of a stem cell origin of human cancer. In this model, replacement of the permanently-marked repressive (H3K27me3) state from the flexible bivalent (H3K4me3 and H3K27me3) state after differentiation may predispose these sites for DNA hypermethylation due to epigenetic cross-talk between DNA methylation and chromatin machinery early during tumorigenesis. However, DNA methylation and H3K27me3 marks appear to be mutually exclusive in ES cells, and are not always coupled in human cancers. Experiments from Gal-Yam et al. [96] support 3 models of epigenetic switching in human cancers in which: 1) genes originally silenced by H3K27me3 marks acquire DNA hypermethylation but then lose the H3K27me3 mark in human cancers; 2) DNA hypermethylation occurs at genes not originally marked by H3K27me3 occupancy; and 3) H3K27me3 marks are added without DNA hypermethylation. Importantly, an appreciable number of demethylated promoter regions after treatment with DNA methylation inhibitors remain silenced by the retained presence or gain of H3K27me3 marks.[15,97] This is a critical aspect of epigenetic therapy, and suggests that treatment of cancer cells with DNA methylation inhibitors alone may not effectively activate genes that are epigenetically silenced in human cancers.

Somatic mutations of epigenetic modifier genes in human cancers

Both DNA methylation and chromatin modifications function in concert to regulate gene expression, with crosstalk between these 2 aspects of epigenetics as important components of the cancer epigenome. Indeed, the preponderance of mutations in epigenetic-modifying genes has been identified across multiple human cancer types suggests that cancer cells are epigenetically deregulated, leading to aberrant gene expression and subsequent signaling pathway alterations. Somatic mutations of genes involved in DNA methylation (*DNMT3A, TET, IDH*), histone methylation (*MLL* family, *EZH2, SUZ12, SETD2*), histone demethylation (*KMD* family), and chromatin remodeling (*CHD* family, *ARID* family, *ATRX, SMARC* family) have been described in multiple forms of human cancer (reviewed in ref.98), and are highlighted in Table 1.

Methylated DNA binding proteins

Proteins with methylated CpG DNA binding specificity have been known for over 2 decades (reviewed in [99]). Proteins with a methylated CpG binding domain (MBD) include MeCP2, and the MBD family (MBD1–6), and function by recognizing, reading and binding to methylated CpG marks, as well as binding to other proteins for transcriptional and chromatin structure regulation. For example, MBD2 is a member of the Mi-2/NuRD chromatin remodeling complex, and functions as a transcriptional repressor by interacting with methylated DNA and deacetylated histones. MBD4 recognizes methylated CpG marks; in addition, it recognizes and repairs mCpG/TpC mismatches due to 5mC deamination to thymine on one DNA strand. Other binding proteins include UHRF1 and Kaiso, among others.[99] UHRF1 binds to hemimethylated DNA in recruiting DNMT1 to replication forks for maintenance DNA methylation, and also binds to H3K9me3 and H3 Arginine 2 (H3Ar2) marks via its histone reader domain. Kaiso is a transcription factor that recruits histone deacetylases and the nuclear co-repressor (N-CoR complex), which contains SWI/SNF chromatin remodelers, to methylated DNA regions for gene repression.

microRNAs

microRNA (miRNA) expression changes are frequent events in human cancers. microRNA expression has profound effects on signaling pathways and cell growth, since miRNAs regulate the translation rate of the majority of coding genes in the genome, and can be categorized as having tumor suppressive or oncogenic capabilities (reviewed in[100]). miR-15a and miR-16–1 were the first miRNAs to be associated with human cancer pathogenesis, as both are frequently deleted in chronic lymphocytic leukemia (CLL), and are therefore referred to as tumor suppressors (reviewed in ref.101). Subsequent reports identified miRNA amplifications and deletions in nearly every cancer type, with deletion of miRNAs that repress oncogenic signaling or activate tumor suppressors, as well as amplification of miRNAs that negatively regulate tumor suppressors or activate oncogenes.

Adding to the complexity of miRNA-based gene regulation, miRNA expression alterations as a result of aberrant DNA methylation have been described (reviewed in refs. 102,103), in which reduced miRNA expression results in increased tumor invasiveness and metastases and reduced growth control that can be reversed by treatment with DNA methylation inhibitors, implicating individually-characterized miRNAs as tumor suppressors. The first evidence described miR-127 DNA hypermethylation and silencing in primary human colon and prostate tumors.[104] miR-127 targets the proto-oncogene *BCL6* by translational repression. BCL6 is an important oncogene as it suppresses p53 expression and is involved in DNA damage-based apoptotic programs in B cells. Treatment of tumor cells with DNA methylation inhibitors resulted in the re-activation of miR-127 and subsequent inhibition of *BCL6*, thus providing additional evidence of the utility of DNA methylation inhibition in reversing oncogenic signaling for cancer treatment. Recently, epigenetic silencing of miR-200c was found to be associated with lymph node metastases in breast cancer.[105] Likewise, DNA hypermethylation-based silencing of miR-490–3p in colorectal cancer is linked to cell proliferation, invasiveness, and the epithelial-mesenchymal transition (EMT).[106] Other examples of miRNA epigenetic silencing include miR-148a and miR-199a, which are silenced by DNA hypermethylation in nasopharyngeal carcinomas[107] and testicular germ cell tumors,[108] respectively.

Additional sets of miRNAs, termed epi-miRNAs, regulate epigenetic modifiers by specifically targeting DNMTs, histone deacetylases (HDACs), histone modifiers, repressors and other epigenetic regulators across a variety of tissue types, and, thereby have wide reaching effects on shaping the epigenomes in both normal and tumor cells.[103] Aberrant epi-miR expression is common in a variety of human cancers. For example, miR-101, which suppresses EZH2 translation, is downregulated in human cancers, resulting in increased EZH2 translation and aberrant placement of repressive H3K27me3 marks.[109] Another example is mir-29a/b/c, which directly inhibits DNMA3A and DNMT3B translation and/or transcription. miR-29a/b/c expression is inversely correlated with DNMT3A and DNMT3B mRNA expression in lung tumors.[110] Interestingly, lung cancer patients with low DNMT3A mRNA expression show increased survival compared with those with high DNMT3A mRNA expression, and are related to miR-29 expression in these tumors. These data suggest miR-29a/b/c function as tumor suppressors in restoring normal DNA methylation, and their silencing is associated with DNA hypermethylation.

Five-azacytidine-based inhibitors of DNA methylation

Currently, chemical inhibitors of several epigenetic modifiers have been developed (reviewed in[111]) (Table 1). The first DNA methylation inhibitors, 5-azacytidine (5-Aza-CR) and 5-aza-2′-deoxycytidine (5-Aza-CdR), were synthesized over 50 y ago by Sorm, Piskala et al. (reviewed in ref.112) as a cytotoxic anticancer drug, much like 5-fluorouracil (5-FU), for the treatment of AML and acute lymphoblastic leukemia (ALL). Early trials evaluating the performance of high doses of 5-Aza nucleotides

Table 1. Epigenetic regulator genes altered in the major forms of human cancer. Genes are listed as their HUGO Gene Nomenclature IDs.

DNA Methylation	Description	Function	Mutation in Cancer	Chemical Inhibitor[b]
DNMT1	DNA (cytosine-5-)-methyltransferase 1	Maintenance DNA methylation		5-Aza-CR (Vidaza), 5-Aza-CdR (Decitabine), Zebularine, SGI-110, RG108
DNMT3A	DNA (cytosine-5-)-methyltransferase 3α	*de novo* DNA methylation	AML, MDS, lymphoma	5-Aza-CR (Vidaza), 5-Aza-CdR (Decitabine), SGI-110
DNMT3B	DNA (cytosine-5-)-methyltransferase 3β	*de novo* DNA methylation		5-Aza-CR (Vidaza), 5-Aza-CdR (Decitabine), SGI-110, Nanaomycin A, RG108
IDH1	Isocitrate dehydrogenase 1	isocitrate to α-ketoglutarate	AML, glioma, MDS, prostate, thyroid	AGI-5198
IDH2	Isocitrate dehydrogenase 2	isocitrate to α-ketoglutarate	AML, MDS, glioma, melanoma	AGI-6780
TET1	Tet methylcytosine dioxygenase 1	5mC to 5hmC, 5fC, 5caC	AML, CML, colon, MDS	
TET2	Tet methylcytosine dioxygenase 2	5mC to 5hmC, 5fC, 5caC	AML, glioma, MDS	
TET3	Tet methylcytosine dioxygenase 3	5mC to 5hmC, 5fC, 5caC		
Histone Lysine Methylation				
EHMT2 (G9a)	Euchromatic histone-lysine-N-methyltransferase 2	HeK3me2 methylase		UNC0642, A-366
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit	H3K27 methylase, component of PRC2	AML, lymphoma, MDS, melanoma	GSK-126, EPZ-6438, UNC1999, GSK2816126, CPI-1205
KDM1A (LSD1)	Lysine-specific histone demethylase 1A	H3K4me1, H3K4me2, H3K9me1, H3K9me2 demethylase		GSK-LSD1, TCP, GSK2879552, ORY-1001
KDM5C (JARID1C)	Lysine demethylase 5C	H3K4me3 and H3K4me2 demethylase	kidney, meningioma, stomach	
KDM5D (JARID1D)	Lysine demethylase 5D	H3K4me3 and H3K4me2 demethylase		
KMD6A (UTX)	Lysine demethylase 6A	H3K27me demethylase	bladder, esophagus, glioma, kidney	
KMT2A (MLL1)	Lysine methyltransferase 2A	H3K4 methylase	ALL, AML, bladder, liver, lung, stomach	
KMT2B	Lysine methyltransferase 2B	H3K4me3 methylase		
KMT2C (MLL3)	Lysine methyltransferase 2C	H3K4 methylase	bladder, breast, liver, pancreas, stomach	
KMT2D (MLL2)	Lysine methyltransferase 2D	H3K4 methylase	breast, head and neck, kidney, lung, lymphoma, prostate	
SETD2	SET domain containing 2	H3K36 trimethylase	ALL, kidney	
SUZ12	Suppressor of zeste binding 12 homolog (Drosophila)	Component of PRC2	ALL, MDS	
Chromatin Remodeler				
ARID1A (SMARCF1)	AT-rich interaction domain 1A	Component of SWI/SNF complex; mutually exclusive with ARID1B	bladder, breast, colon, endometrial, liver, lung, lymphoma, melanoma, ovarian, pancreas, prostate, stomach	
ARID1B	AT-rich interaction domain 1B	Component of SWI/SNF complex; mutually exclusive with ARID1A	breast, liver, melanoma	
ARID2	AT-rich interaction domain 2	Subunit of PBAF complex	breast, liver, lung, melanoma	
ATRX	α thalassemia/mental retardation syndrome X linked	Binds to telomeric sequences, involved in alternative lengthening of telomeres	glioma	
CHD1	Chromodomain helicase DNA binding protein 1	H3K4me3 recognition component of histone acetylation complex	breast, lung, prostate	
CHD2	Chromodomain helicase DNA binding protein 2	Helicase that binds to promoter regions and is involved in H3.3 deposition	lymphoma	
CHD3	Chromodomain helicase DNA binding protein 3	Component of Mi2/NuRD histone deacetylase complex		

Table 1. (*Continued*)

DNA Methylation	Description	Function	Mutation in Cancer	Chemical Inhibitor[b]
CHD4	Chromodomain helicase DNA binding protein 4	Component of nucleosome remodeling and histone deacetylase complex	endometrial	
CHD5	Chromodomain helicase DNA binding protein 5	Neuron-specific chromatin remodeling and transcriptional regulator		
CHD6	Chromodomain helicase DNA binding protein 6	Component of chromatin remodeling complex for cell-specific gene regulation	bladder	
CHD8	Chromodomain helicase DNA binding protein 8	Binds β-catentin and inhibits WNT signaling	lung	
JARID2	Jumonji and AT-rich interaction domain containing 2	Binds to PRC2	MDS, prostate	
SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 1	Member of SWI/SNF protein family		
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2	Transcriptional activator, member of npBAF and nBAF complexes		
HTLF (SMARCA3)	Helicase-like Transcription Factor	Member of SWI/SNF protein family		
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4	Transcriptional activator, component of CREST-BRG1 complex	bladder, lymphoma, melanoma	
SMARCA5	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5	Helicase, member of SWI/SNF family		
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily B, member 1	Component of SWI/SNF complex		
SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily C, member 1	Transcriptional activator, member of npBAF and nBAF complexes		
SMARCC2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily C, member 2	Member of SWI/SNF protein family		
SMARCD1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 1	Transcriptional activator, member of npBAF and nBAF complexes	breast	
SMARCD2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 2	Member of SWI/SNF protein family		
SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily D, member 3	Transcriptional activator, member of npBAF and nBAF complexes		
SMARCE1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily E, member 1	Transcriptional activator, member of npBAF and nBAF complexes		
Histone				
HIST1H3B	Histone Cluster 1 H3 Family Member B	Histone H3.1 variant	K27M - glioma	
H3F3A	Histone H3 Family 3A	Histone H3.3 variant	K27M - pediatric glioma	
H3F3A	Histone H3 Family 3A	Histone H3.3 variant	G34V - pediatric glioma	

[a]A complete list of mutations and copy number alterations is detailed in Shen and Laird (2013) *Cell* 153-38-53.
[b]A complete list of chemical inhibitors for epigenetic modifers is detailed in Shortt *et al* (2017) *Nat Rev Cancer* 17:160-183.

in leukemia patients resulted in only short-term effects accompanied by extensive cytotoxicity.[112]

Work from Jones and Taylor[113] showed that *in vitro* application of 5-Aza-CR led to mouse embryo (10T1/2) differentiation into muscle cells due extensive genome-wide DNA hypomethylation and subsequent cellular reprogramming. 10T1/2 cell differentiation to myoblast-like cells is attributed to *MyoD1* promoter DNA methylation changes.[114] Further research showed that upon delivery into the nucleus, Aza-substituted analogs are converted to Aza-triphosphate and incorporated into newly synthesized genomic DNA during DNA replication. Aza-incorporated DNA traps DNMTs to the site of purported DNA methylation, and effectively removes free DNMT from the nucleus, leading to passive DNA demethylation.[41] An additional model was described by Ghoshal et al.,[115] who showed that upon binding to aza-substituted cytosines, DNMT1 becomes poly-ubiquitinated and then degraded by the 26S proteasome, suggesting that multiple means of DNMT1 inactivation occur in response to 5-Aza treatment.

Both 5-Aza-CR and 5-Aza-CdR are effective DNA methylation inhibitors.[116] While 5-Aza-CR is activated by uridine-cytidine kinase and can be incorporated into both DNA and RNA, 5-Aza-CdR is activated by deoxycytidine kinase and is only incorporated into of newly synthesized DNA strands after replication. As a result, lower doses of 5-Aza-CdR are required for DNA demethylation, even though 5-Aza-CdR treatment results in high cellular toxicity and DNA damage. Aza-based drug treatments in cancer cell cultures and mouse models of cancer resulted in reduced numbers of viable tumor cells and tumor volumes. Both 5-Aza-CR and 5-Aza-CdR are FDA-approved for the treatment of MDS and leukemia patients under the trade names Vidaza and Dacogen/Decitabine, respectively.

Zebularine and SGI-110 show novel attributes as DNA methylation inhibitors

In addition to 5-Aza-CR and 5-Aza-CdR, other DNA methylation inhibitors, including zebularine and SGI-110, have been characterized. Zebularine, 1-(β-D-Ribofuranosyl)-2(1H)-pyrimidinone, was originally designed as a transition state inhibitor of cytidine deaminase, and has structural similarity to cytidine, but lacks the C-4 amino group present on cytidine nucleosides. Much like 5-azacytidine analogs, zebularine is also incorporated into nascent DNA strands and shows a similar mechanism of covalent DNMT trapping for passive DNA methylation inhibition,[117] although zebularine appears to preferentially inhibit DNMT1 over DNMT3A and DNMT3B.[118] Zebularine inhibits cell growth of a variety of cancer types, and re-activates genes silenced by DNA hypermethylation, including tumor suppressor and tumor antigen genes.[118-120] Moreover, zebularine improves radiosensitivity in both *in vitro* and *in vivo* settings.[121] Zebularine showed stability in aqueous solution and can be delivered orally *in vivo*, an important attribute for potential clinical utility. However, 1–2 orders of magnitude greater dosing is required for the same effect seen from 5-Aza-CdR treatments.[118-120] Additional pharmacokinetic studies[122] concluded that zebularine has low

bioavailability and frequent or continuous intravenous infusion is required for effective DNA demethylation.

Guadecitabine (SGI-110), is a modified version of 5-Aza-CdR, consisting of 5-Aza-CdR covalently bound to deoxyguanosine via phosphodiester linkage.[123,124] SGI-110 shows promising clinical utility, as it is less subject to deamination by cytidine deaminase, and therefore, displays improved stability and lower toxicity over 5-Aza-CdR alone.[123] SGI-110 is highly tolerated upon delivery, and is effective by both intraperitoneal and subcutaneous delivery methods in mice and patient derived xenograft (PDX) models of cancer.[123] Kuang et al. evaluated SGI-110 in hepatocellular carcinoma cell lines and PDX mouse models to guide future phase I/II clinical trials, either alone or in conjunction with oxaliplatin,[125] a platinum-based cytotoxic compound that inhibits DNA synthesis by forming cross-links with DNA. Low-dose SGI-110 treatment alone resulted in reduced cell proliferation, and pretreatment of HCC cells with low-dose SGI-110 resulted in significantly increased oxaliplatin sensitivity as well as inhibition of WNT/EGF/IGF signaling.

SGI-110 is currently being evaluated in 17 ongoing clinical trials across multiple cancer types, including AML, CMML, colorectal, germ cell, MDS, melanoma, lung and ovarian cancers, in which SGI-110 is administered either as a single agent or in combination with chemotherapeutic agents (www.clinicaltrials.gov). SGI1–110 clinical trials in liver and non-small cell lung cancer patients have recently completed. Issa and colleagues[126] reported a completed phase I trial evaluating the efficacy of SGI-110 dose escalation and drug delivery frequencies in AML and MDS patients (either 5 consecutive days, once weekly for 4 weeks or twice per week for 4 weeks), with the goal of determining drug tolerance and the extent of DNA demethylation. SGI-110 was well tolerated, had a significantly longer half-life than 5-Aza-CdR and was most biologically effective when administered for 5 consecutive days. In agreement, global DNA demethylation, as measured by *LINE-1* repetitive elements, was maximized at day 8 of the daily dosing schedule, and clinical response correlated with increased *LINE-1* DNA demethylation. These experiments show promise for using SGI-110 in the clinic; however, combining SGI-110 with other chemotherapies has not yet been accomplished.

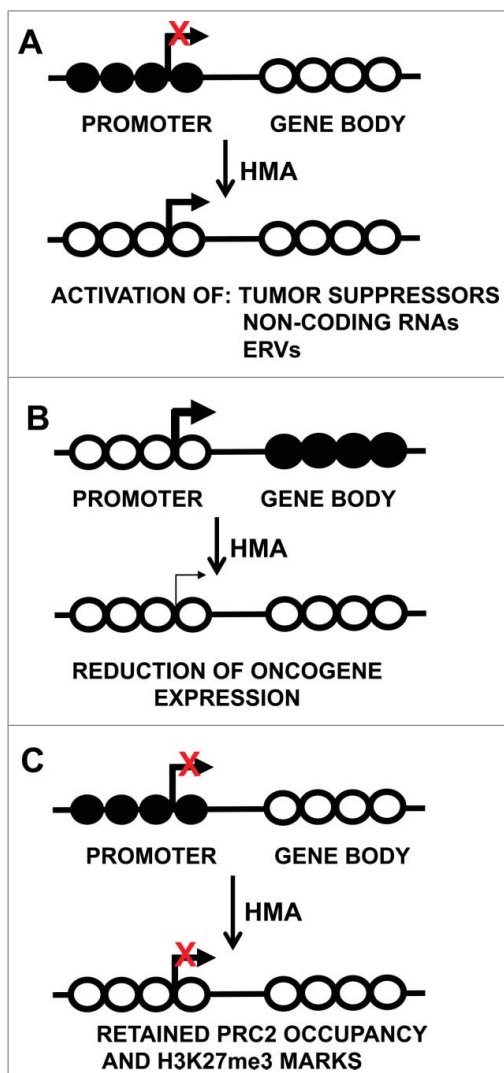### Non-nucleoside DNA methylation inhibitors

Non-nucleoside DNA methylation inhibitors have also been described, namely 5-fluoro-2′-deoxycytidine, (−)-epigallocatechin-3 gallate (EGCG), hydralazine, mitoxanthrone, *N*-acetylprocainamide, psammaplin A, procainamide and procaine (summarized in ref. Chuang, 2005 #788). Hydralazine is clinically administered for hypertension and was shown to reduce DNMT1 and DNMT3A expression. Procainamide has clinical utility in treating arrhythmia and was shown to inhibit DNMT activity. EGCG is a polyphenol in green tea that has shown DNA methylation inhibition and chemopreventive activities. However, comparative analyses showed that these non-nucleoside inhibitors are not as effective as 5-Aza-CdR in DNA demethylation and gene reactivation;[127] therefore, their clinical utility may be limited.

Mechanisms of 5-Aza-CdR-based DNA demethylation based on preclinical studies

Five-Aza-CdR induced DNA demethylation

Even though Aza-based DNA methylation inhibitors display anti-tumor attributes, they are not stable in aqueous solution, and a single high-dose treatment results only in transient effects of DNA demethylation and increased population doubling times, since DNA remethylation occurs within days after a single dose.[12,128] It is well known that Aza-nucleotide treatments of cancer cells result in the re-expression of genes silenced by promoter DNA hypermethylation (Fig. 1A). Epigenetically-silenced genes have been well described in human cancers (reviewed in ref.129), and include *CDKN2A (p16)*, *MGMT*, *MLH1*, *SFRPs* and *BRCA1/2*. Epigenetic silencing of these genes contributes to tumorigenesis by allowing the cell to escape cell cycle control [*CDKN2A (p16)*], DNA repair (*BRCA1/2, MGMT, MLH1*), and ligand-receptor based signaling (*SFRPs*).

Figure 1. DNA methylation and gene expression consequences after treatment with DNA methylation inhibitors. Circles represent CpG sites, with closed circles indicative of methylated CpGs and open circles representing unmethylated CpGs. Transcription start sites are indicated by the bent arrow. (A) Demethylation of CpG island promoter regions, resulting in gene activity. (B) Gene-body demethylation results in reduced expression. (C) Retained gene silencing by PRC2 occupancy after DNA methylation inhibitor treatment. HMA: Hypomethylating agent.

MGMT promoter DNA hypermethylation results in silencing of the gene and subsequently, the inability of the MGMT enzyme to methylate the O-6 position of guanine residues. *MGMT* DNA methylation is an important diagnostic in glioma patient care to determine if temozolomide can be administered. Temozolomide is an alkylating agent, and is effective in glioma treatment only when *MGMT* is silenced. As a result of *MGMT* silencing, DNA damage incurred by the drug cannot be repaired, and the cell enters into apoptotic programmed cell death.[130]

While identifying epigenetically silenced genes reactivated by 5-Aza-CR and 5-Aza-CdR is important for identifying potential epigenetic drivers, gene regions with both 5mC and H3K27me3 marks remain silenced after treatment with DNA methylation inhibitors due to retention of the H3K27me3 repressive mark [15,97] (Fig. 1C). This is a critical issue for using DNA methylation inhibitors for therapeutic approaches, as treatment of cancer cells with DNA methylation inhibitors alone may not effectively activate genes that are epigenetically silenced in human cancers. Treatment schemes with DNA methylation and EZH2 inhibitors may help to effectively activate a larger set of epigenetically silenced genes, and ultimately bring the tumor cell epigenome closer to a normal epigenetic state.

Single low-dose, 24 h delivery of 5-Aza-CdR has more prolonged cell growth inhibition and reduced toxicity, although DNA remethylation still occurs. A report from Tsai a et al.[131] showed that human leukemia cells treated with low-dose (10–500 nM) 5-Aza-CdR displayed significantly reduced tumorigenicity, cellular toxicity, DNA damage and apoptosis. Low-dose 5-Aza-CdR was also effective in reducing tumorigenicity and tumor size in mouse xenograft models of breast cancer. Moreover, single low dose 5-Aza-CdR treatment resulted in loss of DNMT1 protein, together with sustained DNA demethylation of gene promoters and activation of epigenetically-silenced genes.[131] Pathway analyses of re-expressed genes showed increased cyclin dependent kinase (CDK) based cell cycle control and decreased AKT signaling.[131] The AKT pathway mediated epithelial to mesenchymal transition (EMT), which is associated with tumor progression and invasiveness. TGF-β signaling was also increased in the Aza-treated leukemia cells. In this pathway, TGF-β drives cell maturation and inhibits the renewal of progenitor cells. These features highlight the promise for DNA methylation inhibitors in clinical practice.

The reactivation of epigenetically-silenced genes after 5-Aza-CdR treatment has been well characterized. However, such treatment also reduces the overexpression of genes through DNA demethylation of gene bodies and transcribed regions, which are normally methylated in actively expressed genes [12] (Fig. 1B). Downregulated genes include oncogenes and those involved in c-MYC regulated processes. This finding revealed an unexpected therapeutic advantage of 5-Aza-CdR and was the first evidence that DNMT inhibitors not only reactivate tumor suppressor genes, but also down-regulate overexpressed oncogenes in human cancer cells.

Gene-body DNA remethylation after 5-Aza-CdR treatment

While the mechanisms of DNA demethylation are becoming well understood, DNA remethylation kinetics is not universal

for all genes across the genome. Indeed, Yang and colleagues[12] showed that DNA demethylation occurs evenly across the human genome; however, DNA remethylation is differentiated by location and genomic context. DNA remethylation can be categorized by "Fast" or "Slow" kinetics after 5-Aza-CdR treatment of HCT116 colon cancer cells (Fig. 2). Among these, the Fast CpG sites quickly and completely remethylate to pre-treated levels, while the Slow CpGs remain demethylated for at least 60 d after 5-Aza-CdR treatment (Fig. 2). Interestingly, most of the Fast CpG sites were mostly located in non-CpG island gene-body regions and were positively correlated with gene expression. DNMT3B is required for remethylation of Fast sites after 5-Aza-CdR treatment, in which DNMT3B recognizes H3K36me3 marks as a beacon for DNA remethylation.

Since H3K36me3 marks are localized at gene bodies and are positively correlated with active gene expression, SETD2, which is responsible for H3K36me3 marks, is thought to interact with DNMT3B in this regard. Meta analyses showed that genes harboring Fast CpGs are overexpressed in human cancers and are enriched for pathways in which cell growth is stimulated. Similar numbers of Slow genes were located in gene promoter and gene-body regions, and most Slow CpG islands are cancer-associated targets of *de novo* DNA methylation. Slow loci located in gene bodies were not associated with H3K36me3 occupancy, but rather increased occupancy of H3K27me3 and H2A.Z marks, which are anti-correlated with DNA methylation. As a result, these chromatin modifications may block DNMT3B activity required for gene-body DNA remethylation. These findings highlight the importance of gene-body DNA methylation in gene regulation, as well as a novel mechanistic attribute of 5-Aza-CdR-based DNA demethylation.

Viral mimicry induced by 5-Aza-CdR treatment

The reactivation of epigenetically-silenced genes by 5-Azacytidine has been well described, however, additional mechanistic details were recently discovered. Low-dose 5-Aza-CdR treatment does not result in cellular toxicity but rather delayed DNA demethylation, suggesting that pathways unrelated to cytotoxicity may be activated. In general, endogenous retroviruses (ERVs) and other repetitive elements located in gene bodies are commonly silenced by DNA methylation in somatic cells, but can be reactivated after treatment with DNA methylation inhibitors.[132-134] Activated ERVs stimulate an immune response after 5-Aza-CdR treatment by allowing the expression of double stranded RNAs (dsRNAs), which are targeted by melanoma differentiation-associated protein 5 (MDA5). In turn, MDA5 becomes activated and recruits mitochondrial anti-viral signaling protein (MAVS), which induces interferon regulatory transcription factor 7 (IRF7), and subsequent antiviral response pathways. Importantly, these data show that activation of an interferon response is triggered by 5-Aza-CdR via ERV reactivation. Essentially, 5-Aza-CdR induces viral mimicry by deceiving the cancer cell to function in a viral-infected state. These paradigm-shifting findings not only support the efficacy of 5-Aza-CdR as a therapeutic tool, but also highlight the exploitation of dsRNA activation, ERV activation and MDA5 signaling as novel therapeutic targets and biomarkers for gauging treatment response.
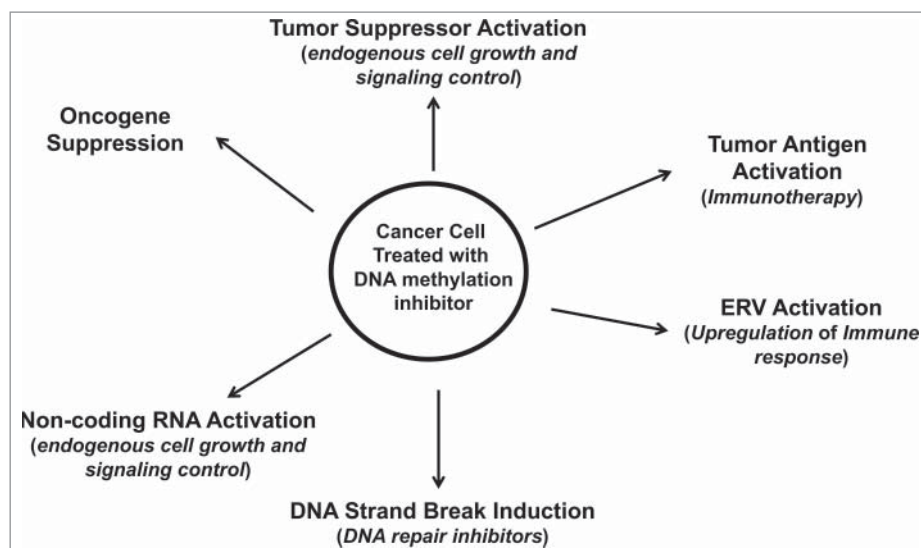
Viral mimicry is also activated by vitamin C, a required co-factor for TET-based DNA demethylation. *In vitro* delivery of physiologic doses of vitamin C has a pronounced effect on DNA demethylation and gene reactivation when combined with low doses of 5-Aza-CdR, most notably, ERVs, dsRNAs and an interferon response.[135] Moreover, this is accompanied by increased apoptosis and inhibition of cancer cell proliferation. The synergistic boost in DNA demethylation is likely due to stimulation of both passive (Aza) and active (TET) DNA demethylation mechanisms. For clinical benefit, vitamin C is water soluble, inexpensive, abundant, orally available and easily administered.

Clinical efficacy of combining DNMT inhibitors with conventional chemotherapies based on preclinical testing

Preclinical models, including cell culture, 3-D organoids, patient derived xenografts (PDX) and genetic mouse models, provide time- and cost-effective means of determining potential clinical efficacy for the development of new treatments, schedules, and doses. These can also be used to determine whether the inclusion of DNA methylation inhibitors result in increased sensitivity to conventional cytotoxic chemotherapeutics or those used for specific pathway inhibition (Fig. 3). One example is the report from Ikehata et al.,[136] in which human colon cancer cell lines were treated with chemotherapies (5-fluorouracil, irinotecan, oxaliplatin), DNA methylation inhibitors
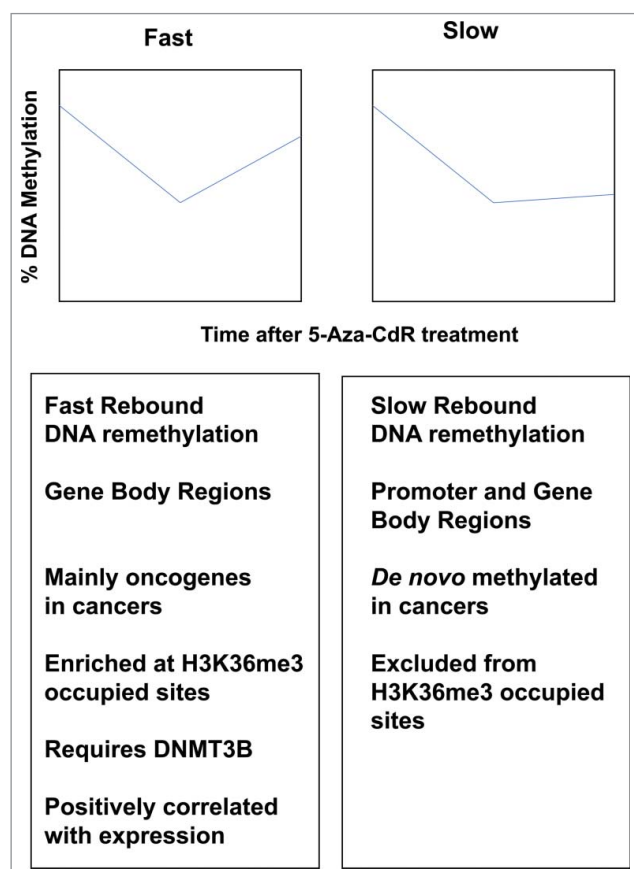
Figure 2. DNA remethylation kinetics after 5-aza-CdR treatment. Top: DNA remethylation of "Fast" (left) and "Slow" (right) CpG sites. Graphs are plotted as percent methylation (y-axis) vs. time after 5-Aza-CdR treatment (x-axis). Bottom: details of Fast and Slow rebounding genes.

Fast

% DNA Methylation

Slow

Time after 5-Aza-CdR treatment

Fast Rebound DNA remethylation

Gene Body Regions

Mainly oncogenes in cancers

Enriched at H3K36me3 occupied sites

Requires DNMT3B

Positively correlated with expression

Slow Rebound DNA remethylation

Promoter and Gene Body Regions

***De novo* methylated in cancers**

Excluded from H3K36me3 occupied sites

Figure 3. Therapeutic approaches accompanying DNA methylation inhibition for cancer patient treatment. Cancer cells treated with DNA methylation inhibitors have activated tumor suppressors, ERVs and miRNAs, with reduced expression of oncogenes. Activated ERVs promote an immune response that can be further targeted by immune-based therapies. Activated genes may help promote sensitivity to conventional chemotherapies. DNA methylation inhibitors, most notably 5-Aza-CR and 5-Aza-CdR, cause DNA damage, thus sensitizing the treated cell to DNA repair inhibitors.

(5-Aza-CR, 5-Aza-CdR, zebularine) and/or histone deacetylase inhibitors (Trichostatin A, SAHA, valproic acid) to determine if epigenetic therapies improve tumor toxicity. Indeed, the addition of DNA methylation inhibitors resulted in synergistic effects incurred by chemotherapy, and in particular, 5-Aza-CdR showed the most potent synergistic effect when administered with oxaliplatin.

Combining DNA methylation inhibitors with immune-based therapies also shows clinical promise. Priming non-small cell lung cancer (NSCLC) cells with 5-Aza-CR (Vidaza) results in the activation of immune-related genes as well as those related to immune evasion.[137] Specifically, 5-Aza-CR upregulates PD-L1, a key mediator of immune tolerance, suggesting that epigenetic therapy combined with PD-L1 inhibitors to block immune checkpoints may be efficacious in driving immune activation and reducing immune inhibition.

DNA methylation inhibition may also have clinical efficacy when coupled with DNA repair inhibitors (Fig. 3). DNA methylation inhibitors cause DNA strand breaks, and cancer cell lines treated with 5-Aza-CdR showed prolonged γH2A.X expression, a marker of double-strand DNA breaks.[138] Therefore, DNMT inhibitors may radiosensitize cells for improved response to radiotherapies. The increase in DNA strand breaks after 5-Aza-CdR treatment of cancer cells is especially important in the clinical setting, as DNA repair inhibitors that inhibit poly-ADP ribose polymerase (PARP), a DNA repair protein that binds to double-strand breaks, are used in the clinic for cancer treatment. DNA mismatch repair systems are challenged in colorectal and endometrial cancers, suggesting that epigenetic therapy may be efficacious in these tumor types.

Findings from clinical trials

Clinical trials involving DNA methylation inhibitors, either implemented alone, in combination with conventional chemotherapies, have been mostly performed for MDS and AML patients (reviewed in [139,140]). Indeed, a query of clinicaltrials.

gov showed over 500 cancer-related clinical trials involving 5-Aza-CR or 5-Aza-CdR (query: "5-aza" and "cancer"). Approximately, 400 trials involved patients with MDS, leukemia [AML, chronic myeloid leukemia (CML), and chronic myelomonocytic leukemia (CMML)] and lymphoma, while most of the remaining 100 trials involve patients with solid tumors. A comparison of the clinical trials in patients with solid or liquid malignancies is shown in Fig. 4.

Five-Aza-CR and 5-Aza-CdR are FDA-approved for treatment of MDS and AML patients, and 5-Aza-CR is regarded as the standard of care for treating MDS patients who are not eligible for allogenic stem cell transplantation, the only known curative treatment of MDS. 5-Aza-CR treatment of MDS patients in the AML-001 trial resulted in a nearly 10-month increased OS and also increased the time of progression of MDS to AML. In agreement, AML patients enrolled in the same trial also showed similar increases in OS after receiving 5-Aza-CR as compared with conventional therapies.[140-143] In support of this, a large phase III trial evaluating 5-Aza-CR vs. conventional care in 488 newly diagnosed AML patients of advanced age and >30% bone marrow blasts[144] concluded that patients treated with 5-Aza-CR showed improved median overall survival, improved one-year survival rates after treatments and improved response metrics; however, remission rates were similar between both treatments.

Five-Aza-CR and 5-Aza-CdR are currently in clinical trials for patients with solid tumors, including lung, breast, ovarian and colon cancers, among others (Fig. 4). A phase I/II study of low-dose 5-Aza-CR and the HDAC inhibitor entinostat for metastatic non-small cell lung cancer (NSCLC) patients showed objective responses in a subset of patients.[145] DNA demethylation of *APC, RASSF1A, CDH13*, and *CDKN2A (p16)* in serial blood samples from NSCLC patients collected during treatment correlated with improved progression free survival (PFS) an OS.[145]

Increased global DNA demethylation in treated tumor biopsies also correlated with longer survival in a similar phase I/II
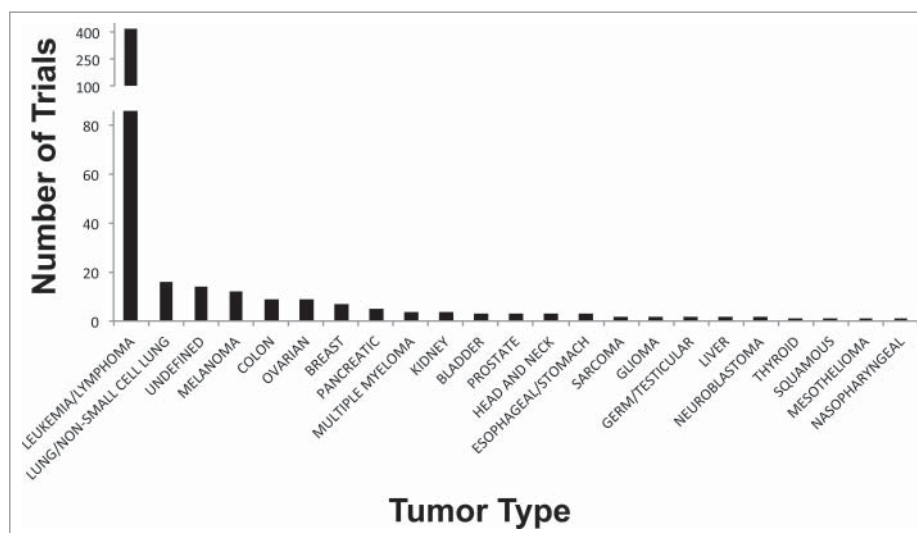
Figure 4. Clinical trials involving 5-Aza nucleotides in human cancers. Plot shows the number of trials completed, withdrawn, or in progress (y-axis) vs. individual tumor type (x-axis).

trial evaluating 5-azacitidine and entinostat in breast cancer patients.[146] While this drug combination was well tolerated in patients with HER2-negative tumors, it did not result in clinical activity in women with triple-negative breast cancer. Clinical response was evident in only a subset of women with hormone-resistant disease; however, *ESR1* DNA hypomethylation and gene expression activation occurred in nearly one-half of the treated hormone-resistant patients, suggesting that these patients may benefit from both endocrine and epigenetic therapies.

DNA demethylating agents have also been used in trials for ovarian cancer patients. In general, ovarian cancer patients receive platinum-based therapies; however, resistance to platinum-based drugs is common. Fu et al.[147] reported on a phase I/II clinical trial of ovarian cancer patients with platinum-resistant or platinum refractory disease, in which patients were given sequential treatments of 5-Aza-CR followed by carboplatin. Pretreatment with 5-Aza-CR agent helped to overcome platinum resistance and inhibit the development of liver metastases in some patients, and demonstrates promise as a means of ovarian cancer treatment.

Colon cancer patients with metastatic disease have few treatment options. Agents targeting vascular endothelial growth factor receptor (VEGFR), epidermal growth factor (EGFR), and tyrosine kinase have improved outcomes for metastatic CRC patients. However, the survival benefit achieved with each novel therapeutic is modest, and only a fraction of patients demonstrate a measurable or durable response. Despite immense preclinical and clinical research efforts, only *RAS* mutation status exists to guide therapeutic decisions. *KRAS* mutations predict resistance to EGFR-based antibodies, including panitumumab and cetuximab, since KRAS is involved in signal transduction from ligand-bound EGFR from the cell membrane to the nucleus.[147]

EGFR silencing may also be involved in treatment resistance for colorectal cancer patients. Scartozzi et al.[148] identified *EGFR* promoter DNA hypermethylation in 58% of primary colon tumors, and patients with *EGFR* promoter DNA methylation showed a 5 month shorter PFS and an 11.7-month shorter OS compared with those patients without *EGFR* promoter DNA methylation. In addition, a phase I/II trial[149] to assess the performance of 5-Aza-CdR and panitumumab in 20 metastatic colorectal cancer patients with wild-type *KRAS* tumors showed tolerance and activity to this drug combination. Partial responses were observed in 2/20 (10%) of patients and stable disease was observed in 10/20 (50%) of patients, suggesting that this drug combination may improve survival and quality of life in patients with metastatic colon cancer. Finally, a phase II study of 5-Aza-CR and entinostat in colon cancer patients,[150] was tolerated and a subset of patients showed DNA demethylation correlated with improved PFS.

Conclusions

Epigenetic therapies, namely DNA methylation and histone modifier inhibitors, show promise as effective anti-cancer agents. Aza-based DNA methylation inhibitors have been tested for nearly 50 years; however, new mechanistic details have recently emerged after combining preclinical drug treatments with genome-wide platforms for measuring DNA methylation changes. Treatment with DNA methylation inhibitors not only results in re-activation of tumor suppressor genes, but also repression of oncogenes and gene with demethylated genebody/transcribed regions. In addition, the reactivation of endogenous retroviruses (ERVs) after 5-Aza-CdR results in viral mimicry and the initiation of an immune response that can be targeted with immune-based therapies. Combining DNA methylation inhibitors with conventional chemotherapies and those that specifically inhibit DNA repair show promise in pre-clinical models. Finally, integrating DNA methylation inhibitors with inhibitors of histone modifiers provides synergistic enhancement of DNA methylation inhibition, as does the addition of vitamin C, a cofactor of TET-based oxidative DNA demethylation. These combined methodologies (Fig. 3) suggest that DNA methylation inhibitors serve as primers for activating epigenetically silenced genes that can be further targeted with

complementary therapies. Evaluation of these treatment schemes in preclinical models and clinical trials will shed light on the efficacy for routine use in treating patients with a wide variety of human cancers.

Disclosure of potential conflicts of interest

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